Cloning of Linoleate Diol Synthase Reveals Homology with Prostaglandin H Synthases

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Linoleate diol synthase is a homotetrameric ferric hemeprotein, which catalyzes dioxygenation of linoleic acid to (8R)-hydroperoxylinoleate and isomerization of the hydroperoxide to (7S,8S)-dihydroxylinoleate. Ferryl intermediates and a tyrosyl radical are formed in the reaction. Linoleate diol synthase was digested with endoproteinase Lys-C, and internal peptides were sequenced. The sequence information was used for reverse transcription-polymerase chain reaction analysis, and a cDNA probe was obtained. Northern blot analysis of linoleate diol synthase suggested a 3.7-kilobase pair (kb) mRNA. A full-length clone of the linoleate diol synthase gene was obtained by screening of a genomic λ-ZAP II library of the fungus Gaeumannomyces graminis. The 5′-untranslated region contained CAAT- and TATA-like boxes. The gene contained three short introns and spanned over 3.2-kb. The deduced open reading frame consisted of 2.9-kb, which corresponded to 978 amino acids and a molecular subunit mass of 108,000. Data base analysis with the gapped BLAST algorithm showed that 391 residues of linoleate diol synthase was 23–24% identical and 36–37% positive with the catalytic domain of mammalian prostaglandin H (PGH) synthase-2. Based on homology with PGH synthases, the proximal heme ligand of linoleate diol synthase was tentatively identified as His-379 and the important tyrosine for catalysis as residue 376 (apparent consensus EFXXXXYW). The distal heme ligand was tentatively identified as His-203 (apparent consensus THXXFXT). We conclude from catalytic and structural similarities that linoleate diol synthase and PGH synthases likely share common ancestry and may belong to a gene family of fatty acid heme dioxygenases.

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phases are prominent peroxidases (2–3, 25), whereas linoleate diol synthase catalyzes isomerization of (8R)-hyperoxepro C18 fatty acids into 7,8-dihydroxy C18 fatty acids (5–6, 13, 26). Linoleate diol synthase and PGH synthases thus have two related features in common, viz. formation of a tyrosyl radical and ferryl intermediates.

PGH synthases show ~20% amino acid identity with the mammalian peroxidase gene family (23, 25). PGH synthases also appear to have regions of amino acid similarity with two plant proteins (27–28) but lack apparent homology to all previously sequenced dioxygenases. Whether PGH synthases and linoleate diol synthase are homologous proteins has not yet been determined.

The aim of the present investigation was to clone and sequence the linoleate diol synthase gene, to determine the deduced primary structure of linoleate diol synthase precursor, and to compare it with PGH synthases and with peroxidases. We report as our main finding that linoleate diol synthase and PGH synthases are structurally related. The similar oxygenation mechanism of PGH synthases and linoleate diol synthase may therefore not be a coincidence.

**Experimental Procedures**

**Materials**—35S-dATP (3000 Ci/mmole), [α-32P]dCTP (3000 Ci/mmole), dNTPs, [α-33P]dNTPs, Hybond-N membranes, and DNA labeling beads, T-primed first-strand kit, T7 sequencing, and Thermo Sequenase kit were from Amersham Pharmacia Biotech. TA cloning kits were from Invitrogen. DNA polymerases for PCR were from Perkin-Elmer (AmpliTaq) and from New England Biolabs (Vent (exo -)). Moloney murine leukemia virus reverse transcriptase was from Life Technologies, Inc., and Amersham Pharmacia Biotech. Restriction enzymes were from New England Biolabs. G. graminis was obtained and grown as described (5, 7). Plant RNasey mini and agarose gel extraction kits were from Qiagen. Primers for PCR were used without further purification and obtained from TIB MOLBIOL (Berlin, Germany), Life Technologies, Inc., and CyberGene (Huddinge, Sweden).

**Amino Acid Sequencing and Oligonucleotides for PCR**—Purified linoleate diol synthase was subject to in situ digestion in the SDS-polyacrylamide gel with Lys-C. Peptides were isolated by narrow bore reverse phase liquid chromatography on the Smart System (Amersham Pharmacia Biotech) and subjected to amino acid sequencing (Perkin-Elmer ABI 456 Sequencer) as described (29). Three peptide sequences were from Qiagen. Primers for PCR were used without further purification and obtained from TIB MOLBIOL (Berlin, Germany), Life Technologies, Inc., and CyberGene (Huddinge, Sweden).

