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STRUCTURE AND FUNCTION OF FATTY ACID AMIDE HYDROLASE

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■ **Abstract** Fatty acid amide hydrolase (FAAH) is a mammalian integral membrane enzyme that degrades the fatty acid amide family of endogenous signaling lipids, which includes the endogenous cannabinoid anandamide and the sleep-inducing substance oleamide. FAAH belongs to a large and diverse class of enzymes referred to as the amidase signature (AS) family. Investigations into the structure and function of FAAH, in combination with complementary studies of other AS enzymes, have engendered provocative molecular models to explain how this enzyme integrates into cell membranes and terminates fatty acid amide signaling in vivo. These studies, as well as their biological and therapeutic implications, are the subject of this review.

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INTRODUCTION

In addition to serving as the core structural components of cell membranes, lipids also function as signaling molecules that regulate a number of physiological processes by acting on specific receptors in the nervous system and periphery. Examples of lipid transmitters include sphingosine 1-phosphate (S1P) (1), the lysophospholipids (2), and the fatty acid amides (FAAs) (3). Interestingly, each of these groups of lipids acts on a distinct set of G protein–coupled receptors (GPCRs) that themselves share significant sequence homology (2). S1P and lysophospholipids bind members of the endothelial differentiation gene (EDG) family of GPCRs to regulate a range of physiological processes in the cardiovascular, reproductive, immune, and nervous systems (4). A prototype FAA *N*-arachidonoyl ethanolamine, or anandamide (Figure 1), serves as an endogenous ligand for the central cannabinoid (CB1) receptor (5), which recognizes Δ^9-tetrahydrocannabinol (THC), the active component of marijuana (6). Hence, anandamide, along with other endogenous CB1 receptor agonists such as 2-arachidonoyl glycerol (7), are considered to be endogenous cannabinoids, or "endocannabinoids," and have been shown to regulate numerous neurobehavioral processes, including pain, motility, cognition, and feeding. Other FAAs, despite also producing provocative behavioral effects, do not bind the CB1 receptor and are, therefore, suspected of acting on distinct receptor systems (8, 9).

Like other chemical transmitters, lipid messengers are regulated by enzymes to ensure tight spatial and temporal control over their signaling activity. In the case of S1P and the lysophospholipids, signal termination may involve the independent and/or concerted action of several different enzymes. In contrast, the chemical inactivation of FAAs appears to be mediated primarily by a single enzyme—fatty acid amide hydrolase (FAAH) (10, 11). Here, we review how FAAs were originally identified as endogenous constituents of mammalian tissues, which led to subsequent discoveries of enzymatic activities for their production and degradation.

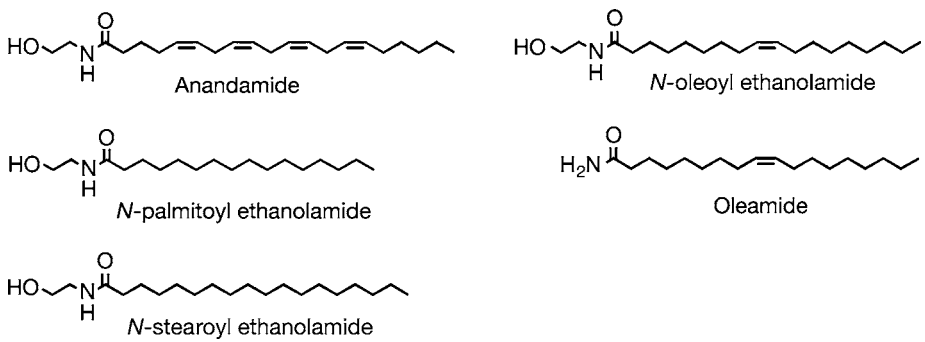


Figure 1 Structures of representative endogenous FAAs.

We then focus on the characterization of FAAH, highlighting how a confluence of multidisciplinary approaches, including enzyme kinetics, structural biology, mammalian genetics, and behavioral pharmacology, has enabled the construction of provocative molecular and cellular models to explain the mechanism by which this enzyme controls FAA signaling in vivo. Finally, we consider the therapeutic implications of these findings for the treatment of a variety of human disorders.

THE FAA FAMILY OF ENDOGENOUS SIGNALING LIPIDS

Two general classes of endogenous FAAs have been identified—the *N*-acyl ethanolamines (NAEs) and fatty acid primary amides (FAPAs). The discovery and characterization of each of these families of signaling lipids occurred independently and will, therefore, be separately discussed.

The Discovery and Pharmacological Characterization of NAEs

NAEs were first discovered as endogenous constituents of mammalian tissues by Bachur and colleagues (12), who identified low levels of *N*-palmitoyl ethanolamine (PEA) and *N*-stearoyl ethanolamine (SEA) (Figure 1) in brains and peripheral tissues of rats and guinea pigs. Subsequently, Schmid and colleagues (13, 14) reported the accumulation of NAEs and their biosynthetic precursors, *N*-acyl phosphatidylethanolamines (NAPEs), in infarcted dog heart muscle.

Although preliminary studies suggested that NAEs may possess specific bioactivities, such as the inhibition of calcium release from mitochondria (15), the classification of this unusual family of lipids as signaling molecules remained obscure until the discovery by Devane and colleagues (5) of anandamide as an endogenous ligand for the CB1 cannabinoid receptor. The CB1 receptor also recognizes THC, the psychoactive component of marijuana (6), and mediates most of its neurobehavioral effects (16, 17). Anandamide has since been shown to mimic several of the pharmacological effects of THC, including the induction of hypothermia, analgesia, and motor defects (18). Notably, however, the magnitude and duration of anandamide's behavioral effects are vastly inferior compared to THC, a difference that can now be attributed to the rapid FAAH-mediated catabolism of this lipid, as is discussed below.

With the exception of anandamide, most other endogenous NAEs do not bind the CB1 receptor (19, 20). Several of these lipids, including PEA (21–23), SEA (24, 25), and *N*-oleoyl ethanolamine (OEA) (26) (Figure 1), have been shown to produce specific cellular and behavioral effects, suggesting that they may represent a large family of endogenous signaling lipids that act on distinct receptor systems in vivo. Consistent with this premise, OEA reduces feeding by activating peroxisome proliferator-activated receptor α (27), and PEA inhibits pain and inflammation, possibly through an unidentified CB2-like receptor (21, 22).

