An Ancient Relative of Cyclooxygenase in Cyanobacteria Is a Linoleate 10S-Dioxygenase That Works in Tandem with a Catalase-related Protein with Specific 10S-Hydroperoxide Lyase Activity*

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In the course of exploring the scope of catalase-related hemoprotein reactivity toward fatty acid hydroperoxides, we detected a novel candidate in the cyanobacterium Nostoc punctiforme PCC 73102. The immediate neighboring upstream gene, annotated as “cyclooxygenase-2,” appeared to be a potential fatty acid heme dioxygenase. We cloned both genes and expressed them in Escherichia coli, confirming their hemoprotein character. Oxygen electrode recordings demonstrated a rapid (>100 turnovers/s) reaction of the heme dioxygenase with oleic and linoleic acids. HPLC, including chiral column analysis, UV, and GC-MS of the oxygenated products, identified a novel 10S-dioxygenase activity. The catalase-related hemoprotein reacted rapidly and specifically with linoleate 10S-hydroperoxide (>2,500 turnovers/s) with a hydroperoxide lyase activity specific for the 10S-hydroperoxy enantiomer. The products were identified by NMR as (8S)-10-oxo-decenoic acid and the C8 fragments, 1-octen-3-ol and 2Z-octen-1-ol, in ~3:1 ratio. Chiral HPLC analysis established strict enzymatic control in formation of the 3R alcohol configuration (99% enantiomeric excess) and contrasted with racemic 1-octen-3-ol formed in reaction of linoleate 10S-hydroperoxide with hematin or ferrous ions. The Nostoc linoleate 10S-dioxygenase, the sequence of which contains the signature catalytic sequence of cyclooxygenase and fungal linoleate dioxygenases (YRWH), appears to be a heme dioxygenase ancestor. The novel activity of the lyase expands the known reactions of catalase-related proteins and functions in Nostoc in specific transformation of the 10S-hydroperoxylinoleate.

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This study was instigated as part of a project to characterize novel catalase-related genes that metabolize fatty acid hydroperoxides rather than hydrogen peroxide. In 1997, we described the prototypical catalase-related enzyme of this type as part of a biosynthetic pathway in the coral Plexaura homomalla (1). This catalase-related allene oxide synthase (cAOS) occurs as the N-terminal domain of a natural fusion protein with a lipoxygenase comprising the C-terminal domain (1, 2). The x-ray crystal structure of the allene oxide synthase domain establishes its close structural homology to true catalases (3). The P. homomalla lipoxygenase domain converts arachidonic acid to 8R-hydroperoxyeicosatetraenoic acid, and the cAOS domain transforms this to a fatty acid allene oxide, a highly unstable epoxide that can cyclize to form a cyclopentenone, a potential precursor of various prostanoid-related products of corals (4).

In a search for relatives of the P. homomalla allene oxide synthase that might exhibit novel catalytic activity, among the hits identified via a TBLASTN search (5) was a catalase-related protein (annotated as Npun_R5468) in the cyanobacterium Nostoc punctiforme PCC 73102. Despite a low overall sequence homology of ~20% to the P. homomalla cAOS, this “hypothetical protein” exhibited two characteristics that marked it as a potential fatty acid hydroperoxide-metabolizing catalase. The comparatively small size of the protein monomer (45 kDa; cf. 56 kDa for “small subunit” conventional catalases such as human catalase (6)) is in common with the known catalase-related proteins that metabolize fatty acid hydroperoxides (1, 7–9). More significantly, the amino acid immediately preceding the putative distal heme His-44 in Npun_R5468 is not the typical Val of...
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FIGURE 1. Segment of the gene map of *N. punctiforme* PCC 73102.

a catalase consensus sequence, the residue is Thr-43. A Thr–His combination occurs in CAOS, its close homologs, and all the catalase-related relatives exhibiting metabolism of a specific fatty acid hydroperoxide (1, 7, 9, 10). Mutation of the equivalent Thr-66 residue in *P. homomalla* CAOS to the typical Val of true catalases allows reaction of the protein with hydrogen peroxide (11). Computational studies on the Thr-66 to Val (T66V) mutation in *P. homomalla* CAOS have invoked a role for this Thr residue in binding H₂O₂ and preventing it from interacting with the heme iron (12).

Inspection of the *N. punctiforme* genes neighboring Npun_R5468 revealed that the immediate upstream gene, designated as Npun_R5469, is annotated as a heme peroxidase and "cyclooxygenase-2" (Fig. 1). As neighboring genes in microorganisms often function together, it appeared very possible that the heme peroxidase/cyclooxygenase-2 might metabolize a polyunsaturated fatty acid (linoleate and ω-linolenate being prominent polyunsaturated fatty acids in *Nostoc* (13, 14)), and the catalase-related protein might specifically metabolize the initial oxygenation product. With this concept in mind, we cloned and expressed the two *N. punctiforme* genes and characterized their catalytic activities. Their reactions mimic fatty acid hydroperoxide (1, 7, 9, 10). Mutation of the equivalent Thr-His residue in binding H₂O₂ and preventing it from interacting with the heme iron (12).

