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PpoC from *Aspergillus nidulans* is a fusion protein with only one active haem

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In *Aspergillus nidulans* Ppos [psi (precocious sexual inducer)-producing oxygenases] are required for the production of so-called psi factors, compounds that control the balance between the sexual and asexual life cycle of the fungus. The genome of *A. nidulans* harbours three different *ppo* genes: *ppoA*, *ppoB* and *ppoC*. For all three enzymes two different haem-containing domains are predicted: a fatty acid haem peroxidase/dioxygenase domain in the N-terminal region and a P450 haem-thiolate domain in the C-terminal region. Whereas PpoA was shown to use both haem domains for its bifunctional catalytic activity (linoleic acid 8-dioxygenation and 8-hydroperoxide isomerization), we found that PpoC apparently only harbours a functional haem peroxidase/dioxygenase domain. Consequently, we observed that PpoC catalyses mainly the dioxygenation of linoleic acid ($18{:}2^{\Delta 9Z,12Z}$), yielding 10-HPODE (10-hydroperoxyoctadecadienoic acid). No isomerase activity was detected. Additionally, 10-HPODE was converted at lower rates into 10-KODE (10-keto-octadecadienoic acid) and 10-HODE (10-hydroxyoctadecadienoic acid). In parallel, decomposition of 10-HPODE into 10-ODA (10-octadecynoic acid) and volatile C-8 alcohols that are, among other things, responsible for the characteristic mushroom flavour. Besides these principle differences we also found that PpoA and PpoC can convert 8-HPODE and 10-HPODE into the respective epoxy alcohols: 12,13-epoxy-8-HOME (where HOME is hydroxyoctadecenoic acid) and 12,13-epoxy-10-HOME. By using site-directed mutagenesis we demonstrated that both enzymes share a similar mechanism for the oxidation of $18{:}2^{\Delta 9Z,12Z}$; they both use a conserved tyrosine residue for catalysis and the directed oxygenation at the C-8 and C-10 is most likely controlled by conserved valine/leucine residues in the dioxygenase domain.

Key words: *Aspergillus nidulans*, fatty acid dioxygenase, oxylipin formation, psi-producing oxygenase (Ppo).

INTRODUCTION

In *Aspergillus nidulans* (teleomorph *Emericella nidulans*) oxidized unsaturated fatty acids, so-called oxylipins, derived from endogenous fatty acids, have been described to regulate the developmental life cycle of the fungus [1–7]. In *A. nidulans* these oxylipins are collectively called psi (precocious sexual inducer) factors. Psi factors are a mixture of oxidized $18{:}1^{\Delta 9Z}$, $18{:}2^{\Delta 9Z,12Z}$ and $18{:}3^{\Delta 9Z,12Z,15Z}$ (where $x{:}y^{\Delta z}$ is a fatty acid containing x carbons and y double bonds in position z, counting from the carboxy group [8]) and are termed psiβ, psiα and psiγ respectively. The number and the position of the hydroxy groups on the fatty acid backbone leads to further classification of psi factors: psiB [OH at C-8, e.g. (8*R*)-HODE], psiA [OH at C-5 and C-8, e.g. (5*S*,8*R*)-DiHODE] and psiC [OH at C-8 and on the δ-lactone ring] (where HODE is hydroxyoctadecadienoic acid and DiHODE is dihydroxyoctadecadienoic acid) [1,2].

The genes encoding enzymes that are involved in the production of psi factors were first identified by Keller and co-workers and were termed Ppos (psi-producing oxygenases) [9–11]. The genome of *A. nidulans* harbours three different genes possibly encoding Ppos: *ppoA*, *ppoB* and *ppoC* [9–11]. The same group showed that deletion of these genes had a significant effect on: (i) the developmental ratio between asexual conidiospores and sexual ascospores; (ii) the production of psi factors; and (iii) the production of secondary metabolites, the mycotoxins [9,11–13]. Ppo enzymes show a significant sequence identity with mammalian PGHS (prostaglandin endoperoxide H synthase; 25–29 % identity) and fungal 7,8-linoleate diol synthase (40–45 % identity) [10]. In contrast with 7,8-linoleate diol synthase a search for conserved domains using the NCBI (National Center for Biotechnology Information) database predicted that Ppo enzymes contained two different haem domains: a haem peroxidase/dioxygenase domain in the N-terminal region and a P450 haem-thiolate domain in the C-terminal region. Indeed, we have previously shown, on the basis of sequence alignments and spectroscopic analysis, that PpoA consists of those different haem domains [14]. Furthermore, we demonstrated that both domains are essential for the two different catalytic activities of PpoA; in the N-terminal peroxidase/dioxygenase domain of PpoA the dioxygenase reaction takes place and $18{:}2^{\Delta 9Z,12Z}$ is oxidized to (8*R*)-HPODE (hydroperoxyoctadecadienoic acid). This intermediate product is then isomerized by the P450 haem-thiolate domain of PpoA to 5,8-DiHODE [14] (Figure 1). On the basis of these results PpoA was assigned as CYP6001A1, a first member of a new P450 subfamily.

Previously Garscha et al. [15] reported that homogenates of *A. nidulans* mutants that are deficient in PpoC failed to form (10*R*)-HPODE and (10*R*)-HODE. From that they concluded that PpoC is a (10*R*)-dioxygenase. Recombinant expression of *Aspergillus fumigatus* PpoC in insect cells confirmed this hypothesis [16]. Moreover mutational studies of 7,8-linoleate

Abbreviations used: CmΔP, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl; DiHODE, dihydroxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; HOME, hydroxyoctadecenoic acid; HOTrE, hydroxyoctadecatrienoic acid; HPODE, hydroperoxyoctadecadienoic acid; HPOME, hydroperoxyoctadecenoic acid; HPOTrE, hydroperoxyocadecatrienoic acid; KODE, keto-octadecadienoic acid; NCBI, National Center for Biotechnology Information; NIST, National Institute of Standards and Technology; ODA, octadecynoic acid; PGHS, prostaglandin endoperoxide H synthase; Ppo, psi-producing oxygenase; psi, precocious sexual inducer; RP, reversed-phase; SPME, solid-phase micro-extraction; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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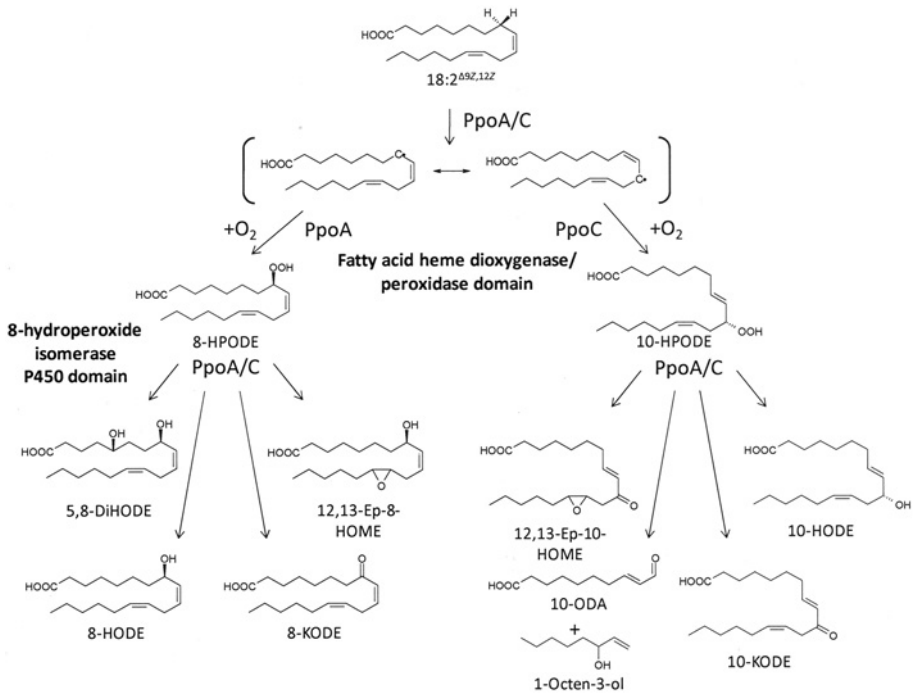

Figure 1 Psi factor formation in *A. nidulans and A. fumigatus*

Ppo enzymes abstract a hydrogen atom from the C-8 of the backbone of unsaturated C-18 fatty acids and form a carbon-centred radical at this position, a reaction that is presumably accomplished via a tyrosyl radical mechanism within the N-terminal fatty acid haem peroxidase/dioxygenase domain. In the case of enzymes belonging to PpoA subfamily molecular oxygen attacks the C-8-centred radical and forms (8*R*)-HPODE. This intermediate product is either used as a substrate for peroxidase reaction, yielding 8-HODE, or it serves as a substrate for the 8-hydroperoxide isomerase reaction, yielding mainly 5,8-DiHODE. The isomerase reaction is believed to be accomplished by the C-terminal P450 haem-thiolate domain of PpoA [14]. Thirdly the fatty acid hydroperoxide is reduced to 8-HODE or transformed to small amounts of 12,13-epoxy-8-HOME respectively. In case of PpoC enzymes the carbon-centred radical migrates to C-10 and molecular oxygen is inserted at this position, yielding 10-HPODE. This product appears to be unstable and either oxidizes to 10-KODE or decomposes to 10-ODA and volatile compounds, such as 1-octen-3-ol. Furthermore, 10-HPODE can be reduced to 10-HODE or transformed to small amounts of 12,13-epoxy-10-HOME respectively. Ep, epoxy.

diol synthase and PpoC implied a general mechanism for the directed oxygenation of $18{:}2^{\Delta 9Z,12Z}$ at the C-8 and C-10 by fungal dioxygenases. It was proposed that conserved leucine/valine residues in the dioxygenation site of both dioxygenases determine the oxygenation site [16]. In the same study the formation of low amounts of the epoxy alcohol 12,13-epoxy-(10*R*)-HOME (where HOME is hydroxyoctadecenoic acid) was observed. It was suggested that, in analogy with the isomerization activity of the bifunctional 7,8-linoleate diol synthase that transforms 8-HPODE to (7*S*,8*S*)-DiHODE, PpoC is also a bifunctional enzyme with epoxy alcohol synthase activity that converts 10-HPODE into 12,13-epoxy-(10*R*)-HOME [16].

In the present study we aimed to compare the biochemical properties of the two Ppo- enzymes PpoA and PpoC from *A. nidulans*, which share a sequence identity of 44 %, to elucidate the basis for differences in the produced compounds. Therefore, we expressed PpoC in the same expression system as reported for PpoA [14], purified PpoC and analysed the biochemical properties of this enzyme.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma–Aldrich and Carl Roth & Co. Agarose was from Biozym Scientific GmbH. All fatty acids were purchased from Sigma–Aldrich or Cayman Chemicals. Acetonitrile was from Fisher Scientific. Restriction enzymes were provided by MBI Fermentas.

