Structural analysis of a plant fatty acid amide hydrolase provides insights into the evolutionary diversity of bioactive acylethanolamides

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Running Title: Structural Features of Arabidopsis FAAH

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ABSTRACT

N-Acylethanolamines (NAEs) are fatty-acid derivatives that in animal systems include the well-known bioactive metabolites of the endocannabinoid signaling pathway. Plants use NAE signaling as well, and these bioactive molecules often have oxygenated acyl moieties. Here, we report the three-dimensional crystal structures of the signal-terminating enzyme fatty acid amide hydrolase (FAAH) from Arabidopsis in its apo and ligand-bound forms at 2.1 Å and 3.2 Å resolutions, respectively. This plant FAAH structure revealed features distinct from those of the only other available FAAH structure (rat). The structures disclosed that although catalytic residues are conserved with the mammalian enzyme, AtFAAH has a more open substrate-binding pocket that is partially lined with polar residues. Fundamental differences in the organization of the membrane-binding “cap” and the membrane access channel also were evident. In accordance with the observed structural features of the substrate-binding pocket, kinetic analysis showed that AtFAAH efficiently uses both unsubstituted and oxygenated acylethanolamides as substrates. Moreover, comparison of the apo and ligand-bound AtFAAH structures identified three discrete sets of conformational changes that accompany ligand binding, suggesting a unique “squeeze and lock” substrate-binding mechanism. Using molecular dynamics simulations, we evaluated these conformational changes further and noted a partial unfolding of a random-coil helix within the region 531–537 in the apo structure, but not in the ligand-bound form, indicating that this region likely confers plasticity to the substrate-binding pocket. We conclude that the structural divergence in bioactive acylethanolamides in plants is reflected in part in the structural and functional properties of plant FAAHs.

NAE signaling is a conserved lipid signaling pathway that has been functionally diversified in multicellular organisms to regulate specific developmental, physiological and behavioral processes. These include wide-ranging processes such as lifespan in C. elegans (1), seedling development in Arabidopsis (2, 3), neurotransmission in mammals (4), and satiety in vertebrates (5). In all organisms examined to date, hydrolysis of the ethanolamine moiety by fatty acid amide hydrolase (FAAH) terminates the signaling
functions of the NAE (6). However, important differences in fatty acid composition among organisms indicate that there are differences in the types of NAEs employed for signaling, and this may be reflected in as yet undetermined differences in the signal-terminating enzyme FAAH. For example, higher plants generally do not contain arachidonic acid, and so anandamide (the ethanolamide conjugate of arachidonic acid) is not a common NAE signaling molecule in plants (7). Instead, plants utilize NAEs with shorter acyl chains (8), and it is the oxylipin metabolites of polyunsaturated NAEs that represent the actual bioactive molecules that modulate seedling development (2,3). Hence, endocannabinoid signaling in animals depends primarily on the regulation of the levels of C20, unsubstituted NAEs by FAAH, while NAE signaling in plants is primarily driven by shorter-chain, often oxygenated NAEs.

In plants, NAEs are most abundant in desiccated seeds and their levels decline dramatically during seed germination and seedling establishment (9). The decline in NAE levels is primarily dependent upon hydrolysis by FAAH, where FAAH activity in Arabidopsis was shown to increase during seedling establishment, consistent with the timing of NAE depletion (10). In addition to hydrolysis by FAAH, polyunsaturated NAEs (e.g. NAE 18:2 and NAE 18:3) in plants are oxygenated by various lipoxygenases (LOXs) to generate a series of NAE oxylipin derivatives with oxygenation substitutions at either position 9 or 13 of the acyl chain (11). It had been assumed that like in mammals, the parent, unsubstituted NAE molecules were the biologically active components in plants; however, recent evidence suggested that it was actually the oxylipin derivatives of NAE 18:2 and NAE 18:3 that negatively impacted seedling growth (2,3,9). This represents a major difference in acylethanolamide signaling between plants and animals, and raises the question of whether FAAH in plants has structurally diverged to accommodate the hydrolysis of both unsubstituted and oxygenated NAEs in order to regulate NAE signaling in plant systems.

The three-dimensional structure of rat FAAH has been instrumental in understanding the catalytic features of this enzyme, and in developing small molecule therapeutic inhibitors for manipulation of the endocannabinoid system in humans (12-14).

However, the evolutionary distribution of diverse acylethanolamide signaling molecules outside of vertebrates, and the lack of any structural information for FAAH enzymes beyond that of rat FAAH (or humanized variants), leaves an important gap in knowledge about a fundamental lipid signaling pathway in eukaryotes. Herein, we address this gap by reporting the three-dimensional structure for full-length, recombinant Arabidopsis (At)FAAH, in both a ligand-free form and complexed with an irreversible inhibitor, methyl α-linolenyl fluorophosphonate (MLnFP), allowing for a mechanistic understanding of the interaction of plant FAAH with its acylethanolamide substrates.

Results and Discussion

The 3D structure of AtFAAH

Full-length AtFAAH was expressed in E. coli, purified and crystallized in the presence of the detergent n-dodecyl-β-D-maltoside (DDM). Both the apo- and MLnFP-bound AtFAAH were crystallized in a space group P21, with 2 and 12 molecules per asymmetric unit, respectively. The crystal structure of the apo-AtFAAH was solved at 2.1 Å by molecular replacement using the transamidosome structure (PDB: 3KFU) (15) as a search model. The structure of AtFAAH complexed with MLnFP was determined at 3.2 Å using the apo-enzyme structure as template.