**Preparation of Total RNA and cDNA Synthesis**—Total RNA was prepared using the RNeasy kit (Qiagen). ~5 μg of total RNA was used in a 25-μl reaction mixture for first strand cDNA synthesis (1 h at 37 °C). The reaction mixture contained reverse transcriptase buffer with 500 units of Moloney murine leukemia virus reverse transcriptase, 15 mM dithiothreitol, 2.5 mM of oligo(dT)16 primer (Perkin-Elmer), and 20 units of RNase inhibitor (Perkin-Elmer). Two μl were used as templates in each PCR. It was later found that these samples were contaminated by genomic DNA.

**PCR Analysis and Cloning**—The PCR (50 μl) contained 0.4 μM of each primer, 10 mM Tris/HCl, pH 8.3, 50 mM KCI, 2.5 mM MgCl2, 0.2 mM dNTPs, and 1.5 units of AmpliTaq DNA polymerase. The PCR protocol was 94 °C for 1 min, 1 cycle; 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min, 30 cycles, and a final extension step (72 °C, 10 min) and then cooled to 8 °C. The amplified cDNAs were cloned in the T vector pcR2.1. Sequencing of both strands was initially performed using dideoxynucleotide sequencing with T7 DNA polymerase but changed to cycle sequencing using the ABI Sequenase Kit and radioactively labeled dNTPs or ABI Prism Big-Dye terminator cycle sequencing kit (Perkin-Elmer).

**Hybridization Screening of a Genomic Library**—The genomic library of G. graminis was constructed by partial digestion of genomic DNA with Tsp6 and ligation into the EcoRI site of λ-ZAP II (Stratagene, Heidelberg, Germany) as described (30). A cDNA probe (0.75 kb) was generated by the reverse transcription PCR using primers Su16 (identical with nucleotides 397–421 of the linoleate diol synthase gene) and Su31 and labeled with 32P using the random primer method (31–32). Control experiments with untranscribed RNA showed that this probe was formed by amplification of genomic DNA. Hybridization screening of the genomic library was performed in QuikHyb (Stratagene) as described (31). The Bluescript plasmid was rescued from positive phage clones with helper phage as suggested by the manufacturer.

**Restriction Analysis and Subcloning**—Restriction analysis of the Bluescript plasmids was performed by cleavage with EcoRI, HindIII, EcoRV, XhoI, PstI, SalI, and NotI and size fractioning in 0.8–1.5% agarose gels (31). Fragments generated by HindIII/EcoRI (1.6 kb) and EcoRI (2.8 kb) were subcloned in pCR2.1 for sequencing.

**Northern Blot Analysis**—Total RNA was size-fractionated by electrophoresis in 1% agarose, 0.22 M formaldehyde gels, transferred to Hybond-N membranes, and hybridized in QuikHyb (Stratagene) with 32P-labeled 0.75-kb linoleate diol synthase cDNA by published methods (31–32).

**Homology Search**—The gapped BLAST algorithm (33) was used for data base search and for pairwise alignments, whereas the Lasergene Megalign program (Dnastar, Madison, WI) was used for multiple alignments.

**Total Amino Acid Analysis**—Total amino acid analysis was performed after acidic hydrolysis at the amino acid laboratory of Uppsala University (courtesy Dr. D. Eaker).

**Results**

**Internal Amino Acid Sequences of Linoleate Diol Synthase**—Purified linoleate diol synthase has an apparent subunit relative molecular mass of ~130,000 (SDS-polyacrylamide gel electrophoresis (7)). In situ digestion with endoprotease Lys-C, peptide separation, and amino acid sequencing (29) yielded three useful internal peptide sequences ((K) KTVTVPYP-NAPGN; (K) FRHPHPK; (K) NEGGFRK, where (K) denotes the fact that Lys-C cleaves peptides at the C-terminal side of Lys residues), which were used for design of degenerate oligonucleotide primers. The N terminal appeared to be blocked. Other internal peptide sequences were (K) AVDWGHVTYK, (K) TLGAGTQSGDAFDD, (K) ARAPI, (K) LAXMY, (K) VTX GPA, and (K) LHQASK. Some peptide sequences were ambiguous or weak but nevertheless useful for finding the correct reading frame (e.g. LVFFVQ/ΤNΨ, NIPD/EPEK, PVAYF (I) F/N/D/ΤLMVR, (K) XXXXDPATXFAAXD, (K) XXIF/Ψ/DT, (K) HLPMEK, and (K) LPPASREGK).

