Crystal Structures of Physcomitrella patens AOC1 and AOC2: Insights into the Enzyme Mechanism and Differences in Substrate Specificity1[W][OA]

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In plants, oxylipins regulate developmental processes and defense responses. The first specific step in the biosynthesis of the cyclopentanone class of oxylipins is catalyzed by allene oxide cyclase (AOC) that forms cis(+)-12-oxo-phytodienoic acid. The moss Physcomitrella patens has two AOCs (PpAOC1 and PpAOC2) with different substrate specificities for C18- and C20-derived substrates, respectively. To better understand AOC’s catalytic mechanism and to elucidate the structural properties that explain the differences in substrate specificity, we solved and analyzed the crystal structures of 36 monomers of both apo and ligand complexes of PpAOC1 and PpAOC2. From these data, we propose the following intermediates in AOC catalysis: (1) a resting state of the apo enzyme with a closed conformation, (2) a first shallow binding mode, followed by (3) a tight binding of the substrate accompanied by conformational changes in the binding pocket, and (4) initiation of the catalytic cycle by opening of the epoxide ring. As expected, the substrate dihydro analog cis-12,13-EOT did not cyclize in the presence of PpAOC1; however, when bound to the enzyme, it underwent isomerization into the corresponding trans-epoxide. By comparing complex structures of the C18 substrate analog with in silico modeling of the C20 substrate analog bound to the enzyme, we were able to identify three major molecular determinants responsible for the different substrate specificities (i.e. larger active site diameter, an elongated cavity of PpAOC2, and two nonidentical residues at the entrance of the active site).

Oxylipins comprise a large family of oxidized fatty acids and metabolites thereof (Acosta and Farmer, 2010). They are abundant in mammals (Funk, 2001) and flowering plants (Creelman and Mulpuri, 2002). In addition, they have been found in fungi (Brodhun and Feussner, 2011) as well as nonflowering plants like mosses and algae (Andreou et al., 2009). In plants, these lipids serve as signaling molecules regulating developmental processes and mediating defense responses (Howe and Jander, 2008; Browse, 2009; Acosta and Farmer, 2010). The first committed step in oxylipin biosynthesis is the peroxidation of a polyunsaturated fatty acid containing a 1Z,4Z-pentadiene system by lipoxygenase (LOX) or the peroxidation at the C2 position of a fatty acid by α-dioxygenase. These reactions start the so-called LOX or oxylipin pathway (Feussner and Wasternack, 2002) and are followed by further enzymatic reactions in which the hydroperoxy fatty acid is converted to a set of different secondary products. In the case of LOX-derived hydroperoxy fatty acids, such conversions are mainly catalyzed by members of the cytochrome P450 subfamily Cyp74 (i.e. fatty acid hydroperoxide lyase, divinyl ether synthase, epoxy alcohol synthase, and allene oxide synthase [AOS]; Stumpe and Feussner, 2006; Lee et al., 2008). Additional conversions of the fatty acid hydroperoxide are catalyzed by other proteins, such as LOX or peroxygenase (Mosblech et al., 2009).

Jasmonic acid (JA) biosynthesis is one specific branch of the oxylipin pathway. It may start with the release of α-linolenic acid [18:3(n-3)] from membrane lipids by a lipase (Schaller and Stintzi, 2009). This free fatty acid is subsequently oxidized by a 13-LOX to yield 13-hydroperoxy octadecatetraenoic acid (13-HPOTE) and converted by the action of AOS into the unstable allene oxide 12,13-EOPA. In the presence of AOC, the epoxide

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is hydrolyzed into ketols and racemic 12-oxo-phytodienoic acid (OPDA). cis(+)OPDA is the first cyclic and biologically active compound in that pathway (Dave and Graham, 2012). While the reactions leading from 18:3 (n-3) to cis(+)OPDA occur in the plastid, all further enzymatic steps resulting in the formation of JA are localized in the peroxisomes (Wasternack, 2007). Here, cis(+)OPDA is reduced in a NADPH-dependent reaction by cis(+)OPDA reductase isoform 3 (OPR3), the octanoic or hexanoic side chain is shortened by $\beta$-oxidation cycles.

The conversion of 13-HPOTE into cis(+)OPDA was first observed using a flaxseed (Linum usitatissimum) acetone powder preparation and was suggested to take place via a hypothetical epoxide intermediate (Vick et al., 1980). Later studies unequivocally demonstrated that 12,13-EOT (Hamberg, 1987; Brash et al., 1988), an allene oxide formed from 13-HPOTE by AOS (Song and Brash, 1991; Song et al., 1993), serves as substrate for the cyclization reaction catalyzed by AOC (Hamberg and Fahlstadius, 1990). The enzyme was purified (Ziegler et al., 1997), characterized with regard to the substrate specificity (Ziegler et al., 1999), and cloned and recombinantly expressed (Ziegler et al., 2000; Stenzel et al., 2003). In 2006, the crystal structure of an AOC from Arabidopsis (Arabidopsis thaliana; AtAOC2) was solved (Hofmann et al., 2006), and the reaction mechanism as well as the subcellular...
localization were studied (Schaller et al., 2008). The enzyme crystallized as a homotrimer, with each subunit forming an eight-stranded antiparallel β-barrel harboring a hydrophobic cavity in which the active site of the enzyme is located. While the exterior loops showed a high degree of flexibility, the central part of the enzyme was very rigid, and no induced-fit mechanism could be observed upon binding of a substrate analog (Hofmann et al., 2006). Based on the structure of AtAOC2 in complex with vermic acid [cis(+/-)-12,13-epoxy-9Z-octadecenoic acid (12,13-EM)] as an inert substrate analog, the following reaction mechanism has been proposed (Fig. 1, box): the allene oxide substrate binds with its fatty acid backbone deep in the barrel, where it interacts with hydrophobic amino acid residues, while the polar carboxy head group is located on the exterior of the cavity. One particular Glu residue (Glu-23 in AtAOC2) pointing to the Δ15Z-double bond of the substrate may induce a partial charge separation that leads to a delocalization of the π-electron system, thereby facilitating opening of the epoxide ring. The oxyanion thus formed is stabilized via polar interactions with a catalytic, structurally conserved water molecule that is positioned in the polar cavity of the enzyme formed by two Asn residues (Asn-25 and Asn-53 in AtAOC2, respectively), one Ser (Ser-31 in AtAOC2), and one Pro (Pro-32 in AtAOC2). The ring closure that leads to the formation of the cyclopentenone derivative is achieved by a conformational reorganization of the C10-C11 substrate bond from the trans- to the cis-geometry. Due to steric limitations in the active site, this rotation may be accompanied by a cis/trans-isomerization of the C8-C9 substrate bond. Since the enzyme dictates the stereochemistry of the final ring closure, the released product is exclusively the (+)-enantiomer, cis(+)-OPDA (Schaller et al., 2008). Notably, this reaction competes with the spontaneous decomposition of the allene oxide substrate that leads to the formation of racemic OPDA as well as α-ketols and γ-ketols. This hints toward a low-energy barrier of the cyclization reaction and suggests that AOC does not need much of a catalytic functionality in terms of lowering this barrier (Schaller and Stintzi, 2009). It has been proposed that the enzymatic cyclization reaction is achieved according to the rules of Hoffmann and Woodward (1970) via a concerted pericyclic ring closure while spontaneous cyclization proceeds through a dipolar ring closure (Grechkin et al., 2002). The facts that the allene oxide formed by AOS has a very short half-life in aqueous solution and that natural OPDA is found in its enantiopure cis(+)-configuration suggest that AOS and AOC are coupled. However, no physical interaction of both enzymes may be necessary to form cis (+)-OPDA in vitro (Zerbe et al., 2007).