AtFAAH is a homodimer with a buried surface of 1636 Å² per monomer. Each monomer consists of a twisted β-sheet surrounded by 23 α-helices (Figs. 1 and S1). Its core structure resembles that of other amidase signature (AS) enzymes, with high similarity to glutamine amidotransferase subunit A (PDB: 3KFU or 2DF4) (15,16), allophanate hydrolase (4ISS) (17), and aryl acylamidase (4YJ6; 18) (Z-score=44.6-41.6; higher Z-score indicates greater structural similarity), but shows lower similarity to rat FAAH (Z-score=37.6), according to a comparative analysis with the DALI search server (19). The relative similarity between AtFAAH and these other amidases is shown visually as structure overlays (Fig. 2). AtFAAH contains an additional N-terminal region (1-72) with three helices and a long loop that distinguishes it from other AS family enzymes, and also from rat FAAH (Fig. S2).
AtFAAH is a membrane-associated protein, and its N-terminus likely plays a key role in membrane binding. A ~50 Å (~25 Å per monomer) long hydrophobic rim/port formed by 12 hydrophobic residues, including Leu33, Leu37, Leu41, Leu44, Ile47, and Leu50 which are arranged like teeth on a comb on α-helices α1 and α2 from both subunits, most likely forms the hydrophobic membrane-binding “cap” of AtFAAH (Fig. 3). This region is predicted to directly interact with the membrane and anchor the enzyme into the cytoplasmic leaflet of the lipid bilayer. Both sides of this hydrophobic rim/port are bounded by both hydrophobic (e.g., Leu30, Ala44, Pro45, Ile51) and hydrophilic residues (e.g., Ser34, Lys36, Asn40, Asp523, and Lys526). The latter set likely interacts with phospholipid head groups in the membrane. By contrast, rat FAAH lacks such a long N-terminal region, and its membrane-binding cap is formed by hydrophobic helices α18 and α19, more toward the C-terminus of the protein (Fig S3).

In the structure of the ligand-bound AtFAAH, the C18 substrate analogue MLnFP was located in a long, deep pocket which defines the AtFAAH acyl-binding channel (ABC) (Figs. 4-6 and S4). The entrance of ligand-binding pocket is composed of a set of hydrophobic amino acids (Ala27, Pro28, Leu30, Phe38, Ile51, Leu55) and two charged residues (Lys26, Asp58) on α-helices α1 and α2 of the unique N-terminal region. This arrangement suggests that hydrophobic ligands access the active site through the membrane “cap”, which may itself be regarded as a component of the entrance channel (Fig. 3). By contrast, the putative substrate entry site of the rat FAAH is formed with hydrophobic residues on the helices α18 and α19 and charged residues Arg486 and Asp403, and this alters the position of the membrane access channel (MAC) relative to the interior core of the substrate-binding pocket (Figs. 5 and S3).

The AtFAAH substrate-binding pocket is mostly hydrophobic, formed with amino acids from the N-terminal long loop (Met25, Ala27), and α2-3 (Leu55, Asn59, Met59), α17 (Val442, Ile445), α18 (Ser472, Ile475, Phe476, Phe579), α21 (Ile532, Thr535, Thr536, Met539), Met540, and Thr545 (Fig. 4). These residues define a long ABC, with the portion near the entrance regarded as the MAC. The MLnFP acyl chain primarily engages in Van der Waals interactions with the hydrophobic residues in the ABC (Fig. 4). Notably, several hydrophilic residues (Asn59, Thr258, His441, Ser472, Thr535, and Thr536) also are present in the substrate-binding pocket (Fig. 4). This feature results in an ABC that is considerably more polar than that of rat FAAH (Figs. 5E and 5F), and endows the AtFAAH ABC the capacity to interact with a diverse array of aliphatic substrates without or with polar functional groups. Primary sequence comparisons of higher plant and mammalian FAAHs showed that a subset of these polar residues are conserved only in higher plant FAAHs, while hydrophobic substituents are conserved in the mammalian enzymes (Fig. S5). The active site is located deep in the bottom of the long substrate-binding pocket, and the phosphorus atom of the inhibitor MLnFP is covalently bonded to the catalytic nucleophile, Ser305 (Figs. 4 and 5E). Ser305, Ser281 and Lys205 form the catalytic triad in the AtFAAH active site, similar to rat FAAH and other AS enzymes. These findings are consistent with previous site-directed mutagenesis studies that demonstrated key catalytic roles for these residues (20).

AtFAAH dimer formation involves a network of hydrogen bonds and Van der Waals interactions contributed by several amino acid residues located at the interface between both subunits. Specifically, residues from the long loop region of the N-terminus (Gln5, Arg46, Thr44, Phe43), α17 (Thr454, Pro455), α20 (Phe479, Ala481), and Asp225 primarily form the oligomerization domain in AtFAAH (Fig. 7). Although different regions of the protein contribute to dimer formation in Arabidopsis and rat FAAH, both enzymes assume a symmetric pattern of dimerization that aligns the protein subunits in a similar orientation so that the membrane-binding cap and the membrane access channel of each subunit is placed on the same face of the dimer (Fig. 1). Such parallel monomer orientations should enhance membrane binding and allow both subunits to function concurrently.

**Unique characteristics of AtFAAH ABC and MAC**

The AtFAAH substrate-binding pocket is more open and relatively more polar than that of rat FAAH (Figs. 5 and 6). In rat FAAH, there are two distinct channels for substrate access and acyl chain binding with two hydrophobic residues (Phe432 and Trp531) forming the so-called “dynamic paddle” located at the junction of these two regions (Figs. 5D and 6B). In AtFAAH, there are no residues that
correspond to Phe\textsuperscript{432} and Trp\textsuperscript{531}, and the substrate is presumed to access directly from the membrane to the acyl chain-binding channel. That is, the MAC and ABC in AtFAAH are not separate structural elements, but rather one long access channel (Figs. 5\textit{C} and 6\textit{A}). Moreover, a detailed comparison of several selected residues lining the substrate-binding pockets of AtFAAH and rat FAAH (Figs. 5\textit{E} and 5\textit{F}) revealed substantial structural differences between the plant and rat enzymes. Although both substrate-binding pockets are lined with aliphatic and aromatic amino acids which surround and interact with the acyl chain of the substrate, the AtFAAH substrate-binding pocket has several polar residues not present in rat FAAH (e.g. Ser\textsuperscript{472} vs. Leu\textsuperscript{404}, Thr\textsuperscript{258} vs. Phe\textsuperscript{194}, as well as other polar residues in AtFAAH such as Thr\textsuperscript{535}) (Figs. 5\textit{E}, 5\textit{F}, and S5). Overall, the more open and polar substrate-binding pocket of AtFAAH suggest a molecular rationale for its ability to accommodate “bulkier” oxylipin substrates.