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**EXPERIMENTAL PROCEDURES**

**Preparation of Fatty Acid Hydroperoxides**—Fatty acids were purchased from NuChek Prep Inc. (Elysian, MN). Racemic 10-HPODE was prepared by reaction of linoleic acid (150 mg) with singlet oxygen using methylene blue as sensitizer (1 ml of a 0.6-mg/15-ml methanol solution) and a strong source of visible light at 0 °C overnight (16). After fractionation on an open-bed silica column to remove unconverted linoleic acid and the methylene blue, the HPODEs were separated by semi-preparative SP-HPLC using a Beckman 5-μm silica column (25 × 1 cm) and a solvent of hexane/isopropyl alcohol/glacial acetic acid (100:1:0.1 by volume). The 10-hydroperoxylinoleate enantiomers were resolved by chiral HPLC using a Chiralpak AD column (25 × 0.46 cm) and a solvent of hexane/methanol/ethanol/glacial acetic acid (100:4:1:0.1 by volume) at a flow rate of 1 ml/min; 0.5-mg aliquots of 10RS-HPODE were injected giving complete resolution of the peaks of 10S (at ~12.5 min) and 10R (~14.0 min). 9-Hydroperoxy- and 10-hydroperoxyoleic acids were prepared from oleic acid by singlet oxygen as described above. The 9RS and 10RS enantiomers were resolved in a single run using a Chiralpak AD column as above and eluting in the order 10S (13.5 min), first 9 enantiomer (not designated, 18.5 min), and 10R (26 min), second 9 enantiomer (48 min). Standards of racemic conjugated diene-containing HPODEs were prepared by vitamin E-controlled autoxidation (17) and the chiral HPODEs using potato (9S) (18), *Anabaena* (9R) (19), or soybean (13S) lipoygenases (20).

**Bacterial Strains, Growth Condition, and Materials**—*Escherichia coli* strains were grown at 37/20 °C in Luria Bertani (LB)/Terrific Broth (TB) media supplemented with the appropriate amount of antibiotics (ampicillin 100 μg ml⁻¹), pCR2.1 vector (Promega) and pET-17b (+) (Novagen) were used for the cloning of PCR products and for the expression of the gene, respectively. *E. coli*-DH5α (Invitrogen) was used as a host cell for the preparation of recombinant plasmid and manipulation of DNA, whereas *E. coli* BL21 (DE3) (Novagen) was used as the host for the expression.

**Cloning, Expression, and Purification of the Full-length Proteins of N. punctiforme PCC 73102**—The putative heme dioxygenase (Npun_R5469, cyclooxygenase-2, 542 amino acids, 63 kDa) annotated as heme peroxidase and the immediate downstream gene, a putative catalase-related protein (Npun_R5468, 393 amino acids, 45 kDa) annotated as a hypothetical protein, were each amplified from the genomic DNA of *N. punctiforme* PCC 73102 (obtained from ATCC) and cloned by PCR. Forward and reverse primers in PCR contained NdeI and XhoI restriction sites, with an N- and C-terminal His6 tag, respectively. The sequence of the forward primer of the heme dioxygenase was 5’-TCCATATGCATCACCACATCACCATACGCTGGGAAAAGAGATACATCG-3’ and that of the reverse primer was 5’-TGGTACGAGCTTTCGAAC-3’. The sequence of the forward primer of minicatalase was 5’-TCCATATGAGCTTTTGGCTTATAGG-3’ and that of the reverse primer was 5’-GACTCGATTCCACATGCACTGACATGAACAGTGTCCCTGCT-TTTTGAC-3’. The correct PCR products were subsequently cut with NdeI and XhoI restriction enzymes and inserted into the same sites of the expression vector pET17b. DNA sequencing confirmed the identity to the published sequence in GenBank (accession numbers YP_001868719 and YP_001868718 respectively). Expression of both genes with N- and C-terminal His6 tags in *E. coli* BL21 (DE3) cells (Novagen) was accomplished with methods described previously (2). A single colony was grown in 1 ml of LB + 400
μg/ml ampicillin for 4 h at 37 °C with shaking at 250 rpm. In a 50-ml conical tube, 10 ml of Terrific Broth was added containing 400 μg/ml ampicillin and 200 μl of the 1-ml culture. This was incubated at 37 °C for 3 h and shaking at 250 rpm. Into a 500-ml flask was added 40 ml of Terrific Broth and the 3-h cultures, and only to the flask containing the Np-cat was added isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. The cultures of both proteins were incubated at 21 °C for 43 h with shaking at 250 rpm. Purification of the Histagged proteins followed the protocol of Imai et al. (21) and Boutaud and Brash (2) with some modifications (22).