Cloning and expression of recombinant PpoC in *Escherichia coli*

A. nidulans ppoC (GenBank™ accession number AY613780.1) was amplified from fungal cDNA of sexual and vegetative stages using gene-specific primers containing NdeI and NotI recognition sites (forward primer, 5′-CCATATGTTGCGAAGATTTTCTACCTTCAG-3′; reverse primer, 5′-AGCGGCCGCTCAAGCAGAATCAATCTGCTTCTTAG-3′) via the Phusion® hot-start high-fidelity DNA polymerase system (Finnzymes). PCR amplification were performed under the following conditions: 98 °C for 3 min followed by 30 cycles of 98 °C for 30 s, 53 °C for 30 s and 72 °C for 3 min, and the PCR was terminated by 5 min at 72 °C. The resulting fragment was cloned into pJET2.1/blunt (Fermantas), yielding the plasmid pJET-PpoC. For expression in *E. coli ppoC* was cloned into the pET24a expression vector (Novagen) using NdeI and NotI, yielding the plasmid pET24a-PpoC and it was transformed together with the plasmid pGro7 (encoding the chaperone groES–groEL; Takara Bio) into *E. coli* BL21 Star™ cells (Invitrogen).

Site-directed mutagenesis

In vitro mutagenesis was carried out using Phusion® hot-start high-fidelity DNA polymerase system using the protocol from the QuickChange® mutagenesis kit (Stratagene). The following

primers were used: PpoA(V328L) sense (5′-CAAATATTATCCTAAAAGATTATUCUTUAUCGTACGATTTTGAATATAAAC-3′) and antisense (5′-GTTTATATTCAAAATCGTACGTAGATAATCTTTTAGGATAATATTTG-3′); PpoA(V328A) sense (5′-GTACGCAAATATTATCCTTAAUGUGATTATGCACGAACGATTTTGAATATAAA-3′) and antisense (5′-GTTTATATTCAAAATCGTTCGTGCATAATCCTTAAGGATAATATTTGCGTAC-3′); PpoC(Y427F) sense (5′-GAAACCAATGTTCTGTCGAATTTAACCTGGCCTTTCGGTGGCACTCTGCTATCAGTG-3′) and antisense (5′-CACTGATAGCAGAGTGCCACCGAAAGGCCAGGTTAAATTCGACAGAACATTGGTTTC-3′); PpoC(G1039C) sense (5′-GGCATCGGTTCTCAAATCTGCCTAGGCAAGGACGCCACACTG-3′) and antisense (5′-CAGTGTGGCGTCCTTGCCTAGGCAGATTTGAGAACCGATGCC-3′); PpoC(L385V) sense (5′-CATCACCCTGTACGATTACGTACGCACGATCGTTAACCTGAACCGAGTGAACAG-3′) and antisense (5′-CTGTTCACTCGGTTCAGGTTAACGATCGTGCGTACGTAATCGTACAGGGTGATG-3′); and PpoC(L385A) sense (5′-CATCACCCTGTACGATTACGCACGCACGATCGTTAACCTGAACCGAGTGAACAG-3′) and antisense (5′-CTGTTCACTCGGTTCAGGTTAACGATCGTGCGTGCGTAATCGTACAGGGTGATG-3′).

Expression of recombinant Ppo enzymes in *E. coli*

E. coli BL21 Star™ cells containing pET24a-PpoC and the plasmid pGro7 (encoding the chaperone groES–groEL) were cultivated in 2YT medium [1.6 % (w/v) tryptone/1 % (w/v) yeast extract/0.5 % (w/v) NaCl] containing 50 μg/ml chloramphenicol, 25 μg/ml kanamycin and 25 μg/ml arabinose to induce the expression of the chaperone, and 0.02 mM δ-aminolaevulinic acid. Cells were grown to a D_{600} of 0.6–0.8. Induction of the expression of the recombinant protein was performed by adding IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 0.1 mM and cultures were cultivated for 3 days with shaking at 16 °C. Cells were harvested by centrifugation at 4000 ***g*** at 6 °C for 10 min. The pellet was shock-frozen in liquid nitrogen and stored at −20 °C.

Cell lysis and protein purification

Harvested cells from 500 ml of culture expressing recombinant PpoC were resuspended in 50 ml of buffer (50 mM Tris/HCl, pH 8.0). After addition of lysozyme to a final concentration of 0.1 mg/ml and PMSF to a final concentration of 0.2 mM, cells were incubated for 30 min on ice. Cells were disrupted by sonification (three times, each for a 30 s duration) on ice using a sonifier cell disruptor B15 (Branson Sonic Power) and this extract was centrifuged at 54000 ***g*** at 4 °C for 20 min to clear debris, yielding crude bacterial extract. The supernatant obtained from cell lysis was loaded on to a Source 30Q resin (a 25 ml XK 16/20 column; GE Healthcare) at a flow rate of 1 ml/min using an ÄKTAprime™ system (GE Healthcare). The column was washed with 50 ml of 50 mM Tris/HCl, pH 8.0 and protein was eluted with a linear gradient of 0–0.3 M NaCl in 50 mM Tris/HCl, pH 8.0, within 30 min at a flow rate of 2 ml/min. Fractions of 2 ml were collected. Fractions with highest activity (as judged by a Clark-type O_2 electrode; Rank Brothers) and highest purity (as judged by SDS/PAGE) were combined and concentrated to a final volume of 2 ml using Vivaspin 20 (100000 molecular-mass cut-off; Sartorius Stedim Biotech). For gel filtration the concentrated protein extract was loaded on to a Superdex S200 26/60 pg column (GE Healthcare) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 1 M NaCl. Elution was performed at a flow rate of 2 ml/min. Fractions containing enzyme activity were combined and loaded on to a desalting 26/10 column (GE Healthcare) equilibrated with 50 mM Tris/HCl, pH 8.0. The desalted protein solution was loaded on to a MonoQ 10/10-column (GE Healthcare). After loading and washing with 50 mM Tris/HCl, pH 8.0, the protein was eluted at 3 ml/min with a linear gradient to 50 mM Tris/HCl, pH 8.0, containing 1 M NaCl within 30 min. Recombinant PpoA and related mutants [PpoA(V328A) and PpoA(V328L)] were purified as described previously [14].

UV–visible spectroscopy

UV–visible spectroscopy was performed in 50 mM Hepes, pH 7.4, at room temperature (approx. 24 °C) using a dual-beam Varian Cary 100 Bio UV/Vis Spectrophotometer.

Activity assays

Fatty acids (250 μg of $18{:}1^{\Delta 9Z}$, $18{:}2^{\Delta 9Z,12Z}$ and $18{:}3^{\Delta 9Z,12Z,15Z}$) were dissolved in 1 ml or 800 μl of 50 mM Tris/HCl, pH 8.0. The reactions were started by the addition of either 15 μg of purified PpoC or 200 μl of PpoC bacterial crude extract and were incubated for 30 min at room temperature. Incubations were stopped by adding 2 ml of diethyl ether to extract fatty acids and their derivatives. A second extraction was performed additionally with 2 ml of diethyl ether and 100 μl of acetic acid. After evaporating under a nitrogen stream the sample was dissolved in 30 μl of HPLC-eluent (water/acetonitrile/acetic acid; 50:50:0.1, by vol.).

Incubations of $18{:}2^{\Delta 9Z,12Z}$ and $^{18}O_2$ (Campro Scientific) with PpoC were performed in 50 mM Tris/HCl, pH 8.0. The buffer (900 μl) was degassed and supplied with $^{18}O_2$ on ice until the system was saturated. PpoC bacterial crude extract (100 μl) and 250 μg of $18{:}2^{\Delta 9Z,12Z}$ were added and the mixture was incubated for 30 min at room temperature. The reaction system was further enriched with $^{18}O_2$ during the incubation. The reaction was terminated by addition of diethyl ether and reaction products were extracted as described above.

Incubations of $18{:}2^{\Delta 9Z,12Z}$ with purified PpoA, PpoA(V328A) and PpoA(V328L) were performed in 50 mM Hepes, pH 7.4, as described previously [14]. Incubations with 8-HPODE and 10-HPODE were either performed in 200 μl of PpoC crude bacterial extract (in 50 mM Tris/HCl, pH 8.0) or with 200 μl of 50 μM Hepes, pH 7.4, containing 1–5 μM PpoA.

RP (reversed phase)-HPLC/MS analysis

The HPLC assembly was a Surveyor HPLC system (Thermo Finnigan) with an EC250/2 100–5 C_{18} column (2.1 mm × 250 mm, 5 μm particle size; Macherey-Nagel). The solvent system for the following gradient was (A) acetonitrile/water/acetic acid (40:60:0.1, by vol.), and (B) acetonitrile/acetic acid [100:0.1, by vol.]. The gradient elution profile was as follows: 0–10 min a flow rate of 0.2 ml/min using 80 % (A) and 20 % (B); 10–30 min from 80 % (A) and 20 % (B) to 100 % (B); 30–35 min a flow rate increase to 0.3 ml/min; 35–40 min 100 % (B); 40–44.5 min from 100 % (B) to 80 % (A) and 20 % (B); and 44.5–45 min 80 % (A) and 20 % (B) and flow rate decreased to 0.2 ml/min. The mass spectrometer was an LCQ ion-trap mass spectrometer (Thermo Finnigan) with electrospray ionization and monitoring of negative ions. The capillary temperature was 300 °C and the capillary voltage was 27 kV. For tandem MS analysis, the collision energy was 1 V.

Analysis of volatile compounds

Volatile compounds were measured from the reaction of 10 ml of crude bacterial extract incubated with 2.5 mg of fatty acid (18:1$^{\Delta 9Z}$, 18:2$^{\Delta 9Z,12Z}$ or 18:3$^{\Delta 9Z,12Z,15Z}$) by SPME (solid-phase micro-extraction). Incubation of the reaction mixture was performed in a vial, sealed with a teflon septum and with an inserted poly(dimethylsiloxane/divinylbenzene)-coated fibre (Supelco). After 30 min the reaction products were extracted as described previously [17]. The analysis of the hydroxy fatty acids was carried out using an Agilent 5973 Network mass-selective detector connected to an Agilent 6890 gas chromatograph equipped with a capillary DB-23 column (30 m × 250 μm; 0.25 μm coating thickness). Helium was used as carrier gas (1 ml/min). The injection temperature was 250 °C, the temperature gradient was 40 °C for 2 min, 150–200 °C at 15 °C/min and 200 °C for 3 min. Electron energy of 70 eV, an ion-source temperature of 230 °C and a temperature of 260 °C for the transfer line was used.

Lipid-derived radical trapping by nitroxy radicals and peroxidase assay

Lipid-derived radicals generated during the incubation of 18:2$^{\Delta 9Z,12Z}$ with PpoC were trapped and analysed as nitroxy radicals as described previously [14,18]. We used the peroxidase assay according to Kulmacz [19] to demonstrate peroxidase activity of PpoC as described previously [14].

