Position 713 Is Critical for Catalysis but Not Iron Binding in Soybean Lipoxygenase 3†

Jeffrey A. Kramer,‡ Keith R. Johnson,§ William R. Dunham,|| Richard H. Sands,|| and Max O. Funk, Jr.*,‡

Departments of Chemistry, Biology, and Medicinal and Biological Chemistry, University of Toledo, 2801 West Bancroft Street, Toledo, Ohio 43606, and Biophysics Research Division, University of Michigan, Ann Arbor, Michigan 48109

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ABSTRACT: The role of asparagine-713 in iron atom incorporation and catalysis in soybean lipoxygenase 3 was investigated using site-directed mutagenesis. A full-length cDNA for the lipoxygenase isoenzyme was obtained from a library derived from soybeans cv. Provar. Protein with native specific activity at pH 7.4 was obtained from expression in *Escherichia coli*. Two recent structure reports provided conflicting views about the participation of the side chain of asparagine-694 in the coordination of the iron atom required for catalysis by lipoxygenase 1. Oligonucleotide-directed mutagenesis was employed to modify residue 713 in lipoxygenase 3 which corresponds to asparagine-694 in the sequence of lipoxygenase 1. It was found that for enzyme expressed in bacteria, asparagine was not required for iron incorporation. Histidine, alanine, and serine substitutions for asparagine-713 all produced proteins that contained iron. The histidine mutant had specific activity and catalytic characteristics comparable to the wild-type enzyme. By contrast, the alanine- and serine-substituted lipoxygenases had no detectable catalytic activity. When oxidized by product, the histidine mutant also displayed the characteristic g6 signal of the soybean enzyme in its EPR spectrum. The possibilities that the residue at position 713 acts as a metal ligand, an acid−base catalyst, and a hydrogen bonding group are considered and discussed.

Lipoxygenase plays a central role in polyunsaturated fatty acid metabolism in both plants and animals. In a variety of mammalian cells, the enzyme inaugurates the biosynthesis of numerous eicosanoids including the leukotrienes, the lipoxins, and various hydroxyeicosatetraenoic acids (Samuelsson et al., 1987). In plants, the primary substrates are linoleic and linolenic acids, and the biosynthetic products have both growth promotion and pest resistance properties (Siedow, 1991). While lipoxygenase 1 from soybeans has been available in a highly purified state since at least 1947 (Theorell et al., 1947), the structure and mechanism of action of the enzyme have only recently begun to be elucidated. The catalytic activity of all lipoxygenases depends on the presence of a unique non-heme iron cofactor (Feiters et al., 1990).

The iron environment has been probed in a variety of ways: in spectroscopic studies, by site-directed mutagenesis experiments, and in recent X-ray crystallographic investigations. According to electron paramagnetic resonance (EPR),[1] UV−visible, magnetic circular dichroism and Mössbauer spectroscopy, and magnetic susceptibility experiments, lipoxygenases obtained from soybeans contain non-heme iron as high-spin iron(II) (De Groot et al., 1975; Whittaker & Solomon, 1988; Dunham et al., 1990). The spectroscopic parameters as well as data for EXAFS are consistent with a six-coordinate iron atom consisting of nitrogen and/or oxygen atom ligands in an octahedral geometry (Navaratnam et al., 1988). Treatment of the native enzyme with 1 equiv of the product of lipoxygenase catalysis converts the cofactor to iron(III). In this state, samples of the enzyme manifest an EPR signal at g6 reflecting an axial heme-like environment (De Groot et al., 1975). The results of variable-temperature EPR and magnetic circular dichroism spectroscopic experiments have been interpreted as reflecting a minimal change in coordination geometry upon oxidation (Zhang et al., 1991). There is evidence that one ligand to the iron(III) in lipoxygenase 1 is a water molecule (Nelson, 1988). Treatment of the oxidized from of the enzyme with substrate in the absence of oxygen results in reduction of the metal atom to an iron(II) form that differs from that in the native enzyme (Funk et al., 1990). These observations taken together have provided strong evidence for a redox role for the iron in the mechanism of lipoxygenase catalysis. Therefore, the characterization of the non-heme iron site has become a high priority in the study of lipoxygenases.