The frozen pellet from a 50-ml bacterial culture was resuspended in 10 ml of BugBuster® protein extraction reagent (Novagen) by sonication and homogenized using a glass Dounce homogenizer (Wheaton). The 16,000 × g supernatant was loaded on a nickel-nitrioltriacetic acid column (0.5-ml bed volume, Qiagen) equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole. The His tag proteins were then eluted with 50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole. The Histagged proteins followed the protocol of Imai et al. (21) and Boutaud and Brash (2) with some modifications (22).

Incubation, Extraction, and HPLC Product Analysis—Small scale incubations were performed at room temperature in a 1-ml quartz cuvette in 1 ml of 50 mM Tris, pH 7.5, 150 mM NaCl using the fatty acids C18:1, C18:2, C18:3, C18:4, or C20:4(10–30 μg) or fatty acid 10S-hydroperoxides added in 5 μl of ethanol, and the reaction was monitored by repetitive scanning from 350 to 200 nm using a Lambda-35 spectrophotometer (PerkinElmer Life Sciences). Larger scale incubations were conducted in 50 ml of oxygenated buffer with an appropriate amount of N. punctiforme enzyme and 10 mg of fatty acid substrate. Reaction was complete within 5 min, whereupon the samples were acidified to pH 6 with 1 N HCl and extracted with methanol using a 1-ml Oasis HLB cartridge (Waters) or 1 g of C18 Bond-Elut for the 50-ml incubations. Volatile reaction products from 10-HPODE were extracted with pentane as described below under chiral analysis of 1-octen-3-ol.

Products were analyzed initially by RP-HPLC using a C18 Waters Symmetry 5-μm column (0.46 × 25 cm) at a flow rate of 1 ml/min with methanol/water/acetic acid (90:10:0.01, by volume). Peaks were monitored using an Agilent 1100 diode array detector. For larger scale samples, a semi-preparative 5-μm Beckman ODS column (10 mm × 25 cm) was used for the separation of incubation products; the major peaks were collected and taken to dryness under a stream of nitrogen and stored in methanol at −20 °C. The major products collected from RP-HPLC were further purified as the methyl esters by SP-HPLC using a Beckman 5-μm Ultrasphere silica column (25 × 0.46 cm or 25 × 1 cm for milligram quantities of product) with a solvent system of hexane/isopropyl alcohol (100:1.5 v/v). Chiral analysis of 10-HODE methyl ester used a Chiralpak AD column (25 × 0.46 cm) and a solvent of hexane/methanol (100:2 by volume).

Chiral Analysis of 1-Octen-3-ol—Analysis of 1-octen-3-ol from Np-cat, hematin, or FeSO₄ incubations or mushroom homogenates was initiated by mild alkalization of the aqueous sample (pH 9, to minimize extraction of fatty acids) and extraction with pentane. The pentane extract was chromatographed on SP-HPLC using a Beckman 5-μm silica column (25 × 0.46 cm) and a solvent of hexane/isopropyl alcohol (100:1 by volume) run at 1 ml/min as follows: 1-octen-3-ol eluted at ~8 min and 2-octen-1-ol at 16 min, both weakly detectable at the low microgram level at 205 nm. The peaks were collected and derivatized to the benzyol ester by treatment with 1 μl of benzyol chloride, 1 μl of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU), and ~1 mg of dimethylaminopyridine in 50 μl of acetonitrile overnight at room temperature. Subsequently, the ester derivative was extracted with pentane, which was washed with copper sulfate solution (to remove the DBU and dimethylaminopyridine), brine, and water and then to dryness. Purification (prior to NMR) was achieved by RP-HPLC using a Waters Symmetry C18 column and a solvent of methanol/water (90:10 by volume). Chiral analysis was performed on the 1-octen-3-ol benzyol ester using a Chiralcel OD-H column (25 × 0.46 cm) eluted with hexane/isopropyl alcohol (100:0.04 v/v) at a flow rate of 1 ml/min, with UV detection at 205, 220, 235, and 270 nm. Racemic 1-octen-3-ol was purchased from Sigma.

Derivatization and GC-MS Analysis—Methyl esters were prepared using ethereal diazomethane in methanol. Catalytic hydrogenations were performed in 100 μl of ethanol using about 1 mg of palladium on carbon and bubbling with hydrogen for 2 min at room temperature. The hydrogenated products were recovered by the addition of water and extraction with ethyl acetate. Trimethylsilyl ether derivatives were prepared using bis(trimethylsilyl)-trifluoroacetamide (10 μl) at room temperature for 2 h or overnight. Subsequently, the reagents were evaporated under a stream of nitrogen, and the samples were dissolved in hexane for GC-MS. Analysis of the methyl ester trimethylsilyl derivatives of the products were carried out in the positive ion electron impact mode (70 eV) using a ThermoFinnigan DSQ mass spectrometer. The initial temperature was set for 100 °C, held for 2 min, and then increased to 280 °C at a 10 °C/min increment and held at 280 °C for 3 min.