Recently, it was shown that the moss Physcomitrella patens harbors and metabolizes not only C18 but also C20 polyunsaturated fatty acids to form oxylipins (Fig. 2; Stumpe et al., 2010). In particular, it was shown that (12S)-hydroperoxy eicosatetraenoic acid (12-HPETE) is endogenously formed by a bifunctional LOX as the major hydroperoxy fatty acid of arachidonic acid [20:4 (n-6)] (Wichard et al., 2004). 12-HPETE serves as a
substrate for further conversions either leading to the formation of C₄₀ and C₃₀ volatiles (e.g. octenals, octenols, and nonenals) or the cyclopentenone derivative 11-oxo prostatrienic acid (11-OPTA; Stumpe et al., 2010). Whereas the volatiles are formed by at least two bi-functional LOXs with an additional hydroperoxide lyase activity (Wichard et al., 2004; Senger et al., 2005; Anterola et al., 2009) or by a Cyp74-derived hydroperoxide lyase (Stumpe et al., 2006), 11-OPTA is formed in analogy to the octadecanoids by one particular AOC, PpAOC2, via the allene oxide intermediate formed by PpAOS (Bandara et al., 2009). In contrast, PpAOC1 does not accept the 12-HPETE-derived C₂₀-allene oxide and thus converts only the 13-HPOTE-derived allene oxide.

In this study, the crystal structures of PpAOC1 and PpAOC2 were solved. Data were also obtained for mutated forms of PpAOC1 and for PpAOC1 and PpAOC2 in complex with the allene oxide stable analog 12,13-EOD. In this way, detailed information about the allene oxide-to-cyclopentenone conversions promoted by the two AOCs was obtained.

RESULTS
Expression and Purification
In order to optimize the protein yield in *Escherichia coli* of previously reported PpAOC1 and PpAOC2 constructs (Stumpe et al., 2010), both open reading frames were expressed as glutathione S-transferase (GST) fusion proteins. They could be obtained in high yield by affinity chromatography using reduced glutathione (GSH)-Sepharose. N-terminally fused GST was subsequently cleaved and separated from AOC by anion-exchange chromatography. The last purification step using gel-filtration chromatography was used to remove large and small protein aggregates. Purification was followed by SDS-PAGE (Supplemental Fig. S1) and yielded 20 to 30 mg AOC L⁻¹ culture and resulted in nearly homogenous protein.

Overall Structure
The crystal structures of both PpAOC isoforms have been solved by molecular replacement, resulting in the location of four trimers of PpAOC1 and of two trimers of PpAOC2 per asymmetric unit (for PpAOC2, see Supplemental Fig. S2A). The structure of apo PpAOC1 has been refined at 1.35 Å resolution to crystallographic R factors of 13.9% and 17.5% for R_work and R_free, respectively. The PpAOC2 diffracted to the highest resolution limit of 1.98 Å and has been refined to R_work and R_free factors of 18.9% and 23.3%, respectively. The 12,13-EOD complex structures of both isoforms have been determined at similar resolution as the apo structures and refined to good crystallographic R factors (Table I). The monomers of PpAOC1 and PpAOC2 fold into an eight-stranded antiparallel β-barrel, forming an elongated cavity containing the ligand-binding site with the overall structure very similar to that of AOC2 from Arabidopsis (AtAOC2) described previously (Hofmann and Pollmann, 2008; Fig. 3, A and B, the entrance of the substrate-binding site is marked by the gray substrate molecule). The calculated root mean square deviation (RMSD) against the monomer of AtAOC2 (Protein Data Bank [PDB] accession code 2BRJ) amounts to 0.51 Å (163 matched Cα atoms, 105 conserved in sequence) and 0.80 Å (163 matched Cα atoms, 100 conserved in sequence) for the monomers of PpAOC1 and PpAOC2, respectively. Similar RMSD values have been obtained when comparing the trimer of AtAOC2 with the trimers of both PpAOCs isoforms (PpAOC1, 0.74 Å for 515 matched Cα positions; PpAOC2, 0.85 Å for 504 equivalent Cα positions), revealing exactly the same trimeric quaternary structure. The largest differences between AtAOC2 and PpAOC2 monomers can be seen in the conformations of two out of three flexible loops (AtAOC2, 149–151 and 38–46; PpAOC1, 161–170 and 33–45; Fig. 3A). The later loop in both PpAOCs isoforms contains a seven-amino-acid-long insertion in comparison with AtAOC2 (Supplemental Fig. S3) and is not traceable in some monomers due to its high flexibility.

Comparison of PpAOC1 and PpAOC2
The two PpAOC isoforms share 76% sequence identity and are structurally very similar. The calculated RMSD between the monomers of the two structures amounts to 0.83 Å for 175 matched Cα positions, of which 139 are identical in sequence (Fig. 3, A and B; Supplemental Fig. S3), but the sequence of PpAOC2 is one amino acid shorter in comparison with PpAOC1 due to a deletion at position 39 localized in one of the three flexible loops (Fig. 3A; Supplemental Fig. S3). As a consequence, there is an offset in numbering between the equivalent residues of both isoforms starting from that position. Sequence comparison of the two PpAOCs reveals 46 not conserved residues, with half of them representing conservative changes (Supplemental Fig. S3). The differing amino acid positions are located mostly on the protein surface, predominantly in the loop regions. In PpAOC1, these are the following amino acids: 33 to 45, 79 to 83, and 161 to 170. Interestingly, two amino acid positions (PpAOC1 Leu-72 [PpAOC2 Ile] and PpAOC1 Leu-90 [PpAOC2 Gln]) are found on the β-barrel surface, just between the regions responsible for trimerization (Fig. 3B, red asterisks). The side chain of PpAOC2 Ile-71 forms a weak (distances vary between 3.3 and 3.5 Å for different monomers) interaction with the side chain of Thr-73 from the neighboring monomer. However, this weak interaction does not affect formation of the trimer, which appears to be a relevant oligomerization state also in vivo (Ziegler et al., 1997). This hypothesis is supported by the fact that all AOCs determined structurally so far crystallized as trimers (Hofmann and Pollmann, 2008). Another differing amino acid position,
PpAOC1 Ile-56 (PpAOC2 Val) in the b-strand 2 (Fig. 3A), occurs on the inner surface of the b-barrel, toward the N terminus. Interestingly, two differences in amino acid positions are located near the entrance of the active site, namely PpAOC1 Phe-29 (PpAOC2 Ile) and PpAOC1 Phe-140 (PpAOC2 Val; Fig. 3). Because of their localization, the latter may be candidates for determinants that cause the observed difference in substrate specificity.

In order to analyze this hypothesis, we generated PpAOC1 single and double variants with (1) a Phe-140-Val substitution (PpAOC1_F140V) and (2) an additional Phe-29-Ile exchange (PpAOC1_F29I/F140V). Both variants were purified as described for the wild-type enzymes. The catalytic activity of both variants was evaluated by incubating a mixture of PpAOS and PpAOC (molar ratio of 0.001) with either 12-HPETE or 13-HPOTE as the control experiment. The formed products were analyzed and quantified (Fig. 4). Incubations of 18:3(n-3)-derived 13-HPOTE with the different enzyme variants yielded significant amounts of OPDA (47%–65%), indicating that all enzyme variants were active with the 13-HPOTEderived allene oxide (Fig. 4, bottom). However, when incubated with 20:4(n-6)-derived 12-HPETE, we observed the formation of 11-OPTA only with PpAOC2 (Fig. 4, top). Neither PpAOC1, the single PpAOC1_F140V variant, nor the double PpAOC1_F29I/F140V variant was able to convert the 12-HPETE-derived allene oxide into 11-OPTA.