Determination of kinetic parameters

Kinetic experiments were performed in 50 mM Tris/HCl, pH 7.9, at room temperature with a Clark-type O_2 electrode. Initial rates of oxygen consumption were measured for different substrate concentrations in buffer that was pre-equilibrated against air at 101.325 kPa. The reaction was started by the addition of 200 μl of PpoC bacterial crude extract to 800 μl of rapidly stirring buffer containing a defined amount of substrate (sodium salts of 18:1$^{\Delta 9Z}$, 18:2$^{\Delta 9Z,12Z}$ or 18:3$^{\Delta 9Z,12Z,15Z}$). Kinetic parameters were determined by fitting seven data points to Michaelis–Menten equations by using the software Origin 6.0.

Determination of the pH optimum

The pH optimum was determined by measuring initial rates of oxygen consumption at a defined pH value using a Clark-type O_2 electrode. PpoC bacterial crude extract (200 μl) was added to 800 μl of buffer containing 100 μM of the sodium salt of 18:2$^{\Delta 9Z,12Z}$. The following buffers were used for the different pH ranges: 0.2 M acetate buffer (pH 4.5–5.5), 0.2 M phosphate buffer (pH 5.5–8.0) and 0.2 M borate buffer (pH 8.0–10.5).

RESULTS

PpoC is a fusion protein of the CYP6001 family

BLAST search analysis of PpoC predicts the presence of two domains in its sequence. Within the N-terminal region of the protein a haem peroxidase/dioxygenase domain is predicted, whereas a P450 haem-thiolate domain is proposed for the C-terminal part. Therefore PpoC from *A. nidulans* was assigned as CYP6001C1 by Dr D.R. Nelson of the P450 Nomenclature Committee (Department of Molecular Sciences, University of Tennessee, Memphis, TN, U.S.A.). Similar domain architecture has also been described recently for PpoA, another member of the Ppo family of *A. nidulans*, for which functionality of both domains has been proven [14]. Sequence alignments of the predicted N-terminal haem peroxidase/dioxygenase domain of *A. nidulans* PpoC with those of *A. fumigatus* PpoC, *A. nidulans* PpoA, *Gaeumannomyces graminis* 7,8-linoleate diol synthase, sheep PGHS-1 and sheep PGHS-2 (Figure 2A) showed that residues reported to be essential for the catalytic dioxygenase activity are conserved in all six sequences. However, sequence alignments of the predicted C-terminal P450 domain of PpoC with those of known P450 haem-thiolate enzymes, i.e. the recently described *A. nidulans* PpoA [14] and *A. fumigatus* PpoC [16], showed that the crucial cysteine residue, which is known as the fifth haem iron ligand, is neither conserved in PpoC from *A. nidulans* nor from *A. fumigatus*. Instead of a cysteine residue a glycine residue and a phenylalanine residue respectively, were found at the particular position of the PpoC sequences (Figure 2B). This may indicate that the P450 domain is not functional and does not show catalytic activity (see below).

Cloning and functional expression of PpoC

In order to analyse the enzymatic reaction catalysed by PpoC from *A. nidulans*, its cDNA was cloned from vegetative and sexual stages of *A. nidulans* by using specific primers. Sequencing of the resulting plasmid confirmed the ORF (open reading frame) was 3933 bp and 1117 amino acids respectively, showing one amino acid residue exchange (P-215) compared with the published genome sequence [20]. Functional expression of PpoC was performed in *E. coli* BL21 Star™ cells and optimal cultivation conditions (16 °C for 3 days) were determined. Expression of PpoC could be enhanced by co-expression of chaperone groES–groEL and the addition of 0.2 mM δ-aminolaevulinic acid before induction (aminolaevulinic acid is a precursor of haem biosynthesis). Protein expression was verified by SDS/PAGE (Figure 3A).

Purification of PpoC

In order to purify PpoC a protocol was used based on that described previously for PpoA purification [14]. Extraction and solubilization of PpoC using crude extract from bacteria was performed with 50 mM Tris/HCl, pH 8.0. The addition of detergents and NaCl to the extraction buffer did not improve protein extraction. For the initial purification step a Source 30Q resin was chosen to isolate and concentrate PpoC from the crude cell extract. Fractions showing the highest activity were combined and purified further using size-exclusion chromatography. The native molecular mass of PpoC using gel-filtration chromatography was 480 kDa. This was 4-fold larger than that determined by SDS/PAGE. Therefore the native protein is a homotetramer, as has been demonstrated previously for 7,8-linoleate diol synthase [21] and PpoA [14]. With the final chromatography step using a MonoQ column most of the remaining contaminants were removed. After each purification step the protein fractions showing the highest activity were combined and subjected to SDS/PAGE (Figure 3A). A protein band at approx. 120 kDa became subsequently more prominent. Using this protocol we were able to purify approx. 0.5–2 mg of protein from 500 ml of cell culture. The activity of the pure protein was estimated to be 6.5 μmol [O_2]/(min × mg) using the Clark-type O_2 electrode. However, we found that the activity of the purified protein decreased significantly within a few hours. Neither the addition of 10 % (v/v) glycerol nor 5 mM EDTA to the buffer improved PpoC stability.

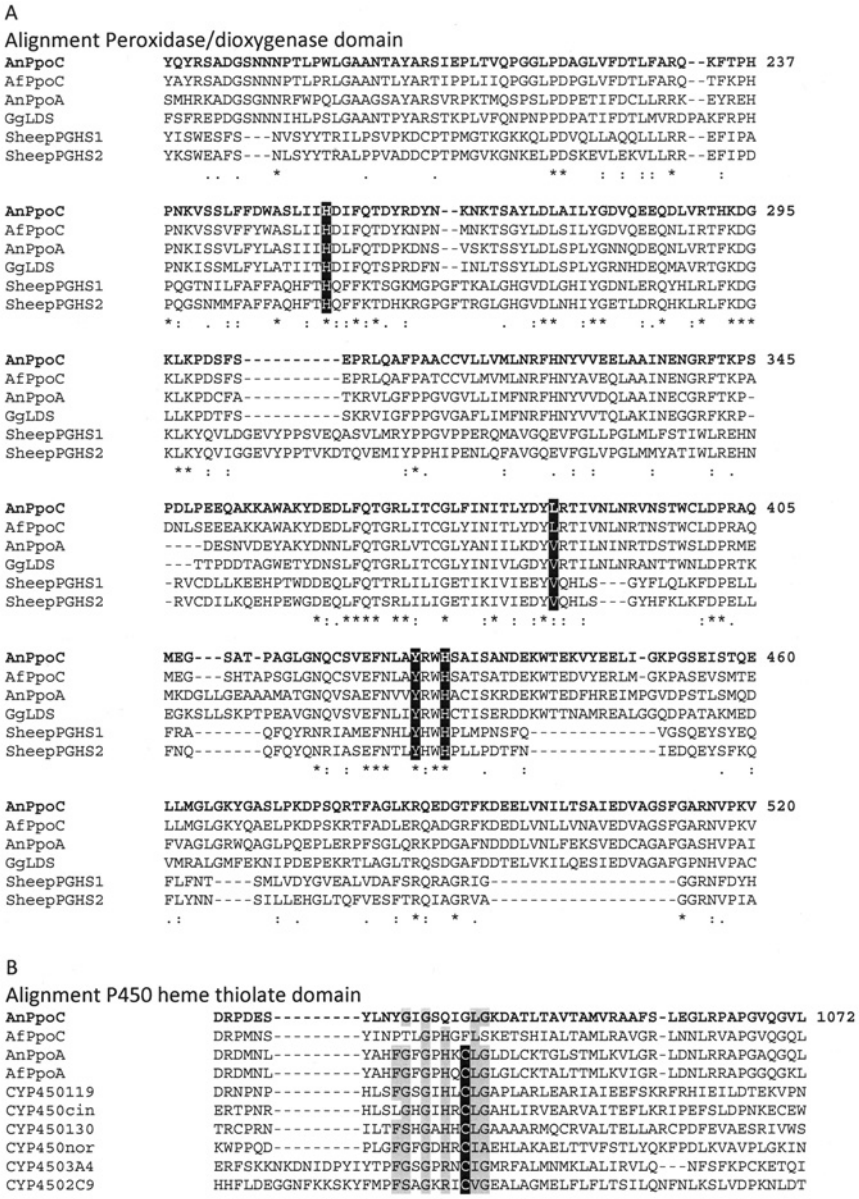

Figure 2 Partial alignment of the predicted N-terminal haem peroxidase/dioxygenase domain and C-terminal P450 domain from PpoC with sequences from different fatty acid haem dioxygenases and P450 enzymes

(**A**) Partial amino acid alignment of the predicted haem peroxidase/dioxygenase domain from *A. nidulans* PpoC (AnPpoC; RefSeq XP_662632) with *A. fumigatus* PpoC (AfPpoC; GenBank® ABV21633), *A. nidulans* PpoA (AnPpoA; RefSeq XP_659571), *G. graminis* 7,8-linoleate diol synthase (GgLDS; Swiss-Prot Q9UUS2.1;), sheep PGHS-1 (Swiss-Prot P05979.2) and sheep PGHS-2 (Swiss-Prot P79208.1). The catalytic tyrosine residue as well as the proximal and distal histidine residues, which are conserved in the six sequences, and have been identified previously [30,41], are shown on a black background. Additionally, leucine/valine residues that are responsible for direction of oxygenation in *A. fumigatus* PpoC and *G. graminis* 7,8-linoleate diol synthase are on a black background. (*), conserved residues; (:) and (.), similar residues. (**B**) Partial amino acid alignment of the predicted P450 haem-thiolate domain from *A. nidulans* PpoC with known P450 enzymes. The predicted CYP450 domains from different Ppo enzymes: *A. nidulans* PpoC, *A. fumigatus* PpoC; *A. nidulans* PpoA [all as given in (**A**)] and *A. fumigatus* PpoA (AfPpoA; RefSeq XP_751750) are aligned with known P450 enzymes. CYP450119, PDB code 1F4T:B; CYP450cin, PDB code 1T2B:B; CYP450130, PDB code 2UUQ:A; CYP450nor, PDB code 1JFB:A; CYP4503A4, PDB code 1TQN:A and CYP4502C9, PDB code 1R9O:A. Amino acids matching the P450 consensus sequence according to the PROSITE database are shown on a grey background. Additionally the cysteine residue responsible for the co-ordination of haem iron is shown on a black background. The sequences were aligned using the ClustalW algorithm.

Spectral analysis and prosthetic groups

The UV–visible absorption spectrum of purified PpoC is shown in Figure 3(B). The highest absorption maxima were at 280 nm (probably due to aromatic amino acid site chains; results not shown) and 403 nm (γ, the Soret band) indicating the presence of haem. Additionally two weaker maxima at approx. 530 nm and 630 nm were detected. However, the haem absorption spectrum appeared to be weak, a fact that might indicate that haem was partially lost during the purification procedure.