The Discovery and Pharmacological Characterization of FAPAs

FAPAs were originally isolated as endogenous substances by Arafat and colleagues (28), who identified several of these lipids in human plasma. Subsequently, one of these substances, 9-Z-octadecenamide, or oleamide (Figure 1), was detected in the cerebrospinal fluid (CSF) of sleep-deprived cats and shown to induce sleep in rats (29). Oleamide has also been found to accumulate three- to fourfold in the CSF of rats following six hours of sleep deprivation (30). The sleep-inducing effects of oleamide are absent in mice lacking the β subunit of the GABA(A) receptor (31) or in mice treated with CB1 antagonists (32), suggesting that oleamide may act through one or both of these receptor systems in vivo. Consistent with this premise, oleamide has been shown to activate both GABA(A) (33, 34) and CB1 receptors (35) in vitro, although the interactions between oleamide and the CB1 receptor remain controversial (20). Additional postulated sites of action for oleamide include serotonin receptors (36) and gap junctions (37), the latter of which is also sensitive to anandamide (38).

ENZYMATIC ROUTES FOR THE BIOSYNTHESIS AND DEGRADATION OF FAAS

As our understanding of the signaling functions of FAAs has increased over the past two decades, so has interest in elucidating the enzymatic mechanisms for their biosynthesis and degradation. However, as will be highlighted below, many of the enzymes involved in FAA metabolism still remain poorly characterized.

FAA Biosynthesis

In a remarkable series of studies during the early 1980s, Schmid and colleagues (39, 40) elucidated a general route for the biosynthesis of NAEs (Figure 2*a*). Using a series of phospholipid probes radiolabeled on specific acyl chains, these researchers demonstrated the presence of a dog heart enzyme activity that transferred, in a calcium-dependent manner, the *sn*-1 fatty acyl group of phospholipids to the primary amine of phosphatidylethanolamine to form NAPEs (39, 40). They proceeded to show that these NAPEs were hydrolyzed by a phospholipase D enzyme to give NAEs (41). This two-step calcium-dependent transacylase/phospholipase D pathway has since been found in the rodent nervous system and peripheral tissues, where it is capable of producing most, if not all, of the endogenous NAEs, including anandamide (42). Recently, a candidate enzyme responsible for the phospholipase D step of NAE biosynthesis was purified, and its cDNA was cloned (43). The molecular identity of the transacylase enzyme remains unknown.

The enzymatic route for FAPA biosynthesis is less clear, but has been suggested to occur via the peptidyl glycine α-amidating monooxygenase (PGAM) (44) (Figure 2*b*). In this mechanism, FAPAs would originate from the PGAM-catalyzed

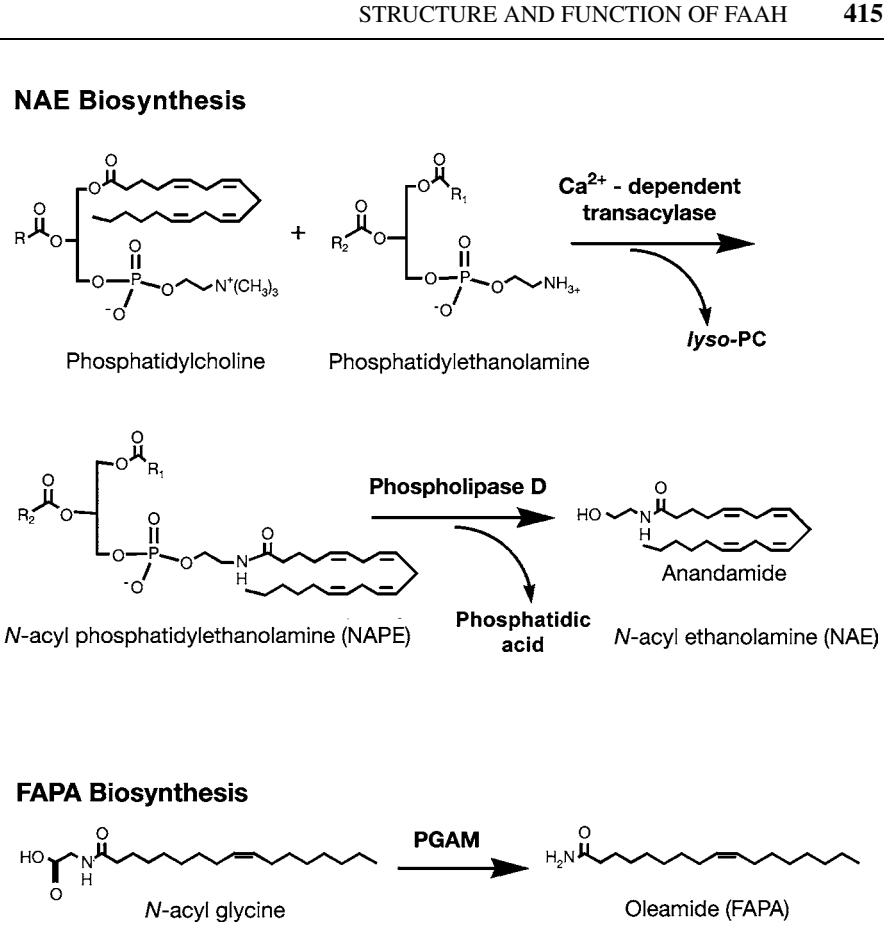


Figure 2 Proposed biosynthetic routes for (*a*) NAEs and (*b*) FAPAs.

oxidative cleavage of *N*-fatty acyl glycines, thus following a biosynthetic route analogous to the production of C-terminally amidated neuropeptides. In vitro studies have shown that PGAM efficiently generates oleamide from its corresponding glycine adduct (45). Also consistent with this route for FAPA biosynthesis, *N*-fatty acyl glycines have been identified as natural constituents of rat and bovine brain (46). Future studies with specific inhibitors of PGAM should confirm if this enzyme is responsible for FAPA biosynthesis in vivo.