The superposition of PpAOC1 and PpAOC2 monomers revealed three regions that exhibit significant differences: two flexible loops (PpAOC1 from amino acids 33–45 and 79–83) and the N terminus (including the loop from amino acids 161–170; Fig. 3A). The N-terminal 10 residues of PpAOC1 are not defined in the electron density map. In contrast, these residues are traceable in PpAOC2 and fold into an amphipathic a-helix protruding out of the monomer (Fig. 3A; Supplemental Fig. S2). Three helices of one PpAOC2

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**Table I. Data collection and refinement statistics**

| Data | Apo PpAOC1 | PpAOC1 12,13-EOD Complex | Apo PpAOC2 | PpAOC1 12,13-EOD Complex |
|------|------------|--------------------------|------------|--------------------------|
| Space group | P1 | P1 | P2 | P2 |
| Cell dimensions | | | | |
| a (Å) | 67.32 | 67.42 | 67.51 | 67.42 |
| b (Å) | 67.43 | 67.51 | 115.40 | 115.57 |
| c (Å) | 161.78 | 161.88 | 87.03 | 87.04 |
| α (˚) | 84.61 | 84.36 | 90.00 | 90.00 |
| β (˚) | 79.32 | 79.15 | 91.58 | 92.29 |
| γ (˚) | 61.99 | 62.13 | 90.00 | 90.00 |
| Resolution (Å) | 19.80–1.35 | 19.85–1.35 | 19.50–1.95 | 19.56–2.00 |
| Rmerge (%) | (1.45–1.35) | (1.45–1.35) | (1.95–2.05) | (2.10–2.00) |
| I/%s | 99.6 (73.0) | 99.9 (79.3) | 99.7 (99.9) | 99.6 (99.9) |
| Completeness (%) | 82.4 (79.3) | 92.8 (84.2) | 99.7 (99.9) | 99.6 (99.9) |
| Redundancy | 444,547 | 480,301 | 96.747 | 90.061 |
| No. of reflections | 13,78 (24.80) | 14,13 (26.70) | 96.747 | 90.061 |
| Rwork (%) | 19.80–1.35 | 19.85–1.35 | 19.50–1.95 | 19.56–2.00 |
| Rfree (%) | (1.39–1.35) | (1.39–1.35) | (1.97–1.95) | (2.02–2.00) |
| No. of atoms | 19,588 | 19,972 | 9,169 | 9,169 |
| Protein | 16,861 | 17,101 | 8,457 | 8,457 |
| Ligand/ions | 133 | 300 | 77 | 77 |
| Water | 2,594 | 2,571 | 635 | 635 |
| B factors (Å²) | 24.7 | 27.1 | 38.9 | 38.9 |
| Protein | 31.8 | 37.5 | 52.6 | 52.6 |
| Ligand/ions | 42.6 | 43.2 | 43.2 | 43.2 |
| Water | 0.015 | 0.009 | 0.003 | 0.003 |
| Bond lengths (Å) | 1.430 | 1.537 | 0.794 | 0.794 |
| Bond angles (˚) | 98.87 | 99.2 | 97.1 | 97.1 |
| Ramachandran plot, favored/disallowed (%) | 0.05 | 0.00 | 0.40 | 0.40 |

*Values in parentheses are for the highest resolution shell. * From Karplus and Diederichs (2012).
Trimer form a closely packed symmetrical three-helix bundle with the hydrophobic side chains half-buried in the hydrophobic core (Supplemental Fig. S2A). The three hydrophobic grooves on the surface of that bundle are covered by the hydrophobic sides of the three N-terminal α-helices belonging to the second PpAOC2 trimer present in the asymmetric unit (Supplemental Fig. S2B). The observed hexameric complex is most likely an artifact of crystal packing and probably biologically irrelevant, as most of the interactions between the PpAOC2 trimers are formed by nine N-terminal residues, of which five originate from the GST tag overhang (Supplemental Fig. S2, dark green and orange). The N-terminal amino acids of native PpAOC2 could form helices composed of at most 2.5 α-helical turns, which, however, would be too short to form such a network of intertrimer interactions.

Substrate Binding by PpAOCs

In order to further analyze the putative differences in the ligand-binding mode of PpAOC1 and PpAOC2 and to detect possible ligand-induced changes of the active site, crystals of both apo enzymes were soaked with the competitive inhibitor and substrate analog 12,13-EOD. The calculated difference electron density maps confirmed the presence of ligand molecules in one active site of each PpAOC1 trimer and in five active sites of two PpAOC2 trimers (Fig. 5). The active sites of PpAOC1 as well as of PpAOC2 are very similar to that of AtAOC2, which was previously characterized by the crystal structure of AtAOC2 complexed with the competitive inhibitor published as vernolic acid (PDB code 2DIO; Hofmann et al., 2006). Overall, the amino acids forming the active site of AOC are highly conserved. It is composed of mainly aromatic and hydrophobic residues and harbors three patches of polar amino acids (Figs. 5 and 6A). The first patch is formed by a Glu (AtAOC2 Glu-23, PpAOC1 Glu-18) positioned at the bottom of the β-barrel. The second one consists of a Ser (AtAOC2 Ser-31, PpAOC1 Ser-26), a Pro (AtAOC2 Pro-32, PpAOC1 Pro-27), and two Asn residues (AtAOC2 Asn-25 and Asn-53, PpAOC1 Asn-20 and Asn-55). All amino acids from this second patch bind a catalytic water molecule present in all known AOC crystal structures. The third patch is a Cys (AtAOC2 Cys-71, PpAOC1 Cys-73). The binding mode of the ligand is very similar in the two PpAOC structures as well: the carboxylic group of the ligand is located on the protein surface outside of the cavity (Fig. 3, A and C). This is similar to what has been observed

(less than $-7 \, k_B T$) colors indicate the positively and negatively charged areas, respectively, where $k_B$ is the Boltzmann constant and $T$ is the absolute temperature. The substrate analog 12,13-EOD is shown in a ball-and-stick presentation. The blue mesh indicates the position of Arg-22, and the white mesh indicates the positions of Phe-140/Val-139 and Phe-29/Ile-29 in PpAOC1 and PpAOC2, respectively.
in AtAOC2 (Hofmann et al., 2006), and it seems not to be fixed in a defined position by a specific determinant in AtAOC2 and PpAOC1 (Supplemental Fig. S6). However, in four of the five occupied active sites of PpAOC2, the carboxylic group of 12,13-EOD is bound via Arg-22 that is localized at the substrate entrance (Figs. 3C and 5B; Supplemental Fig. S5). The epoxy group of 12,13-EOD is positioned in hydrogen-bonding distance to the catalytic water molecule bound by the second hydrophilic patch described above (Figs. 5B and 6A; Supplemental Figs. S4–S6). The D15-double bond is positioned in all three structures close to the conserved catalytic Glu (AtAOC2 Glu-23, PpAOCs Glu-18; Supplemental Fig. S4). Most of the protein-ligand interactions contributing to the binding are hydrophobic. Importantly, careful inspection of the AtAOC2 structure (PDB code 2DIO; Hofmann et al., 2006) reveals a cis-double bond geometry between the C15 and C16 atoms of the modeled ligand, a feature not existing in vernolic acid. Based on the observed ligand geometry, the substrate analog published as vernolic acid was most probably identical to 12,13-EOD used in our soaking experiments.

In one active site of the PpAOC2 complex structure, the observed difference in the mFo-DFc electron density map suggested a new ligand-binding mode showing the substrate molecule bound less deep in the active site (Supplemental Fig. S7). In this binding mode, the Glu interacts with a water molecule (Supplemental Fig. S7, red and marked by HOH) mimicking the position of the C15 atom of the ligand bound in the heretofore known binding mode. C18 of the substrate analog in the new binding mode reaches the position known to be occupied by the epoxide oxygen. Another water molecule that may represent the catalytic water molecule is still bound similarly in close proximity to the epoxide ring (Supplemental Fig. S7, cyan), and the carboxylic moiety of the ligand does not form any polar interactions at the cavity entrance. Most of the side chains that line up the cavity adopt identical side chain conformations, as observed for the unoccupied and fully occupied active site of PpAOC1 and AtAOC2. There are, however, three notable exceptions: Tyr-106 (PpAOC1 Tyr-107), Phe-137 (PpAOC1 Phe-138), and Gln-134 (PpAOC1 Gln-135; Supplemental Fig. S7). These residues are located close in space at the side opposing the trimerization interface of the β-barrel, and one may suggest that this structure represents an early stage of substrate binding.