Comparisons of the various lipoxygenase primary sequences as they became available (e.g., from the isoenzymes in soybean seeds, from lipoxygenases from other plant sources, and from mammalian cells) led to hypotheses about the side chains that might be acting as iron ligands. For example, a highly conserved cluster of histidines was found in sequence alignments, and these residues (494, 499, 504, 522, 531, and 690 in lipoxygenase 1) were excellent candidates for the iron ligands (Shibata et al., 1987, 1988). Site-directed mutagenesis studies have subsequently shown

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* Address correspondence to this author at the Department of Chemistry, University of Toledo. Telephone: (419) 537-4569. Fax: (419) 537-4033.

‡ Department of Chemistry and Department of Medicinal and Biological Chemistry, University of Toledo.

§ Department of Biology, University of Toledo.

|| Biophysics Research Division, University of Michigan.

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[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride.

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that mutant proteins in which the histidine residues 499, 504, and 690 were substituted with noncoordinating amino acids were not active and contained no iron (Steczko & Axelrod, 1992; Steczko et al., 1992). These findings implicated these three histidine residues in iron binding. Deletion and mutagenesis experiments on the C-terminal isoleucine residue have been carried out on murine platelet and spleen/leukocyte 12-lipoxygenases (Chen et al., 1994). Alteration of the C-terminal residue by deletion or substitution with most amino acids abolished catalytic activity. The iron content of the site-directed mutants was not reported.

Two reports on the three-dimensional structure of lipoxygenase 1 have appeared very recently. Both investigations addressed the critical question of the non-heme iron site in the protein, but came to somewhat different conclusions with respect to the coordination of the iron by amino acid side chains. In one of the crystal structures, the iron was found to be four-coordinate in the shape of a distorted tetrahedron (Boyington et al., 1993). The ligands included the histidines at residues 499, 504, and 690, and the carboxyl group from the C-terminal Ile, residue 839. In the second reported crystal structure, the iron was found to be five-coordinate in the shape of an octahedron with one coordination site vacant (Minor et al., 1993). The ligands were identified as His-499, His-504, His-690, Asn-694, and Ile-839. The discrepancy in the two structures centers around the involvement of Asn-694 as an iron ligand. It is important to note that the two structures were obtained from crystals that were prepared under significantly different conditions of pH and ionic strength. The differences between the observations therefore may reflect differences in the conditions of the experiments. In this report, we provide an independent investigation of the role of the asparagine residue as an iron ligand using the site-directed mutagenesis approach on an isoenzyme of lipoxygenase from soybeans. We have developed an expression system for lipoxygenase 3 in *Escherichia coli* that yields sufficient quantities of active enzyme for a detailed physical and chemical characterization of the mutants. Here we report on the attributes of this system and its application to the substitution of the appropriate amino acid, Asn-713, in site-directed mutagenesis experiments. We find that the asparagine is not required for iron binding in L-3, but that the residue at this position is crucial for efficient catalytic activity.