NMR Analysis—1H NMR and 1H,1H COSY NMR spectra were recorded on a Bruker DXR 500- or 600-MHz spectrometer at 298 K. Analysis of the benzyol ester of octenol was conducted at 298 K in d6-benzene. The ppm values were calibrated using residual nondeuterated solvent (δ = 7.26 ppm for CHCl₃ and 7.16 ppm for C₆H₆).

RESULTS

Sequence Homologies—The heme peroxidase, Npun_R5469, designated herein as Np-diox, shows a meaningful alignment with several COX-2 sequences (Fig. 2), while only sharing about 27% sequence identity. Compared with COX-2, the Nostoc sequence (predicting a 63-kDa protein) is missing about 40 of the first 100 amino acids near the N terminus; these represent an EGF domain and membrane-binding helices in the animal
proteins. Although a few of the well studied amino acids in COX-2 are not conserved in the Nostoc protein (e.g. Arg-120, Val-349, and Ser-530), the critical amino acids on the distal and proximal heme and the catalytic Tyr in the fatty acid binding channel are all present, with the caveat that the distal heme His is represented as Asp-145. A model of Npun_R5469 constructed with the Swiss-Model program shows a striking resemblance to COX-2 (Fig. 3).

Although the hypothetical protein, Npun_R5468, shows low overall sequence identity with catalases, it has several regions of homology in the correct sequential order in the protein. These include structurally important residues representing the distal heme His residue in the context RDTHSK (cf. catalase consensus RVVHAK), tentative assignment of the distal heme Asn residue as Asn-147, and the signature sequence near the C terminus representing the proximal heme Tyr ligand RSIVYPIVANDR (catalase consensus: R(three hydrophobic residues)Y(any six residues)R).

**Cloning and Expression of the Two Genes**—The PCR-cloned cDNAs, each with an added His_6 tag, were transferred into a pET17b vector and expressed individually in E. coli. Partial purification was achieved by nickel affinity chromatography (Fig. 4). The UV-visible spectra of the individual proteins were characteristic of ferric hemoproteins with the main Soret band λ_max at 410 nm (Np-diox) and 406 nm (Np-cat). Comparison of the main Soret signal to the 280 nm absorbance indicated ~30–50% incorporation of heme in the holoenzymes.

**Oxygen Uptake Recordings of Np-diox Reaction with Unsaturated Fatty Acids**—Using three potential substrates that occur in Nostoc (13, 14), studies of oxygen uptake showed similar rates of reaction of Np-diox with oleic and linoleic acids (reaching 100 turnovers/s) and a lower maximal rate with α-linolenic acid (Fig. 5A and Table 1). The k_cat/K_m values (1.6 × 10^6 s^-1 M^-1 for oleate, 2.1 × 10^6 s^-1 M^-1 for linoleate, and 2.4 × 10^6 s^-1 M^-1 for α-linolenate) tend to favor α-linolenic acid as the most efficient substrate. This, however, does not take into account an additional factor, namely that fatty acid oxygenation results in the suicide inactivation of Np-diox. Illustrated in Fig. 5B are a series of oxygen uptake recordings with increasing concentrations of each of the three fatty acids, all using identical aliquots of Np-diox. It can be seen that oleic and linoleic acids attain a similar magnitude of oxygen consumption over the range of fatty acid concentrations tested, whereas α-linolenic acid gives a much lower final oxygen consumption due to more rapid enzyme inactivation. Overall, our conclusion is that Np-diox is best described as an oleate or linoleate dioxygenase, albeit with α-linolenic acid very competitive at low substrate concentrations.
Three additional fatty acids were evaluated as Np-diox substrates. The C18:3/H9253 6 isomer -linolenic acid is not oxygenated and does not inactivate Np-diox. The C20:2/H9253 6 analog of linoleate, 11Z,14Z-eicosadienoic acid, is oxygenated similarly to linoleate, at about a third the rate of reaction. Arachidonic acid elicits a slow uptake of oxygen (at ~5% of the rate with oleic and linoleic acids), achieving only about 15% of the extent of O2 uptake and resulting in inactivation of Np-diox. The best substrates are C18 fatty acids with the first double bond at C9 (Fig. 5).