Identification of products formed by recombinant PpoC

As we found purified PpoC to be highly unstable under our assay conditions, we decided to investigate most of the biochemical

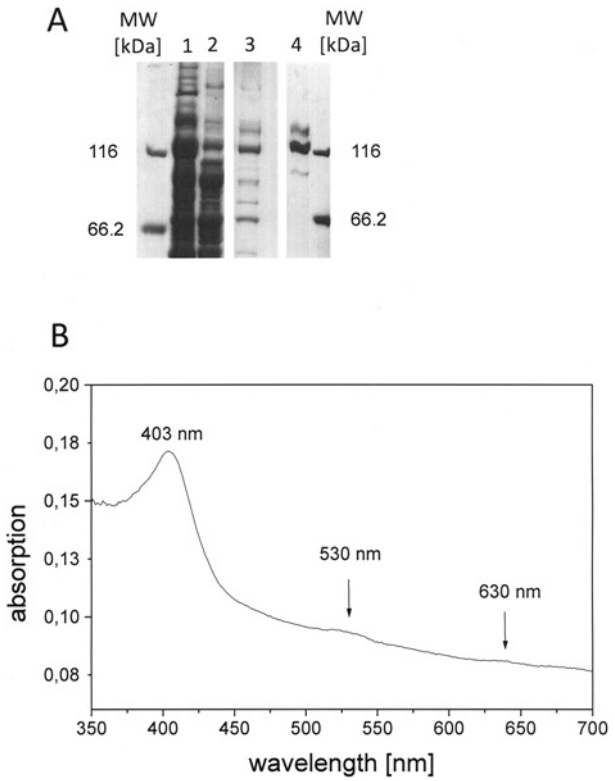

Figure 3 SDS/PAGE at different stages of purification and UV–visible absorption of purified PpoC

(**A**) Aliquots after each purification step were analysed by SDS/PAGE (8 % gels). Lane 1, crude cell extract; lane 2, after Source 30Q purification; lane 3, after Sephadex S200 (60/20) purification; and lane 4, after MonoQ (10/10) purification. MW, molecular mass markers in kDa. (**B**) A spectrum of the native enzyme in 50 mM Hepes, pH 7.4, is shown. The UV–visible spectrum of the purified enzyme showed absorption maxima at 403 nm (γ, Soret band) indicating the presence of haem. Additionally two weaker maxima at approximately 530 nm and 630 nm are indicated by arrows.

properties of PpoC by using bacterial crude extract of PpoC-expressing *E. coli* cells and using cells expressing the empty vector as controls.

Bacterial crude extract of PpoC-expressing cells was incubated with $18{:}2^{\Delta 9Z,12Z}$ for 30 min on a shaker. After extraction of fatty acids and their derivatives we analysed product formation with RP-HPLC/tandem MS. We detected the formation of 10-HPODE (*m*/*z* 311; retention time 22.1 min), 8/10-HODE (*m*/*z* 295; retention time 19.5 min/20.9 min), 10-KODE (where KODE is keto-octadecadienoic acid; *m*/*z* 293; retention time 22.6 min) and 10-ODA (where ODA is octadecynoic acid; *m*/*z* 183; retention time 3.8 min) (Figures 4A–4D). Additionally we found the formation of 12,13-epoxy-10-HOME (*m*/*z* 311; retention time 9.9 min; Figure 4A). The corresponding spectra of 12,13-epoxy-10-HOME, 8-HODE, 10-HODE, 10-KODE and 10-ODA are shown in Supplementary Figure S1(A–E), available at http://www.BiochemJ.org/bj/425/bj4250553add.htm, and are in agreement with previously reported spectra [15,16]. Bacterial crude extract of empty vector controls did not metabolize $18{:}2^{\Delta 9Z,12Z}$ to any of these products. Comparing the relative amounts of different products formed during the incubation, we found that 10-HODE and 10-HPODE appeared to be the main products that are formed from $18{:}2^{\Delta 9Z,12Z}$ by PpoC (Figure 4E), whereas 12,13-epoxy-10-HOME and 10-ODA accumulated to lesser extends.

Table 1 Oxygenation products of *A. nidulans* PpoC

Products were identified using RP–HPLC/tandem MS analysis and by detection of characteristic signals (8-hydroperoxide derivative with *m*/*z* 157 (cleavage between C-8 and C-9) and 10-hydroperoxide derivative with *m*/*z* 183 (cleavage between C-10 and C-11, as also shown in Supplementary Figure S2). Volatile products were extracted by SPME and identified by GC/MS analysis using the NIST database (Figure 6).

Substrate	Hydroperoxide products	Hydroperoxide derived products	Volatile products
$18{:}1^{\Delta 9Z}$	8-HPOME	8-HOME	1-octanol, 2-undecenal
	10-HPOME	10-HOME	
$18{:}2^{\Delta 9Z,12Z}$	8-HPODE	8-HODE	
	10-HPODE	10-HODE	1-octen-3-ol
		12,13-Ep-10-HOME	2-octen-1-ol
		10-ODA	2-octenal
		10-KODE	3-octanone
$18{:}3^{\Delta 9Z,12Z,15Z}$	8-HPOTE	8-HOTrE	
	10-HPOTE	10-HOTrE	Octadiene isomers
		2-decenedioic acid	

We next examined which products are formed by PpoC from other fatty acid substrates. We were particularly interested in products formed from fatty acids that occur endogenously in *A. nidulans,* such as other unsaturated C-18 fatty acids. As expected, $18{:}1^{\Delta 9Z}$ and $18{:}3^{\Delta 9Z,12Z,15Z}$ were mainly converted into their corresponding 10-hydroxy/10-hydroperoxy and 8-hydroxy/8-hydroperoxy derivatives (Table 1). Interestingly, $18{:}1^{\Delta 9Z}$ was predominantly oxidized at the C-8, yielding 8-HOME/8-HPOME (HPOME is hydroperoxyoctadecenoic acid), whereas $18{:}3^{\Delta 9Z,12Z,15Z}$ was mainly oxidized at the C-10, yielding 10-HOTrE/10-HPOTrE (where HOTrE is hydroxyoctadecatrienoic acid and HPOTrE is hydroxyoctadecatrienoic acid).

Additionally, we observed that during incubations of $18{:}3^{\Delta 9Z,12Z,15Z}$ with PpoC a polar product was formed that eluted at 3.8 min from RP-HPLC. The corresponding tandem MS spectrum (*m*/*z* 199) of this compound is shown in Supplementary Figure S1(F) and shows the following signals: *m*/*z* 181 (M^-–H_2O), 155 (M^-–44, probably loss of CO_2), 139 (M^-–60) and 137 (M^-–62). Due to similarity to previously reported tandem MS spectra of 10-ODA [15] this product was tentatively assigned as (2*E*)-decendioic acid.

Identification of volatile products

10-ODA, formed during the incubation of PpoC and $18{:}2^{\Delta 9Z,12Z}$, has been described as a cleavage product of 10-HPODE [22]. It is known that this compound is formed from β-scission of the hydroperoxide, a reaction which is catalysed by metals [22] and by still unidentified fungal lyases [23]. As additional cleavage products volatile octenol isomers have also been reported [23–25]. To elucidate whether similar volatile compounds are also formed during the reaction of PpoC with $18{:}1^{\Delta 9Z}$, $18{:}2^{\Delta 9Z,12Z}$ and $18{:}3^{\Delta 9Z,12Z,15Z}$, we analysed the gas phase above the reaction mixture using SPME and GC/MS analysis. Using the NIST (National Institute of Standards and Technology) library and spectra reported previously [26,27], we were able to identify most of these compounds.

We found that during the reaction of PpoC and $18{:}1^{\Delta 9Z}$ two volatile compounds were formed that could be identified as 1-octanol and 2-undecenal respectively (Figure 5A and Table 1). The latter compound has been described as an odorant formed from 8-HPOME [28]. Volatile compounds that were formed during the reaction of PpoC and $18{:}2^{\Delta 9Z,12Z}$ were identified as 1-octen-3-ol, 2-octen-1-ol, 3-octenone and 2-octenal respectively (Figure 5B

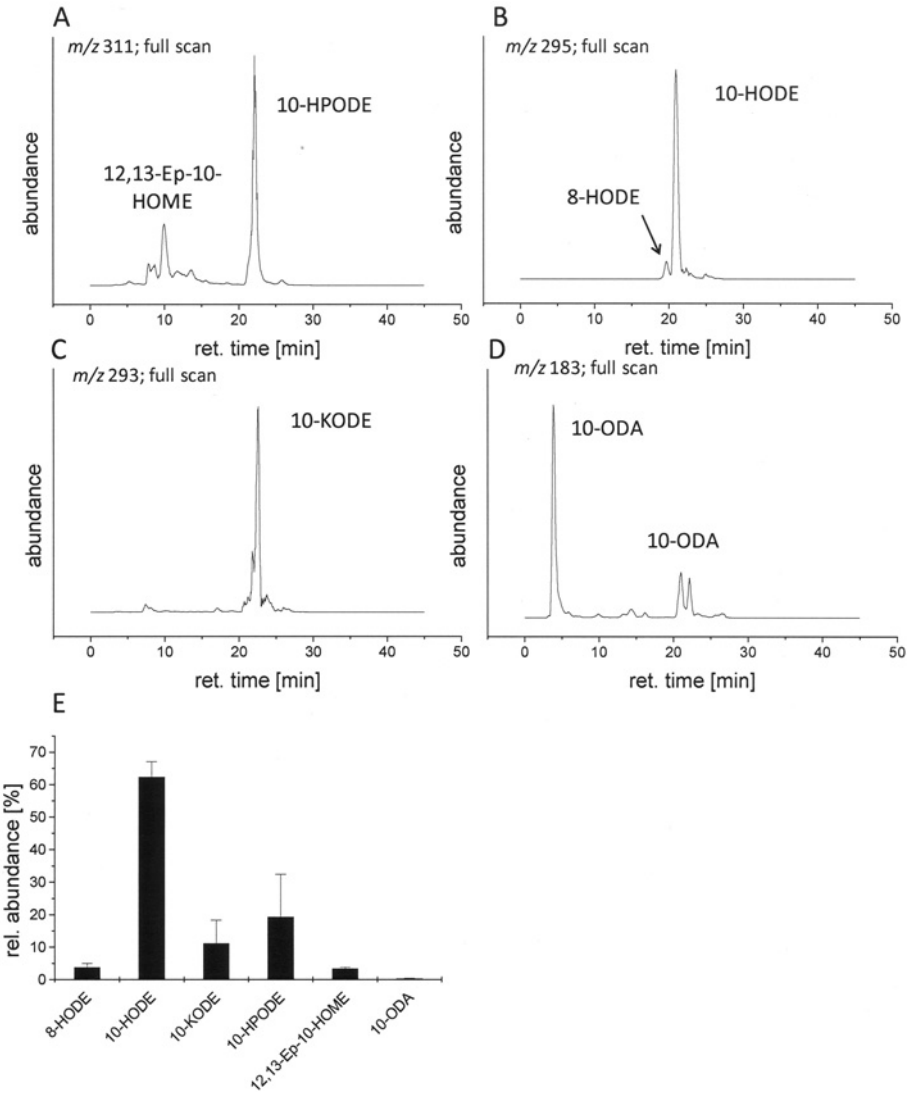

Figure 4 RP–HPLC/MS analysis of metabolites formed from 18:2$^{\Delta 9Z,12Z}$ by recombinant PpoC

RP–HPLC/MS analysis after 30 min incubation of 18:2$^{\Delta 9Z,12Z}$ with PpoC in 50 mM Tris/HCl buffer, pH 8.0, and extractive isolation. Shown are: (**A**) 10-HPODE and 12,13-epoxy-10-HOME (*m/z* 311; full scan); (**B**) 8-HODE and 10-HODE (*m/z* 295; full scan); (**C**) 10-KODE (*m/z* 293; full scan); and (**D**) 10-ODA (m/z 183; full scan). As reported previously [15], 10-HPODE decomposed in the mass spectrometer into 10-ODA which co-elutes with 10-HPODE at 20.9 min, whereas pre-formed 10-ODA elutes at 3.8. (**E**) Relative amounts of products formed during the incubation. Results are means ± S.D. calculated from three different experiments. Ret. time, retention time; Ep, epoxy.

and Table 1). Furthermore, we also detected the formation of two volatile products during incubations of PpoC with 18:3$^{\Delta 9Z,12Z,15Z}$ (Figure 5C). These products could not be reliably identified by using the NIST library. However, incubation with [$^{13}C_{18}$]18:3$^{\Delta 9Z,12Z,15Z}$ indicated that these products are octadiene-isomers (results not shown).