\textit{AtFAAH accommodates NAE oxylipins}

The 9-lipoxygenase (9-LOX) metabolite of NAE 18:2 (acyleinolamide with 18C and 2 double bonds in the acyl chain) (Figs. 8\textit{A} and 8\textit{B}) is the bioactive molecule that interacts with abscisic acid signaling to invoke a so-called secondary dormancy in \textit{Arabidopsis}. This response is a survival mechanism that enables seedlings to arrest growth under unfavorable environmental conditions (3). We considered the possibility that AtFAAH hydrolyzes both oxylipin and unsubstituted NAE substrates in order to regulate NAE signaling. To determine whether AtFAAH hydrolyzes NAE oxylipins, enzyme kinetic studies and computational docking experiments were performed (Figs. 8\textit{C}-\textit{F}). Initial velocities were measured for recombinant AtFAAH (Fig. 8\textit{C}) and rat FAAH (Fig. 8\textit{D}) as a function of increasing concentrations of either [1-\textsuperscript{14}C]-NAE 18:2 or [1-\textsuperscript{14}C]-NAE-9-HOD. Indeed, AtFAAH hydrolyzed NAE-9-HOD with equal or better catalytic efficiency than the enzyme hydrolyzed NAE 18:2. By contrast, the ethanolamide oxylipin was a decidedly inferior substrate for rat FAAH when measured against the unsubstituted NAE. This in-vitro enzymatic activity of AtFAAH toward NAE-9-HOD is consistent with previous findings that \textit{AtFAAH} overexpressors (OE) and knock-out mutants accumulated lower and higher levels of endogenous NAE-9-HOD, respectively, and that seedlings of \textit{AtFAAH OE} were tolerant to the root-growth-inhibiting effect of exogenous NAE-9-HOD (3). Moreover, docking experiments with NAE-9-HOD in the substrate-binding pocket of AtFAAH revealed that the acyl chain of the substrate can assume two distinct energetically-favorable poses in the active site (Fig. 8\textit{F}). Specifically, MD simulation of the AtFAAH::NAE-9-HOD complex visualized a transition in the binding of NAE-9-HOD between these two poses with the 9-hydroxyl group interacting with either Ser\textsuperscript{472} or Thr\textsuperscript{535} (Movie S1). The identification of potential hydrogen bonding interactions of the oxylipin ligand with the side-chain oxygen of either Ser\textsuperscript{472} or Thr\textsuperscript{535} was consistent with the theoretical possibility that AtFAAH accommodates the binding of NAE oxylipins.

\textit{Conformational changes upon ligand binding}

Determination of AtFAAH structures without and with bound ligand identified three discrete sets of conformational changes in AtFAAH that accompanied ligand binding. First, the movement of amino acids 25-28 that form one side of the MAC, as well as movement of Leu\textsuperscript{55} on the opposite side of the MAC, resulted in MAC closure upon ligand binding (Fig. 9). Second, a conformational rotation of the Asn\textsuperscript{59} side chain had the dual effects of extending the substrate-binding pocket so as to support ligand binding and contributing to MAC closure (Fig. 9). These gating residues in AtFAAH (especially Ala\textsuperscript{27}, Pro\textsuperscript{28}, Leu\textsuperscript{55}, and Asn\textsuperscript{59}) are conserved in sequences of other higher plant FAAH enzymes (Fig. S2). The third set of discrete conformational changes involved the 531-537 region of helix \textit{α}21 which shifted toward, and interacted with, the bound ligand. Particularly prominent in that regard were residues Ile\textsuperscript{532}, Thr\textsuperscript{535} and Thr\textsuperscript{536}, all of which contribute to the AtFAAH ABC (Fig. 9).

These collective conformational changes in AtFAAH “squeeze and lock” the substrate into the binding pocket for hydrolysis. Consistent with this idea, comparison of solvent accessible surface areas (SASA) and cavity volumes of ‘apo’ and ligand-bound conformations indicated that there were ca. 8% and 16% reductions in SASA and substrate cavity volumes upon ligand binding, respectively.
On the contrary, similar calculations for rat FAAH in ‘apo’ (PDB: 3QJ8; (14)) and inhibitor-bound (PDB: 3QJ9; (14)) conformations showed 15% and 10% increase in SASA and cavity volume, respectively, further suggesting the different mechanism of ligand entry and binding. Indeed, the “squeeze and lock” mechanism for AtFAAH differs fundamentally from that posited for rat FAAH, where Phe\textsuperscript{332} rotates its side chain orientation from the ABC to the MAC upon ligand binding, and is proposed to guide the substrate toward the active site for hydrolysis (13,14,21).

To further dissect the conformational transitions in the MAC and ABC regions of AtFAAH upon ligand binding, all-atom molecular dynamics (MD) simulations were carried out. In the AtFAAH apo-structure, a partial unfolding of the 531-537 helix region was observed (Fig. 10A and Movie S2). By contrast, that structural element was stabilized and shifted toward the ligand in the AtFAAH::MLnFP bound structure. In that conformation, the 531-537 helix region enclosed the ABC in a manner consistent with the crystal structure (Fig. 10B and Movies S3 and S4). These 531-537 helical region dynamics were analyzed in further detail in two additional sets of independent MD simulations where: i) MLnFP was removed from the co-crystal structure, resulting in derived ‘apo’ structure ii) MLnFP was docked into the ‘apo’ crystal structure to produce a docked co-crystal structure. In both sets of simulations, the conformational dynamics of this helical region were reproduced (Figs. 10C and 10D; Movies S5 and S6). The presence of this random coil motif in AtFAAH likely confers more plasticity to the substrate-binding pocket, which in turn makes the enzyme more able to accommodate a broader range of substrates. Moreover, a noticeable shift and uncoiling in the N-terminus α1 and α2 helices was also observed in these MD simulations (especially in the absence of ligand; Figs. 10A and 10C), suggesting concerted movements of these key regions in the MAC and ABC are required to accommodate ligand binding.