**Identification of Np-diox Products—**RP-HPLC analysis of a reaction of [14C]linoleic acid with the heme peroxidase showed production of a single more polar peak (Fig. 6); the UV spectrum of this product showed only end absorbance, indicating no conjugated double bonds. Corresponding products were seen with oleic and a-linolenic acids. The linoleic acid product was reduced with triphenylphosphine, hydrogenated, and converted to the methyl ester trimethylsilyl ether derivative. On GC-MS analysis, the electron ionization mass spectrum showed structural diagnostic ions at m/z 371 (2% relative abundance, M-15), 355 (3%, M-31), 339 (10%, M-47), 273 (91%, C1-C10), 244 (15%), 215 (base peak, C10-C18), 169 (37%), 159 (13%), 129 (19%), 103 (13%), 82 (23%), and 73 (70%), indicative of a C18:2 hydroxy fatty acid. The prominent a-cleavage ions at m/z 273 and 215 unambiguously place the hydroxyl group at C-10. A similar analysis of the same derivative of the nonhydrogenated product gave a spectrum dominated by the a-cleavage ion at m/z 271 (base peak), with weak ions at m/z 367 (M-15, 0.5% abundance) and 335 (M-47, 1%), indicating a double bond on either side of C10, and a predicted structure of the enzymatic product of 10-hydroperoxyoctadec-8,12-dienoic acid (10-HPODE).

A method for steric analysis of the C-10 hydroxyl configuration was developed, using 10-HODE purified from a singlet oxygen reaction with linoleate as a racemic standard (16, 23) and 10S-HODE from an incubation of linoleate with mushroom homogenate as the standard of known chirality (24, 25). The results using a Chiralpak AD column for resolution showed that the Nostoc heme peroxidase formed almost exclusively the linoleate 10S enantiomer (99.5% enantiomeric excess) (Fig. 7).

Oleic and a-linolenic acids were also efficiently converted to their respective 10-hydroperoxides, identified by HPLC, UV, and GC-MS exactly as described for 10-HPODE. [1-14C]Oleate and [1-14C]a-linolenate were each converted by Np-diox to a single more polar radiolabeled product, detected on RP-HPLC with absorbance at 205 nm. GC-MS of the hydrogenated products as the methyl ester trimethylsilyl ether derivative each gave an identical mass spectrum to the 10-hydroxyoctadecanoate obtained from linoleate and described above. Analysis of the minor amounts of product formed from arachidonic acid using both conventional and chiral HPLC systems indicated the formation of multiple racemic hydroperoxyeicosatetraenoic acid isomers and a more prominent peak of 12S-hydroperoxyeicosatetraenoic acid with a 30% enantiomeric excess of 12S (data not shown).

**Reactions of Np-cat with Fatty Acid Hydroperoxides—**The catalase-related protein Np-cat at 2 nM concentration reacted rapidly with 10S-HPODE, the product of the heme peroxidase, to form a product with strong UV absorbance characteristic of an enone chromophore with \( \lambda_{\text{max}} \) at 227 nm in the pH 7.5 Tris buffer (Fig. 8A). The initial rate was too fast to record accurately with a conventional spectrophotometer (Fig. 8B), but a few s after addition of substrate the rate corresponded to ~2,500
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FIGURE 5. Kinetic analyses of Np-diox with fatty acid substrates. A, rates of reaction versus fatty acid concentration with oleic acid (●), linoleic acid (○), and α-linolenic acid (□). Error bars represent the S.E. from three independent experiments. B, representative series of recordings of O₂ uptake upon addition of the fatty acid concentrations indicated on the top line. All reactions were initiated by addition of 75 nm Np-diox. Oleic and linoleic acids give similar extents of O₂ uptake before inactivation of Np-diox halts further reaction. α-Linolenic acid oxygenation leads to a more rapid enzyme inactivation and lower extent of O₂ uptake. C, substrate specificity of Np-diox.

TABLE 1
Kinetic parameters for Np-diox with C18 fatty acid substrates

| Substrate     | \( k_{cat} \) (μM) ± S.E. | \( k_{cat}/K_m \) (s⁻¹) ± S.E. | \( k_{cat}/K_m \) (s⁻¹·M⁻¹⁻) |
|---------------|--------------------------|-----------------------------|-----------------------------|
| Oleic acid    | 113 ± 15                 | 178 ± 13                    | 1.6 \( \times \) \( 10^6 \) |
| Linoleic acid | 63 ± 9                   | 135 ± 8                     | 2.1 \( \times \) \( 10^6 \) |
| α-Linolenic   | 13 ± 2                   | 31 ± 1                      | 2.4 \( \times \) \( 10^6 \) |

FIGURE 6. RP-HPLC analysis of the metabolism of [14C]linoleic acid by Np-diox. The sample was chromatographed on a Waters Symmetry C18 column (25 × 0.46 cm) using a solvent of methanol/water/glacial acetic acid (85:15:0.01 by volume) with a flow rate of 1 ml/min with on-line detection of radioactivity using a Radiomatic Flo-One detector (and also UV detection at 205 nm; data not shown). The UV profiles at 205 nm are illustrated. The Np-diox product. 10-HODE methyl ester from the Np-diox reaction with linoleic acid.