FAA Catabolism

In the mid-1980s, Schmid and coworkers (47) described a membrane-associated enzyme from rat liver that hydrolyzed saturated and monounsaturated NAEs to their corresponding fatty acids. Later, an enzyme with similar properties that converted anandamide to arachidonic acid was characterized in N18TG2 neuroblastoma cells (48) and mammalian brain tissue (49, 50). Coincidental with the identification of

oleamide as a sleep-inducing substance, a brain enzyme that hydrolyzed this FAA was also reported (29). These various FAA hydrolytic activities all shared similar properties, including tissue distribution, membrane association, and sensitivity to inhibitors of both serine and cysteine hydrolases, and it was suggested that they could be attributed to the same enzyme (51). Considering the potentially important role that this hydrolase played in regulating FAA signaling in vivo, its molecular identification was intensely pursued.

MOLECULAR CHARACTERIZATION OF FAAH

In 1996, an "oleamide hydrolase" activity was affinity purified to near homogeneity from rat liver membranes using an oleoyl trifluoromethyl ketone inhibitor-derivatized column (52). Peptide sequence information on the purified 65-kDa protein provided sufficient information for the cloning of its cDNA. Transfection of this cDNA into COS-7 cells resulted in robust oleamide hydrolase activity in the membrane fraction of these cells. Importantly, the transfected cells also displayed high levels of anandamide hydrolase activity, confirming that a single enzyme was indeed capable of degrading both NAEs and FAPAs (Figure 3). Hence, this enzyme was named FAA hydrolase, or FAAH, in recognition of the large number of endogenous FAAs that it accepted as substrates. Shortly thereafter, human (53), mouse (53), and pig (54) orthologues of FAAH were also cloned and shown to share over 80% sequence identity, as well as similar substrate selectivities and inhibitor sensitivities.

FAAH and the Amidase Signature Family

Database searches with the FAAH sequence revealed that this enzyme was the first and, still to date, only characterized mammalian member of a large group of enzymes referred to as the amidase signature (AS) family (55). AS enzymes share strong sequence homology over a contiguous stretch of approximately 160 amino acids, termed the amidase signature sequence (55). More than 100 members of this enzyme family can be found in public databases, most of which are bacterial and fungal in origin. In addition to FAAH, representative members of the AS class include the fungal enzyme acetamidase (56), indoleacetamide hydrolase from bacterial plant pathogens (57), prokaryotic Glu-tRNAGln amidotransferase (58), a nylon-degrading enzyme from *Flavobacterium* (59), mandelamide

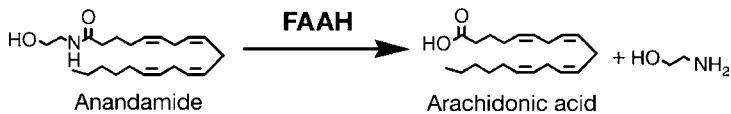


Figure 3 FAAH-mediated hydrolysis of FAAs.

hydrolase from *Psuedomonas putida* (60), malonamidase E2 (MAE2) from *Bradyrhizobium japonicum* (61), and the C-terminal peptide amidase (PAM) from *Stenotrophomonas maltophilia* (62). These examples underscore an intriguing feature of the AS family—despite sharing significant sequence homology, members of this enzyme class exhibit markedly different substrate selectivities. We revisit this subject in subsequent sections and discuss how structural studies have begun to shed light on the molecular basis for diverse substrate recognition among the AS enzymes.

Despite the impressive size and functional diversity of the AS family, insights into the structure and mechanism of this enzyme class were sparse at the time of FAAH's initial characterization. Thus, enzymatic and structural studies of FAAH offered an opportunity to understand not only the catalytic properties of this enzyme, but also of the AS family as a whole. Moreover, the cloning of the FAAH cDNA provided the requisite molecular information to create specific reagents and animal models to test the function of this enzyme in vivo. In the following sections, we review how a series of biochemical, structural, genetic, and pharmacological studies of FAAH have illuminated its central role in regulating FAA signaling in vivo.

FAAH STRUCTURE AND CATALYTIC MECHANISM

FAAH is unusual among AS enzymes in that it is an integral membrane enzyme. Sequence comparisons identified a predicted N-terminal transmembrane (TM) domain (amino acids 9–29) that was not present in other AS enzymes (52), thus suggesting a relatively straightforward mechanism for membrane binding. However, deletion of FAAH's first 30 amino acids produced a catalytically active mutant (ΔTM-FAAH) that was still bound to membranes even in the presence of strong base, indicating that FAAH possesses multiple modes for membrane integration (63). Despite this added complexity, bacterial systems for the efficient recombinant expression of both WT- and ΔTM-FAAH were established.

Recombinant Expression and Purification of FAAH

Optimal conditions for the recombinant expression and purification of FAAH proteins in *Escherichia coli* provided yields of 1–2 mg of purified enzyme per liter culture volume (63). Detergents were required for the solublization and purification of both FAAH variants. A comparison of the biophysical properties of WT- and ΔTM-FAAH revealed that the TM domain can self-associate, thereby increasing the oligomerization state of FAAH (63). Although ΔTM-FAAH existed in solution as a single detergent-protein complex with a sedimentation coefficient of 11.2S, WT-FAAH comprised a heterogeneous mixture of species between 15S and 28S. These findings suggested that the ΔTM-FAAH variant, owing to its homogenous biophysical properties, might constitute a preferred form of the enzyme for X-ray crystallographic studies.

Structure of FAAH

In 2002, the X-ray crystal structure of rat ΔTM-FAAH in complex with an active site-directed inhibitor, methoxy arachidonyl fluorophosphonate (MAFP), was solved to 2.8 Å resolution (64) (Figure 4). For these studies, the detergent lauryl dimethylamine *N*-oxide was included throughout the purification and crystallization process to maintain ΔTM-FAAH in solution. In the same year, the crystal structures of two soluble bacterial AS enzymes, MAE2 (61) and PAM (62), were also determined, thus providing the first opportunity to compare and contrast the three-dimensional structures of widely divergent members of this enzyme class. All three AS enzymes share a common core fold comprised of a twisted β-sheet consisting of 11 mixed strands, surrounded by a number of α-helices (shown for FAAH in Figure 4). However, FAAH and the bacterial amidases also display several key structural differences that may account for FAAH's unique biochemical properties.

