Fatty Acid Amide Hydrolase Competitively Degrades Bioactive Amides and Esters through a Nonconventional Catalytic Mechanism†

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ABSTRACT: The greater reactivity of esters relative to amides has typically been reflected in their faster rates of both solvolysis and enzymatic hydrolysis. In contrast to this general principle, the serine hydrolytic enzyme fatty acid amide hydrolase (FAAH) was found to degrade amides and esters with equivalent catalytic efficiencies. Mutation of a single lysine residue (K142) to alanine (K142A) abolished this property, generating a catalytically compromised enzyme that hydrolyzed esters more than 500-fold faster than amides. Conversion of this same lysine residue to glutamic acid (K142E) produced an enzyme that also displayed severely diminished catalytic activity, but one that now maintained FAAH's ability to react with amides and esters at competitive rates. The significant catalytic defects exhibited by both the K142A and K142E mutants, in conjunction with their altered pH−rate profiles, support a role for lysine 142 as a general base involved in the activation of FAAH's serine nucleophile. Moreover, the dramatically different amide versus ester selectivities observed for the K142A and K142E mutants reveal that FAAH's catalytic efficiency and catalytic selectivity depend on distinguishable properties of the same residue, with the former relying on a strong catalytic base and the latter requiring coupled general acid−base catalysis. We hypothesize that FAAH's unusual catalytic properties may empower the enzyme to function effectively as both an amidase and esterase in vivo.

Fatty acid amide hydrolase (FAAH)[1] is a mammalian integral membrane enzyme responsible for the catabolism of the fatty acid amide family of endogenous signaling lipids (*1, 2*). Representative fatty acid amides include the endocannabinoid anandamide (*3*) and the sleep-inducing lipid oleamide (*4, 5*). Fatty acid amides generate an intriguing array of pharmacological effects in mammals, including sleep (*4*−*6*) and analgesia (*7*−*9*), indicating that FAAH might serve as an attractive target for pharmaceutical efforts aimed at influencing endogenous pain and/or sleep−wake systems (*10*−*12*). The elucidation of FAAH's catalytic and structural features would provide a valuable foundation upon which to launch such medicinal chemistry endeavors.

FAAH is the only characterized mammalian member of a large group of enzymes termed the amidase signature family (*13, 14*). These enzymes share a highly conserved linear sequence rich in serine and glycine residues that spans approximately 50 amino acids in length. Despite the presence of amidase signature enzymes in several kingdoms of life, their catalytic features and possible relatedness to other classes of amidolytic enzymes remain poorly understood. Our recent efforts to characterize the catalytic properties of FAAH have identified the enzyme as a nonconventional amidase that utilizes a serine nucleophile (serine 241) and a nonhistidine catalytic base (*15*). We now report the surprising discovery that FAAH reacts with structurally similar amide and ester substrates at equivalent rates and that this unusual property of the enzyme depends on a single catalytic residue.

EXPERIMENTAL PROCEDURES

Synthesis of FAAH Substrates. ^{14}C-Labeled substrates were synthesized by reaction of ammonia (oleamide), methylamine (OMA), or methanol (OME) with [^{14}C]oleoyl chloride as described (*1*). OpNA was synthesized from oleoyl chloride and 4-nitroaniline.

Enzyme Assays. FAAH hydrolysis rates with oleamide, OME, and OMA were measured by following the conversion of ^{14}C-labeled substrates using a TLC assay essentially as described (*15, 16*). Oleamide and OMA were separated from oleic acid with 50% ethyl acetate/hexanes, while OME was separated from oleic acid with 30% ethyl acetate/hexanes. The conversion of OpNA was monitored by the change in UV absorbance at 382 nm caused by the release of 4-nitroaniline. An extinction coefficient of 13 500 M^{-1} was used for the rate calculations.

Construction of FAAH Point Mutants. Mutant enzymes were constructed using the Quickchange procedure (Stratagene) as described (*15*). The gel filtration profiles and far-UV circular dichroism spectra of the mutant enzymes matched those of wild-type FAAH (*15*).