Chiral HPLC analysis of the Np-diox product 10-HODE. A, 10-HODE methyl ester from the Np-diox reaction with linoleic acid, B, racemic 10-HODE methyl ester; the 10S/10R designation was made using 10S-HODE from mushroom (data not shown). C, co-injection of racemic 10-HODE and the Np-diox product. 10R5-HODE methyl ester was resolved using a Chiralpak AD column (25 × 0.46 cm) with a solvent of hexane/methanol (90:10 by volume) at a flow rate of 1 ml/min. The UV profiles at 205 nm are illustrated. AU, absorbance units.

turnovers per s. The UV absorbing product was extracted, purified, and identified by GC-MS and 1H NMR. GC-MS of the trimethylsilyl ester methoxime derivative gave a pair of GC peaks (syn- and anti-oxime isomers) each with a prominent molecular ion at \( m/z \) 285 (25 and 18% relative abundance); the mass spectrum of the first eluting isomer included \( m/z \) 254 (M−31, 26%) and 239 (M−47, 98%) with no other ions above 2% abundance until \( m/z \) 164, 147, 136, and 129 and a base peak at \( m/z \) 112 (ions not recorded below \( m/z \) 100). The molecular weight is compatible with the trimethylsilyl ester methoxime derivative of a C10 fatty acid containing one double bond and a ketone or aldehyde and is in accord with the published mass spectrum of the same derivative of 10-oxo-8E-decenoic acid (26). The 1H NMR spectrum of the free acid confirmed the C10 aldehyde in conjugation with the 8,9-trans double bond as follows: signals in CDCl₃ were \( \delta \) 9.50 ppm 1H d, \( J_{9,10} \) = 7.9 Hz, H10; 6.84 1H dt, \( J_{7,8} \) = 6.8 Hz, H8; 6.12 1H dd, \( J_{9,10} \) = 7.9, \( J_{8,9} \) 15.7 Hz. The doublet at 9.5 ppm (H10) identifies the aldehyde functionality and its coupling to the trans double bond.
bond \(J_{8,9} = 15.7\) Hz, defining the product as the 10-oxo-8E-decenoic acid.

Cleavage of 10S-HPODE at C10–11 will also produce a C8 fragment (expected as octenol (23, 27)). Pentane extraction of a reaction of Np-cat with 100 \(\mu\)g of 10S-HPODE recovered this potentially volatile C8 fragment. After derivatization to the benzyl ester to decrease volatility and improve the detectability by HPLC-UV, two C8 alcohol isomers were separated by RP-HPLC (7.8- and 9.2-min peaks in \(\sim 2–3:1\) ratio using a 25 \(\times\) 0.46-cm C18 Waters Symmetry column and 90:10 (v/v) MeOH/water solvent run at 1 ml/min). The peaks were identified by \(^1\)H NMR (with two-dimensional COSY) as the more prominent 1-octen-3-ol and 22-octen-1-ol, respectively. 1-Octen-3-ol benzyl ester in \(CDCl_3\): 8.195 2H d, 7.10 1H t, 7.04 2H t (aromatic signals); 5.64–5.73 2H m, H2 and H3; 4.87 2H d, H1; 2.155 2H q, H4; 1.395 2H p, H5; 1.31 4H m, H6, H7; 0.885 3H t, H8. (2Z) 2-octen-1-ol benzyl ester in \(CDCl_3\): 8.05 2H d, 7.55 1H t, 7.435 2H t (aromatic signals); 5.64–5.73 2H m, H2 and H3; 4.87 2H d, H1; 2.155 2H q, H4; 1.395 2H p, H5; 1.31 4H m, H6, H7; 0.885 3H t, H8. Decoupling of the 4.87 doublet collapsed H2 centered at 5.662 ppm to a doublet \(J_{2,3} = 11\) Hz, the latter coupling constant established the cis configuration of the 2,3 double bond.

A method for chiral analysis of the 1-octen-3-ol was developed with the benzyl ester chromatographed on a Chiralcel OD-H column (the Chiralpak AD column giving poor resolution). This established the \(R\) configuration of the 3-alcohol (Fig. 9, A and B), identical to the product extracted from mushroom (Fig. 9, C and D). The tandem reactions of Np-diox and Np-cat can thus be summarized as in Scheme 1.

We questioned whether the \(R\) chirality of the 1-octen-3-ol is inherent to the use of a chiral 10S-hydroperoxide as substrate, or is the enzyme controlling both the cleavage and “oxygen-rebound” that produces the C8 alcohols? To examine this issue, which to the best of our knowledge has not been addressed previously, we reacted 10S-HPODE with free heme and also with free ferrous iron (cf. Ref. 28) and recovered the C8 fragment by pentane extraction and derivatized as before. The results (Fig. 9, E and F) clearly show that the 1-octen-3-ol from 10S-HPODE is racemic, thus implicating the Np-cat and the as yet uncharacterized enzyme in mushroom as controlling both the carbon chain cleavage and transfer of oxygen to the C8 fragment.