QUATERNARY STRUCTURE FAAH crystallized as a dimeric enzyme (Figure 4), consistent with chemical cross-linking and analytical ultracentrifugation studies, indicating that the enzyme is at least a dimer in solution (63). The MAE2 enzyme was also found to be a dimer (61). Interestingly, although the dimer interfaces of both FAAH and MAE2 are formed by roughly the same regions of the AS fold, these enzymes display different relative monomer orientations, which produce a

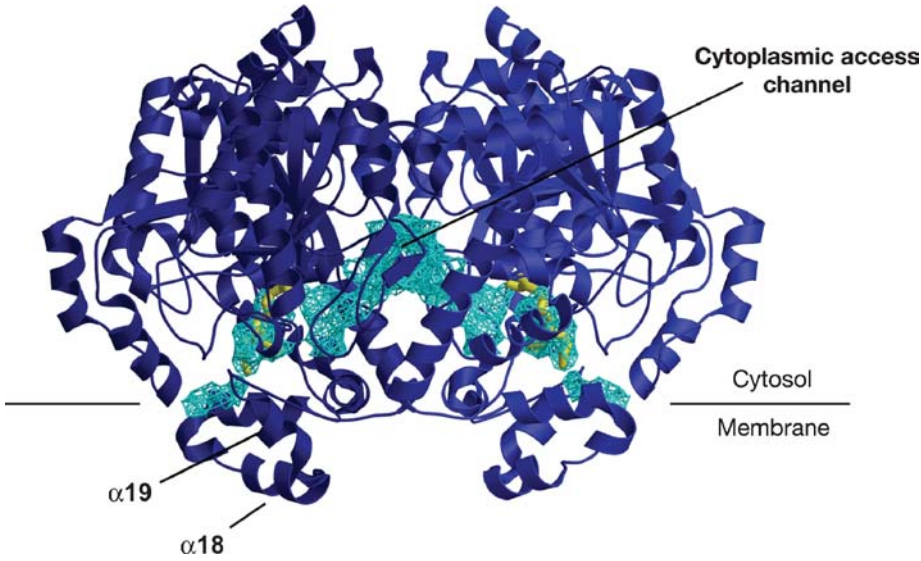


Figure 4 Crystal structure of the FAAH dimer with a bound MAP inhibitor (*yellow*). Channels that traverse the FAAH structure are shown (*light blue*). The proposed membrane-binding domain of FAAH is also noted (α18–α19).

parallel and antiparallel alignment of the FAAH and MAE2 active sites, respectively. The parallel arrangement of FAAH monomers, which appears to derive from the insertion of a few discrete structural elements, has important biological implications as described below.

MEMBRANE BINDING Unlike other AS enzymes, which are mostly soluble proteins, FAAH behaves as an integral membrane enzyme, even in the absence of its single predicted N-terminal TM domain (63). Examination of the ΔTM-FAAH structure revealed a helix-turn-helix motif (α18 and α19, amino acids 410–438) that interrupts the AS fold and is comprised mainly of hydrophobic residues interspersed with a handful of basic amino acids. This motif forms a hydrophobic plateau domain that caps the active site of FAAH (Figure 5) and may represent a mode for monotopic integration into membranes (insertion into one leaflet of the lipid bilayer). Consistent with this idea, neither MAE2 nor PAM possesses this hydrophobic cap domain (Figure 5). Additionally, the predicted N-terminal TM domain of FAAH should be properly positioned, on the basis of the crystal structure, to reinforce the membrane interactions of the α18 and α19 hydrophobic cap (64). Finally, the quaternary structure of FAAH places the α18–α19 cap of each monomer on the same the face of the dimer, thereby reinforcing membrane binding and possibly permitting both subunits to function concurrently by recruiting substrates from the same membrane.

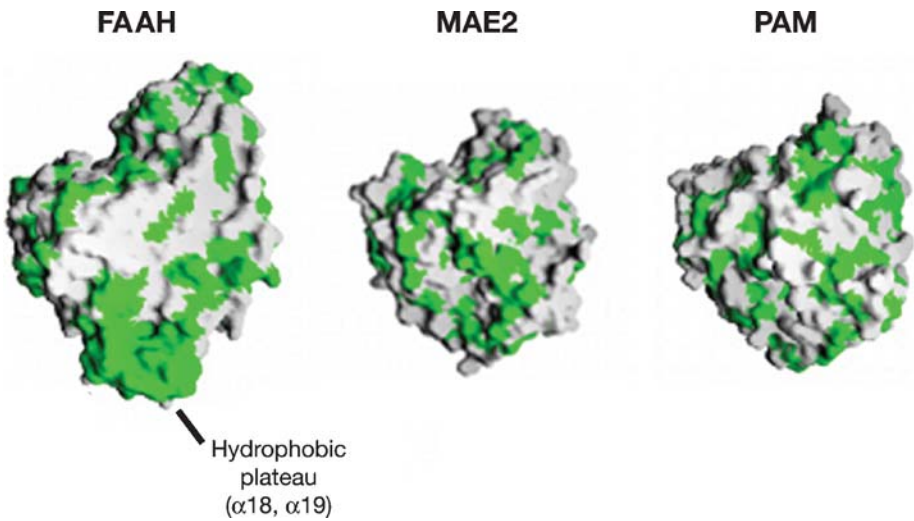


Figure 5 A comparison of the structures of FAAH, MAE2, and PAM reveals a hydrophobic plateau domain (α18–α19 helices) that constitutes the putative membrane face of FAAH. Hydrophobic residues are shown in green. (For FAAH and MAE2, only one subunit of each dimer is shown.)

The overall structure of the $\alpha 18$–$\alpha 19$ hydrophobic cap of FAAH resembles the membrane-binding domains of two additional monotopic integral membrane enzymes, prostaglandin H2 synthase (65) and squalene cyclase (66). All three enzymes are dimeric proteins with active sites that are capped by a hydrophobic plateau domain surrounded by basic amino acids to interact with negatively charged phospholipids. Notably, these enzymes share no sequence or fold homology, indicating that they have independently evolved similar modes for membrane integration (67), possibly reflecting their shared commitment to performing chemistry on hydrophobic substrates that are embedded in the lipid bilayer of the cell. For FAAH, in particular, its intricate mode for membrane binding may facilitate movement of FAAs directly from the bilayer to the active site without the need for transport of these lipids through the aqueous cytosol. This intriguing model for substrate recruitment and product release is further supported by a series of channels observed in the FAAH structure.