As these volatile compounds were also detected during incubation of 10-hydroperoxy derivatives with empty vector controls, we suggest that these cleavage reactions were mainly caused by non-enzymatic reactions, or they may at least be catalysed independently from PpoC. Similar observations have been reported previously [15].

Peroxidase activity and pH optimum

In order to determine whether the high amounts of 10-HODE (Figure 4E) can be formed by PpoC within an enzymatic peroxidase reduction, we performed a peroxidase assay using TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) as a co-substrate. When purified PpoC was incubated with 100 μM TMPD and 100 μM 18:2$^{\Delta 9Z,12Z}$, we were able to follow the characteristic increase at 611 nm for several minutes. A typical progress curve is shown in Supplementary Figure S2 (available at http://www.BiochemJ.org/bj/425/bj4250553add.htm) and is indicative for the peroxidase activity of PpoC.

We analysed the pH optimum by measuring the initial rates of oxygen consumption for the reaction of PpoC crude extract with 100 μM 18:2$^{\Delta 9Z,12Z}$. The initial rate of oxygen consumption reached a maximum between pH 7.4 and 8.0. pH values >8.0 led to an rapid decrease in enzymatic activity.

Kinetic properties

Kinetic parameters were determined by measuring the initial rates of oxygen consumption for the reaction with different substrate concentrations using the Clark-type O_2 electrode. The reaction

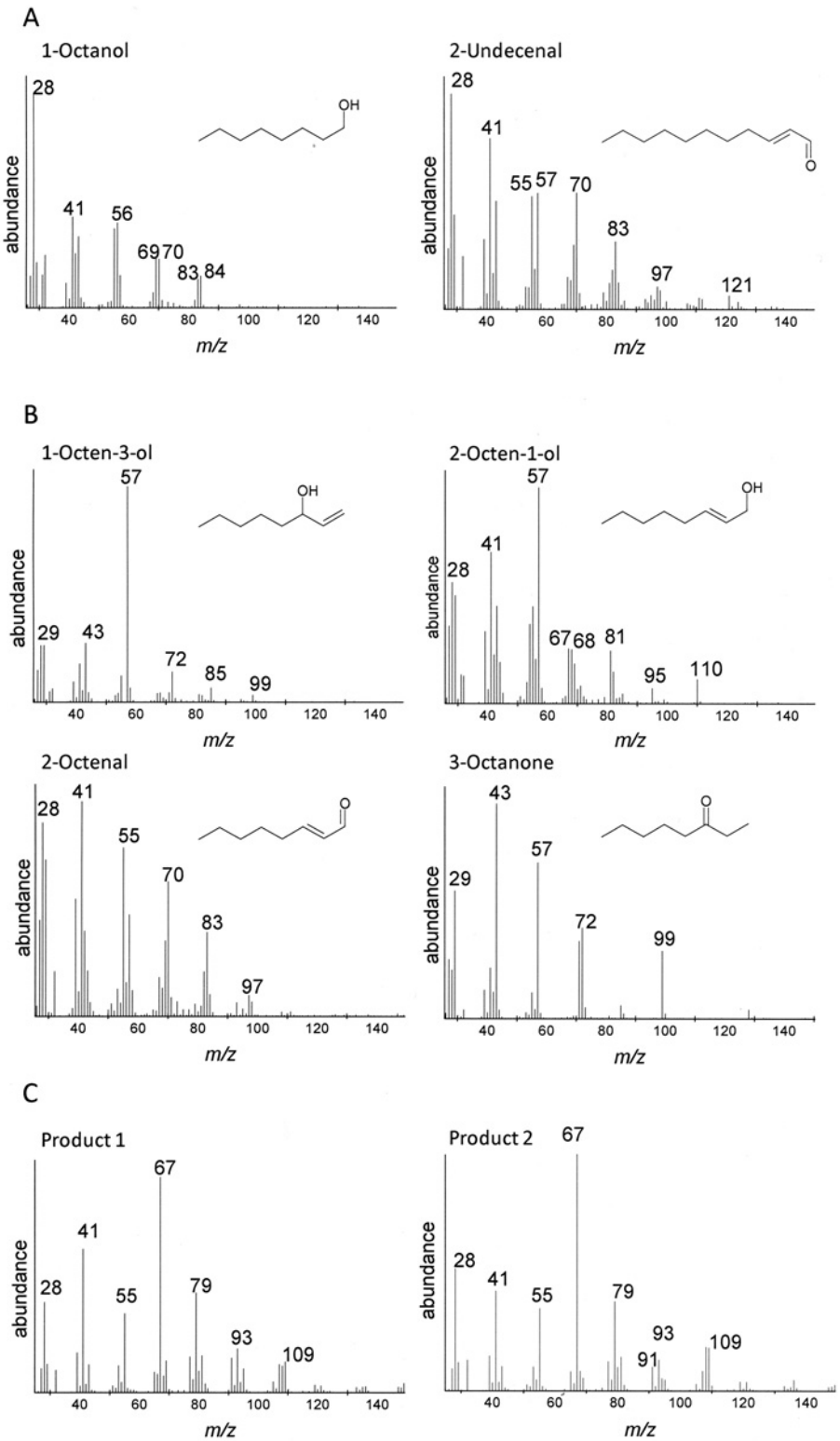

Figure 5 GC/MS analysis of volatile compounds of PpoC and different fatty acid substrates

Volatile compounds formed during the incubation of PpoC with (**A**) 18:1$^{\Delta 9Z}$, (**B**) 18:2$^{\Delta 9Z,12Z}$ and (**C**) 18:3$^{\Delta 9Z,12Z,15Z}$. The products were identified with SPME, GC/MS and by using the NIST library and spectra reported previously [26,27]. Incubations of PpoC with 18:1$^{\Delta 9Z}$ led to the production of 1-octanol and 2-undecenal, whereas during incubations with 18:2$^{\Delta 9Z,12Z}$ 1-octen-3-ol, 2-octen-1-ol, 2-octenal and 3-octenon were predominantly formed. 18:3$^{\Delta 9Z,12Z,15Z}$ was converted by PpoC into two volatile products which could not be identified using the NIST library. By experiments with [^{13}C]-labelled 18:3$^{\Delta 9Z,12Z,15Z}$ these products were tentatively assigned as octadiene isomers.

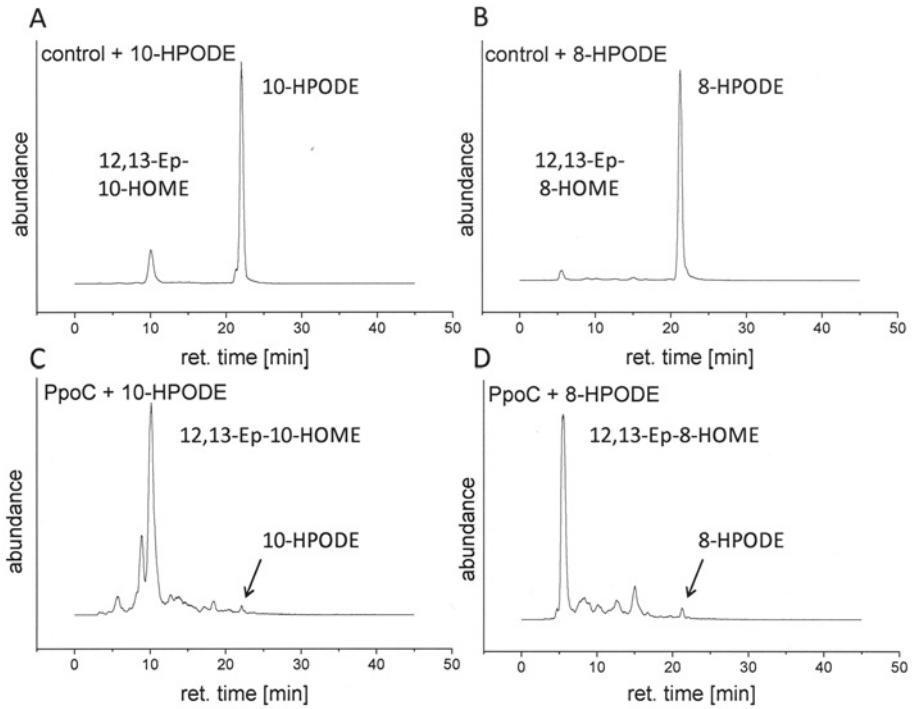

Figure 6 Conversion of 8-HPODE and 10-HPODE into epoxy alcohols by PpoC

The results from RP-HPLC/MS analysis after incubation of (**A** and **C**) 10-HPODE or (**B** and **D**) 8-HPODE with (**A** and **B**) empty vector controls or (**C** and **D**) recombinant PpoC bacterial crude extract after 40 min incubation at room temperature. Ret. time, retention time; Ep, epoxy.

Table 2 Kinetic parameters for different substrates

Kinetic analysis was performed with a Clark-type O_2 electrode. Initial time-dependent rates of oxygen consumption were measured with different substrate concentrations and K_m values were determined by fitting seven data points to the Michaelis–Menten equation using the software Origin 6.0. To prevent mycel formation the sodium salts of each fatty acid were used as a substrate for the kinetic analysis. Results are means ± S.D. calculated from three different experiments.