**Conclusions**

Taken together, our results outline the structural organization of AtFAAH that supports its efficient activity toward a range of acylethanolamides that include oxygenated derivatives. Our data project that this is a result of a “malleable”, easily accessible, and relatively more polar substrate-binding pocket that supports a “squeeze and lock” substrate-binding mechanism (Fig. 11A). This is fundamentally distinct from the case for rat FAAH that is proposed to consist of discrete membrane access and substrate-binding channels, and requires a conformational “flip” of dynamic paddle residues to correctly orient the substrate for catalysis (Fig. 11B). Moreover, these results add new structural and functional information to the family of amidase signature enzymes more broadly, and provide a mechanistic explanation for the structural divergence of acylethanolamide signaling in plants and endocannabinoid signaling in mammals.

The utilization of NAEs as signaling molecules is a common feature of most, if not all, multicellular organisms (9). The formation and turnover of this group of lipids is broadly conserved across animal and plant kingdoms, particularly with regard to signal termination by the action of FAAH (6). Herein, we identify the structural differences in the signal-terminating enzyme AtFAAH that facilitate the efficient hydrolysis of ethanolamide oxylipins. This diversification in ligand specificity represents an important evolutionary adaptation of NAE signaling in higher plants. These collective results not only support an expanded view of NAE signaling beyond that of the intensively-studied endocannabinoid pathway of mammals, but also support the concept that oxylipin derivatives of NAEs have important signaling functions with activities that are terminated by FAAH enzymes in higher plants.

FAAH recently was suggested to play a key role in plant responses to a class of bacterial quorum-sensing (QS) signals, namely N-acyl l-homoserine lactones (AHLs). FAAH hydrolysis of AHLs was shown to be pivotal for plants to perceive these QS signals, and hence mediate plant-microbe interactions (22,23). These studies emphasized the concept that AtFAAH can utilize a broad range of substrates, which is consistent with our structural findings with respect to the openness and flexibility of AtFAAH substrate-binding pocket. The AtFAAH structure presented here will form the basis for future studies to understand how plant FAAHs recognize and utilize AHLs as substrates, and to explore the diversity of FAAH-like enzymes in various plant species.
Experimental procedures

Arabidopsis FAAH protein expression and purification

Recombinant AtFAAH (At5g64440, Uniprot # Q7XJJ7) was expressed in E.coli TOP10 cells from the pTrcHis2 plasmid as described previously (24), with a few modifications in expression, enzyme extraction and purification to support crystallization studies. Overnight cultures grown in LB medium containing 100 µg/mL ampicillin (37 °C) were inoculated into fresh medium and grown at 37 °C to an OD₆₀₀ of 0.5-0.7. Recombinant protein expression was induced with 1 mM IPTG for 20 hrs at 22 °C. Cells were harvested by centrifugation (4000 X g, 10 min, 4 °C), and frozen at -20 °C for at least 24 hrs. Frozen cells were thawed and suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% v/v Triton X-100) supplemented with 1 mg/mL lysozyme and 25 U/mL Benzonase Nuclease (Sigma-Aldrich; St. Louis, MO). After incubation on ice for 30 mins, cells were sonicated on ice with fifteen 30-sec bursts at 50% intensity, with 30-sec cooling period between bursts (Microson MS-50 Ultrasonic Cell Disruptor; 50 Watts). The crude lysate was centrifuged at 14000 X g for 45 mins at 4 °C, and the supernatant was loaded onto Ni-NTA Agarose beads (Qiagen). The beads were successively washed with three wash buffers of increasing imidazole and n-dodecyl-β-D-maltoside (DDM) concentrations to elute nonspecific proteins and to exchange detergents: (1) 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% v/v Triton X-100, 10 mM imidazole; (2) 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% v/v Triton X-100, 25 mM imidazole; (3) 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.01% w/v DDM, 40 mM imidazole. Finally, recombinant His-tagged proteins were eluted with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.03% DDM, 250 mM imidazole, and concentrated using Amicon Ultra 30K centrifugal filter devices (Millipore; Bedford, MA) with the buffer exchanged to BTP buffer (50 mM Bis-Tris propane, pH 9.0, 100 mM NaCl, 0.03% w/v DDM). Purified recombinant FAAH protein was fractionated by size-exclusion FPLC (Amersham Pharmacia Biotech) using Superdex 200 Increase 10/300 GL columns (GE Healthcare Life Sciences). The column was equilibrated with BTP buffer (50 mM Bis-Tris propane, pH 9.0, 100 mM NaCl, 0.03 % w/v DDM), and the eluted proteins were monitored by UV absorbance at 280 nm. Arabidopsis FAAH was eluted in two size fractions (of about 200 and 400 kDa), compared with molecular weight standards. FAAH was confirmed to be in these fractions by SDS-PAGE analysis and enzyme activity assays. Only the AtFAAH fractions in the 200 kDa range (Fig. S1; fraction 2) were collected, concentrated as above, aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C until use. The molecular mass calibration of the column was performed using a commercially-available gel filtration standard (BioRad; Hercules, CA), which is a mixture of molecular weight markers ranging from 1,350 to 670,000 daltons. The concentration of the purified protein was determined by Pierce BCA protein assay kit (Thermo-Fisher Scientific; Rockford, IL). High yields of the full-length AtFAAH (5-7 mg of purified protein per liter of culture) enabled a large number of crystallization experiments.