**Isolation of a Linoleate Diol Synthase Clone by PCR Cloning**—The primers Su20 (sense) and Su11 (antisense) generated a band of 550 base pairs, whereas semi-nested PCR, first round Su20 and Su31, second round Su10 (sense), and Su31 (antisense), yielded a band of 480 base pairs. Primers designed downstream of Su20 and upstream of Su31 generated a cDNA band of the expected size (about 1 kb), which contained the deduced amino acid sequence FRHPNK (peptide 2) in one of its reading frames.

**Northern Blot Analysis**—The cDNA probe (0.75 kb) yielded a strong signal at 3.7 kb (data not shown). A weak signal at 2 kb was also noted at lower stringency in some experiments.

**Isolation and Characterization of Linoleate Diol Synthase Genomic Clones**—About 100,000 plaques were screened with a cDNA probe (0.75 kb), and 8 positive clones were obtained. Positive plaques were subject to three rounds of screening. All rescued Bluescript plasmids seemed to contain inserts of the same size (~7 kb). Restriction analysis and sequencing showed that the coding region of linoleate diol synthase started ~2 kb from the 5′-end of the insert.

**Organization and Sequence of the Linoleate Diol Synthase Gene**—The linoleate diol synthase gene with a restriction map is shown in Fig. 1. The 5′-untranslated region of the linoleate diol synthase gene contained a TATA-like box (at nucleotides ~31 to ~24, TAATATAAT) and a CAAT-like box (at nucleotides ~76 to ~72, TCAAT) located upstream of the tentative translation start point (GGGGCCTA'TGA). The gene appeared to be divided into 4 exons and 3 short introns (Table I). Short introns
are characteristic of filamentous fungi (34). The introns were confirmed by sequencing of cDNA. The introns contained a motif similar to the consensus sequence of lariat formation ((C/T)NCT(A/G)Al(C/T)) during splicing of RNA of filamentous fungi (34). The GC content of the coding region was ~60%. About 0.8 kb of the 3’-untranslated region was sequenced, and a polyadenylation signal (AATTAT) (35) appeared to be present 54 base pairs from the stop codon.

**Predicted Amino Acid Sequence of Linoleate Diol Synthase**

The predicted amino acid sequence of the linoleate diol synthase precursor is shown in Fig. 2. By structural homology with PGH synthases (see below), His-203 and His-379 were tentatively identified as distal and proximal heme ligands, respectively. The sequence contained five possible N-glycosylation sites, and the sequence NGNGVY(H,P)T(A) was repeated five consecutive times starting at amino acid residue 862. The total amino acid composition of linoleate diol synthase was determined after hydrolysis. These figures were similar to the deduced amino acid composition of the protein precursor (Table II).

**Sequence Homology to PGH Synthases**

When the predicted amino acid sequence of linoleate diol synthase was subject to BLAST (33) search of GenBank™, PGH synthase-2 of horse, rabbit, and chick obtained the highest scores (and lowest E values) as follows: 3

**Sequence Homology to Other Proteins**

The BLAST search of GenBank™ suggested that 70–100 residues of exons 1 and 2 of linoleate diol synthase had sequence similarity with many peroxidases, e.g. human thyroid peroxidase (36) and the peroxidase precursor of Drosophila melanogaster (37). This sequence similarity included and ended near the His residue, which is the distal heme ligand of peroxidases.