Substrate Binding May Be Accompanied by Structural Changes in the Active Site of AOCs

Previously determined structures of the ligand-bound and ligand-free AtAOC2 (PDB codes 2DIO and 2GIN, respectively), both determined at 1.7 Å resolution, revealed no significant conformational changes of the active site residues caused by ligand binding (Hofmann et al., 2006). However, the high-resolution structure of ligand-free AtAOC2 (PDB code 2BRJ), determined at 1.5 Å resolution, revealed two disordered residues that are involved in the active site surface formation (AtAOC2 Tyr-105 and Gln-133). In order to investigate the internal flexibility of residues forming the AOC active site, we performed a superposition of 12 monomers of the apo PpAOC1 structure determined at 1.35 Å resolution. It revealed differences in conformations of three side chains: Tyr-107 (AtAOC2 Tyr-105), Phe-138 (AtAOC2 Phe-136), and Gln-135 (AtAOC2 Gln-133), residues...
**Figure 5.** The active sites of PpAOC1 (A) and PpAOC2 (B). A. The residues forming the active site and located within a 4-Å distance from the ligand are depicted as sticks. The SA mFo-DFc omit electron density map contoured at 3σ shows the position of the 12,13-EOD molecule in the active site. cis-12,13-EOD and trans-12,13-EOD are colored orange and tan, respectively. The difference mFo-DFc map (green; contoured at 3σ) calculated after refinement of the modeled cis-12,13-EOD indicates the presence of the trans-12,13-EOD. Residues forming the hydrophilic patch and harboring the catalytic water molecule (cyan sphere) required for catalysis are labeled, and their interactions with the water molecule are indicated as dashed lines. B. The residues forming the active site and located within a 4-Å distance from the ligand are depicted as sticks. The SA mFo-DFc omit electron density map contoured at 3σ confirms the position of the 12,13-EOD molecule in the active site. The catalytic water molecule is shown as a cyan sphere. Polar interactions of the ligand molecule with Arg-22 and the conserved water molecule are shown as dashed lines.

structurally equivalent to those displaying conformational differences observed for the new, shallow ligand-binding mode in the PpAOC2 complex structure (Supplemental Fig. S7). Side chain conformations of those three residues are coupled and adopt two possible states: a major one, observed in most ligand-bound and ligand-free AOC structures (group A in tan in Fig. 6A); and a minor one (group B in pale green in Fig. 6A), observed in the PpAOC2 active site with the new ligand-binding mode, in the high-resolution crystal structure of apo AtAOC2 (PDB code 2BRJ) and in five out of 12 monomers of apo PpAOC1 structure. The PpAOC1 crystal soaked with the substrate analog 12,13-EOD revealed identical grouping (groups A and B) of the 12 monomers as observed for the nonsoaked crystal. The presence of ligand molecules has been confirmed only in four monomers belonging to group A. Obviously, no ligand molecules have been identified in the active sites of monomers belonging to group B of the soaked PpAOC1 structure, which are partially covered by the loops of the other monomers. A similar comparison of differences in conformations of the side chains comprising the active site has been performed for PpAOC2 structures and revealed consistent results as for the lower resolution structures of AtAOC2. Together, these data may support the hypothesis of a structural change within the active site from the first contact of the ligand with the enzyme and tight ligand binding: whereas most
Structural Changes of the Catalytic Center Lead to an Open and Closed Conformation of PpAOC2

The proposed cyclization mechanism assumes an opening of the epoxide ring controlled by the protein environment followed by the formation of a pentadienyl cation, which is stabilized by the tightly bound catalytic water molecule. The second hydrophilic patch is responsible for binding the water molecule involved in catalysis. Mutagenesis studies on AtAOC2 revealed that only two out of four residues constituting the hydrophilic patch II (Pro-32 and Asn-25) are essential for the enzymatic activity (Schaller et al., 2008). These two residues are located on a longer loop between β-strands 1 and 2, forming one side of the cavity entrance (AtAOC2 from Asn-25 to Gly-44, PpAOC1 from Asn-20 to Ser-45; Fig. 6A). The first part of that loop (up to a highly conserved Leu: PpAOC1 Leu-30 and
AtAOC2 Leu-35) adopts an extended conformation and forms a number of hydrogen bond interactions with β-strand 2, while the remaining part (PpAOC1 amino acids 31–45, AtAOC2 amino acids 36–44) is neither conserved in sequence nor in length between the AOCs from flowering plants and those two from the moss (Supplemental Fig. S3). The hydrophilic patch II is partially formed by the first part of the mentioned loop connecting β-strands 1 and 2 (Fig. 6A; Supplemental Fig. S8). It is one of the structural elements forming the AOC active site and is thought to be its integral part. The structure of apo PpAOC2, however, revealed an unexpected conformational flexibility of the essential Pro residue (PpAOC2 Pro-27, AtAOC2 Pro-32), resulting in covering the active site entrance by a flexible loop, and only Val-28 and Ile-29 following the conserved Pro are traceable (Supplemental Fig. S8, yellow structure). The Val-28 side chain points toward Arg-22, and the first eight carbon atoms of 12,13-EOD in the PpAOC2 complex (as well as in all other complex structures) occupy exactly the same position as the two hydrophobic residues (Val-28 and Ile-29) covering the active site entrance in the apo PpAOC2 structure. The observed structural rearrangements have been identified in three out of six apo PpAOC2 monomers occupying the asymmetric unit, and in one of them no electron density has been observed for the catalytic water molecule. Upon ligand binding, however, the disordered loop covering the active site adopts the conformation known from the other AOC structures, and the conserved water molecule is bound by all monomers of the PpAOC2 complex structure (Supplemental Fig. S8, orange structure).

cis/trans-Isomerization of 12,13-EOD Bound to PpAOC1

The quality and shape of the difference electron density, calculated at 1.35 Å resolution for the soaked PpAOC1 crystal, allowed modeling of the 12,13-EOD molecule in one active site of each PpAOC1 trimer. Subsequent refinement resulted in the appearance of positive peaks of the difference mFo-DFc electron density map at the 3σ level, close to the C11 atoms. Interestingly, the density peaks, which appeared in three active sites, could only be explained by modeling 12,13-EOD in trans-configuration of the epoxide ring (Fig. 5A, green area), although the 12,13-EOD that has been used for soaking experiments was in the cis-configuration at the epoxide ring. No evidence for the presence of trans-epoxide as an impurity has been obtained based on HPLC/mass spectrometry (MS) and gas chromatography-MS analysis (Supplemental Fig. S9).

Careful examination of electron density maps allowed modeling of single ligand conformers in three active sites of the PpAOC1 complex structure: one with cis-12,13-EOD and two with trans-12,13-EOD. The fourth’s active site has been modeled with the ligand in two almost equally populated, alternate configurations of the epoxide ring: cis and trans. Despite differences in the epoxide ring configuration, the overall binding mode of the cis/trans-12,13-EOD in PpAOC1 is very similar also when compared with the published AtAOC2 complexed with most likely the identical ligand (PDB code 2DIO). Thus, the observed isomerization of the epoxide ring affects neither the conformation nor the positioning of the pentenyl moiety comprising the Δ12-double bond. The epoxide ring of the trans-12,13-EOD is only slightly reoriented relative to its position observed for the cis-12,13-EOD, preserving the hydrogen bonding distance to the catalytic water molecule (Supplemental Fig. S6). The largest difference was observed for the position of atom C11 and consequently the Δ2-double bond, albeit the changes were very limited. The carboxyl moiety is known to be flexible and does not form any polar interactions with PpAOC1 atoms comprising the active site entrance (vide supra; Supplemental Fig. S6). Interestingly, the electron density maps calculated for the structure of 12,13-EOD complexed by PpAOC2 did not confirm the presence of trans-12,13-EOD in any active site. This could be due to a limited number of details that could be seen at 2 Å resolution or the lack of geometrical isomerization caused by the different time and pH of soaking conditions (pH 7.5 for PpAOC2 versus pH 4.5 for PpAOC1).