CHANNELS IN THE FAAH STRUCTURE The crystal structure of FAAH revealed a number of channels that appear to grant the enzyme simultaneous access to both the membrane and cytoplasmic compartments of the cell (64) (Figure 4). One channel is adjacent to the $\alpha 18$–$\alpha 19$ hydrophobic cap and leads from the surface of the protein to the tail of the arachidonyl chain of the bound MAP inhibitor. This putative substrate entryway is amphipathic, possibly to accommodate the admission and movement of polar FAA head groups toward the FAAH active site. The MAP alkyl chain is bound in a second channel that leads to the FAAH active site and is comprised almost entirely of hydrophobic residues, including I491, which has been identified by photocross-linking and mutagenesis to participate in substrate recognition (68). Similar regions of MAE2 and PAM also appear to be responsible for substrate binding, although distinct sets of residues line their channels, presumably reflecting the unique substrate selectivity of each enzyme. Finally, a third channel is evident in the FAAH structure; this channel emerges from the active site at about an 80° angle from the substrate-binding cavity to create a solvent-exposed cytosolic port (Figure 4). The overall architecture of the FAAH channels may provide a streamlined mechanism for substrate binding and product release. In this model, FAAs would first enter via the membrane to the active site. Following hydrolysis, the liberated fatty acid (hydrophobic) and amine (hydrophilic) products would then exit through the membrane-access and cytosolic-access channels, respectively. The cytoplasmic port may serve the additional function of providing an entry for a water molecule required for deacylation of the FAA-FAAH acyl-enzyme intermediate, which has been biochemically characterized by liquid chromatography-mass spectrometry methods (69).

Catalytic Mechanism of FAAH

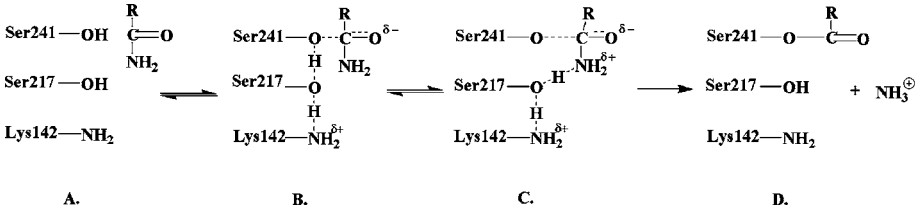
The crystal structures of FAAH, MAE2, and PAM also provided a framework for interpreting a growing body of biochemical studies aimed at characterizing the core

catalytic residues of the AS family. Initial efforts to identify the AS nucleophile were obscured by the sensitivity that this family of enzymes displayed to serine- and cysteine-directed reagents (50, 70). However, through a series of mutagenesis, kinetic, and chemical labeling studies, the FAAH nucleophile was determined to be serine 241 (S241) (71), a residue that is completely conserved among the AS family. Mutagenesis of the corresponding residue in the *Rhodococcus rhodochrous* J1 amidase also produced a catalytically inactive enzyme (72). In the crystal structures of FAAH, MAE2, and PAM, S241 and the equivalent serine residues were either covalently bound (61, 64) or in close proximity (62) to the electrophilic center of an active-site-directed inhibitor. These findings indicate that AS enzymes are members of the serine hydrolase superfamily.

Additional mutagenesis studies revealed that FAAH (69, 73) and, by extension, other AS enzymes employ a catalytic mechanism distinct from the prototypical serine-histidine-aspartic acid catalytic triad utilized by most serine hydrolases. Instead, a completely conserved lysine residue (K142 in FAAH) appears to play a critical role as both a base and acid in the hydrolytic cycle (69). Several lines of evidence support K142 playing a role as a base that activates the S241 nucleophile in FAAH. First, mutation of K142 to alanine yields a catalytically deficient enzyme that shows linear dependence on solvent hydroxide concentration (69, 74). Second, the K142A mutant exhibits severely reduced fluorophosphonate (FP) reactivity (69, 74), indicative of a weakened nucleophile. Both of these properties are similar to those observed in serine proteases lacking their respective histidine bases (75–77). Finally, a K142E mutant yields a catalytically deficient enzyme with a shifted pK_a value from 8 to ~5–6 (69), consistent with the conversion of an active site base from lysine to glutamate.

Other kinetic evidence supported a role for K142 as an acid that participates in the protonation of the substrate leaving group. For example, FAAH exhibits an unusual ability to hydrolyze structurally similar amide and ester substrates at equivalent rates, and this property is lost in the K142A mutant, which hydrolyzes esters at much greater rates than amides (69). The relative importance of acid-catalyzed leaving group protonation for amide hydrolysis compared to ester hydrolysis has been emphasized previously in semiempirical studies by Fersht (78). Notably, the K142E mutant, despite showing greatly reduced nucleophile strength as judged by slower FP-reactivity rates, still hydrolyzed amides and esters at equivalent rates (69). These results suggest that the catalytic efficiency and catalytic selectivity of FAAH depend on distinguishable properties of the same residue (K142), with the former relying on a strong base and the latter requiring coupled acid-base catalysis.

The simplest mechanistic interpretation of the kinetic data highlighted above is that FAAH would employ a S241-K142 dyad. However, mutagenesis studies also invoked the participation of additional residues in the catalytic mechanism of FAAH. In particular, S217, a residue conserved among all AS enzymes, when mutated to alanine, produced a FAAH mutant with significant reductions (~2000-fold) in hydrolytic activity and FP reactivity (71, 74). Mutation of this residue in PAM (62) and MAE2 (79) also resulted in similar losses in catalytic activity.



Scheme 1 Proposed mechanism for the acylation step of amide (and ester) hydrolysis catalyzed by FAAH (74).

For all three enzymes, these catalytic defects were much less severe than those observed with mutants lacking the serine nucleophile or lysine base/acid. The crystal structures of FAAH, MAE2, and PAM revealed that these three conserved residues compose a novel serine-serine-lysine catalytic triad (S241-S217-K142 in FAAH; Figure 6). This structural arrangement of catalytic residues indicates that, in FAAH, the impact of K142 on S241 nucleophile strength and leaving group protonation likely occurs indirectly via the bridging S217 of the triad, which may act as a "proton shuttle," as depicted in Scheme 1. In this mechanism, FAAH would force protonation of the substrate leaving group early in the transition state of acylation concomitant with nucleophile attack on the substrate carbonyl. Consistent with the general predictions of Fersht (78), such tight coupling of base-catalyzed nucleophile activation and acid-catalyzed leaving group protonation would enable FAAH to normalize the acylation/hydrolysis rates of amide and ester substrates.

