Identification of a Naturally Occurring Peroxidase-Lipoxygenase Fusion Protein

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A distant relative of catalase that is specialized for metabolism of a fatty acid hydroperoxide was identified. This heme peroxidase occurs in coral as part of a fusion protein, the other component of which is a lipoxygenase that forms the hydroperoxide substrate. The end product is an unstable epoxide (an allene oxide) that is a potential precursor of prostaglandin-like molecules. These results extend the known chemistry of catalase-like proteins and reveal a distinct type of enzymatic construct involved in the metabolism of polyunsaturated fatty acids.

The Caribbean sea whip coral *Plexaura homomalla* is noted for its high content of prostaglandin esters, which account for 2 to 3% of the tissue dry weight (*1*). The mechanism of biosynthesis of these prostanoids remains unresolved, although *P. homomalla* and other corals can metabolize arachidonic acid to an allene epoxide (allene oxide) that is a potential precursor of prostaglandins, clavulones, and other marine eicosanoids (Fig. 1) (*2–4*). This type of transformation also occurs in plants, where an allene oxide has a crucial role in synthesis of the five-membered carbon ring of the growth hormone jasmonic acid (Fig. 1) (*5*). The biosynthetic pathway in plants involves a distinct lipoxygenase and an allene oxide synthase that belongs to the cytochrome P450 family of hemoproteins (*6*). The pathway in coral is initiated by an 8*R*-lipoxygenase and proceeds with a peroxidase type of reaction that yields the allene epoxide. Here we describe the cloning of a fusion protein that catalyzes both steps of this transformation.

We recently cloned and characterized a 76-kD soluble 8*R*-lipoxygenase from *P. homomalla* that has properties typical of animal lipoxygenases (*7*). In the course of subsequent polymerase chain reaction (PCR) experiments with the coral cDNA and primers based on conserved lipoxygenase sequences, we detected another lipoxygenase-related cDNA distinct from the previously identified transcript (*8*). This cDNA was cloned with 3′ RACE and 5′ RACE procedures (*9*), and a full-length clone corresponding to the 3.2-kb open reading frame (ORF) was obtained by PCR and sequenced.

The predicted polypeptide of 122 kD showed homology in its COOH-terminal two-thirds to mammalian and plant lipoxygenases (Fig. 2), including the conserved amino acids that bind iron in the enzyme active site (*10*). The lipoxygenase domain of the coral protein most closely resembles that of the mammalian enzymes (*11*), both in size (79 kD) and in sequence (41% amino acid identity to the human 5*S*-lipoxygenase and 32 to 35% identity to the human 12*S*- and 15*S*-lipoxygenases). Like other plant and animal lipoxygenases, the sequence has limited resemblance (<20% identity) to the *P. homomalla* soluble 8*R*-lipoxygenase (*7*).

BLAST searches on the 43 kD of sequence NH_2-terminal to the lipoxygenase domain revealed substantial homology with catalase, a highly conserved hemoprotein in aerobic organisms that eliminates hydrogen peroxide. This domain had five regions of homology with catalase, in the correct sequential order in the protein (Fig. 3, A and B). These sequences include several conserved amino acids of the catalase active site. There are three important residues in the distal face of the heme group of catalase: His-74, Asn-147, and Phe-160 (Fig. 3C) (*12*). The first two are included in the regions identified in the BLAST search (Fig 3B, bold), and the third (equivalent to Phe-160) is located 13 residues COOH-terminal to the conserved Asn-147. On the basis of this alignment, it appears that Leu-150 is the Phe-160 equivalent in the coral protein. The proximal heme ligand in catalase is Tyr-357, and Arg-353 has an important structural role (*12*); Fig. 3B shows a potential sequence alignment of catalase with the coral protein.