Comparison of PpAOC1 and PpAOC2 Active Sites

Notwithstanding structural similarities between active sites, the two PpAOC isoenzymes differ in their substrate specificity. The ability of PpAOC2 to accept both C18 and C20 fatty acid derivatives indicates at least slight structural differences within the binding cavities of the two PpAOC isoforms. Those differences must allow PpAOC2 to accommodate, deep in the active site, an octenyl side chain of the substrate derived from 12-HPETE and still not impair the enzymatic activity for the substrate originating from 13-HPOTE having a pentenyl side chain. Subtle changes could easily escape detection when comparing very similar macromolecules. Our attention, however, was riveted by different RMSD values calculated on Ca positions between monomers of PpAOCs and individually against the monomer of AtAOC2. Surprisingly, based on RMSD values, one could expect higher structural similarity between PpAOC1 and AtAOC2 (having the same substrate specificity) than between both PpAOCs (RMSD values of 0.51 Å and 0.83 Å, respectively). In comparison, the RMSD calculated between monomers of PpAOC2 and AtAOC2 amounts to 0.80 Å, suggesting that the β-barrel of the PpAOC2 monomer is indeed slightly different. In order to address this aspect, we compared the active site diameters of both PpAOC isoforms as measured across the β-barrel between amino acids forming the inner part of the binding cavity, mostly responsible for binding of the pentenyl side chain of the C18-derived substrate molecule. We have chosen amino acids lying on opposite sides of the active site, namely PpAOCs Glu-18, PpAOC1 Leu-142 (PpAOC2 Leu-141), and PpAOC1 Leu-133 (PpAOC2 Leu-132) on
DISCUSSION

In plants, oxidized cyclic fatty acids are important signaling molecules (Acosta and Farmer, 2010). The first specific step in their biosynthesis is catalyzed by AOC that forms enantipure cis(+)–OPDA, the first cyclic bioactive signaling molecule (Fig. 1; Dave and Graham, 2012). In contrast to Arabidopsis, which has \( C_{16} \) and \( C_{18} \) fatty acid-derived pathways leading to the formation of cyclopentenones, \( P. patens \) has \( C_{18} \) and \( C_{20} \) fatty acid-derived pathways (Fig. 2; Wichard et al., 2004; Stumpe et al., 2010). In addition, PpAOC2 metabolizes the allene oxides from both pathways, while PpAOC1 has the same substrate specificity as AtAOC2 and other AOCs from flowering plants. It does not accept the 12-HPETE-derived \( C_{20} \)-allene oxide and thus converts only the 13-HPOTE-derived \( C_{18} \)-allene oxide. Building on the published studies of AtAOC2 that described the structure of the apo enzyme and an AtAOC2 12,13-EOD complex in which the ligand is tightly bound to the active site (Hofmann et al., 2006; Schaller et al., 2008), the aim of this study was to expand our knowledge of the structural details of the cyclization process catalyzed by AOCs and to identify the structural determinants that are responsible for the different specificities of PpAOC1 and PpAOC2. Therefore, both enzymes were crystallized and their crystal structures solved. The PpAOC isoforms are structurally very similar and have the same overall fold and oligomerization state as known for AtAOC2 (Fig. 3; Hofmann et al., 2006). By solving and analyzing the crystal structures for all monomers of both apo and 12,13-EOD complexes of PpAOC1 (24 monomers) and PpAOC2 (12 monomers), this study now expands our understanding of the intermediate steps of AOC catalysis. (1) In their resting state, AOCs most likely employ a closed conformation, as observed for PpAOC2. (2) Upon substrate binding, the active site opens, first to a shallow binding mode, again

The distances were measured for all monomers of both apo and 12,13-EOD complexes of PpAOC1 (24 monomers) and PpAOC2 (12 monomers), and their averaged values with accompanying SD are presented in Table II. The analysis revealed that in some directions (e.g. PpAOC2 Leu-141 \( \rightarrow \) Tyr-90, Leu-141 \( \rightarrow \) Tyr-106, Leu-141 \( \rightarrow \) Ala-88, Leu-132 \( \rightarrow \) Tyr-106, Leu-132 \( \rightarrow \) Ala-88), the active site of PpAOC2 is significantly (around 0.5 Å) larger in diameter than that of PpAOC1. Those small differences are consistent in all compared monomers regardless of the presence or absence of a substrate molecule in the active site. In addition, a detailed comparison of the inner part of PpAOC active sites revealed the presence of an elongated narrow cavity positioned close to the C18 atom of the bound substrate analog exclusively in PpAOC2 (Fig. 6B). This cavity is lengthening the active site and is long enough to accommodate additional carbon atoms present in a substrate derived from a \( C_{20} \)-allene oxide (Supplemental Fig. S10). In the apo PpAOC2 monomer, the additional cavity is still too narrow to avoid van der Waals clashes between the terminal carbons of the octenyl side chains and the protein; however, slight movements (approximately 0.2 Å) of surrounding side chains should broaden the cavity enough to avoid van der Waals clashes.

### Table II. Comparison of the active site diameters of both PpAOC isoforms as measured across the β-barrel between amino acids forming the inner part of the binding cavity

| Parameter       | PpAOC1 Average (Å) | PpAOC2 Average (Å) | Difference (Å) |
|-----------------|--------------------|--------------------|----------------|
| L133/L132-Y91/Y90 | 12.42              | 12.60              | 0.179          |
| L133/L132-Y107/Y106 | 13.55              | 13.70              | 0.140          |
| L133/L132-C73/C72 | 15.50              | 15.44              | -0.055         |
| L133/L132-A89/A88 | 14.88              | 15.09              | 0.208          |
| L142/L141-Y91/Y90 | 14.05              | 14.46              | 0.408          |
| L142/L141-Y107/Y106 | 10.94              | 11.33              | 0.380          |
| L142/L141-C73/C72 | 15.16              | 15.32              | 0.157          |
| L142/L141-A89/A88 | 14.32              | 14.85              | 0.53           |
| L133/L132-Y91/Y90 | 12.58              | 12.90              | 0.321          |
| L133/L132-Y107/Y106 | 7.65               | 8.13               | 0.480          |
| L133/L132-C73/C72 | 14.18              | 14.33              | 0.153          |
| L133/L132-A89/A88 | 11.51              | 12.03              | 0.522          |
as described for the PpAOC2 12,13-EOD complex. (3) Next, tight binding of the substrate occurs by conformational changes of at least three amino acid side chains in the binding pocket, as demonstrated by comparing different structures of PpAOC2 12,13-EOD complexes. (4) The catalytic cycle is initiated by the opening of the epoxide ring. This is supported by the observation of an epoxide cis/trans-isomerization of the PpAOC1 12,13-EOD complex.