MATERIALS AND METHODS

Preparation of Plasmids Bearing the Lipoxygenase 3 cDNA. A full-length lipoxygenase 3 cDNA (Yenofsky et al., 1988) (graciously provided by Professor David Hildebrand, University of Kentucky) labeled with [^{32}P]dCTP (Feinberg & Vogelstein, 1984) was used to screen a cDNA library from soybean seeds cv. Provar (kindly provided by Professor John Thompson, Cornell University) inserted between the *Eco*RI and *Xho*I sites in λZAP. Restriction fragments large enough to harbor the entire coding region were sequenced to identify DNA containing the lipoxygenase start and stop codons. The selected DNA was inserted into pUC19 and sequenced (Sanger et al., 1977). The plasmid bearing the full-length lipoxygenase cDNA was referred to as pUC LOX. For the purpose of expression, the L-3 cDNA was transferred to pET3a (Studier, 1990) in a two-part process. A *Bam*HI restriction fragment from pUC LOX containing the N-terminal two-thirds of the cDNA was inserted into pET3a. The resulting plasmid was cut with *Esp*I, filled in with the Klenow fragment of DNA polymerase I to yield a blunt end, then cut with *Bst*XI, and finally ligated to a *Bst*XI/*Sma*I restriction fragment from pUC LOX which contained the remainder of the coding sequence. This construct referred to as pET LOX contained 75 extra nucleotides between the start codon of phage T7 gene 10 present in pET3a and the L-3 start codon. Removal of these nucleotides was required for the expression of active protein. This was achieved by oligonucleotide-directed mutagenesis of the plasmid that introduced a restriction site (*Nde*I) that could be used to remove the extra nucleotides. An *Nde*I restriction site spans the start codon in pET3a. Briefly, an *Eco*RI restriction fragment from pET LOX containing the lipoxygenase start codon was inserted into pBluescript KS-(+). Isolated single-stranded DNA was annealed with an oligonucleotide (Oligos etc.) that introduced an *Nde*I restriction site spanning the ATG start codon of the enzyme. Double-stranded DNA was synthesized with T4 DNA polymerase, and the preparation was used to transform JM109 cells. Small-scale plasmid preparations (Johnson, 1990) were digested with *Nde*I and run on agarose gels. From a selected preparation, the band corresponding to 3325 bp was isolated, religated, and used for the transformation of JM109 cells. Separately, the *Bgl*II/*Hin*dIII restriction fragment from pET LOX containing only the promoter region and 5′ end of the lipoxygenase cDNA was inserted into pBluescript. Digestion of the two pBluescript constructs with *Nde*I and *Eco*RI provided fragments (346 and 4785 bp) that when ligated lacked the 75 nucleotides between the start codons of pET and lipoxygenase. The new sequence was exchanged for the sequence in pET LOX by *Xba*I/*Bst*XI digestion of the two plasmids, ligation of the fragments, and selection of cells bearing the modified DNA. The presence of the shortened DNA was confirmed by sequencing.

Expression and Purification of Active Lipoxygenase 3. The final plasmid, termed pFTB, was used to transform BL21(DE3) cells for the expression of protein. Culture medium (500 mL, SOB, with 50 μg mL^{-1} ampicillin) was inoculated with pFTB in BL21(DE3) (1.0 mL) and shaken at 37 °C for 90 min. Ethanol (15 mL; 3%, v/v, final content) (Steczko et al., 1991) and IPTG (0.4 mL, 0.4 mM final concentration) were added, and the mixture was shaken at 20 °C for 24 h. The cells were collected (10000*g*, 15 min), washed once with deionized water, and suspended in an equal volume of histidine buffer (25 mM, pH 5.5, 1 mM PMSF). The cells were disrupted by two passes through a chilled French pressure cell at 8000 psi. The supernatant (15000*g*, 40 min) was fractionated with ammonium sulfate. The protein precipitating between 30% and 60% ammonium sulfate was resuspended in the histidine buffer and dialyzed against the same buffer. The solution was applied to a chromatofocusing column for final purification as previously described (Funk et al., 1986). The lipoxygenases were assayed with linoleic acid as the substrate at pH 7.4 in a final volume of 1.0 mL using the procedure of Schewe et al. (1983). Kinetics studies were conducted in Tris buffer at pH 9.0 in a final volume of 1.0 mL using the procedures of Axelrod (Axelrod et al., 1983). The determination of iron was carried out using a Perkin-Elmer Plasma II Inductively Coupled Plasma Emission spectrometer calibrated with SPEX multielement plasma standard and Iron Reference Standard Solution (Fisher). Each determination was carried out in

triplicate. The largest standard deviation for any set of data was 6% of the mean value. The reproducibility between independently prepared batches of an expressed protein was ±0.04 mol of iron mol^{-1}. The protein concentrations were obtained with the Bradford assay (Bradford, 1976). Electrophoresis was performed in a 12% polyacrylamide gel in the presence of SDS using the discontinuous buffer system of Laemmli (Laemmli et al., 1970). The protein bands were visualized with Coomassie blue staining. The molecular weight standards were phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase.