Substrate	K_m (μM)
$18{:}1^{\Delta 9Z}$	13.1 ± 1.7
$18{:}2^{\Delta 9Z,12Z}$	3.6 ± 0.97
$18{:}3^{\Delta 9Z,12Z,15Z}$	7.9 ± 1.6

was performed with PpoC crude extract; thus we were able to determine kinetic parameters that are independent of the enzyme concentration, such as the K_m. We used sodium salts of $18{:}1^{\Delta 9Z}$, $18{:}2^{\Delta 9Z,12Z}$ and $18{:}3^{\Delta 9Z,12Z,15Z}$ as substrates and measured the initial rate of oxygen consumption for the different concentrations in triplicate. The reaction was performed at pH 7.9 (pH optimum) and was started by the addition of substrate to the oxygen-saturated stirring solution. The K_m values are summarized in Table 2 and suggest that $18{:}2^{\Delta 9Z,12Z}$ may be the preferred substrate.

Investigating the epoxy alcohol synthase activity of Ppo enzymes

We detected the formation of 12,13-epoxy-10-HOME (approx. 3 % of all products formed) when PpoC was incubated with $18{:}2^{\Delta 9Z,12Z}$. It has been suggested that this product is formed from 10-HPODE by *A. fumigatus* PpoC [16]. To clarify the enzymatic formation of epoxy alcohols by PpoC, we incubated 10-HPODE with PpoC-containing bacterial crude extract. Clearly, formation of 12,13-epoxy-10-HOME could be detected. Next, we also incubated 8-HPODE with PpoC and detected the formation of the corresponding 12,13-epoxy-8-HOME. The individual RP-HPLC/MS chromatograms of both experiments are shown in Figure 6. The corresponding tandem MS spectra of both epoxy alcohol derivatives, which are in agreement with those published previously [16], are shown in Supplementary Figure S3(A) and S3(B) (available at http://www.BiochemJ.org/bj/425/bj4250553add.htm). It should be noted, however, that we also detected small amounts of both epoxy alcohols when using cell lysates of empty vector controls (Figures 6A and 6B). As the detected amounts in control assays were much lower compared with incubations with PpoC, we conclude that the major part of the detected epoxy alcohols are formed by an enzymatic reaction as proposed previously in [16], but by the peroxidase/dioxygenase domain of Ppo enzymes, rather than by the isomerase domain.

To investigate the origin of both oxygen atoms in the epoxy alcohol derivatives, we incubated $18{:}2^{\Delta 9Z,12Z}$ with bacterial crude lysate under an atmosphere enriched in $^{18}O_2$. The MS spectrum of the epoxy alcohol product is shown in Figure 7 and displays two major signals: *m/z* 311 corresponding to [^{16}O]-labelled 12,13-epoxy-10-HOME and *m/z* 315 for [^{18}O]-labelled 12,13-epoxy-10-HOME. As no abundant signal with *m/z* 313 could be detected, we conclude that the epoxy and the hydroxy group contain either two atoms of ^{16}O or two atoms of ^{18}O. This indicates that both oxygen atoms of the epoxy alcohol group are derived from the same substrate molecule, the fatty acid hydroperoxide.

We next examined a potential epoxy alcohol synthase activity of the related PpoA enzyme (purified to homogeneity). Therefore we incubated 10-HPODE with different concentrations of PpoA. In Figure 8 the relative amounts of 12,13-epoxy-10-HOME, 10-ODA and 10-HPODE are plotted against PpoA concentrations. We observed a linear correlation between the PpoA concentrations used and product formation or substrate consumption respectively. Thus we suggest PpoA exhibits

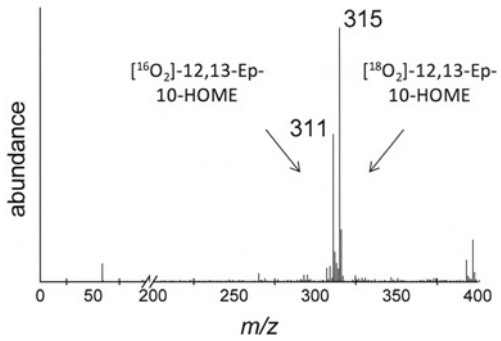

Figure 7 MS analysis of 12,13-epoxy-10-HOME formed from 18:2$^{\Delta 9Z,12Z}$ by PpoC under $^{18}O_2$

RP-HPLC/MS analysis of the 12,13-epoxy-10-HOME obtained after incubation of 18:2$^{\Delta 9Z,12Z}$ with recombinant PpoC bacterial crude extract under an enriched $^{18}O_2$ atmosphere.

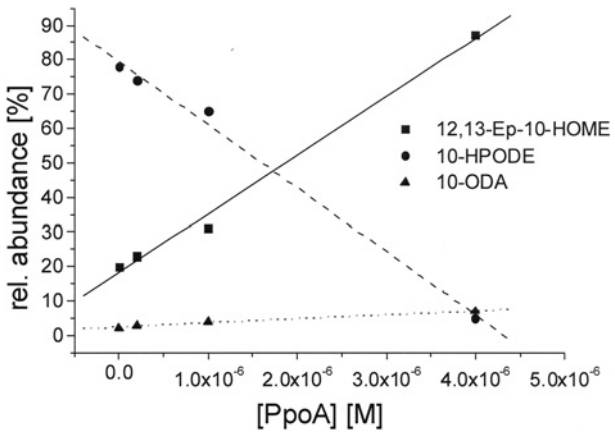

Figure 8 Conversion of 10-HPODE into 12,13-epoxy-10-HOME and 10-ODA as a function of PpoA concentration

The reaction system contained 500 μM 10-HPODE and the indicated concentration of purified PpoA in 50 μM Hepes, pH 7.4. The reaction was terminated after 40 min. Ep, epoxy.

epoxy alcohol synthase activity with 10-HPODE as a substrate, paralleling the results obtained with PpoC (see above). As reported previously 8-HPODE is predominantly converted into 5,8-DiHODE by the 8-hydroperoxide isomerase P450 domain of PpoA [14]. The tandem MS spectrum of this product is shown in Supplementary Figure S3(C). Additionally, we detected traces of 12,13-epoxy-8-HOME formed by PpoA from 8-HPODE.

Site-directed mutagenesis of determinants that control site of dioxygenation

Val^{385} is a conserved residue in the peroxidase/dioxygenase domain of PpoC, PpoA (Val^{328}), 7,8-linoleate diol synthase (Val^{330}) and PGHS (Val^{349} in sheep PGHS-1). In the latter, Val^{349} is known to be important for substrate positioning and the dioxygenase reaction [29]. To compare the oxygenation mechanisms of *A. nidulans* PpoA and PpoC, we mutated the corresponding amino acids in both enzymes; Leu^{385} of PpoC was replaced with either valine or alanine, whereas Val^{328} of PpoA was replaced with either alanine or leucine. Crude bacterial extract from wild-type or mutated PpoC and purified wild-type or mutated PpoA was incubated with 18:2$^{\Delta 9Z,12Z}$. The relative amounts of hydroxy products formed were determined by RP-HPLC/MS and the results are visualized as column diagrams (Figure 9). For

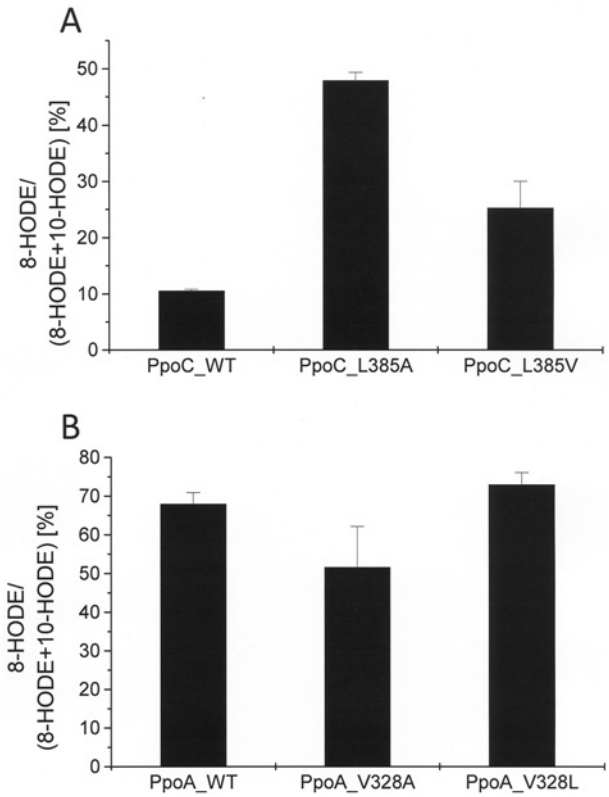

Figure 9 Relative amounts of 8-HODE formed by wild-type PpoC and PpoA compared with their corresponding mutants

(**A**) Relative amounts of 8-HODE formed by recombinant wild-type PpoC (PpoC_WT), PpoC with the L385A substitution (PpoC_L385A) and PpoC with the L385V substitution (PpoC_L385V) bacterial crude extract in 50mM Tris/HCl, pH 8.0. (**B**) Relative amounts of 8-HODE formed by purified wild-type PpoA (PpoA_WT), PpoA with the V328A substitution (PpoC_V328A) and PpoA with theV328L substitution (PpoC_V328L) in 50 mM Hepes, pH 7.4. Results are means ± S.D. calculated from three different experiments.

PpoC (Figure 9A) we observed that replacement of Leu^{385} with the smaller valine residue enhanced the formation of 8-HODE by 2–3-fold compared with the wild-type enzyme. Reducing the size to a smaller residue, alanine, enhanced the formation of 8-HODE by 3–4-fold as judged by RP-HPLC/MS analysis.

We previously reported that we were unable to detect formation of any 10-HODE/10-HPODE during incubations of 18:2$^{\Delta 9Z,12Z}$ and PpoA by RP-HPLC. Using an improved RP-HPLC/MS approach we now observed the formation of 10-HODE/10-HPODE and especially found that approx. 30 % of the formed hydroxides were 10-HODE (Figure 9B). The replacement of Val^{328} in PpoA with an alanine residue reduced the formation of 8-HODE and increased the production of 10-HODE, whereas replacement with leucine only led to a modest increase in 8-HODE formation compared with the wild-type enzyme (Figure 9B).

Site-directed mutagenesis of the catalytic tyrosine residue

As shown in Figure 1(A) Tyr^{427} of *A. nidulans* PpoC is conserved in the enzymes analysed. Previous mutagenesis studies have indicated that this tyrosine residue is important for catalytic activity [14,29–31] presumably by generating a tyrosyl radical [32,33]. Replacement of PpoC Tyr^{427} with phenylalanine (Y427F) led to an inactive enzyme that showed no detectable dioxygenase activity when incubated with 18:2$^{\Delta 9Z,12Z}$. The expression of the PpoC(Y427F) mutant was confirmed by SDS/PAGE

analysis (see Supplementary Figure 4 available at available at http://www.BiochemJ.org/bj/425/bj4250553add.htm).