It should be noted that the serine-serine-lysine triad may function differently in other AS enzymes. In contrast to FAAH, which is labeled rapidly by electrophilic inhibitors, PAM shows negligible reactivity with such agents (62). These results indicate that FAAH possesses a highly activated serine nucleophile, whereas the PAM nucleophile is relatively inert, and have been interpreted to reflect differences in the basal protonation state of the lysine residues in these enzymes (62). In this model, K142 in FAAH would be deprotonated (basic) in the absence of bound substrate, leading to a constitutively activated nucleophile; conversely, the corresponding lysine residue in PAM would be protonated, resulting in a deactivated nucleophile prior to substrate binding. Determining which, if either, of these mechanisms is the predominant mode of action for AS enzymes will require the kinetic analysis of additional members of this family. It also remains unclear if FAAH's unusual ability to normalize the hydrolysis rates of amides and esters will extend to other members of the AS class. Regardless of its generality, this property may have evolved in FAAH to enable the enzyme to function efficiently as an amidase in vivo. For example, in the nervous system, FAAH must bind and hydrolyze its FAA substrates in the background of a large excess of structurally similar ester substrates such as monoacylglycerols (6). If FAAH behaved as most serine proteases and hydrolyzed its specific ester substrates in a deacylation rate-limiting manner with acylation rates that exceed those of amides by 2–3 orders of magnitude (75, 80, 81), then FAAH might encounter difficulty accessing its FAA

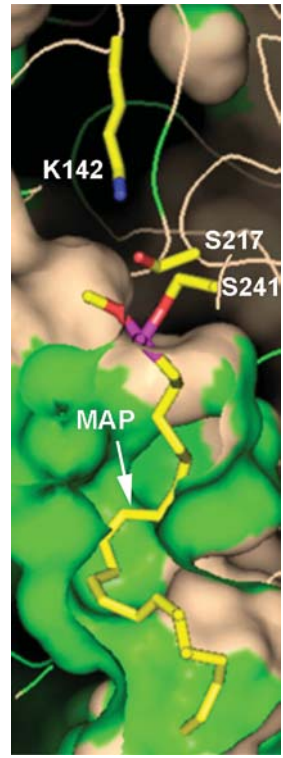


Figure 6 The Ser-Ser-Lys catalytic triad of FAAH (S241-S217-K142) is shown with MAP inhibitor bound in the hydrophobic acyl chain-binding channel. Hydrophobic residues colored in green.

substrates in vivo. Instead, FAAH has evolved an alternative catalytic mechanism in which both amide and ester substrates are hydrolyzed in an acylation rate-limiting manner with comparable efficiencies (69). This strategy for competitive degradation may empower FAAH to function as a lipid amidase in vivo.

FAAH KNOCKOUT MICE

The behavioral effects of anandamide and other FAAs are very weak and transient compared to the activity of exogenous cannabinoids like THC (18). The limited in vivo activity of FAAs appears to be the result of their rapid catabolism in vivo [e.g., the half-life of anandamide in rodent brains is less than five minutes (82)]. However, the specific role that FAAH plays in this degradative process remained unclear until the generation and characterization of a transgenic mouse model lacking this enzyme. These FAAH knockout [FAAH(−/−)] mice were generated by standard targeted gene disruption procedures and were viable, healthy, and

fertile (83). Tissue extracts from FAAH(−/−) mice displayed 50- to 100-fold reductions in hydrolysis rates for anandamide and other FAAs, indicating that FAAH was the primary enzyme responsible for the hydrolytic degradation of these lipids in vivo. Consistent with this premise, the pharmacological administration of anandamide produced greatly exaggerated behavioral effects in FAAH(−/−) mice compared to wild-type mice, including hypomotility, analgesia, hypothermia, and catalepsy. All of the effects of anandamide in FAAH(−/−) mice were blocked by a CB1 antagonist, indicating that this FAA acts as a selective CB1 ligand in these animals.

The endogenous levels of anandamide and other FAAs were elevated over 10-fold throughout the nervous system of FAAH(−/−) mice (83–85). This striking change in brain chemistry correlated with a CB1-dependent analgesic phenotype in FAAH(−/−) mice observed in several assays of pain sensation (Table 1) (83, 86). More recently, FAAH(−/−) mice have been found to exhibit reduced inflammation in both the carrageenan-induced paw edema (85, 86) and dinitrobenzene sulfonic acid (DNBS)-induced intestinal colitis models (87). Interestingly, neither a CB1 nor a CB2 antagonist, nor a combination of the two agents, fully reversed this anti-inflammatory phenotype in the carrageenan model (85, 86), suggesting that it is not mediated by the endocannabinoid system (the effects of CB receptor antagonists were not examined in the DNBS model). These findings implicate FAAs other than anandamide as mediators of the reduced inflammation in FAAH(−/−) mice, possibly acting through either peripheral or central mechanisms (or both). To explore this premise further, an advanced mouse model was recently introduced in which the central and peripheral FAA-signaling systems have been functionally uncoupled (85). Mice that contain FAAH specifically in the nervous system (FAAH NS mice) were generated by crossing FAAH(−/−) mice with transgenic mice expressing

TABLE 1 Comparison of behavioral effects of CB1 agonists versus the genetic [global (−/−) or peripheral (NS)] or chemical (inhibitor) inactivation of FAAH

	CB1 agonist	**FAAH(−/−) mice**	**FAAH NS mice**	**Chemical FAAH inhibitor**
Potential therapeutic effects				
Analgesia	Yes	Yes	No	Yes
Anxiolysis	Yes/no	Unknown	Unknown	Yes
Anti-inflammation	Yes	Yes	Yes	Unknown
Antispasticity	Yes	Unknown	Unknown	Unknown
Anti-emesis	Yes	Unknown	Unknown	Unknown
Decrease intraocular pressure	Yes	Unknown	Unknown	Unknown
Side Effects				
Hypomotility	Yes	No	No	No
Hypothermia	Yes	No	No	No
Catalepsy	Yes	No	No	No

FAAH under the neural specific enolase promoter. These FAAH NS mice possessed wild-type levels of FAAs in the nervous system but significantly elevated concentrations of these lipid messengers in several peripheral tissues. Interestingly, this anatomically restricted biochemical phenotype correlated with a reversion of the reduced pain sensitivity, but not anti-inflammation observed in FAAH(−/−) mice (Table 1), indicating that these activities were mediated by the central (cannabinoid) and peripheral (noncannabinoid) FAA signaling systems, respectively.