Reactivity of FAAH Enzymes with FP−Biotin. The synthesis of FP−biotin will be reported elsewhere. FP−biotin-

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[1] Abbreviations: FAAH, fatty acid amide hydrolase; OME, oleoyl methyl ester; OMA, oleoyl methyl amide; OpNA, oleoyl *p*-nitroanilide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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labeled FAAH samples were analyzed with a Western blot assay, and values of k_{obs}/[I] were determined as follows. FAAH samples were incubated at 5 μg/mL (80 nM) with several concentrations of FP−biotin (1−50 μM), quenched with SDS−PAGE loading buffer at various times, resolved by SDS−PAGE and electroblotting, and visualized with an avidin−horseradish peroxidase conjugate. The time required to reach 50% labeled protein was estimated by comparing time points to a standard consisting of a 2-fold dilution of FAAH fully labeled with FP−biotin. The values of k_{obs}/[I] for the K142E and K142A mutants represent the average of duplicate trials at a minimum of four concentrations of FP−biotin.

Isolation of FAAH Acyl-Enzyme Intermediates. Acyl-enzyme intermediates of FAAH were isolated essentially as described previously for the isolation of ethoxy oleoyl fluorophosphonate-labeled FAAH (*15*). Briefly, each enzyme (2 μM) was incubated with the indicated substrate (150 μM) for a time equal to roughly 5−10 turnovers in order to reach steady state before quenching the reaction with trichloroacetic acid. The proteins were subjected to SDS−PAGE and digested in gel with trypsin, and the resulting peptides separated by reverse-phase HPLC.

Determination of k_2 Values for the K142E Mutant. Saturating concentrations of ^{14}C-labeled substrates (150 μM) were incubated with the K142E mutant (6 μM) for various times before quenching with SDS−PAGE loading buffer. The protein samples were bath sonicated for 5 min, heated at 90 °C for 30 s, resolved by SDS−PAGE, transferred to a poly(vinylidene difluoride) membrane, and protein-associated radioactivity quantified by phosphorimaging (Packard). The kinetics of acyl-enzyme accumulation were fit using the assumption that the equilibration of the noncovalent ES complex is a rapid process such that enzyme freed through deacylation rapidly returns to the ES state. Thus, the kinetics for accumulation of acylated enzyme can be simplified to the kinetics of a simple equilibrium between two states: the noncovalent Michaelis complex ES and the acylated enzyme ES*. The kinetics of this scheme can be described by the following equation: $[ES^*]_t = [E]_T - \{[E]_T k_2/(k_2 + k_3)\}\{(e^{-(k_2+k_3)t}) + (k_3/k_2)\}$; where $[ES^*]_t$ is the amount of radioactivity observed at time, *t*, corresponding to acylated enzyme; $[E]_T$ is the maximal amount of radioactivity observed for 100% acylated enzyme (i.e., the plateau value for oleamide or OME, since $k_2 \gg k_3$); and k_2 and k_3 are the acylation and deacylation rate constants, respectively. The value of k_3 was determined from steady-state measurements of oleamide hydrolysis. $[E]_T$ and k_2 were independent variables minimized by the fitting process in the case of oleamide and OME. The value of $[E]_T$ obtained was then used for the fitting of k_2 for OMA and OpNA.

RESULTS

In all studies described herein, a rat FAAH protein lacking its N-terminal 39 amino acids was used. This modification removed FAAH's predicted transmembrane domain, the deletion of which was previously found to leave FAAH's catalytic and membrane-binding properties unaltered while at the same time facilitating the recombinant enzyme's purification (*16*). For the sake of clarity, we refer to this N-terminal deletion protein as FAAH throughout the manuscript.

Table 1: Catalytic Properties of FAAH at pH 9.0

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
oleamide	9 ± 1	37 ± 7	0.24
OMA	1.9 ± 0.1	9 ± 2	0.21
OpNA	0.17 ± 0.02	22 ± 3	7.7×10^{-3}
OME	2.8 ± 0.1	21 ± 3	0.13

Scheme 1: Standard Kinetic Scheme for Serine Hydrolases[a]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} ES^* \xrightarrow[P_2]{k_3} E$$

[a] In this scheme, where acylation is controlled by k_2, deacylation by k_3, and dissociation constant $K_s = k_{-1}/k_1$, E is the enzyme, S is the substrate, ES is the noncovalent Michaelis complex, ES* is the acyl-enzyme intermediate, and P_1 and P_2 are products.