**TABLE I**

| Intron | Donor | Intron size | Lariat | Acceptor |
|--------|-------|-------------|--------|----------|
| 1      | AGttcG | 85          | tggtagc | tagC     |
|        | Ser-128|             |        | TTC CJG  |
| 2      | AACgtatg | 74       | tggtaac | cag     |
|        | Thr-209 |             |        | TCA CCT  |
| 3      | Aagtasg | 74          | tggtagc | cagG    |
|        | Lys-283 |             |        | ATC AAC  |

The first region of apparent homology (26% identical) corresponded to the core helix H2 (residues 196–207 of ovine PGH synthase-1, see Ref. 23) with the distal His heme ligand (consensus TLLXXFXT, where boldface indicates the distal His) at the edge. The second and third regions contained helices H3 and H5 of PGH synthases with 38 and 32% identical residues. Helix H5 is of interest since it harbors the highly conserved decapetide sequence (consensus TLWLRHENRL) of mammalian peroxidases (25). The fourth region contained 54% identical residues and corresponded to core helix H6. The fifth region (33% identical) contained the core helix H8 with the proximal heme ligand and the important Tyr residue (consensus EFNXXYXWH) of PGH synthases. The sixth region contained helices H11–H16, and linoleate diol synthase was aligned for more than 60 amino acids without gaps, with 35% identical amino acids in helices H15 and H16.

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**Sequence Homology to Other Proteins**

The set of PGH synthases was followed on the BLAST search by three proteins with apparent homology to linoleate diol synthase (E values <6 × 10⁻⁴), viz. a peroxidase-related protein² of Caenorhabditis elegans, and two closely related plant proteins, the feebly protein of tomato (27) and the oxygenase of Nicotiana tabacum (28). Their sequence similarities extended over at least 450, 240, and 380 residues of linoleate diol synthase. All sequences appeared to be homologous around the predicted proximal heme ligand of diol synthase. A partial alignment of the peroxidase-like proteins of C. elegans, D. melanogaster, and N.

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² The GenBank™ accession number is Z66520.
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The predicted amino acid sequence of linoleate diol synthase precursor. Amino acids are numbered beginning with the methionine residue (Met-1). Internal peptides, which were generated by cleavage with endoproteinase Lys-C and subjected to amino acid analysis, are underlined, and the accurately determined amino acids are double underlined. One internal peptide sequence was apparently formed by acid-catalyzed cleavage between Asp and Pro residues (sequence (D)PATIFDTL...int he 3rd line). The predicted amino acid sequencing yielded the peptide AVDWGHVIYK (9th line), whereas the determined amino acid peptide contained a Thr instead of an Ile (at position 602). This difference might be due to a sequencing error.

**Table II**

| Amino acids | Abundance in mol % | Measured<sup>a</sup> | Predicted<sup>b</sup> |
|-------------|--------------------|----------------------|----------------------|
| Ala         | 9.2                | 8.4                  |                      |
| Arg         | 6.0                | 5.2                  |                      |
| Asx         | 11.0               | 12.4                 |                      |
| Cys         | 1.1                | 0.7                  |                      |
| Gix         | 7.6                | 7.4                  |                      |
| Gly         | 7.9                | 7.9                  |                      |
| His         | 3.0                | 3.3                  |                      |
| Ile         | 5.0                | 4.9                  |                      |
| Leu         | 9.7                | 9.8                  |                      |
| Lys         | 4.9                | 5.3                  |                      |
| Met         | 1.6                | 2.3                  |                      |
| Phe         | 4.4                | 4.6                  |                      |
| Pro         | 7.0                | 6.7                  |                      |
| Ser         | 6.2                | 6.0                  |                      |
| Thr         | 6.4                | 6.4                  |                      |
| Tyr         | 2.6                | 2.8                  |                      |
| Val         | 6.2                | 5.9                  |                      |

<sup>a</sup> Measured after acidic hydrolysis of extensively purified linoleate diol synthase.<sup>b</sup> Calculated from the deduced amino acid composition of the linoleate diol synthase precursor excluding Trp. The predicted Trp content was 1.2%, and the measured content was 1.4% (courtesy of Drs. H. Jörnvall and T. Bergman, Karolinska Institute).

Tabacum with the amino acids around the predicted proximal heme ligands of linoleate diol synthase is shown in Fig. 4A. Some sequence similarity in the region of the distal heme ligand might also be present (cf. Ref. 28). A phylogenetic tree of linoleate diol synthase, sheep PGH synthase-1, chick PGH synthase-2, and the oxygenase of *N. tabacum*, is shown in Fig. 4B. As discussed above, the amino acid identity between linoleate diol synthase and PGH synthases was most apparent in certain α-helices of PGH synthases. A schematic drawing of some of these α-helices and the position of the heme is shown in Fig. 4C.