The central part of the enzyme harboring the active site is very rigid, thus limiting possible conformational changes induced upon ligand binding. However, a part of the active site is not entirely formed by residues constituting the \( \beta \)-barrel, namely, the hydrophilic patch \( \beta \)-I binding the catalytic water molecule (Fig. 5B). It is partially formed by part of a loop between \( \beta \)-strands 1 and 2 comprising the essential Pro-27 (Supplemental Fig. S8). The structure of the apo PpAOC2 revealed a conformational flexibility of Pro-27 leading to closure of the active site entrance by this flexible loop and consequently destroying the hydrophilic patch. The observed structural rearrangements indicate that the enzyme can also exist in a nonfunctional or resting state, which may be required for protecting the hydrophobic cavity from being exposed to the solvent if there is no substrate around or in a very hydrophilic environment. In addition, the loop connecting \( \beta \)-strands 1 and 2, due to its flexibility, amino acid composition, length, and localization at the active site entrance, may play a role in the cooperation with AOS, especially for substrate recruitment (two adjacent Lys residues, of which one, Lys-35, in both PpAOCs is conserved in sequence; Supplemental Fig. S3). The positively charged residues on the flexible loop flanking the active site entrance could be used to anchor the negatively charged substrate molecules without the necessity of forming the AOS/AOC molecular complex, whose existence has not been experimentally proven yet (Schaller et al., 2008).

For a deeper insight into possible differences in the binding modes of AOC substrates, crystal structures may be needed with either the substrate or the product bound within the active site. Due to the short life time of the allene oxides in aqueous solution of about 30 s (Hamberg, 1987), details about ligand binding have only been obtained from a crystal structure of AtAOC2 complexed with 12,13-EOD, a stable substrate analog (a competitive inhibitor) lacking the vinylic double bond present in allene oxides. The flexible carboxylic moiety of the ligand is located in all structures on the protein surface, but only in PpAOC2 is it fixed by forming interactions with Arg-22 located at the active site entrance (Figs. 3C and 5B; Supplemental Figs. S4 and S5). This interaction is probably responsible for a positional shift of the ligand’s epoxide ring and \( \Delta^{15} \)-double bond of approximately 1.2 Å toward the active site entrance, in comparison with PpAOC1 and AtAOC2 complexes. Most interestingly, the structure of the PpAOC2 12,13-EOD complex revealed, in one active site, a new ligand-binding mode with the substrate analog bound less deep in the active site (Supplemental Fig. S7). In this orientation, the methyl group of the pentenyl side chain reaches the position occupied by the epoxide ring in hitherto known ligand-binding mode and the carboxylic moiety is not involved in forming any polar contacts at the active site entrance. The observed accompanying conformational changes of the three side chains constituting the active site, Tyr-106 (PpAOC1 Tyr-107), Phe-137 (PpAOC1 Phe-138), and Gln-134 (PpAOC1 Gln-135; Fig. 6A), reveal the intrinsic dynamics inside the rigid and narrow cavity, which may be important for the substrate binding and/or release of the product. The structurally equivalent residues of PpAOC1 revealed the same conformational flexibility as observed for some monomers of the apo structure. This strongly suggests that the side chain conformations of those three residues are coupled and the enzyme samples between two distinct conformations, of which only one is able to lock the flexible acyl moiety in the position essential for catalysis.

To expand our knowledge of different substrate-binding modes, crystals of both apo enzymes were soaked in this study with three different substrate analogs, 12,13-EOD, vernolic acid [cis(+/-)-12,13-epoxy-9Z-octadecenoic acid (12,13-EOM)], and 11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET), as well as the product, cis(+)-OPDA. However, soaking was only successful for the 12,13-EOD ligand. Interestingly, the crystal structure of the PpAOC1 12,13-EOD complex revealed a cis/trans-isomerization of the epoxide ring, which, however, did not affect the binding mode of the substrate analog (Fig. 5A; Supplemental Figs. S4 and S6). The \( \Delta^{11} \)-double bond present in the natural substrate precludes such isomerization and causes the epoxide ring to be in plane with the C11 atom; therefore, its position would slightly differ from that observed in both cis- and trans-12,13-EOD (Supplemental Fig. S11). The in silico docking experiments of the \( \Delta^{11} \)-epoxide ring being cis- or trans-configured, all structurally characterized complex structures of AOCs share a very similar binding mode of 12,13-EOD. Oxiranes are stable when stored in alcohols; however, in aqueous solution, under both acidic and basic conditions, they can undergo ring-opening reactions leading to geometrical isomerization of the epoxide ring (Yamaguchi et al., 1993). Oxirane ring opening may occur via C-O or C-C bond cleavage, and the preferred route is often determined by the nature of the substituent (MacDonald and Crawford, 1972). The formed intermediates can either spontaneously close back to the original ring form or partake another fragment/compound, yielding a nonepoxide product. The mechanistic features of electrocyclic epoxide ring-opening reactions have been described by Woodward and Hoffmann (1965), who formulated, based on orbital symmetry,
rules predicting the stereochemistry of pericyclic reactions. Considering these rules, the preferred way of epoxide ring reclosure is a conrotatory mode. Thus, the spontaneous reclosing reaction can result in the geometrical isomerization of epoxides, which passes through the open intermediate form, and is likely to yield a more stable epoxide ring structure in the trans-configuration (MacDonald and Crawford, 1972; Davey et al., 2008). In the case of a ligand that cannot form a cyclopentadienyl cation, cyclization is not possible; therefore, the epoxide ring is being formed again, this time in its more stable trans-conformation (Fig. 1). Therefore, the data on the cis/trans-isomerization of 12,13-EOD may support the model published recently in which an opening of the epoxide ring of the allene oxide substrate via the catalytic Glu residue (Glu-23 in AtAOC2) initiates the cyclization reaction (Hofmann et al., 2006). However, analysis of this initial reaction step is hampered by the fact that a mutation in the catalytic Glu-23 (in AtAOC2) resulted in an inactive enzyme (Schaller et al., 2008).

Previously, we have reported the preferential formation of cis(+)OPDA in a coupled AOS/PpAOC assay using 13-HPOTE as the substrate in the presence of either PpAOC1 or PpAOC2 (Stumpe et al., 2010). In contrast, incubation of both PpAOC isoforms with the allene oxide derived from 12-HPETE resulted in cyclopentenone formation (11-OPTA) only when PpAOC2 was used. Due to the different lengths of side chains of substrate molecules derived from both C18- and C20-allene oxide derivatives, slight differences within the binding cavities of the two PpAOCs can be expected (Fig. 2). A detailed analysis of active sites of both PpAOC isoforms revealed differences in active site diameter and an additional cavity in PpAOC2 that might accommodate a longer octenyl side chain of the substrate derived from C20 fatty acid (Fig. 6B). In order to check the possibility of an alleged binding of the longer octenyl side chain inside the narrow active site, we performed in silico docking of 11,12-EET to homomers of both PpAOC isoforms. The docking results clearly demonstrated that slight conformational changes of a few hydrophobic side chains forming the narrow cavity (PpAOC Tyr-90, Phe-92, Val-102, Leu-114, Leu-132, and Phe-145) allow PpAOC2 to accommodate the longer octenyl side chain and simultaneously maintain correct positioning of the epoxide oxygen and the homoallylic double bond required for catalysis (Fig. 6B; Supplemental Fig. S10). In order to find additional factors that may have an influence on the observed differences in substrate specificity between the two PpAOC isoenzymes, we analyzed known plant AOCs by sequence alignment on the basis of the sequence differences observed between the two PpAOCs (Supplemental Fig. S3). Among the analyzed plant AOC sequences, only PpAOC2 has been reported to convert the 12-HPETE-derived substrate. The residues comprising the active site are strictly conserved between the AOCs. However, two amino acid positions located near the cavity entrance differ in size between the PpAOCs: PpAOC1 Phe-29 and Phe-140 correspond to the less space-filling residues Ile-29 and Val-139 in PpAOC2, respectively (Fig. 3C). The substrate C20-allene oxide has a one-carbon shorter carboxylic side chain that is less flexible (one additional double bond in the cis-configuration) in comparison with the saturated octanoyl side chain of C18-allene oxide (Fig. 2). Consequently, its carboxylic moiety will be positioned closer to the negatively charged active site. Therefore, it would be energetically favorable to neutralize its charge by forming interactions with positively charged protein residues (Fig. 3C). The structure of PpAOC2 with bound 12,13-EOD revealed that such interactions are possible. In all active sites, with the substrate molecule bound in hitherto known binding mode, the ligand’s carboxylic group interacts with the side chain of Arg-22 located near the active site entrance (Supplemental Fig. S5). In contrast, the PpAOC1 complex structure revealed disordered carboxyl moieties of the same substrate analog that were not forming any polar interactions with the protein residues, most probably due to a steric hindrance of the Phe-140 side chain (Supplemental Fig. S6). Replacement of PpAOC1 Phe-140 with the less bulky side chain of Val in PpAOC2 should make the side chain of Arg-22 accessible to the substrate carboxylic moiety, which is a unique feature of PpAOC2. In order to check the influence of those two amino acid positions (PpAOC1 Phe-29/PpAOC2 Ile-29 and PpAOC1 Phe-140/PpAOC2 Val-139), we generated the respective PpAOC1 single (PpAOC1_F140V) and double (PpAOC1_F29I/F140V) variants by means of site-directed mutagenesis and analyzed their catalytic activities. Similar to the wild-type enzymes of PpAOC1 and PpAOC2, both variants catalyzed the specific conversion of 18:3(n-3)-derived allene oxide to 47% to 65% of OPDA, as shown in Figure 4. When incubated with 20:4(n-6)-derived allene oxide, specific formation of the respective cyclopentenone was observed only in incubations with PpAOC2 but not with PpAOC1 (as has been reported before) or with PpAOC1_F104V or PpAOC1_F29I/F140V mutants. Although our experimental data thus could not support the influence of these amino acids on different substrate specificities, it should be emphasized here that we also could not exclude the function of those residues in combination with other steric factors (diameter and depth of the cavity) to determine substrate specificity. Cocrystallization of the respective enzyme variant with different substrates might help to tackle this question.