Arabidopsis FAAH protein crystallization

The purified AtFAAH protein was initially screened by the sparse-matrix method (25) using the crystal screen kits from Qiagen at 20 °C, by sitting drop vapor diffusion with a Phoenix crystallization robot (Art Robbins Instruments; Sunnyvale, CA). Protein sample (20-25 mg/mL) was mixed with an equal volume of reservoir solution and the mixture was equilibrated over the reservoir solution. Crystals of AtFAAH were obtained after extensive screening (800 different conditions). Crystallization was optimized further by hanging drop vapor diffusion where larger, high-quality AtFAAH crystals were obtained in hanging drops formed of 1 µL concentrated protein (25 mg/mL) and 1 µL of reservoir solution (0.1 M MES buffer, pH 6.0, 30% PEG 200, 5% PEG 3350) at 20 °C. For the ligand-bound AtFAAH, crystals were obtained by co-crystallization under the same conditions, but the irreversible inhibitor methyl α-linolenyl fluorophosphonate (MLnFP; Cayman Chemical; Ann Arbor, MI) was dissolved in DMSO and added to the reservoir solution to achieve a final protein-to-ligand molar ratio of 1:5.

X-ray diffraction data collection

X-ray diffraction datasets were collected for ligand-free (apo-form) and ligand-bound AtFAAH crystals at 2.1 Å and 3.2 Å, respectively, at beamline 14-1 of the Stanford Synchrotron...
Radiation Lightsource (SSRL). The data were processed by the program HKL2000 (26). Both crystals belonged to the space group P2₁, but with different unit cell parameters; for apo AtFAAH a = 72.08 Å, b = 79.66 Å, c = 132.59 Å, β = 104.4°; while for ligand-bound AtFAAH a = 225.57 Å, b = 83.31 Å, c = 272.81 Å, β = 110.98°. The calculated Matthews coefficients (Vₐₚ = 2.5 Å³Da⁻¹) (27), corresponding to a solvent content of 50%, indicated the presence of two and twelve molecules in the asymmetric unit for apo- and ligand-bound AtFAAH, respectively.

Structure determination and refinement

For structure determination of apo-AtFAAH, molecular replacement studies were carried out with the program Phaser (28), and a good solution was obtained with the transamidosome structure (PDB: 3KFU) (15) as a search model. Interactive model building was carried out with the program Coot (29). Crystallographic structure refinements were performed using the programs Refmac (30) and Phenix (31). The B-factors were refined individually. Water molecules were added with Phenix and checked manually for inclusion. The high resolution and the good quality of the electron density map allowed for model building of all the amino acids except a small gap at 118-125, where the poor electron density in this region did not allow for model building of these 8 amino acids (probably a disordered or flexible region in the apo-AtFAAH structure). Other few missing residues in the structure include the first three amino acids at the N-terminus of one subunit (chain A) and the last two amino acids at the C-terminus of both subunits. The program PROCHECK (32) was utilized to check the model, and all backbone φ-ψ torsion angles are within the allowed regions of the Ramachandran plot.

For ligand-bound AtFAAH, a good molecular replacement solution was obtained by using the apo-enzyme dimer structure as a search model. Model building and refinement were carried out with the same programs. In this case, the structure model has 6 dimers in the asymmetric unit. All amino acid residues were modeled into the electron density map except the first 3 amino acids in chains A, C, E, G, I, and K; and the last two amino acids in chains A-G, I, and K. Three chains (H, J, and L) had an additional map at their C-termini, most likely corresponding to some sequence from the plasmid (pTrcHis2), so about 9 additional amino acid residues were modeled into these regions. All amino acids are within the allowed regions of the Ramachandran plot. The small molecule, MLnFP model was built using the methyl arachidonyl fluorophosphonate (MAFP) from the rat FAAH structure (PDB: 1MT5) (12) as a template, and manually fitted into the electron density map found in the substrate-binding pocket of AtFAAH. The ligand chemical restraints were generated and geometry-optimized with the programs REEL (33) and eLBOW (34) in Phenix. A summary of the data collection and refinement statistics is provided (Table S1).

Rat FAAH protein expression and purification

The recombinant plasmid, rat FAAH1::pTrcHis2 (NCBI accession number NP_077046), was provided by Dr. Benjamin Cravatt’s laboratory (35), and was introduced into E.coli TOP10 cells. In order to compare the kinetic behavior of Arabidopsis and rat FAAH toward NAE 18:2 and NAE-9-HOD, the rat FAAH recombinant protein was purified by the same expression and purification procedures described above for Arabidopsis FAAH.

FAAH enzyme kinetics

FAAH enzyme assays were conducted with radiolabeled substrates as previously described (36) with some modifications. The [1-¹⁴C]-NAE 18:2 was synthesized and purified as described (36), mixed with non-radiolabeled NAE 18:2 (Cayman Chemical) in two different ratios, and the radiospecific activities were used to calculate enzyme activity following radiometric scanning of TLC-separated FAAH reaction products. Two ratios of radioactive to non-radioactive substrates were prepared so that a wide range of substrate concentrations in the final enzymatic reactions could be tested without excessive dilution of the radioactive molecules. In addition, batches of these two NAE 18:2 mixtures (non-radiolabeled + ¹⁴C-labeled) were used to synthesize radiolabeled NAE-9-HOD ([1-¹⁴C]-NAE-9-HOD) using a commercial preparation of potato lipooxygenase (LOX) (with 9-LOX activity; Cayman Chemical), and following previously described procedures (11). Quantities of the purified NAE-9-HOD recovered from each reaction were calculated based on the radiospecific activity of the product following TLC separation.
Parallel reactions were performed with unlabeled NAE 18:2 only and analyzed by GC-MS to confirm the identity of the product (NAE-9-HOD) and evaluate the purity of product yielded from the 9-LOX reactions. The conversion rate of NAE 18:2 to NAE-9-HOD was more than 90% in all reactions. For consistency, all enzymatic assays for both Arabidopsis and rat FAAH were performed with the same batches of synthetic substrates. FAAH assays were performed in triplicate at increasing substrate concentrations (10-120 µM) of either [1-14C]-NAE 18:2 or [1-14C]-NAE-9-HOD in 0.25 mL 50 mM Bis-Tris propane-HCl, pH 9.0, 0.2 mM DDM at 30 °C for 5 mins with shaking (120 rpm). The reactions were initiated by adding 0.1 µg and 0.27 µg of Arabidopsis and rat FAAH, respectively, and terminated by adding hot isopropanol (2 mL, 70 °C, 30 min). After cooling to room temperature, lipids were extracted into chloroform, separated by TLC, and analyzed by radiometric scanning as described elsewhere (36). Initial velocity measurements at increasing substrate concentrations were plotted with Prism software version 3.0 (GraphPad Software; San Diego, CA), where the data were fitted to a non-linear regression (curve fit) using a one site binding (hyperbola) equation. Correlations between actual measurements and the fitted curves were between R² = 0.94 to 0.98.