Site-Directed Mutagenesis of Asn-713. A *Bam*HI/*Nsi*I restriction fragment (491 bp) from pFTB was isolated by agarose gel electrophoresis and inserted into pGEM7Zf(+). Single-stranded DNA was prepared and annealed with oligonucleotides (Oligos Etc.) centered on the specified changes for site-directed mutagenesis. The Asn AAT codon was changed to GCT for N713A (26-mer), AGT for N713S (21-mer), and CAT for N713H (21-mer). The incorporation of the altered bases was confirmed by sequencing from a synthetic primer (Oligos Etc.) situated 48 nucleotides upstream from the mutations. The altered *Bam*HI/*Nsi*I restriction fragments were exchanged for the wild-type fragment, and the resulting constructs were transformed into BL21(DE3). Double-stranded DNA was prepared, and the mutations were recombined with the remainder of pFTB at the *Bam*HI/*Nsi*I sites. The plasmids were used for the transformation of BL21(DE3) cells for the expression of protein. The expression and isolation procedures for the mutants were the same as for the native protein.

EPR Spectroscopy. EPR measurements were made at 9 GHz using a Varian Century Line spectrometer equipped with a liquid helium transfer line and a quartz dewar cavity insert. Spectra were recorded at 25 K under microwave power that avoided saturation. Running conditions were set at 5 mW power, 1 mT modulation amplitude, 4 min scan time, and 0.128 s time constant. The samples were prepared for analysis by dialysis against Tris buffer (0.1 M, pH 7.4, 0.1 M NaCl, 1 mM EDTA). The oxidized enzyme was obtained by treatment with 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (Funk et al., 1976) in a 1:1.1 ratio. Final concentration of the samples for EPR was carried out in a centrifugal concentrator (Amicon Centricon 30).

RESULTS

Cloning the Lipoxygenase 3 cDNA. A full-length lipoxygenase L-3 cDNA was used to screen a cDNA library prepared from soybean seeds cv. Provar. The longest cDNA that was identified was subcloned into pUC19 and completely sequenced. Our nucleotide sequence differed from the published lipoxygenase L-3 sequence in six positions, resulting in five differences in the deduced amino acid sequence. The differences in the deduced amino acid sequence of L-3 and what we have in the past called P4 were (L-3→P4) P57S, L112P, V201I, E382D, and G428D. In each case, the amino acid difference resulted from a single nucleotide change. We attributed these amino acid substitutions to varietal differences rather than to the existence of slightly different isoenzymes. This conclusion was supported by additional sequence comparisons. First, the 5′ and 3′ untranslated regions of our cDNA clone (218 nucleotides) were identical to the L-3 sequence. Second, homologues of the L-3 sequence have been identified in *Pisum sativum* (pea) and *Len culinaris* (lentil). In each of these two cases, the deduced amino acid sequence agrees with our sequence rather than that of L-3 in four of the five positions. By contrast, the deduced amino acid sequence obtained here differs from the published sequences of L-1 and L-2 by 251 (29%) and 218 (25%) residues, respectively. It is therefore justifiable to refer to the soybean seed lipoxygenase from cv. Provar that was cloned and sequenced in the present study as an L-3 isoenzyme.