Site-directed mutagenesis of the predicted P450 haem-thiolate domain

As shown in Figure 2(B) we found that the predicted P450 domain of PpoC does not contain the conserved cysteine residue that is known to be the fifth haem iron ligand in P450 enzymes [34]. Substitution of this cysteine residue (Cys^{1006}) in PpoA for an alanine residue led to an enzyme that showed dioxygenase activity, but no detectable isomerase activity, presumably through distortion of the P450 haem moiety [14]. As PpoC contains a glycine residue instead of a cysteine residue at this particular position, we hypothesized that replacement of Gly^{1039} with a cysteine residue may reconstitute the haem-binding moiety and thereby lead to an enzyme with isomerase activity, as it is known for PpoA. However, we did not detect formation of any DiHODE derivative when PpoC(G1039C) was incubated with $18{:}2^{\Delta 9Z,12Z}$. Moreover, the enzyme showed the same catalytic activity as the wild-type enzyme. Moreover, we also incubated PpoC(G1039C) with 8-HPODE and upon this incubation with 8-HPODE no DiHODE was detectable.

Radical spin-trapping adducts

In order to investigate the formation of carbon-centred fatty acid radicals during the reaction of PpoC and $18{:}2^{\Delta 9Z,12Z}$ we used the method of Koshiishi and co-workers to scavenge the hypothetical radical with the radical scavenger CmΔP (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl) [35,36]. Recently, we adapted this approach for the PpoA/linoleate system and found the formation of two radical spin-trapping adducts, which we tentatively assigned as the trapped C-8 and C-10 radical adducts [14]; this finding that now proves to be consistent with our observation that PpoA is also able to form 10-HODE/10-HPODE (Figure 9B). To test the existence of radical intermediates in the PpoC/linoleate system we performed the trapping reaction with CmΔP using PpoC and $18{:}2^{\Delta 9Z,12Z}$. Linoleate allyl radical CmΔP adducts of *m/z* 463 were identified using RP-HPLC/MS. Two distinct peaks were be detected: a major peak eluting at 28.8 min and a smaller one at 30.8 min [14]. We suggest from this result that the major peak corresponds to the trapped C-8 radical adduct, whereas the smaller peak may correspond to the C-10 radical adduct, in analogy with the PpoA/linoleate system. The MS spectra of both trapping adducts of the PpoC/linoleate system showed a characteristic fragment with *m/z* 185; this fragment arose from the reduced form of CmΔP through heterolysis of the C–O–N bond [36]. We also detected trapping adducts with *m/z* 479 that may correspond to nitroxyl radical–lipid epoxy allyl adducts [36].

DISCUSSION

The present study aimed to characterize *A. nidulans* PpoC, a putative fatty acid dioxygenase, and to compare it with *A. nidulans* PpoA, another member of the *A. nidulans* Ppo-family. The Ppo enzyme family in *A. nidulans* consists of three dioxygenase proteins, PpoA, PpoB and PpoC. Genetic evidence indicated Ppo products were involved in the regulation of the developmental life cycle of *A. nidulans* [13]. Interestingly, an NCBI-conserved-domain search predicted that all three protein sequences seem to be fusion proteins because of the presence of two conserved domains: a haem peroxidase/dioxygenase domain in the N-terminal region of the enzymes and a cytochrome P450 region in the C-terminal part. Recently, we showed that the bifunctional activity of PpoA is indeed related to the presence of both domains: within the peroxidase/dioxygenase domain of PpoA the (8*R*)-dioxygenase reaction takes place, whereas in the P450 domain the (8*R*)-hydroperoxide isomerization is accomplished [14]. Although PpoC is predicted to be a fusion protein with a similar C-terminal P450 domain, we found on the basis of sequence alignments, that the crucial cysteine residue is not conserved in its sequence (Figure 2B). In P450 enzymes the corresponding residue acts as the fifth haem iron ligand and mutation of this cysteine residue leads to an inactive enzyme [37]. Therefore it appeared doubtful that this domain is functional in PpoC and has catalytic activity. To analyse the influence of this changed domain structure we isolated *PpoC* cDNA, expressed the enzyme in *E. coli* and investigated its biochemical properties.

Our results indicate that both fusion proteins, PpoA and PpoC, have many properties in common, but there are also essential differences that are most likely caused by the differences in their C-terminal P450 domain. PpoA and PpoC resemble 7,8-linoleate diol synthase tetrameric haem proteins that catalyse the oxidation of $18{:}2^{\Delta 9Z,12Z}$. Whereas PpoA predominantly oxidizes $18{:}2^{\Delta 9Z,12Z}$ at the C-8, yielding 8-HPODE, PpoC mainly performs oxygenation at the C-10 forming 10-HPODE. These dioxygenase reactions are both catalysed within the peroxidase/dioxygenase domain of the fusion proteins (Figure 9). Sequence alignments showed that PpoA and PpoC contain the conserved tyrosine residue within their haem peroxidase/dioxygenase domain (Tyr^{374} in PpoA and Tyr^{427} in PpoC) that is also found in 7,8-linoleate diol synthase (Tyr^{376}), sheep PGHS-1 (Tyr^{385}) and sheep PGHS-2 (Tyr^{370}). Previous studies showed that dioxygenase activity was reduced or even abolished in these enzymes when this tyrosine residue was mutated [14,29,30]. As also reported previously for PpoA, we now provide evidence that replacement of this tyrosine residue with a phenylalanine residue also abolished the dioxygenase activity of PpoC. Thus we conclude that PpoC also uses a tyrosyl radical for the abstraction of the hydrogen from the C-8 of $18{:}2^{\Delta 9Z,12Z}$ and forms a carbon-centred radical at this position. The unpaired electron is delocalized and can migrate from the C-8 to the C-10 by shifting the C-9–C-10 double bond to C-8–C-9. The formation of carbon-centred substrate radicals at the C-8 and C-10 was also supported by experiments in which we trapped these radicals using the radical scavenger CmΔP as reported before for other enzymes [14,36].

The results thus far led us to ask what the amino acid determinants that directed the oxygenation at C-8 or C-10 respectively were. Recently, Garscha et al. [16] reported the recombinant expression of a PpoC from *A. fumigatus* that catalyses a similar reaction to PpoC. By replacing Leu^{384} and Val^{388} with larger and smaller amino acids they could show that these residues act as determinants that direct oxygenation either at C-8 or C-10 of $18{:}2^{\Delta 9Z,12Z}$ [16]. As similar substitutions of the equivalent residue in *G. graminis* 7,8-linoleate diol synthase (Val^{330}) showed a related effect, they developed a general model for the oxygenation by fungal 8-dioxygenases and 10-dioxygenases respectively. This model proposed that Leu^{384} shields the C-8 of $18{:}2^{\Delta 9Z,12Z}$ in the active site of (10*R*)-dioxygenase or PpoC [16]. To gain further insights into the oxygenation mechanisms, we also mutated the related Val^{328} (in PpoA) and Leu^{385} (in PpoC) in the Ppo enzymes from *A. nidulans*. This experiment enabled, for the first time, the direct comparison of two dioxygenases present in the same species, with highly similar amino acid sequences, but different product specificities. Replacement of PpoC Leu^{385} with an alanine residue or a valine

residue gave similar effects as reported previously [16] that may also indicate that Leu^{385} shields the C-8 of 18:2$^{\Delta 9Z,12Z}$. However, the replacement of Val^{328} in PpoA with a leucine residue or an alanine residue conversely indicated that this residue might shield C-10 rather than C-8. This effect may be explained by a different positioning, or even by a different orientation, of the fatty acid in the active site of both enzymes, leading to the conclusion that our results, in contrast with those of Garscha et al. [16], indicate that there either is no general model for oxygenation by these fungal dioxygenases or that the role of the mutated amino acids is as yet unclear. Clearly a crystal structure is needed to further address this question. A further explanation may be that the isomerase activity of recombinant 7,8-linoleate diol synthase is very weak and, therefore, 8-HODE/8-HPODE can accumulate. This accumulation might influence the equilibrium in a different way compared with PpoA, where almost all 8-HPODE is immediately converted into 5,8-DiHODE.

We investigated further the downstream products of the dioxygenase reaction of PpoC. The 10-HPODE that was formed during incubations of PpoC and 18:2$^{\Delta 9Z,12Z}$ was mainly converted into 10-HODE, which was formed by an enzymatic peroxidase reaction of PpoC within the C-terminal haem peroxidase/dioxygenase domain. Another product of this domain was 10-KODE. However, 10-HPODE appeared to be unstable on its own and 10-ODA and volatile compounds such as 2-octen-1-ol, 2-octenal, 3-octanone and 1-octen-3-ol were formed, most likely by chemical fragmentation from this PpoC product. The latter compound has also been reported to be formed from 18:2$^{\Delta 9Z,12Z}$ by homogenates of *Lentinus decadetes* [38], *Psalliota bispora* [23–25] and *Tricholoma matsutake* [39]. Although the reaction was discovered 25 years ago, the enzyme involved in formation of these compounds, known for the mushroom flavour, was never identified from either of these sources. Interestingly, at first an independent metabolic reaction leading to formation of the same volatiles 2-octen-1-ol and 1-octen-3-ol was described for the moss *Physcomitrella patens* [27]. Here a bifunctional lipoxygenase uses the C-20 fatty acid arachidonic acid and the C-8 alcohols are formed via the (12*S*)-hydroperoxide of this fatty acid [40].

As a further product 12,13-epoxy-10-HOME was formed during the reaction of PpoC and 18:2$^{\Delta 9Z,12Z}$. This product has also been reported to be formed by PpoC from *A. fumigatus* [16]. It was suggested that this enzyme has epoxy alcohol synthase activity and specifically converts 10-HPODE into 12,13-epoxy-10-HOME in analogy with the isomerization reaction that is performed with 8-HPODE by 7,8-linoleate diol synthase [16]. Indeed, by incubating purified 10-HPODE with PpoC bacterial crude extract we confirmed the enzymatic conversion of this substrate into 12,13-epoxy-10-HOME. Thus we showed for the first time that 10-HPODE is in fact the precursor of 12,13-epoxy-10-HOME. Additionally, we found that PpoC can also convert 8-HPODE into 12,13-epoxy-8-HOME but not into 5,8-DiHODE. This finding underlines that a functional P450 domain, as in case of PpoA, is essential for an isomerization reaction but not for epoxy alcohol synthase activity.

As 7,8-linoleate diol synthase displays its bifunctionality by conversion of 18:2$^{\Delta 9Z,12Z}$ into 8-HPODE, followed by the isomerization yielding 7,8-DiHODE, we next investigated whether purified PpoA also harboured a similar epoxy alcohol synthase side activity, besides the described dioxygenase and isomerase activity. Incubations of purified PpoA and 8-HPODE led mainly to the formation of 5,8-DiHODE by the 8-hydroperoxide isomerase P450 domain as reported. As we also detected low amounts (< 1 % of total reaction products) of 12,13-epoxy-8-HOME during these incubations we concluded that, like PpoC, PpoA also harbours an epoxy alcohol synthase side activity. This was further confirmed by incubations of PpoA with 10-HPODE (Figure 8). In contrast with 8-HPODE this substrate was not converted into a dihydroxy fatty acid but rather transformed into 12,13-epoxy-10-HOME. The finding that the amount of 12,13-epoxy-10-HOME showed a linear dependency on the concentration of the PpoA used in the experiment additionally indicated an enzymatic reaction. However, as we also found production of epoxy alcohols in negative controls, in minor amounts, we suggest that epoxy alcohol derivatives can also be formed non-enzymatically from 10-HPODE and 8-HPODE under the used assay conditions.