Computational docking experiment of NAE-9-HOD

The protein models were prepared using the Protein Preparation Wizard panel in the Schrödinger suite (2017-4, Schrodinger, LLC, Mew York, NY, 2017) (37). Complete structure of AtFAAH was optimized with the OPLS_2005 force field in the Schrödinger suite to relieve all atom and bond strains found after adding all missing side chains and/or atoms. The small molecule model structure for the compound 9-hydroxy-10,12-octadecadienoyl-ethanolamide (NAE-9-HOD) was prepared and energy-minimized in MOE (2016.08; Chem. Comp. Group Inc., Montreal, Canada) (38) and the lowest energy conformation was selected for docking.

Computational docking was carried out using the genetic algorithm-based ligand docking program GOLD 5.2.1 (39). GOLD explores ligand conformations fairly exhaustively and also provides limited flexibility to protein side chains. For computational docking, crystal structure of the apo-AtFAAH was used. The active site was defined by taking the centroid of catalytic residues in the crystal structure as a reference center to define protein binding site of radius 10 Å around it, with the GOLD cavity detection algorithm. GOLD covalent docking was carried out to find diverse poses of the NAE-9-HOD ligand with a covalent bond constraint set between the side chain oxygen (OG) of the nucleophilic Ser305 and the carbonyl carbon of the ligand. In order to explore all the possible binding modes, docking was carried out to generate diverse solutions with early termination turned off. All other parameters were as the defaults. NAE-9-HOD was then docked and scored using CHEMPLP scoring function within GOLD as it has been found to give the highest success rates for both pose prediction and virtual screening experiments against diverse validation test sets (40).

Determination of solvent accessible surface area (SASA) and cavity volume

Solvent accessible surface area (SASA) and cavity volume were calculated on the crystal structures of the apo- and ligand-bound AtFAAH. Similarly, for rat FAAH, ‘apo’ (PDB: 3QJ8; (14)) and inhibitor-bound (PDB: 3QJ9; (14)) conformations were used. A 5Å distance cutoff around ligand was used for the calculation. SASA of binding cavity and change in surface area upon ligand binding were calculated with binding_sasa.py script implemented within Schrödinger suite (2017-4, Schrodinger, LLC, Mew York, NY, 2017) (37). Cavity volume was calculated using SiteMap application within Schrödinger suite (2017-4, Schrodinger, LLC, Mew York, NY, 2017) (37).

Molecular dynamics simulation experiments

All-atom MD simulations were carried out for 100 ns on AtFAAH structures. Crystal structures of AtFAAH in apo ‘open’ conformation and in complex with the irreversible inhibitor MLnFP were used to build starting structures for MD simulations. MD simulations were run for 100 ns each using Desmond 2016-3 version of the MD simulation package (41).
Schrodinger version 2016-3. (D.E. Shaw, Schrodinger Inc.), with the OPLS-AA force field as defined in Desmond. System Builder within Desmond/Schrodinger was used to generate a solvated system of protein-ligand complex. An orthorhombic box with 12 Å buffer around protein-ligand complex was used to generate periodic box. An all-atom atomistic scale MD simulation was carried out in periodic box of explicit water molecules. For water, the TIP3P water model that is compatible with the OPLS-AA parameterization was employed. Simulation was carried out at 300 K at physiological salt concentration of 150 mM NaCl. Counter ions were included to neutralize the total charge of the system. The OPLS-AA all-atom force field was used to describe all molecules. For AtFAAH::MLnFP, a force constraint was used between Ser305 and the phosphorus atom of MLnFP head group to account for covalent bond interaction. The system was relaxed using Maestro’s system relaxation protocol before the production run. This includes a multi-stage relaxation protocol with two stages of minimization (restrained and unrestrained) followed by five stages of MD runs with gradually diminishing restraints. All MD production runs were carried out at constant temperature and pressure. Long-range electrostatic interactions were estimated using the particle mesh Ewald, whereas bonds involving protons were constrained using the SHAKE algorithm. A 2-fs time step was used throughout the simulation. For Thermostat Nose-Hoover chain method was applied with relaxation time of 1 ps. Barostat parameters were set according to Martyna-Tobias-Klein with a relaxation time of 2.0 ps with isotropic coupling. A 9 Å cutoff was applied to Lennard-Jones interactions, and the non-bonded list was updated every 1.2 ps. The production run was continued for 100 ns, and snapshots of the coordinates were written out every 1 ps. MD simulations were repeated for n=3, starting from a random seed for each simulation. The results were analyzed and plots were generated within Maestro’s Simulation Event Analysis and Simulation Interaction Diagram modules.