CONCLUSION

The structure-based elucidation of the enzyme mechanism of two AOCs from P. patens obtained detailed information about the allene oxide-cyclopentenone conversions. By solving 36 monomeric AOC structures, we suggest now the following different conformations: (1) a closed conformation for the resting state, (2) a first shallow binding mode, (3) followed by the recently published tight binding of the substrate, and finally (4)
the initiation of cyclization by the ring opening of the epoxide group. In addition, different substrate specificities may be accompanied by different active site diameters and an elongated cavity.

**MATERIALS AND METHODS**

**Cloning and Recombinant Expression of PpAOC1 and PpAOC2**

PpAOC1 and PpAOC2 from *Physcomitrella patens* were cloned from the respective pGEM-T plasmid reported before using the BamHI and HindIII recognition sites as described (Stumpe et al., 2010). The resulting fragment was cloned in the pGEX-6P-1 vector (GE Healthcare), resulting in the plasmids pGEX-6P-1/PpAOC1 and pGEX-6P-1/PpAOC2 that encode the respective AOC with an N-terminal fusion to GST via the PreSciss peptide recognition site. These clones were used as templates for site-directed mutagenesis PCR (AOC1_F140V and AOC1_F291/F140V) using the Phu polymerase.

For expression, the respective plasmid was transformed into B. subtilis Star cells (Invitrogen). Cells were cultivated in Luria-Bertani broth or 2YT broth containing 100 μg ml⁻¹ ampicillin and grown at 37°C to an optical density of 600 nm of 0.6 to 0.8. Protein expression was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside. Bacterial cultures were cultivated by shaking at 28°C for approximately 18 h and harvested by centrifugation (3220g) for 20 min. The resulting cell precipitate was shock frozen in liquid nitrogen and stored at −20°C.

**Protein Purification**

The cell pellet was resuspended in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 2 mM diethanolamine (DTE), and 0.2 mM phenylmethylsulfonyl fluoride. Lysozyme was added (final concentration of approximately 0.1 mg ml⁻¹), and cells were incubated at 0°C for 30 min. Cell lysis was increased by sonication on ice using a SoniC (Gene) device for 20 min at 4°C. The resulting cell-free crude extract was loaded on a G-100 Sephacryl matrix (50 ml of 0.05 M sodium acetate (2 Fast Flow) in an XK16/20 column; GE Healthcare) using an ÄKTAprime system (GE Healthcare) at a flow rate of 2 mL min⁻¹.

The column was reequilibrated by a linear gradient back to 100% buffer A (0.1 M sodium acetate, 2 mM DTE, and 0.2 mM phenylmethylsulfonyl fluoride) and an elongated cavity.

**Crystallization, Soaking Experiments, and Data Collection**

Crystals of PpAOC1 and PpAOC2 were obtained using the sitting-drop vapor-diffusion method on 24-well Cryocry screen plates (Hampton Research) at 20°C and 4°C. The crystallization droplets (2 μL) were prepared by mixing equal amounts of protein (at a concentration of 20 to 30 mg mL⁻¹) and reservoir solution and were equilibrated against a 500-μL reservoir. The initial needle-shaped crystals of PpAOC1 were obtained at 4°C using PEG 3350 in the full-scan mode. The ion spray voltage was set to 4 kV, while the effective drift time was 0.3 mL min⁻¹.

**Activity Assay**

The catalytic activity of different PpAOC variants (AOC1_F140V and PpAOC2_F291/F140V) was analyzed in a coupled assay similar to the method described by Stumpe et al. (2010). The PpAOS cDNA (Bandara et al., 2009) was PCR amplified from a cDNA library of *P. patens* protoplasts and cloned into pQE30, yielding the construct pQE30/PpAOS. The enzyme was expressed in *Escherichia coli* SG13009 (pREP4) (Δd Δe Δf Δg Δh Δi Δj Δk Δl Δm Δn). Protein expression was induced by using reverse-phase HPLC-MS. The analysis was carried out on a Xcalibur software (Thermo Finnigan) device described above with similar MS parameters, scanning for ions in the mass range of mass-to-charge ratio 80 to 350 in the negative electrospray ionization mode. For MS/MS analysis, collision energy of 1 eV was employed.

**Purity Control of the Substrate Analog 12,13-EOD**

The ligand investigated in both structures has been synthesized from 18:3(n-3) containing three double bonds in the cis-conformation. The synthesis of the epoxide ring at position 122 is stereo specific, meaning that stereoisomerism in the alkene will be reflected in the epoxide product. The synthesized substrate analog was stored in ethanol, where it is stable. Its enantiomeric purity was assessed by using reverse-phase HPLC-MS. The analysis was carried out on a Surveyor HPLC system (Thermo Finnigan) equipped with an EC 50/2 Nucleodur C18 gravity column (50 × 2 mm, 1.8-μm particle size; Macherey & Nagel) using a solvent system consisting of acetonitrile/water/acetic acid (40:60:0.1 v/v/v) as solvent system A and acetonitrile/water/acetic acid (100:0.1 v/v/v) as solvent system B. The gradient elution profile started with a linear gradient from 100% solvent A to 100% solvent B within 25 min and continued with an isocratic run for 5 min. The column was reequilibrated by a linear gradient back to 100% solvent system A within 2 min, which was followed by an isocratic run of 3 min. The flow rate was set to 0.3 mL min⁻¹. The MS analysis was performed on an LQ Advantage mass spectrometer (Thermo Finnigan) in the negative electrospray ionization mode scanning for ions in a mass range of mass-to-charge ratio 250 to 350 in the full-scan mode. The ion spray voltage was set to 4 kV, while the capillary voltage was used at 13 V. The capillary temperature was 300°C, and a sheath gas flow rate of 22 arbitrary units and an auxiliary/ sweep gas flow rate of 8 arbitrary units were applied. As the trans-12,13-EOD enantiomer was commercially unavailable, we tested the separation efficiency by using cis-12,13- EOM and trans-12,13-EOM (close homologs of 12,13-EOD) as standards. As shown in Supplemental Figure S9, both enantiomers were chromatographically separated by using this method.
12,13-EOM, and 11,12-EET. The data sets from several crystals (two or three repetitions for each ligand differing in soaking time) were collected. The structures were refined using the available apo structures as starting models. The estimation of the success in soaking experiments was made based on the presence and quality of the difference electron density maps (mFo-DFc and 2mFo-DFc) inside the elongated cavity known to be the ligand-binding site of AOCs. Inspection of those difference electron density maps and later a simulated annealing (SA) omit map confirmed successful soaking only for the 12,13-EOD ligand. The electron density maps calculated for crystals soaked with the remaining substrate analogs did not allow us to unambiguously trace the ligand molecules, as the active sites were also partially occupied by low-M_r alcohols (1,6-hexanediol for PpAOC1, isopropanol and MPD for PpAOC2), which were present in soaking or cryo solutions. Desoaking of 1,6-hexanediol from the PpAOC1 crystals or soaking experiments with reduced amounts of that alcohol resulted in complete crystal damage. Other cryoprotectants, than MPD for PpAOC2 crystals resulted in significant loss of diffraction properties.