The basic kinetic parameters, k_{cat} and K_m, for FAAH-mediated hydrolysis of various ester and amide substrates are presented in Table 1. Interestingly, the k_{cat} and k_{cat}/K_m values for the hydrolysis of oleamide were slightly but significantly higher than those for the hydrolysis of oleoyl methyl ester (OME, Table 1). The k_{cat} and K_m values for the hydrolysis of oleoyl methyl amide (OMA) were comparable to those for OME hydrolysis, indicating that the methyl group of OME did not directly hinder catalysis or productive binding. The overall similarity in measured k_{cat} values for FAAH's amide and ester substrates raised the possibility that the rate-limiting step for their reaction might be the deacylation of a common acyl-enzyme intermediate (ES*, Scheme 1). However, in deacylation rate-limiting reactions, lower K_m values are observed for those substrates with faster acylation rates (k_2 values), in turn leading to higher k_{cat}/K_m values (assuming similar values of K_s; *17, 18*). Thus, the similar K_m and k_{cat} values measured for oleamide, OMe, and OMA supported their comparable acylation rates with FAAH. Substrate competition experiments with oleamide and OME were also conducted, and these studies confirmed the relative K_m and k_{cat}/K_m values reported in Table 1 (data not shown).

FAAH's similar catalytic efficiency with oleamide, OME, and OMA contrasted sharply with the substrate selectivities displayed by most serine proteases, which tend to react with esters at rates several orders of magnitude faster than amides (*18*−*22*). FAAH's peculiar substrate selectivity was further exposed with oleoyl *p*-nitroanilide (OpNA). Due to the electron-withdrawing nature of the *p*-nitrophenyl group, *p*-nitroanilides are typically much more reactive substrates than their unactivated amide counterparts (*18, 19*). However, FAAH hydrolyzed OpNA at a substantially slower rate than oleamide, despite both substrates displaying similar K_m values. The reduced k_{cat} value observed for OpNA indicated that this substrate was acylation rate limiting, thus making $K_m = K_s$. The similarity of OpNA's K_s value to the K_m values for oleamide, OMe, and OMA further supports the conclusion that the comparable k_{cat}/K_m values for these substrates reflect their similar acylation rates (k_2) rather than compensatory differences in K_s and k_2 values among the substrates. In summary, the leaving group dependence of k_{cat} (and analogously, k_2) displayed by FAAH with the substrates shown in Table 1 (amide ≥ ester > anilide) differed markedly from the typical solution hydrolysis and protease acylation rates observed with these classes of compounds (ester > anilide > amide).

Table 2: Catalytic Properties of the K142A Mutant at pH 9.0

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
oleamide	$(2.6 \pm 0.3) \times 10^{-4}$	20 ± 5	1.3×10^{-5}
OMA	$(1.0 \pm 0.1) \times 10^{-4}$	41 ± 8	2.4×10^{-6}
OpNA	0.012 ± 0.001	63 ± 9	1.9×10^{-4}
OME	0.15 ± 0.01	12 ± 3	0.013

Table 3: Catalytic Properties of the K142E Mutant

substrate	k_{cat} (s^{-1})[a]	k_2 (s^{-1})[b]	K_m (μM)
oleamide	$(6.4 \pm 0.8) \times 10^{-4}$	0.024 ± 0.002	<10
OMA	$(3.1 \pm 0.3) \times 10^{-4}$	$(3.1 \pm 0.3) \times 10^{-3}$	<10
OpNA	$(2.0 \pm 0.2) \times 10^{-4}$	$(3.0 \pm 0.3) \times 10^{-4}$	32 ± 4
OME	$(5.2 \pm 0.6) \times 10^{-4}$	0.032 ± 0.003	<10

[a] k_{cat} determined at pH 9.0. [b] k_2 determined at pH 6.5.

Table 4: Relative Substrate Selectivities of FAAH and K142 Mutants

	$k_{cat}/(k_{cat}$ oleamide)		$k_2/(k_2$ oleamide)
substrate	FAAH	K142A	K142E
oleamide	1	1	1
OMA	0.2	0.4	0.2
OpNA	0.02	50	0.02
OME	0.3	600	1.3

To explore the molecular mechanism by which FAAH normalized the acylation rates of its ester and amide substrates, we characterized a series of missense mutants of the enzyme for altered patterns in their relative substrate selectivities. In each of these mutants, a single, highly conserved residue was replaced with alanine (S217A, S218A, S241A, and K142A), resulting in an enzyme with a greatly diminished k_{cat} value for oleamide hydrolysis (*15*). The three serine mutants displayed similar degrees of catalytic deficiency with ester, anilide, and amide substrates (data not shown), indicating that these residues do not significantly impact FAAH's substrate selectivity. In contrast, the K142A mutant exhibited a dramatic loss in oleamide hydrolase activity (a 35 000-fold reduction in k_{cat}), while maintaining 5−10% of wild type activity with OME and OpNA at pH 9.0 (Table 2). The altered substrate selectivity of the K142A mutant resulted in hydrolysis rates for OpNA and OME that exceeded oleamide hydrolysis by approximately 50- and 600-fold, respectively (Table 4). Thus, mutation of a single conserved lysine residue transformed FAAH's unusual substrate selectivity (amide ≥ ester > anilide) into a preferred order of reactivity compatible with the solvolysis rates of these compounds (ester > anilide > amide).