Lipoxygenase 3 Is Expressed in E. coli. The lipoxygenase L-3 cDNA was inserted into the pET3a plasmid for expression in *E. coli* BL21(DE3). As a result of the positions of the restriction sites employed to insert the cDNA, 75 nucleotides were originally incorporated between the start sites of pET3a and the lipoxygenase coding region. These in-frame nucleotides would have coded for an additional 25 amino acid residues at the N-terminus of the protein. Active protein was not obtained in trials of induced expression using the plasmid containing the extra nucleotides. Therefore, these nucleotides were removed from the plasmid by a process involving site-directed mutagenesis to incorporate a restriction site (*Nde*I) at the start codon of the lipoxygenase cDNA. Because an *Nde*I site spans the start codon of the T7 gene 10 present in pET3a, the extra nucleotides were removed as a part of an *Nde*I/*Eco*RI restriction fragment from a portion of the native sequence. When the fragment lacking the nucleotides was recombined with DNA digested at the engineered restriction site, the extra nucleotides had been effectively deleted. This process was carried out in pBluescript, and the DNA was returned to pET3a for the purpose of expression.

The soluble extracts from IPTG-induced and ethanol-treated BL21(DE3) cells had lipoxygenase activity at pH 7.4. The enzyme was readily purified in two steps using a previously published chromatofocusing procedure (Funk et al., 1986). Inoculation of 500 mL of culture medium resulted in the isolation of 1.1 mg of the enzyme with a specific activity of 4.1 μmol min^{-1} mg^{-1}. Under the same conditions, the specific activity for the enzyme isolated from soybean seeds was also 4.1 μmol min^{-1} mg^{-1}.

Site-Directed Mutagenesis of Asn-713. The asparagine residue at position 713 in L-3 was replaced by alanine, serine, and histidine through site-directed mutagenesis. The mutant enzymes were obtained from extracts of induced BL21(DE3) cells as described for the wild-type enzyme. The purification of the proteins is presented in Figure 1. All of the expressed proteins eluted from the chromatofocusing column in peaks centered between pH 5.13 and 5.18. The mutant and wild-type lipoxygenases all appeared as single bands on SDS–polyacrylamide electrophoresis gels with relative molecular masses of approximately 90 kDa. These proteins were immunoreactive on Western blots with antibodies specific for epitopes in lipoxygenase 3 (Wheelock et al., 1991; results not shown). The iron content of the expressed proteins varied from 0.51 mol mol^{-1} for the serine mutant to 1.07 mol mol^{-1} for the wild-type enzyme. The alanine and histidine mutants had 0.90 and 0.96 mol of iron mol^{-1}, respectively. Samples of the lipoxygenase 3 isolated from soybeans and analyzed in the same set of experiments had 1.02 mol of iron mol^{-1}. The serine- and alanine-substituted lipoxygenase 3 had no detectable enzymatic activity at pH 7.4. The low limit of activity that would have been detected

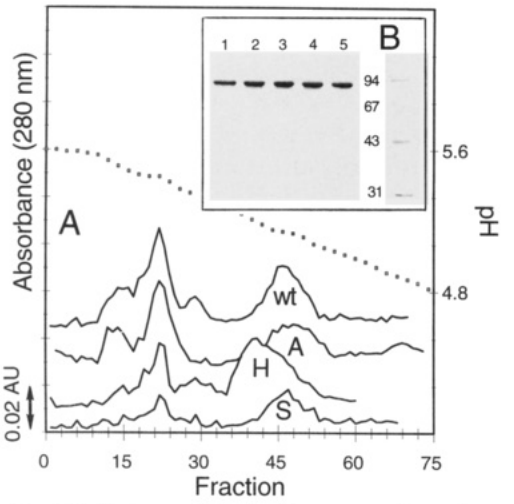

FIGURE 1: (A) Elution profiles for the expressed proteins purified by chromatofocusing: wild-type (wt) and site-directed mutants at position 713 (A, H, and S). (B) SDS−polyacrylamide gel electrophoresis determinations of lipoxygenase 3 from soybeans (lane 1), N713H (lane 2), N713S (lane 3), N713A (lane 4), and wild-type expressed protein (lane 5).