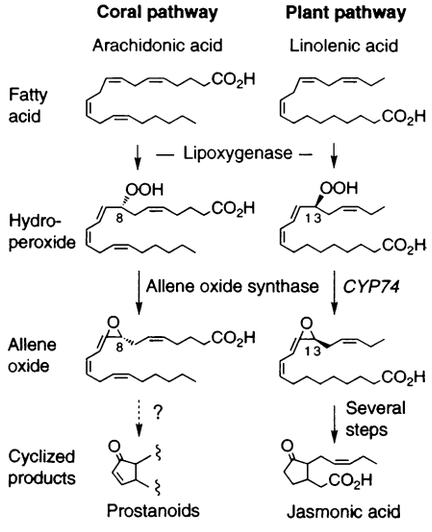

Fig. 1. Allene oxide biosynthesis and metabolism in corals and plants. In coral, the conversion of allene oxide to prostanoid-related products has not been demonstrated.

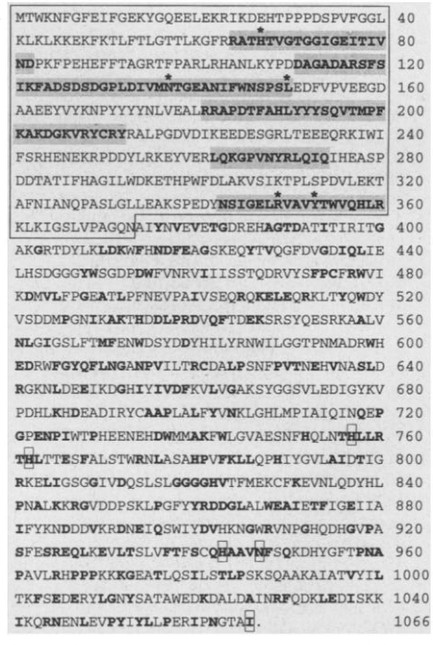

Fig. 2. Deduced amino acid sequence of the fusion protein. The boxed NH_2-terminal domain has several regions of homology with catalase (shaded in bold; see also Fig. 3). Amino acid residues aligning with essential residues of the catalase active site are indicated with an asterisk. Homology of the 8*R*-lipoxygenase of the coral fusion protein extends up to the initiating methionine of mammalian lipoxygenases, allowing delineation of the link between the two domains of the coral protein at amino acid positions 373 to 374. Residues identical to the human 5*S*-lipoxygenase are shown in bold, and the conserved residues implicated in binding iron are boxed. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The cDNA sequence has been deposited with GenBank (accession number AF003692).

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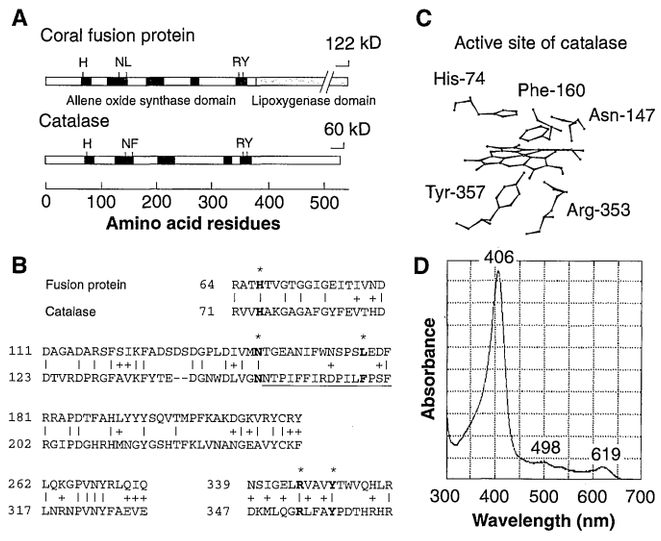

Fig. 3. Sequence alignments of the coral fusion protein NH_2-terminal domain with catalase. (**A**) Map of the sequences (black bars) and the amino acids aligning with critical residues in the heme environment of catalase [H, N, F/L, R, and Y, each indicated with an asterisk in (B)]. (**B**) The first four sequences were reported in BLAST searches, except for the underlined extension of the second sequence. The last alignment, in the region of the tyrosine ligand, was made by inspection. Matches and similarities (+) are indicated according to the rules of the BLAST program. (**C**) Active site of bovine catalase (*12*), showing the heme and conserved residues. (**D**) UV-visible spectrum of the expressed NH_2-terminal domain after purification to electrophoretic homogeneity (*13*).