Data and materials availability

The structures of AtFAAH were deposited in the Protein Data Bank with the accession codes 6DHV (without ligand) and 6DII (complexed with ligand).
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Author contributions: M.A. performed the protein expression and purification, protein crystallization, X-ray diffraction data collection, model building and refinement, and FAAH enzyme kinetics; X.W. guided and assisted with the protein crystallization, X-ray diffraction data collection, and model building and refinement; A.T. performed the computational docking and molecular dynamics simulations under the supervision of V.A.B.; K.D.C. supervised the overall project; M.A., X.W. and K.D.C. drafted the majority of the manuscript, except for sections involving molecular dynamics simulations which were drafted by A.T. and V.A.B; all authors contributed to discussions and interpretations of the data as well as editing the manuscript.
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FOOTNOTES
The abbreviations used are: FAAH, fatty acid amide hydrolase; NAE, N-acylethanolamine; MLnFP, methyl α-linolenyl fluorophosphonate; DDM, n-dodecyl-β-D-maltoside; AS, amidase signature; ABC, acyl-binding channel; MAC, membrane access channel; MD, molecular dynamics; SASA, solvent accessible surface areas; NAE-9-HOD, 9-hydroxy-10,12-octadecadienylethanolamide; OE, overexpressors; QS, quorum-sensing; AHLs, N-acyl γ-homoserine lactones; MAFP, methyl arachidonyl fluorophosphonate; LOX, lipoxygenase; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni-NTA, Nickel-nitrilotriacetic acid; BTP, Bis-Tris propane; BCA, bicinchoninic acid; MES, 2-(N-morpholino)ethanesulfonic acid; SSRL, Stanford Synchrotron Radiation Lightsource; OPLS, optimized potentials for liquid simulations.
Figure 1. *Arabidopsis* FAAH three-dimensional structure. A and B, side (A) and top (B) views of AtFAAH structure. AtFAAH is a homodimer assembled from 66-kDa subunits. Each subunit is shown in a color gradient ranging from blue (N-terminus) to green (C-terminus) for one subunit (Chain A), and from yellowish-green to red for the other subunit (Chain B). The presumed membrane-binding cap (α1 and α2) and the putative substrate entryway (membrane access channel; MAC) are located at the N-terminus of the enzyme. The AtFAAH dimer interface is formed mainly by parts of helices α17 and α20 and some regions of the N-terminus (see Fig. 7).
Figure 2. Comparison of Arabidopsis FAAH structure with other amidase signature (AS) enzymes. A-F, AtFAAH (green; PDB: 6DHV) was superposed to the structures of rat FAAH (gray; PDB: 3QJ8 (14)) (A and D), glutamine amidotransferase subunit A (wheat; PDB: 3KFU (15)) (B and E), and aryl acylamidase (pink; PDB: 4YJ6 (18)) (C and F) and presented as structure overlays from two different views. The N- and C-termini of each enzyme are indicated with letters of the same color as the corresponding protein structure.
Figure 3. The putative membrane-binding cap of AtFAAH. The hydrophobic helices α1 and α2 of the N-terminus (amino acids 27-60) are rich in hydrophobic amino acids (21/34) and are predicted to form the membrane-binding cap of AtFAAH that is presumed to anchor the enzyme into half of the lipid bilayer. A and B, one monomer of apo-AtFAAH (PDB: 6DHV) integrated into the membrane is shown in both cartoon (A) and electrostatic surface (B) depictions with positive charged areas in blue, negative charged areas in red, and hydrophobic areas in white. C and D, electrostatic molecular surface of AtFAAH viewed from the membrane face showing the membrane-binding cap and the membrane access channel (MAC) in the open (C) and closed (D) conformations, in apo- (PDB: 6DHV) and MLnFP-bound (PDB: 6DII) AtFAAH, respectively. Some of the hydrophobic amino acids on the helices α1 and α2 are highlighted as yellow sticks with their numbers and single-letter codes are indicated.
Figure 4. *Arabidopsis* FAAH active site and substrate-binding pocket. The acyl chain of the irreversible inhibitor methyl α-linolenyl fluorophosphonate (MLnFP; cyan sticks) is surrounded by several aliphatic and aromatic amino acids, as well as some polar residues (e.g. Asn\(^{59}\), Thr\(^{258}\), His\(^{441}\), Ser\(^{472}\), Thr\(^{535}\), and Thr\(^{536}\)). All the depicted residues (slate blue lines) are within 5Å distance from the inhibitor. Representative Van der Waals interactions between the ligand and some of the surrounding residues are shown as blue dashed lines with the distance of each potential interaction is indicated; only few selected interactions are shown for simplification. The Ser\(^{305}\)-Ser\(^{281}\)-Lys\(^{205}\) catalytic triad is shown (slate blue sticks) with the nucleophilic Ser\(^{305}\) covalently bound to the phosphorous atom of MLnFP. The 2Fo-Fc electron density map of MLnFP contoured at 1.0 σ is shown in gray.
Figure 5. *Arabidopsis* FAAH substrate-binding pocket is more accessible and relatively more polar than that of rat FAAH. *A* and *B*, chemical structures of the irreversible inhibitors methyl α-linolenyl fluorophosphonate (MLnFP) and methyl arachidonyl fluorophosphonate (MAFP). *C* and *D*, one subunit of the ligand-bound AtFAAH (*C*; PDB: 6DII) and rat FAAH (*D*; PDB: 1MT5) (12) are shown with the protein molecular surface rendered gray and transparent to illustrate the key differences between both enzymes with respect to the membrane access channel (MAC) and the acyl-binding channel (ABC). The putative membrane-binding cap is colored magenta (α1 and α2) and red (α18 and α19) in AtFAAH and rat FAAH, respectively. In rat FAAH, the “dynamic paddle” residues, Phe432 and Trp531, are shown as green sticks. *E* and *F*, comparison of the substrate-binding pockets of AtFAAH (*E*; PDB: 6DII) and rat FAAH (*F*; PDB: 1MT5) complexed with MLnFP (cyan sticks) and MAFP (yellow sticks), respectively. Both enzymes were superposed and the substrate-binding pockets were compared; only few selected residues are shown for simplification. The amino acid residues of AtFAAH are shown as slate blue sticks, while those of rat FAAH are shown as orange sticks. The numbers on the ligands indicate the position of the double bonds.