**Discussion**

We have cloned and sequenced the gene of linoleate diol synthase. We report as our main finding that linoleate diol synthase is homologous to PGH synthases and more distantly related to peroxidases and peroxidase-related proteins.

Mature linoleate diol synthase consists of a homotetramer with a relative subunit mass of ~130,000 as judged from gel filtration and polyacrylamide gel electrophoresis (7). The deduced subunit molecular mass of the linoleate diol synthase precursor was somewhat lower than these estimates (108,000), which might indicate post-translational modifications. Nevertheless, the determined amino acid composition of linoleate diol synthase agreed with the predicted composition (Table II).

The tertiary structure of PGH synthases can be divided into three folding units (23), the EGF-like module (resides 34–72 of ovine PGH synthase-1), a membrane-binding motif (resides 73–116), and a globular catalytic domain (resides 117–587). 391 amino acids of the catalytic domain of PGH synthases were found to have significant (22–24%) sequence identity with linoleate diol synthase. The sequence identity increased to an average of 36% (range 25–54%) in selected core α-helices, which included the proximal and distal heme ligands and the critical tyrosine residue of PGH synthases.

Previous work has shown that linoleate diol synthase is a ferric heme protein, which forms a tyrosyl radical and ferryl oxygen intermediates during catalysis (7, 8). The structural homology and the catalytic similarity between PGH synthases and linoleate diol synthase suggest that His-203 (consensus THXXYXT) could be the distal heme ligand of linoleate diol synthase. His-379 could be the proximal ligand and Tyr-376 an important residue for catalysis (consensus EFNXXYYWH). Additional work including site-directed mutagenesis will be needed to confirm that these His residues indeed are heme ligands and to determine both the position and the catalytic function of the tyrosyl radical of linoleate diol synthase.

Tyrosyl radicals can be formed by many enzymes, e.g. ribonucleotide reductase, bovine liver catalase, galactose oxidase, and photosystem II, but only the tyrosyl radicals of PGH synthases have been demonstrated to be catalytically competent (22). The tyrosyl radical is a powerful oxidant with a redox potential of 0.94 V (22). Indeed, PGH synthases can abstract hydrogen and introduce molecular oxygen at the mononuclear C-13 of 5,8,11-eicosatrienoic acid in an abortive cyclooxygenase reaction (38–39), which is in close analogy with the hydrogen abstraction and dioxygenation at C-8 of linoleic acid by linoleate diol synthase (5, 13). This analogy and the structural homology suggest that the tyrosyl radical of linoleate diol synthase might be catalytically competent.

Linoleate diol synthase also appeared to be homologous to a peroxidase-related protein of *C. elegans* and with two plant proteins and distantly related to various peroxidases. In con-
Contrast to the plant proteins, the peroxidase-related protein of *C. elegans* appears to be homologous with linoleate diol synthase both at the proximal and distal heme ligands.

For many proteins of common ancestry, the tertiary structure has been conserved, whereas the amino acid sequence has changed considerably during evolution. The catalytic domains of PGH synthase-1 and canine myeloperoxidase show approximately 20% amino acid identity, yet their tertiary structures have been described as strikingly similar (23). Linoleate diol synthase and PGH synthases have a somewhat higher degree of homology.

**FIG. 3.** Partial alignment of linoleate diol synthase precursor with PGH synthases. A, alignment of linoleate diol synthase with horse and rabbit PGH synthase-2 over the catalytic domain. B, alignment of linoleate diol synthase with the catalytic domain of four PGH synthases along α-helices H2, H3, H5, H6, H8, and H15–H16 (23). Identical amino acids are shaded black; the distal heme ligands of PGH synthases of helix H2 is marked by ◦, and the proximal heme ligand and the important Tyr residue of helix H8 in PGH synthases are marked ● and ●, respectively.

The abbreviations used are: lds, linoleate diol synthase; c_2, chick PGH synthase-2; E_2, horse (*Equus caballus*) PGH synthase-2; O_2, rabbit (*Oryctolagus cuniculus*) PGH synthase-2; r_2, rat PGH synthase-2; r_1, rat PGH synthase-1; s_1, sheep PGH synthase-1; con, consensus.

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Acknowledgment—We thank Dr. Hans Jornvall (Karolinska Institutet) for generous advice.

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