The hemoprotein nature of the coral NH_2-terminal domain is confirmed by the ultraviolet (UV)-visible spectrum of the recombinant protein (Fig. 3D) (*13*). Additional support for the catalase homology of the NH_2-terminal domain comes from its catalytic activity: Whereas catalase is a peroxidase for hydrogen peroxide, the coral NH_2-terminal domain catalyzes a peroxidase-related reaction on a fatty acid hydroperoxide.

To investigate the catalytic activities of the fusion protein and its separate domains, we overexpressed them in bacteria (Fig. 4) (*13*, *14*). We incubated bacterial pellets expressing the fusion protein with [^{14}C]arachidonic acid and analyzed the products by reversed-phase high-pressure liquid chromatography (HPLC) (Fig. 4A). The bacterial extracts converted arachidonic acid completely to an allene oxide, an unstable epoxide that was recovered from the incubations as its α-ketol hydrolysis product (*4*). This product was identified by comparison to an authentic standard by HPLC, UV spectroscopy, and gas chromatography–mass spectrometry. The formation of an allene oxide depends on the initial production of hydroperoxide by the lipoxygenase activity of the fusion protein. The intermediate lipoxygenase product could be trapped as the hydroxy derivative (8*R*-HETE) by coincubation with a mild reducing agent, $SnCl_2$ (Fig. 5B). This result indicates that the initial oxygenation of arachidonic acid involves the actions of an 8*R*-lipoxygenase, and that a peroxidase activity then converts the hydroperoxide to the allene oxide. The ability to trap the hydroperoxide intermediate, together with the fact that added 8*R*-HPETE is converted to allene oxide, suggest that the two catalytic activities of the protein are not tightly coupled. Confirmation that the coral enzyme is a functional fusion protein of separate catalytic domains was derived from expression of the individual NH_2- and COOH-terminal domains (*13*, *14*). In this way we identified the catalase-like NH_2-terminus as an allene oxide synthase and the COOH-terminal domain as an 8*R*-lipoxygenase (Fig. 5, C and D).

Our results illustrate that a lipoxygenase can exist as a fusion protein with a polypeptide that catalyzes the next step of enzymatic transformation. Certain bacterial cyto-

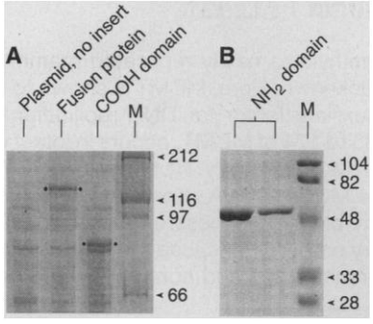

Fig. 4. Expression of the coral fusion protein and the separate domains in *E. coli*. (**A**) SDS–polyacrylamide gel electrophoresis (Coomassie stained) of bacterial extracts expressing the fusion protein and the COOH-terminal (lipoxygenase) domain; black dots indicate the overexpressed proteins at 122 and 79 kD, respectively. This analysis is from bacterial expression at 37°C. Expression at lower temperatures (room temperature and 15°C) resulted in reduced expression of protein, but higher catalytic activities (*22*). (**B**) Similar analysis of the expressed and purified NH_2-terminal domain (*13*); it migrates more slowly than predicted from its molecular mass (43 kD). This protein has high expression (1 to 2 μmol per liter of culture) and allene oxide synthase activity after bacterial expression at 28°C (*13*). M, molecular size markers (sizes shown on the right in kilodaltons).