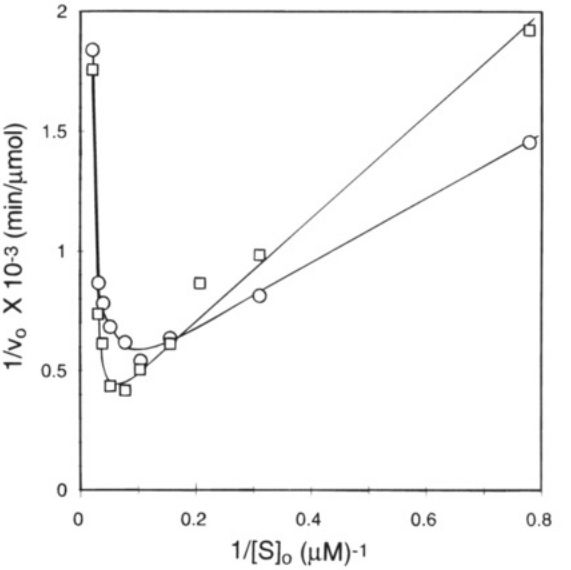

FIGURE 2: Lineweaver−Burk plot comparison of catalysis at pH 9.0 by the wild-type enzyme (open squares) and the site-directed mutant, N713H (open circles).

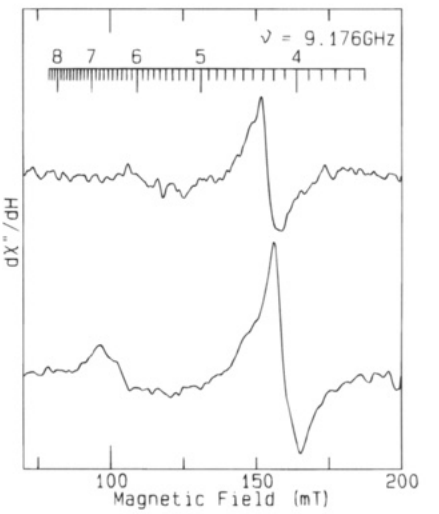

FIGURE 3: 9 GHz EPR spectrum for N713H lipoxygenase 3 as isolated from *E. coli* (top) and after treatment with 13-hydroxyperoxy-9*Z*,11*E*-octadecadienoic acid (bottom).

with these proteins expressed as a percentage of the activity obtained for the wild-type enzyme was 0.02%. The possibility that substitution by these amino acids changed the pH optimum for catalysis in the mutant enzymes was tested. No activity was detected in the accessible range of pH for lipoxygenase assays (7−10) for either the serine or the alanine mutant.

The asparagine at position 713 is not uniformly conserved among all sequence-aligned lipoxygenases. The enzymes that do not contain asparagine at this position contain histidine instead. We reasoned that the N713H substitution would result in a functional mutant if the two amino acids served the same purpose in the lipoxygenases from different sources. Asparagine and histidine have comparable polarity and participate in similar hydrogen bonding networks, but while histidine is commonly found to act as a ligand in metalloproteins, asparagine is not. The lipoxygenase 3 bearing histidine at position-713 was found to be catalytically active. Under the conditions of the standard pH 7.4 assay, the N713H mutant had a specific activity of 2.5 μmol min^{-1} mg^{-1}, or 60% of the wild-type enzyme. In order to compare the characteristics of catalysis by the histidine mutant and the wild-type enzyme, kinetic studies were carried out at pH 9.0 where the substrate linoleic acid has greater solubility. Catalysis by lipoxygenases other than the type-1 enzyme at pH 9.0 has been previously reported (van Os et al., 1979). Lipoxygenase 3 was found to display substrate inhibition at linoleic acid concentrations above 100 μM. The substrate dependence for catalysis by the wild-type and histidine-substituted lipoxygenase 3 is presented in Figure 2. The two enzymes have similar characteristics, and both show the substrate inhibition effect at high linoleic acid concentrations. It is apparent from the plots that the k_{cat} and K_M values are not identical for the wild-type and histidine-bearing enzymes. The histidine mutant has somewhat lower values for both k_{cat} and K_M in comparison to the wild-type enzyme, resulting in a higher overall catalytic efficiency, k_{cat}/K_M, 1.3×10^7 min^{-1} M^{-1} versus 0.6×10^7 min^{-1} M^{-1}. Because of the suboptimal conditions of the kinetics experiments (pH 9), these numbers may not be significantly different. Both are close to the value of k_{cat}/K_M obtained for the soybean enzyme under the same conditions: 1.0×10^7 min^{-1} M^{-1}.