These initial studies with FAAH(−/−) and FAAH NS mice have confirmed that FAAH is a primary regulator of FAA signaling in vivo. In the nervous system, FAAH appears to set an endocannabinoid tone that regulates pain perception, whereas in the periphery, this enzyme may control other, yet undetermined, FAA-signaling pathways that modulate inflammation. Notably, these phenotypes occur in the absence of any defects in motility, weight, or body temperature, indicating that the inactivation of FAAH produces a provocative subset of the behavioral effects caused by direct CB1 agonists (Table 1). These findings suggest that FAAH may represent an attractive therapeutic target for the treatment of pain and inflammatory disorders and have stimulated interest in the development of specific inhibitors of this enzyme.

FAAH INHIBITORS

FAAH(−/−) mice have provided a powerful model system to evaluate the physiological consequences of constitutive elevations in FAA activity. Specific FAAH inhibitors would represent a valuable complement to probe the impact of acute and reversible increases in FAA signaling in vivo. Additionally, these agents may have therapeutic utility for the treatment of pain and other neural disorders.

The Design of FAAH Inhibitors and Their Pharmacological Properties

Initial examples of FAAH inhibitors were based closely on the structures of substrates for this enzyme. For example, oleoyl and arachidonoyl variants of trifluoromethyl ketones (70, 88) and FPs (e.g., OTFMK and MAFP, Figure 7) (71, 89) were shown to be potent reversible and irreversible inhibitors of this enzyme, respectively. However, these agents also inhibit several other lipases, including phospholipase A2 (90), platelet-activating factor acetylhydrolase (91), and monoacylglycerol lipase (92), emphasizing the need for novel structural classes of FAAH inhibitors. Toward this end, Boger and colleagues (93) reported a large group of α-ketoheterocycle inhibitors of FAAH that achieved remarkable potency while avoiding substrate-like structural scaffolds (e.g., OL-92, Figure 7). More recently, Kathuria and colleagues (94) described a series of structurally novel carbamate inhibitors of FAAH and showed that two of these agents, URB532 and URB597 (Figure 7), were highly efficacious in vivo, raising endogenous brain anandamide

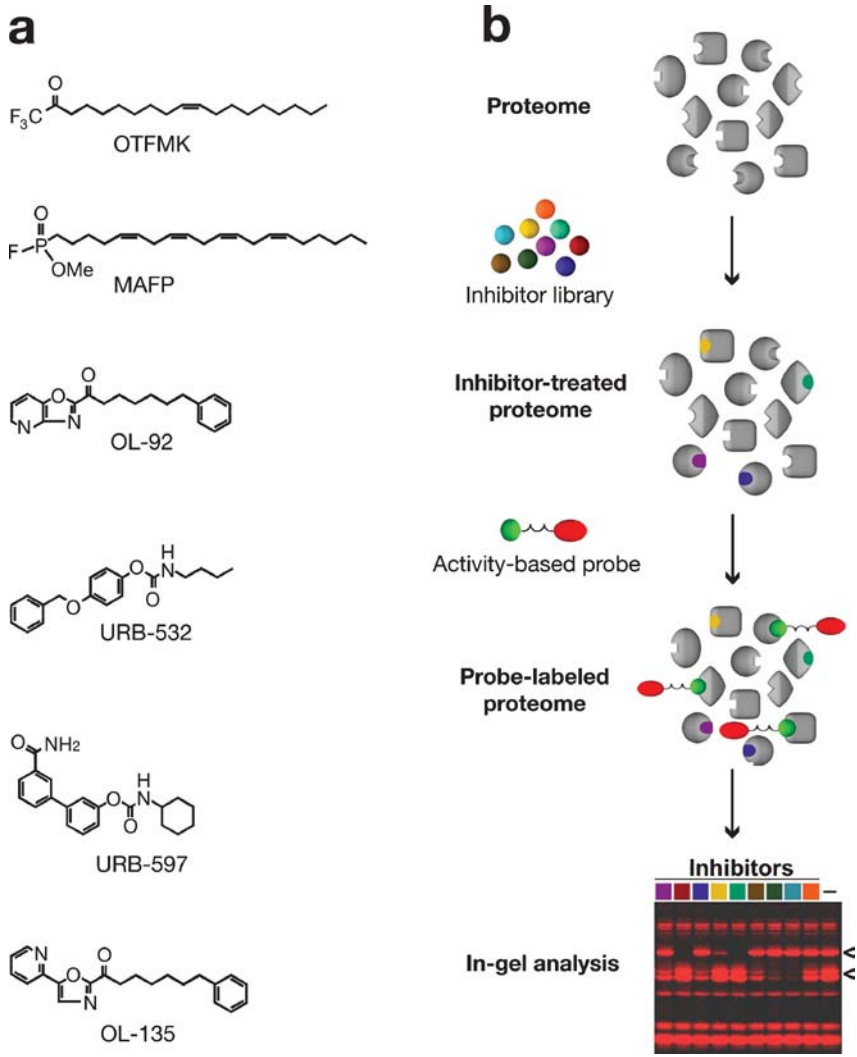


Figure 7 (*a*) Representative inhibitors of FAAH: OTFMK, oleoyl trifluoromethyl ketone; MAFP, methoxy arachidonyl fluorophosphonate. (*b*) Inhibitor discovery by competitive activity-based protein profiling (99). The interactions of inhibitors with many enzymes in the proteome are measured in parallel by the ability of inhibitors to compete activity-based probe labeling (examples of inhibitor-sensitive enzymes marked with arrowheads).

levels and promoting CB1 receptor-dependent analgesia and anxiolytic effects in rodents (Table 1). Interestingly, as was the case with FAAH(−/−) mice, these behavioral effects occurred in the absence of any overt changes in locomotor activity or appetite that are typically observed with direct CB1 agonists. More recently, these carbamate FAAH inhibitors have also been found to reduce blood pressure, cardiac contractility, and vascular resistance in hypertensive rats (95).