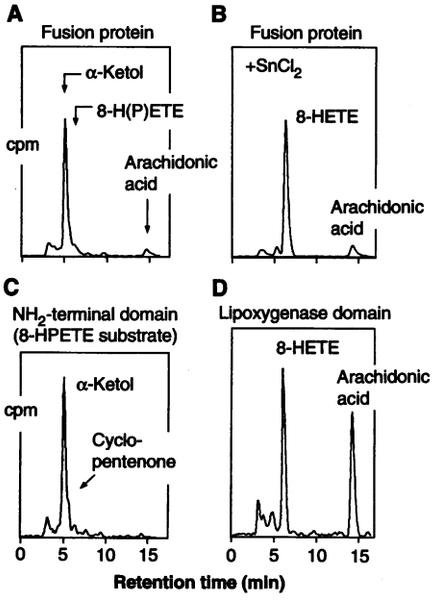

Fig. 5. Catalytic activities of the coral fusion protein and the separate domains. The full-length cDNA (Fig. 2) in the pET3a vector was expressed in *E. coli* (HMS 174) by infection with CE6 phage at an induction temperature of 15°C. Bacterial pellets were sonicated in tris-HCl (pH 8) [cells/buffer, 1:10 (v/v)] and incubated with [^{14}C]arachidonic acid (100 μM) for 30 min at room temperature. Fatty acids were extracted with ethyl acetate and analyzed by HPLC with a Beckman 5S ODS column (25 cm by 0.46 cm), a solvent system of methanol–water–acetic acid [90:10:0.01 (v/v)], and a flow rate of 1 ml/min with online detection of radioactivity. There was no activity in bacteria expressing vector alone. (**A**) Formation of the allene oxide, as evidenced by its α-ketol hydrolysis product, 8-hydroxy-9-ketoeicosa-5,11,14-trienoic acid. The cyclopentenone derivative of the allene oxide formed by nonenzymatic cyclization (*4*) was also detected on HPLC [it lies in the tail of the α-ketol peak in chromatogram (A)], providing an additional line of evidence for formation of the allene oxide. The retention times of standards are indicated with arrows. (**B**) Incubation of the cell sonicate and arachidonic acid in the presence of 0.5 mM $SnCl_2$ led to trapping of the intermediate product, 8*R*-HPETE, as its hydroxy derivative, 8*R*-HETE. (**C**) The purified NH_2-terminal domain (*13*) metabolized [^{14}C]8*R*-HPETE (50 μM) to allene oxide that was recovered from the incubation as its α-ketol and cyclopentenone derivatives. (**D**) The lipoxygenase domain (*14*) converted [^{14}C]arachidonic acid to 8*R*-HPETE (analyzed after reduction with triphenylphosphine to 8-HETE) and minor early eluting derivatives of 8*R*-HPETE.

chrome P450s are natural fusion proteins (*15*), as are the mammalian nitric oxide synthases (*16*). In these examples, a monooxygenase activity is supported by reducing equivalents supplied by a reductase in the other domain. There is no requirement for redox cofactors in either the lipoxygenase or allene oxide reactions.

The allene oxide synthase domain of the coral protein has no sequence similarity to plant allene oxide synthases, which are hemoproteins of the cytochrome P450 superfamily (CYP74A) (*6*). The coral allene oxide synthase is also a hemoprotein, although it is related to catalase rather than the cytochrome P450 proteins. There are other examples of structurally unrelated proteins that mimic the type of chemistry associated with cytochrome P450s, including the chloroperoxidase of the mold *Caldariomyces fumago* (*17*) and the mammalian nitric oxide synthases (*18*). Both enzymes can catalyze P450-like reactions, an activity that has not been associated with catalase itself, possibly because of restricted access of substrates to the catalase active site (*12*, *19*). This active site topology limits the metabolism of alkyl peroxides and leaves the smaller hydrogen peroxide as the only natural substrate.

As regards mechanism, allene oxide synthesis is related to the formation of a wide array of products from fatty acid hydroperoxides. The initial steps of transformation involve a common intermediate (*20*), and thus the different reactions can be catalyzed by related enzymes. The coral allene oxide synthase may therefore be adapted to other catalytic activities. For example, starfish oocytes contain, in addition to 8*R*-lipoxygenase and allene oxide synthase, a hydroperoxide lyase that forms an aldehyde with 8*R*-HPETE as substrate (*21*). The synthesis of allene oxides may be but one manifestation of the catalytic activity of this fusion of lipoxygenase and catalase-related domains.