EPR Spectroscopy. The EPR spectrum obtained for the histidine mutant before and after treatment with the product of catalysis (13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid) is presented in Figure 3. The results obtained closely parallel what is seen for the enzyme isolated from soybeans. The spectrum for the histidine mutant as isolated from *E. coli* contains a signal at g4.3 that accounts for only a small fraction (<3%) of the iron present in the sample. Treatment of the histidine mutant with the product of catalysis results in the appearance of a new signal in the EPR spectrum around g6. The ratio of the concentration required for simulation of this signal to the concentration of lipoxygenase in the sample was 1.4 ± 0.4. The value for the intensity measurement lacks precision compared to the g4.3 signal because the former has a small amplitude and a large spectral width (extending to g2 where intensity from extraneous signals is difficult to suppress) and the latter has a large amplitude and a narrow spectral width. The appearance of

a g6 signal in the EPR spectrum after product treatment is a hallmark physical property of lipoxygenase. The resemblance of the spectrum of the histidine mutant to that of the soybean enzyme extends to the shape of the g6 signal. In the absence of low molecular weight alcohols, the signal for the soybean enzyme is typically composed of two contributions differing in the degree of rhombic distortion to the axial g6 signal. These features are also apparent in the spectrum of the histidine mutant.

DISCUSSION

Lipoxygenase 3 has been successfully cloned and expressed in a bacterial system. The recombinant enzyme had native specific activity at pH 7.4. Site-directed amino acid substitutions revealed that Asn-713 was not required for the expression of protein containing iron. Histidine, alanine, and serine substitutions at position 713 produced proteins that contained 96%, 90%, and 51% of the iron found in native lipoxygenase 3. These observations appear to contradict the recent finding that Asn-713 was a ligand to lipoxygenase iron. It is still possible that the asparagine is weakly bound in the structure and not required for the proper folding of the polypeptide and incorporation of the iron. Alternatively, the amino acid side chain, which is certainly in the vicinity of the iron in both of the reported structures, may be poised to become a ligand during changes that occur in the enzyme as a consequence of catalysis, e.g., oxidation to the iron(III) state. The crystallization conditions used in the two structure determinations were quite different. A weakly coordinating ligand might be sensitive to the experimental variables. The asparagine with its weakly coordinating amide side chain could be a displaceable ligand, but this is difficult to reconcile with the effect of site-directed mutagenesis on catalysis (*vide infra*). The close correspondence of the EPR spectrum obtained for the product-treated histidine mutant to that of the soybean enzyme also argues against a role for the asparagine in coordination, since a change from the weakly coordinating amide to the imidazole group of histidine could be expected to influence the EPR spectral properties of the non-heme iron site.

There is no precedent for iron coordination by asparagine in metalloproteins. This amino acid has been found as a ligand to calcium and zinc, but not iron in proteins (Chakrabarti, 1990). Further, the set of structurally characterized coordination compounds involving amide−iron interactions is not large. The amide group is capable of both N- and O-based transition metal coordination depending on pH (Sigel & Martin, 1982). Deprotonation occurs at high pH, favoring amido nitrogen bond formation. In neutral complexes, O bonding is preferred. Each form of coordination has been reported for the two common oxidation states of iron (Holt et al., 1979; Calderazzo et al., 1980). For example, complexes which mimic the coordination of iron by the antitumor drug bleomycin involving deprotonated amido nitrogens as donors to both iron(II) and iron(III) have been reported (Guajardo et al., 1993). While the coordination proposed for iron by Asn-713 in lipoxygenase is conceivable, it would definitely represent a new form of bonding in an iron-containing protein.