**Reaction of Np-cat with Other Fatty Acid Hydroperoxides**—The reaction rates of 10S-HPODE and 10R-HPODE were compared by monitoring the appearance of the UV enone chromophore of 10-oxo-8E-decenoic acid at 230 nm (Fig. 8B). For the enantiomeric 10R-HPODE (20 \(\mu\)g/ml reacted with 2 \(\mu\)M Np-cat), the rate of increase in absorbance at 230 nm was only 3% of the rate with the same concentration of 10S-HPODE. Similarly, the 10S-hydroperoxide of oleic acid (10S-HPOME) reacted at \(\sim 3\)% the rate of 10S-HPODE, the linoleate analog. Nonetheless, analysis of the reactions of 10R-HPODE and 10S-HPODE using higher concentrations of Np-cat showed complete transformation to a mixture of the cleavage products 10-oxo-decenoic acid (30–40%) and the corresponding 10-ke- to-3,10-diecadienoic acid (60–70%) respectively. By comparison, the preferred substrate 10S-HPODE is converted almost exclusively to the aldehyde 10-oxo-decenoic acid (>97% alde- hyde, <3% ketone).

Four oleate hydroperoxides (10S, 10R, 9S, and 9R) were compared in reaction with each other. The 10S-HPOME reacted abruptly with Np-cat with inactivation of the enzyme within 1–2 min (similar in character to 10S-HPODE), whereas the other three reacted very slowly over the course of 10 min. All four gave a prominent keto-octadecenoic acid with similar amounts of 10-oxo aldehyde acid from the 10S- and 10R-HPOME and only traces of a 1-oxy-2-decenoic acid fragment from the 9R- and 9S-HPOME.

Other fatty acid hydroperoxides tested were not significantly metabolized by the Np-cat, as judged by a scanning in the UV, 200–350 nm. Whereas 10RS-HPODE (20 \(\mu\)g/ml) showed rapid appearance of the obvious enone chromophore at 227 nm, 12RS- HPODE showed no reaction. Various fatty acid hydroperoxides containing a conjugated diene chromophore (9RS-HPODE, 13RS- HPODE, and 9RS-HPOME) were not significantly metabolized by the Np-cat as judged by scanning in the UV, 200–350 nm; they showed no changes other than a slight diminution in the peak absorbance at \(\sim 235\) nm.
DISCUSSION

Np-diox is identified here as an oleate and linoleate heme 10S-dioxygenase. α-Linolenate is also efficiently metabolized at lower concentrations, but it much more rapidly leads to enzyme inactivation (illustrated in Fig. 5B). The immediate downstream gene, Np-cat, is a specific linoleate 10S-hydroperoxide lyase (10S-hydroperoxyoleate being metabolized at only 3% of the rate). The two genes are representative of some highly homologous genes in cyanobacteria, illustrated for Np-diox in Fig. 10, and show the 89% sequence identity of an annotated heme peroxidase in *Cylindrospermum stagnale*. A linoleate 10S-dioxygenase is described in the 42A2 strain of *Pseudomonas aeruginosa*, and the 10S-hydroperoxide is isomerized to a 7,10-dihydroxylinoleate, but genomic sequence is not yet available, and the gene(s) are unidentified (29). Although in catalytic activity Np-diox is related to the plant and fungal linoleate heme dioxygenases (of which 2R-dioxygenase (PIOX, pathogen-inducible oxygenase) (30), 8R-dioxygenase, and 10R-di-

**FIGURE 9. Chiral HPLC analysis of 1-octen-3-ol as the benzoate ester.** 1-Octen-3-ol was analyzed as the benzoate ester using a Chiralcel OD-H column (25 × 0.46 cm) and a solvent of hexane/isopropyl alcohol (100:0.04 by volume) run at 1 ml/min with on-line UV detection at 220 nm. A, 1-octen-3-ol from reaction of 10S-HPODE with Np-cat. B, Np-cat product mixed with racemic 1-octen-3-ol. C, 1-octen-3-ol from a mushroom homogenate. D, mushroom product mixed with racemic 1-octen-3-ol. E, 1-octen-3-ol from a reaction of 10S-HPODE with ferrous sulfate (FeSO₄). F, 1-octen-3-ol from a reaction of 10S-HPODE with hematin. Slight differences in retention times between runs is due to slow equilibration of the column with the low % isopropyl alcohol in the solvent and analyses on different days; in each case assignment was checked by co-chromatography with the racemic standard.
oxygenases are characterized (31), the sequence similarity to these enzymes is quite divergent, only ~15% identity (Fig. 10). The linoleate 10S-dioxygenase/10S-hydroperoxide lyase activity is identical to the metabolism of linoleic acid described in the 1980s in mushroom (23, 24, 32), although not as yet attributed to known protein sequence(s). There are putative heme dioxygenase/cytochrome P450 fusion proteins in two sequenced mushroom genomes (the proteins are related to the characterized fungal linoleate heme dioxygenase/P450 fusion proteins), yet their percent identity to Np-diox is also far distant (11–16%, Fig. 10). In fact Np-diox is more similar to the mammalian cyclooxygenases (27–28% identity, Fig. 10), as the database annotation suggests and as a modeling structure supports (Fig. 3).