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8. For the first round of PCR, we used cDNA from *P. homomalla* total RNA and the primers 5′-GGTTC-CAARTGGYTNATGGCNAA and 5′-CTATGTRTGI-ATRCTRTTIGGIAT (*7*). For the second round of PCR, we used the equivalent of 0.1 μl of the first-round PCR products as template with the same upstream primer and the nested downstream primer 5′-CCAGATCAGIAAICCRTCRTCICKRTA. The 405-bp product included sequences from the previously identified 8*R*-lipoxygenase (*7*) and the lipoxygenase described here.
9. The 5′ end of the sequence was cloned with the Marathon cDNA Amplification kit (Clontech) (*7*) and the 5′ RACE procedure (Gibco-BRL).
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13. The NH_2-terminal domain [amino acids 1 to 373 of the coding sequence, with or without a COOH-terminal $(His)_4$ tag] was expressed in the pET3a vector in *Escherichia coli* (BL21, Novagen) incubated at 28°C in TB medium for 24 hours. This resulted in appearance in the bacterial cytosolic fraction of a hemoprotein detectable by UV-visible spectroscopy (≈1 absorbance unit at 406 nm) that was not present in cells transfected with vector alone. The enzyme was purified by ammonium sulfate precipitation (30 to 55% fraction) and by anion exchange chromatography on Q-Sepharose, and on a nickel affinity column (Qiagen) for the His-tagged protein.
14. The lipoxygenase domain was expressed after deletion of the NH_2-terminus from the 5′ end to nucleotide 1116 of the ORF.
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Human DNA–(Cytosine-5) Methyltransferase–PCNA Complex as a Target for $p21^{WAF1}$

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DNA–(cytosine-5) methyltransferase (MCMT) methylates newly replicated mammalian DNA, but the factors regulating this activity are unknown. Here, MCMT is shown to bind proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA replication and repair. Binding of PCNA requires amino acids 163 to 174 of MCMT, occurs in intact cells at foci of newly replicated DNA, and does not alter MCMT activity. A peptide derived from the cell cycle regulator $p21^{WAF1}$ can disrupt the MCMT-PCNA interaction, which suggests that $p21^{WAF1}$ may regulate methylation by blocking access of MCMT to PCNA. MCMT and $p21^{WAF1}$ may be linked in a regulatory pathway, because the extents of their expression are inversely related in both SV40-transformed and nontransformed cells.

DNA methylation in mammals is involved in imprinting (*1*), regulation of transcription (*2*), and development (*3*). Various diseases, including cancer (*4*) and fragile X syndrome (*5*), are associated with abnormal DNA methylation, which indicates that one or more regulatory mechanisms must exist to ensure the maintenance of precise methylation patterns by MCMT in the mammalian genome.

To investigate whether PCNA, an auxiliary factor for DNA replication and repair, was involved in the regulation of MCMT activity, we first determined whether the two proteins interact in vitro. We incubated human acute lymphoblastic leukemia (CEM) cell extracts with immobilized glutathione-S-transferase (GST) fusion proteins containing fragments of MCMT (Fig. 1, A and B) and analyzed the bound cellular proteins on immunoblots. Results with both cellular and recombinant PCNA (rPCNA, Fig. 1C) indicated that MCMT binds to PCNA directly through amino acids 122 to 322. We refer to this region as hMPBD (human methylase-PCNA binding domain). Further deletion analysis (Fig. 2, A to C) revealed that hMPBD requires only the sequence TRQTTITSHFAKG (*6*). Comparative studies on vertebrate MPBDs, as well as point-mutation analyses (Fig. 2, D to F), indicated that Arg^{163}, Gln^{164}, Thr^{166}, Ile^{167},