The principal finding here is that while histidine substitutes for asparagine at position 713, producing a mutant with similar properties to the wild-type enzyme, alanine and serine substitutions result in protein with no detectable catalytic activity even though they contain iron. This finding was foreshadowed by the observation that sequence-aligned lipoxygenases that do not have asparagine at this position contain histidine instead. The kinetic results show that this change in amino acids has little effect on the characteristics of catalysis by the enzyme. This means that whatever part the asparagine may play in the structure or mechanism of action of lipoxygenase, histidine is also effective in this role. This is not expected if the amino acids are involved in iron coordination where the behavior of asparagine and histidine is expected to be quite different. This is best illustrated by the example of axial iron ligation in heme proteins. Numerous heme proteins contain axial fifth ligands to the iron atom which are histidine residues. For example, the proximal histidine (F8) in hemoglobin and myoglobin is one of the five invariant amino acids in all vertebrate and invertebrate globin chains (Dickerson & Geis, 1983). Therefore, there is no mutant hemoglobin or myoglobin, functional or otherwise, bearing asparagine at position F8. Substitution of the axial histidine residue by asparagine has been either observed or carried out in both the *b*- and *c*-type cytochromes (Meyer et al., 1991; Hampsey et al., 1986). These alterations do not result in biologically active proteins. This results from the fact that the properties of these two amino acids are so greatly different when they are acting as metal ligands. Because the iron atom takes part in redox changes as a part of the catalytic mechanism, it seems likely that if asparagine is a ligand to lipoxygenase iron, a significant change in catalytic behavior would be observed upon histidine substitution.

The only role proposed for an amino acid side chain in lipoxygenase catalysis is for a base to assist in net hydrogen atom abstraction from the substrate. An affinity labeling study pointed to the involvement of a nucleophilic/basic group in close proximity to the substrate binding site (Corey et al., 1986). Coordination of asparagine to the iron would be expected to reduce the pK_a of the side chain into the physiological range. It is tempting to speculate that asparagine accepts the proton, causing it to dissociate. However, because hydrogen abstraction is known to be the rate-limiting step in catalysis, it is difficult to see why there would not be a bigger difference between asparagine and histidine at this position, since the latter amino acid would be expected to have a much lower pK_a, and consequently be much less basic under comparable conditions of coordination.

An alternative explanation that is consistent with the currently available set of observations can also be considered. The presence of asparagine could be required for catalysis through hydrogen bonding interactions. This would be consistent with the histidine substitution results. Both amino acids are capable of simultaneously providing both donor and acceptor hydrogen bonding groups. In a hydrogen bonding role, asparagine and histidine can be considered to have similar properties in contrast to anticipated differences in their behavior when acting in other ways, e.g., metal−ligand or acid/base. The structure of the enzyme at a point on the catalyzed reaction pathway might depend critically on the presence of hydrogen bonding at position 713. The amino acids could participate in the maintenance of a hydrogen-bonded network of residues near the iron site that is necessary for catalysis. Alternatively, the asparagine might be involved in enzyme−substrate and/or transition state

interactions. For example, the distal histidine (E7) in hemoglobin and myoglobin is thought to stabilize the coordinated oxygen molecule through hydrogen bonding (Phillips & Schoenborn, 1981; Nagai et al., 1987). Mutations with glutamine at the E7 position are found naturally in the elephant and shark myoglobins, and several hemoglobins, as well as in a site-directed mutant of sperm whale myoglobin (Yu et al., 1990; Rohlfs et al., 1990). The changes in structure wrought by this substitution may be complex, but certainly result in functional proteins. While there is no evidence for dioxygen−iron binding in either the native or the product-oxidized forms of soybean lipoxygenase (Petersson et al., 1985), this does not rule out the possibility that an interaction of this kind forms transiently as a part of the catalyzed reaction.

The results reported here provide the first evidence for a critical role for a specific amino acid in lipoxygenase catalysis. Functional substitution of the asparagine at position 713 by histidine is difficult to reconcile with the chemical properties of these amino acid side chains in metal ion ligation and basicity. Alternatively, the residue at position 713 may be critical to catalysis for its participation in hydrogen bonding interactions.

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