As the isomerase activity of the bifunctional PpoA is located in the P450 domain and PpoC seems to contain an inactive P450 domain, due to the lack of a highly conserved cysteine residue, we tried to obtain a bifunctional PpoC by mutation analysis. To reconstitute the P450 haem-binding moiety and to obtain a functional 8-hydroperoxide isomerase domain we replaced Gly^{1039} with a cysteine residue and incubated the enzyme with 18:2$^{\Delta 9Z,12Z}$ and 8-HPODE. No functional 8-hydroperoxide isomerase activity was retrieved. This might indicate that several more residues in the PpoC sequence are important for correct binding of haem and a correct folding of the P450 domain. However, for conclusive identification of further essential amino acids which constitute a functional P450 motif a crystal structure will be required. A domain-switching approach may also be suitable to address this question in the future, but the lack of information about the crystal structure of the enzyme is also hindering further investigations in that direction.

Taken together, our results show that PpoA and PpoC are fusion proteins that employ similar mechanisms to catalyse the formation of 8- and 10-hydroperoxy fatty acids. In this context the leucine/valine residues that have been reported recently for *A. fumigatus* PpoC and *G. graminis* 7,8-linoleate diol synthase seem also to determine the position of oxygenation of 18:2$^{\Delta 9Z,12Z}$, i.e. C-8 and C-10, of dioxygenases in *A. nidulans*, but a general mechanism as to how these amino acid residues influence the site of oxygenation remains unclear. PpoC and PpoA both have an epoxy alcohol synthase side activity and can convert 8-HPODE and 10-HPODE into the corresponding epoxy alcohols. However, this is employed by the peroxidase/dioxygenase domain of these enzymes. This is supported further by the fact that PpoA converts 8-HPODE mainly into 5,8-DiHODE, whereas PpoC cannot perform a similar reaction, probably due to a degenerated P450 haem-thiolate domain.

AUTHOR CONTRIBUTION

Florian Brodhun and Ivo Feussner designed the research. Florian Brodhun, Stefan Schneider, Cornelia Göbel and Ellen Hornung performed the experiments. Florian Brodhun, Stefan Schneider, Cornelia Göbel, Ellen Hornung and Ivo Feussner analysed the data. Florian Brodhun, Cornelia Göbel, Ellen Hornung and Ivo Feussner wrote the manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

PpoC from *Aspergillus nidulans* is a fusion protein with only one active haem

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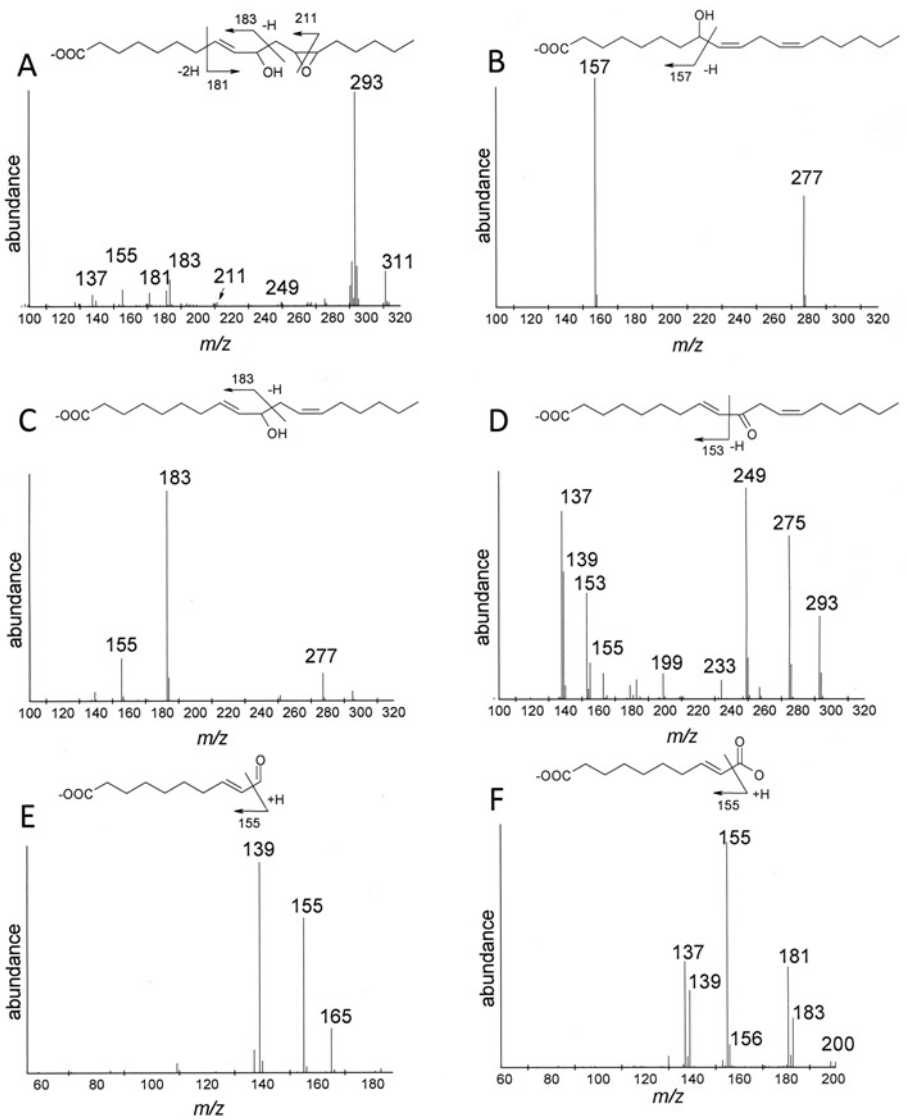

Figure S1 Tandem MS analysis of products formed from 18:2$^{\Delta 9Z,12Z}$ by PpoC

Tandem MS spectra of (**A**) 12,13-epoxy-10-HOME, (**B**) 8-HODE, (**C**) 10-HODE, (**D**) 10-KODE and (**E**) 10-ODA are shown, which were formed by PpoC bacterial crude extract and 18:2$^{\Delta 9Z,12Z}$. Additionally, in (**F**) the tandem MS spectrum of 2-decendioic acid is shown, which was obtained after incubations of PpoC with 18:3$^{\Delta 9Z,12Z,15Z}$. Above each tandem MS spectrum the characteristic fragmentation patterns are indicated, as reported previously [1,2].

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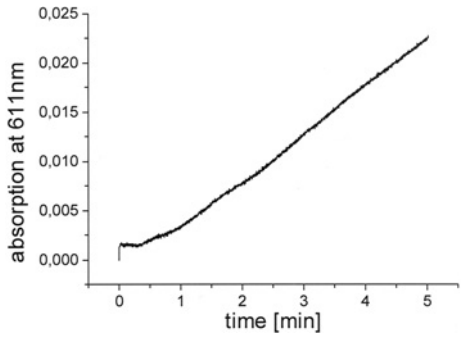

Figure S2 Peroxidase activity assay of recombinant and purified PpoC

For the peroxidase activity assay purified PpoC was incubated with 100 μM 18:2$^{\Delta 9Z,12Z}$ and 100 μM TMPD. The increase in the absorption at 611 nm, due to oxidized TMPD, is visualized as a function of time.

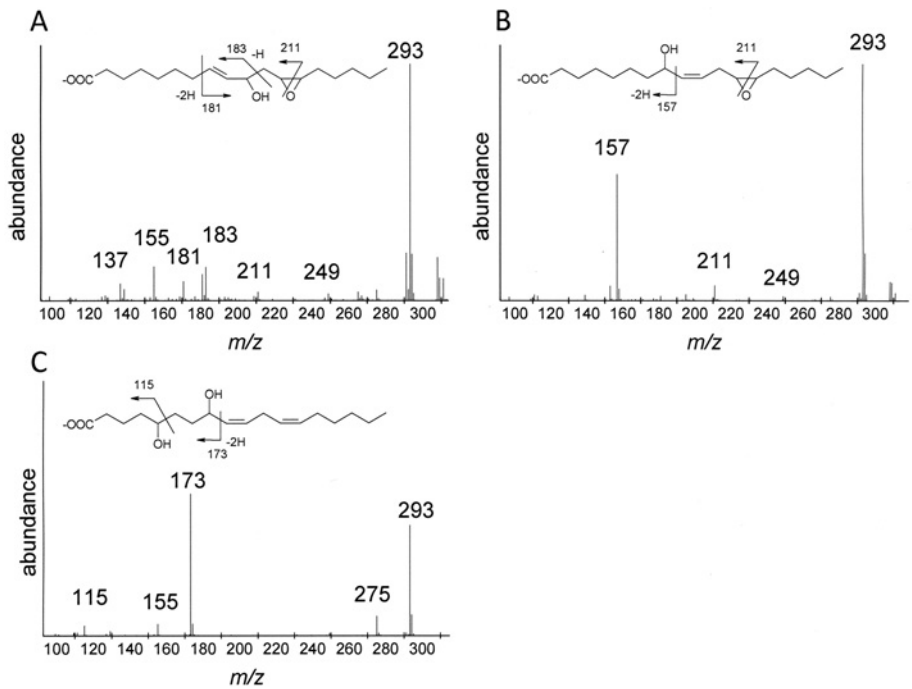

Figure S3 Tandem MS analysis of products formed from 10-HPODE and 8-HPODE by PpoC and PpoA

(**A**) Tandem MS spectrum of 12,13-epoxy-10-HOME that was either formed from incubation of 10-HPODE with purified PpoA or PpoC bacterial crude extract. (**B**) Tandem MS spectrum of 12,13-epoxy-8-HOME that was formed by PpoC bacterial crude extract incubated with 8-HPODE. (**C**) PpoA formed from 8-HPODE predominantly formed 5,8-DiHODE as reported previously [3].

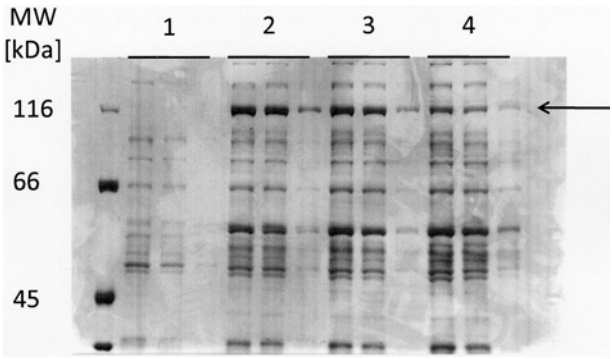

Figure S4 SDS/PAGE of expressed recombinant PpoC mutants

A SDS/PAGE analysis of different PpoC mutants is shown. Lane 1, empty vector control; lane 2: PpoC with the L385A substitution; lane 3: PpoC with the Y427F substitution; lane 4: PpoC with the G1039C substitution. MW, molecular-mass markers in kDa.

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