The data were collected at beamlines BL 14.1 and 14.2 at the BESSY II synchrotron in Berlin (Mueller et al., 2012). The beamlines were equipped with a MARMosaic-CCD detector (225 and 165 mm; MARI RESEARCH) and a MARAdigonostat (MARRESEARCH). The data sets were integrated and scaled using the XDS package (Kabsch, 2010). The details are presented in Table I.

**Structure Solution and Refinement**
PpAOC1 crystallized in the P1 space group with 12 monomers occupying the asymmetric unit with a corresponding solvent content of 54.9%. The structure of PpAOC1 was solved by molecular replacement with the Phaser (McCoy et al., 2007) package using the trimer of AOC2 (PDB accession code 2GIN) as search model. PpAOC2 crystallized in the P2_1 space group with six monomers in the asymmetric unit (corresponding solvent content of 57.6%). The phase problem was solved by molecular replacement using the trimer of PpAOC1 as the search model in Phaser. Prior refinement the R-free set was chosen in thin shells in order to avoid noncrystallographic symmetry (NCS)-based bias of the R-free factor. The structures of PpAOC1 and PpAOC2 (apo and complexes) were refined using PHENIX (Adams et al., 2010) and Refmac5 (Winn et al., 2003). Medium NCS restraints were used at the early stages of refinement of both structures and were released for variable parts of the AOC monomers where appropriate. The final refinement steps were applied without any NCS restraints. Manual model rebuilding and verification were performed against difference electron density maps as well as SA omit electron density maps in Coot (Emsley et al., 2010). Alternative steps of model adjustment and refinement were performed until the crystallographic R factors converged (Table I). The atomic coordinates and the necessary geometry definitions for the ligand molecules were created using the PRODRG server (Schüttekopf and van Aalten, 2004). The quality of the final models was assessed using MolProbity (Chen et al., 2010; Table I). Figures were prepared using Pymol (http://www.pymol.org).

**In Silico Docking Experiments**

Docking experiments were performed using AutoDock Vina (Trott and Olson, 2010). Briefly, the initial ligand’s coordinates (12,13-EOT and 11,12-EET) were obtained from the PRODRG server. Autodock Tools (Adams et al., 2010) was used to set up the docking experiments, which were performed with the catalytically important water molecule bound in the hydrophilic cavity. Both C8 (12,13-EOT) and C6 (11,12-EET) substrates were docked to both PpAOC1 and PpAOC2 monomers. The predicted binding orientations were compared with the crystallographically determined position of the 12,13-EOD and were considered to be reasonable if the following criteria were fulfilled: the differences between positions of epoxide oxygen atoms as well as C15 atom and its equivalent should not exceed 1.2 Å. For docking experiments with C8-derived substrate, the receptor atoms were fixed; thus, the only rotatable degrees of freedom were possessed by the ligand molecule (double bonds were fixed in the cis-configuration). Docking experiments using the standard settings revealed mostly nonsense binding predictions when comparing 10 predictions with the lowest energy. Therefore, several docking experiments were performed changing the exhaustiveness parameter from the default value, determined automatically, up to 1,000 in steps of 50. The exhaustiveness parameter describes the time spent on the search and is varied heuristically depending on the number of atoms, flexibility, etc. Setting it to a larger value increases the time of the calculations linearly and decreases the probability of not finding the minimum exponentially. The program reports at most 20 binding predictions sorted according to the affinity, expressed in kcal mol$^{-1}$. We observed that increasing the value of the exhaustiveness parameter to 100 or higher results in obtaining the highest affinity decays, which are consistent with the orientation of the substrate analog known from the crystal structure. Finally, for the 12,13-EOD ligand, we have chosen the highest affinity binding predictions calculated with the exhaustiveness parameter of 1,000, which fits very well the position of the 12,13-EOD determined experimentally (Supplemental Fig. S11). The calculated affinity predicted for the PpAOC1 receptor (−7.4 kcal mol$^{-1}$) was 0.3 kcal mol$^{-1}$ lower than that calculated for the PpAOC2 monomer. The observed difference in predicted substrate affinity between the two PpAOC isoforms has not been experimentally investigated so far. The 11,12-EET ligand was docked first to the rigid monomers of PpAOC isoforms running 20 individual docking jobs with different values of the exhaustiveness parameter (see above). As expected, no reasonable binding predictions could be found, as 11,12-EET comprises a three-carbon-atom-longer octenyl side chain in comparison with 12,13-EOT (Fig. 2). Binding of three additional carbon atoms inside the narrow active site requires conformational changes of some side chains building its inner surface. Therefore, subsequent experiments were performed with setting selected side chains of both receptor molecules to be flexible during the in silico docking experiments (PpAOC2 Try-90, Phe-92, Val-102, Leu-114, Leu-132, Phe-145, Arg-22, Ile-29, and Val-139, and equivalent residues of PpAOC1). Considering one decry with the highest reported binding affinity (approximately 7.7 kcal mol$^{-1}$) from each individual docking job and applying the distance-based criteria, rational substrate-binding predictions were exclusively obtained for six docking jobs with the PpAOC2 monomer used as the receptor (Supplemental Fig. S10).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: P. patens, AOC1 (Q8H315) and AOC2 (Q8H316); Solanum tuberosum, AOC (Q8H315); Lyopersicon esculentum, AOC (Q9LEG5); Humulus lupulus, AOC4 (Q8H316) and AOC1 (Q8H317); Zn maps, AOC (Q6R9W9); Hordeum vulgare, AOC (Q711R0); Oryza sativa, AOC (Q8L6H4); Medicago truncatula, AOCb (Q59978) and Aoca (Q711Q9); Pismium sativum, AOC (Q3L84); Arabidopsis (Arabidopsis thaliana), AOC4 (Q93ZC5), AOC3 (Q9LS01), AOC1 (Q9LS03), and AOC2 (Q9LS02).

**Supplemental Data**
The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of the purification of PpAOC1 and PpAOC2 by SDS-PAGE.

Supplemental Figure S2. Oligomerization state of PpAOC2.

Supplemental Figure S3. Sequence alignment of different AOCs.

Supplemental Figure S4. Superposition of individual PpAOC2 and PpAOC1 monomers.

Supplemental Figure S5. Representation of the PpAOC2 active site.

Supplemental Figure S6. The PpAOC1 active site.

Supplemental Figure S7. Superposition of the active sites of two PpAOC2 monomers.

Supplemental Figure S8. Superposition of two ligand-free PpAOC2 monomers differing in conformation of the region connecting $\beta$-strands 1 and 2.

Supplemental Figure S9. Reverse-phase HPLC-MS separation of cis/trans isomers of 12,13-EOM.

Supplemental Figure S10. Results of docking experiments of 11,12-EET to a monomer of PpAOC2.

Supplemental Figure S11. The active site of PpAOC1 is shown in cartoon representation.
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