Figure 6. Differences in the organization of the membrane access channel (MAC) and the acyl-binding channel (ABC) between Arabidopsis and rat FAAH. A and B, one subunit of the ligand-bound Arabidopsis FAAH (A) (PDB: 6DII) and rat FAAH (B) (PDB: 1MT5) (12) were superposed, and their cavities/channels are shown as dark-gray shadows. In rat FAAH, there are two distinct channels for substrate access and acyl chain binding with two hydrophobic residues (Phe432 and Trp531; shown in orange, space-filling representation) forming the so-called “dynamic paddle” located at the junction of these two channels. In Arabidopsis FAAH, there are no corresponding residues to Phe432 and Trp531, and the substrate is presumed to access directly from the membrane to the acyl-binding channel. In other words, in AtFAAH, there is only one large cavity for both substrate access and binding (i.e. indistinguishable MAC and ABC). The catalytic triad residues are shown as slate blue and orange sticks in Arabidopsis and rat FAAH, respectively. The irreversible inhibitors methyl α-linolenyl fluorophosphonate (MLnFP; cyan sticks) and methyl arachidonyl fluorophosphonate (MAFP; yellow sticks) are shown bound to the active site of Arabidopsis and rat FAAH, respectively.
Figure 7. Atomic interactions that contribute to dimer formation in AtFAAH. A, the overall dimer of *Arabidopsis* FAAH with the dimerization region indicated by a red circle. B, a close-up view of this dimerization region with some of the key residues depicted as sticks; AtFAAH dimer interface is formed mainly by parts of helices α17 and α20 and some loop regions of the N-terminus. C, detailed representation of the network of hydrogen bonds and Van der Waals interactions at the monomer-monomer interface of AtFAAH. Residues from one subunit (chain A) are shown as green sticks, while those from the other subunit (chain B) are shown as cyan sticks. The single-letter code and the number of each amino acid as well as the distance of each potential interaction are indicated.
Figure 8. Arabidopsis FAAH can accommodate and hydrolyze NAE oxylipins more efficiently than rat FAAH. A and B, chemical structures of linoleoyl ethanolamide (NAE 18:2) and 9-hydroxy-10,12-octadecadienoylethanolamide (NAE-9-HOD). C and D, initial velocities were measured for AtFAAH (C) and rat FAAH (D) with increasing concentrations of either [1-14C]-NAE 18:2 or [1-14C]-NAE-9-HOD. Data points represent means ± S.D. of triplicate enzymatic assays. E, summary of the apparent kinetic parameters of both enzymes. F, docking of NAE-9-HOD in the substrate-binding pocket of AtFAAH with the oxygenated acyl chain of the substrate displayed in two different binding poses; pose 1 shown as orange sticks while pose 2 shown as magenta sticks. Arabidopsis FAAH amino acid residues are depicted as green sticks. In AtFAAH, Ser^{472} is well-positioned to accommodate and form a hydrogen bond interaction with the hydroxyl group at position 9 when the NAE-9-HOD acyl chain exhibits binding pose 1, while Thr^{535} on the opposite side of the substrate-binding pocket can form a hydrogen bond with the 9-hydroxyl group when the substrate is in binding pose 2 (see Movie S1). The numbers of the ligand atoms (9-13) are indicated for clarity.
Figure 9. Conformational changes in Arabidopsis FAAH structure upon ligand binding. Superposed structures of one subunit of AtFAAH in both the apo (green; PDB: 6DHV) and ligand-bound (slate blue; PDB: 6DII) forms. The ligand methyl α-linolenyl fluorophosphonate (MLnFP) is depicted as blue spheres. Regions of the protein that undergo conformational changes are zoomed in on to demonstrate the details of each group of changes. The zoomed in surface region is a close-up view of the protein molecular surface from the membrane face showing the open (green) and closed (slate blue) membrane access channel (MAC) in the apo- and MLnFP-bound AtFAAH, respectively; the protein surface was rendered partially transparent to show the corresponding amino acid residues.
Figure 10. Molecular dynamics simulations of *Arabidopsis* FAAH. *A*, overlay of the first (light-gray) and last (pink) frames of a 100-ns MD simulation of the apo-AtFAAH structure (see Movie S2). *B*, overlay of the first (dark-gray) and last (magenta) frames of a 100-ns MD simulation of the MLnFP-bound structure (see Movie S4). *C*, the ligand, MLnFP was removed from the bound co-crystal structure and the resulting ‘apo’ structure was simulated for 100 ns (see Movie S5); the first and last frames are shown in dark-gray and teal, respectively. *D*, MLnFP was docked into the apo-AtFAAH structure and the resulting AtFAAH::MLnFP complex was simulated for 100 ns (see Movie S6); the first and last frames are shown in light-gray and golden yellow, respectively. In (*A*) and (*C*), partial unfolding of the 531-537 helix region and noticeable shifts in the N-terminus α1 and α2 helices were observed in the absence of the ligand, while in (*B*) and (*D*), these changes were not observed in the presence of MLnFP. The mesh rendering indicates the surface of the cavity.
Structural Features of Arabidopsis FAAH

Figure 11. Schematic representation of the substrate-binding mechanisms in Arabidopsis and rat FAAH. The ligand-free AtFAAH has a long, widely-open acyl-binding channel (ABC) with the part near the entrance regarded as the membrane access channel (MAC). Ligand binding triggers movement of the 531-537 helix region of the ABC toward the substrate, resulting in a “squeezed” ABC. This is accompanied by a concomitant movement of residues 25-28 as well as Leu55, located on the opposite sides of the MAC, resulting in a “locked” MAC. These changes in AtFAAH upon ligand binding “squeeze and lock” the substrate into the binding pocket for hydrolysis. By contrast, rat FAAH has two separate acyl-binding and membrane access channels. In the absence of ligand, the “dynamic paddle” residue Phe432 is oriented toward the ABC, resulting in a short ABC and a long, open MAC. Upon ligand binding, Phe432 changes its side chain orientation from the ABC to the MAC, resulting in an extended ABC and partially closed MAC. This conformational “flip” of the “dynamic paddle” residue is proposed to guide the substrate toward the active site for hydrolysis (13,14,21).
Structural analysis of a plant fatty acid amide hydrolase provides insights into the evolutionary diversity of bioactive acylethanolamides
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