The Np-cat lyase reaction is distinct from the CYP74 plant hydroperoxide lyases that transform conjugated diene-containing fatty acid hydroperoxides (33). The plant P450 lyases convert the fatty acid hydroperoxide to a hemiacetal that then spontaneously fragments into two aldehydes (34). The Np-cat reaction occurs on the other side of the carbon bearing the hydroperoxide and more closely resembles the uncharacterized hydroperoxide lyases of starfish (35) and diatoms (36). The products are one aldehyde and an alkyl alcohol. This type of lyase transformation is also a well known anaerobic reaction of lipoxigenases (albeit associated with the formation of many other products) (37). Aerobically, there is at least one well documented example of a specific lipoxigenase-catalyzed lyase transformation of this type (38).

Superficially, the C10/C8 cleavage by Np-cat mimics the well known nonenzymatic reactions of heme or ferrous iron with linoleate 10-hydroperoxide (28). One of the differences is that free ferrous or hematin form mixtures of the ketone and the cleavage products. In our experiments, this was evident only in the weaker reactions of Np-cat with the slowly reacting 10R-HPODE and the 10S- and 10R-HPOME (oleate hydroperoxides). Free ferrous ion or hematin would also be expected to exhibit no preference for a 10R- or 10S-hydroperoxide enantiomer, whereas Np-cat reacts ~30-fold faster with the 10S-HPODE (Fig. 8B). A more open issue in our eyes was the possibility that there might be an inherent chirality of product formation in the nonenzymatic cleavage reaction with a chiral 10-hydroperoxide. We tested this by analysis of the 1-octen-3-ol formed from 10S-HPODE with ferrous ion and hematin. Both FeSO4 and hematin produced racemic 1-octen-3-ol (Fig. 9, E and F), thus providing strong support for direct and highly

**FIGURE 10.** Phylogenetic tree of representative protein relatives of Np-diox. The numbers represent percent identity to Np-diox. The top four proteins are annotated mushroom heme peroxidase/cytochrome P450 fusion proteins (11–15%), not as yet characterized. The *Aspergillus fumigatus* and *Gaeumannomyces graminis* sequences (10–15%) are representative of the known linoleate dioxygenase/P450 fusion proteins. *C. stagnale* (88%) is representative of cyanobacteria that also have a close relative of Np-cat as the immediate downstream gene. *Methylobacterium sp.* 4-46 (44%) is a proteobacterium that exhibits the heme dioxygenase signature YRWH in the heme peroxidase (although it has no close Np-cat relative). The mammalian cyclooxygenases are represented here by human COX-1 and COX-2 (27–28%), PIOX (10%) is the plant linoleate \( \alpha \)-dioxygenase, which has a relative in the cyanobacterium *Acaryochloris marina* (13%).

**SCHEME 1.** Transformations of linoleic acid by Np-diox and Np-cat.
controlled involvement of Np-cat in the hydroperoxide cleavage.

Cyanobacterial metabolites are the subject of study due to their potential involvement in water quality issues (water odors and algal blooms), and polyunsaturated fatty acids are known as one of the sources. Several fatty acid-derived aldehydes contributing to malodor have been identified (39), but 10-oxodecenoic acid and the alcohol 1-octen-3-ol are not among the recognized products, possibly because homologs of Np-diox/Np-cat are only present in a subset of the cyanobacterial genomes currently sequenced. *N. punctiforme* is a nitrogen-fixing cyanobacterium that can occur in symbiotic associations with fungi to form lichens and with many plants (40). 1-Octen-3-ol is best known as a product of the fruiting body of many fungi (its common name is “mushroom alcohol”), and it is attributed with various biological activities, including stimulating the conidiation of fungi (41, 42) and as a chemical defense (43), so this might be the basis of a connection in the symbiosis.

There is substantial evidence for the concept of gene transfer from cyanobacteria to animals (44–46), although we are not aware of a relevant study regarding heme peroxidase/dioxygenase. As Ni *et al.* (46) conclude, “Most identified algal genes (identified by the authors as potential transfers from alga/cyanobacteria to *Ciona intestinalis*) are related to molecule transport and signaling, suggesting their important role in intercellular communication and possibly the origin of multicellularity in animals.” The fatty acid heme dioxygenases and cyclooxygenases certainly fit into the categories of signaling and intercellular communication. On the basis of percent identities of the proteins, an evolutionary origin of the animal cyclooxygenases from relatives of the cyanobacterial Np-diox appears a realistic possibility.

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