In summary, pharmacological studies with FAAH inhibitors have provided further evidence that FAA/endocannabinoid signaling is under the tonic control of FAAH in vivo and indicated that inhibitors of this enzyme may have therapeutic value for the treatment of a range of disorders in both the nervous system and periphery. Nonetheless, for FAAH inhibitors to achieve clinical utility, they must display high selectivity for this enzyme relative to the numerous other hydrolases present in the human proteome.

A Proteomic Screen for Evaluating the Selectivity of FAAH Inhibitors

All of the FAAH inhibitors described above possess a mechanism-dependent binding group (electrophilic carbonyl) critical for potency. Because compounds containing electrophilic carbonyls typically inhibit serine hydrolases by formation of a covalent reversible intermediate with the conserved serine nucleophile, these agents may interact with multiple members of this large enzyme class. Evaluating the selectivity of FAAH inhibitors is a particularly daunting task given the immense size of the serine hydrolase superfamily [approximately 300+ members in the human proteome (96, 97)]. To address this problem, a functional proteomic screen has recently been introduced to test the activity of FAAH inhibitors against numerous serine hydrolases in parallel (98). This screen is based on a competitive version of the chemical proteomic technology activity-based protein profiling (ABPP) (99, 100), in which active-site-directed probes are used to measure the functional state of enzymes in whole cell/tissue samples (Figure 7*b*).

Activity-based probes have been developed for serine hydrolases based on the FP reactive group (101), a class-selective affinity label that targets numerous members of this enzyme family. FP probes bearing a biotin or fluorescent rhodamine reporter tag have been shown to label numerous members of the serine hydrolase superfamily, including several proteases, esterases, and lipases, directly in whole proteomes. These probes can be used to distinguish active members of this enzyme class from their inactive precursor and/or inhibitor-bound forms (101–103). In the competitive version of ABPP, conditions are identified in which probe labeling for the majority of the serine hydrolases in a proteome can be monitored at a single, kinetically relevant time point (98). Under such conditions of incomplete probe labeling, the binding of competitive inhibitors to enzymes will slow the rate of probe labeling and be detected as a decrease in fluorescence signal intensity (Figure 7*b*). Competitive ABPP was used to examine a panel of FAAH inhibitors, resulting in the discovery of a set of α-ketoheterocycle agents bearing an unfused

pyridyl oxazole that exhibited exceptional selectivity for FAAH compared to other serine hydrolases in mouse tissue proteomes (98, 104). One of these agents, OL135 (Figure 7), which inhibited FAAH with 300-fold greater potency than any of the other hydrolases examined, was found to increase endogenous CNS levels of FAAs and produce CB1 receptor-dependent analgesia in several pain models (104). In contrast, the trifluoromethyl ketone and carbamate inhibitors examined by ABPP, including URB532 and URB597, were not selective for FAAH and exhibited significant activity against other serine hydrolases, which included the peripheral lipase triacylglycerol hydrolase and the uncharacterized brain enzyme KIAA1363 (98, 104). Notably, neither of these serine hydrolases shares any sequence homology with FAAH, indicating that widely divergent enzymes may exhibit similar inhibitor sensitivity profiles and thus highlighting the value of global screening strategies, such as ABPP, that can test the selectivity of inhibitors against numerous enzymes in parallel. Finally, it is interesting to speculate about the structural basis for the remarkable selectivity of the OL135-FAAH interaction. Modeling studies suggest that the pyridyl oxazole substituent of this inhibitor protrudes into the cytoplasmic access channel of FAAH, making key contacts with core catalytic residues (e.g., K142) and neighboring amino acids (105). Inhibitors that exploit this unusual channel in the FAAH active site may achieve exceptional selectivity for this enzyme relative to the numerous other lipases present in mammalian proteomes.

CONCLUSION

Over the past several years, remarkable advances have been made in our understanding of the FAA-FAAH-signaling system. This progress can largely be attributed to the synergy that arises from the implementation of complementary experimental approaches. Indeed, FAAH has been the subject of intense biochemical, structural, transgenic, and pharmacological analysis, and each of these approaches has yielded unique insights into the function of this enzyme. Kinetic studies of FAAH have provided mechanistic insights that explain how this enzyme may have evolved to function as an amidase in vivo. The crystal structure of FAAH uncovered a monotopic membrane-binding domain that may facilitate the movement of FAA substrates directly from the lipid bilayer into the enzyme's active site. Transgenic studies demonstrated that FAAH is a primary regulator of FAA signaling in vivo, setting an endocannabinoid tone that regulates pain sensation. Finally, pharmacological studies, in combination with novel functional proteomic methods, have generated the first selective FAAH inhibitors and shown that these agents induce analgesic and anxiolytic effects in rodents. Thus, studies of FAAH have provided not only key insights into the structure and function of this enzyme, but also powerful research tools to probe the activity of the FAA-signaling system in vivo.

Despite these breakthroughs in our knowledge of FAAH, several important questions remain to be addressed. For example, does FAAH, as structural studies

would suggest, actually recruit its FAA substrates directly from the lipid bilayer, and if so, does this mechanism imply that FAAH must be associated with membranes to function in vivo? Similarly, is each of the channels in FAAH important for catalysis, and if so, what unique purposes do they individually serve? Finally, from a biomedical perspective, will FAAH inhibitors achieve the long-sought goal of selectively exploiting the beneficial effects of the endocannabinoid system without also causing the untoward side effects that are observed with direct CB1 agonists? Results to date in rodents are supportive of this possibility; however, studies in humans are still needed to survey the complete set of behavioral effects that accompany elevations in FAA signaling as a result of FAAH inhibition. The recent discovery of a polymorphism in the human FAAH gene that is overrepresented in patients with problem drug use may suggest that changes in FAAH function could influence addictive behavior (106, 107). This finding indicates that a more thorough understanding of the relationship between the FAA-FAAH system and addiction is warranted.

In conclusion, it is worth considering to what extent the FAA-FAAH system may serve as a paradigm for other lipid-signaling pathways. For example, do specific enzymes also control the activity of monoacylglycerols and lysophospholipids, or alternatively, does the regulation of these lipids involve the concerted function of several enzymes? Given the number of potential enzymatic routes that could lead to the synthesis or catabolism of esterified lipids, their metabolism may prove more complex than is the case for FAAs. Regardless, continued investigations into each of these chemical messengers and the enzymes that regulate them should greatly enrich our understanding of the physiological functions of lipid-signaling systems and offer new therapeutic strategies for the treatment of human disease.

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