The pH dependence of k_{cat} for OME hydrolysis by the K142A mutant showed a linear dependence on [OH^-] with a slope of 0.9 (Figure 1A). Similar pH dependencies were found for the K142A enzyme with oleamide and OpNA (data not shown). These pH−rate profiles contrasted dramatically with those of FAAH whose k_{cat} showed dependence on a basic residue with a pK_a of 7.9 (Figure 1A) (*15*). Neither enzyme exhibited significant changes in K_m values over the pH range investigated. The greatly reduced amidase activity of the K142A mutant coupled with its altered pH−rate profile were consistent with a role for lysine 142 in the base-catalyzed deprotonation of FAAH's serine nucleophile (*19*, *23*−*25*). In support of this notion, the reactivity of the K142A mutant with a biotin-tagged fluorophosphonate inhibitor

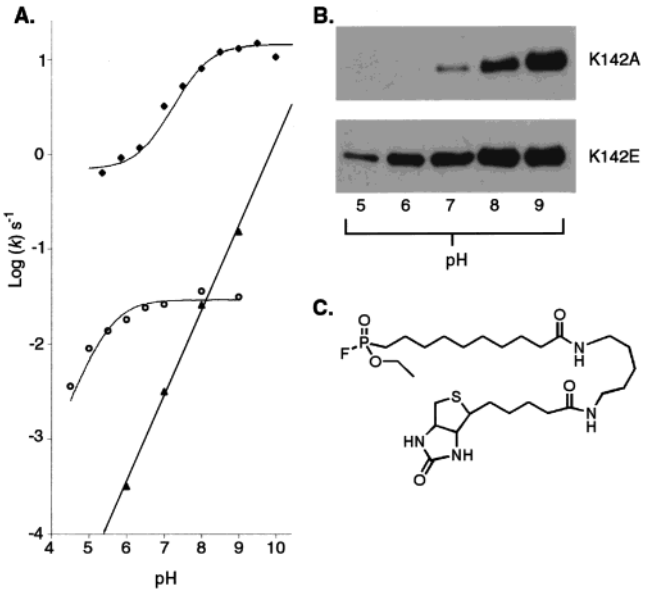

FIGURE 1: pH profiles for kinetic parameters of FAAH and lysine 142 mutants. (A) The pH dependence of k_{cat} observed for FAAH with oleamide (diamonds) and the K142A mutant with OME (triangles), and the pH dependence of k_2 observed for the K142E mutant with oleamide (open circles). Single-residue ionization models were used to fit the pH dependence of FAAH and the K142E mutant, resulting in pK_a values of 7.9 and 5.7, respectively. The pH dependence of k_{cat} for the K142A mutant was fit to a line with a slope of 0.9. (B) Western blot depicting the FP−biotin reactivities of the K142A and K142E mutants at various pH values. For these studies, 50 μM FP−biotin was reacted with 80 nM mutant enzyme for 10 min, and labeling was detected with an avidin−horseradish peroxidase conjugate. (C) Structure of FP-biotin.

(FP−biotin; Figure 1C) was reduced over 1000-fold relative to FAAH at pH 9.0 ($k_{obs}/[I]_{K142A} = 64 \pm 10$ M^{-1} s^{-1} versus $k_{obs}/[I]_{FAAH} > 1.4 \times 10^5$ M^{-1} s^{-1}) and showed a dramatic pH dependence that mirrored the pH dependence of the mutant enzyme's catalytic activity (Figure 1B).

To further investigate the role of lysine 142 in FAAH's catalytic mechanism, a mutant enzyme was generated in which this residue was converted to glutamate (K142E). Mutant enzymes in which lysine 142 was replaced with histidine or arginine were created as well, but these proteins proved structurally defective and were not studied further. The steady-state kinetic properties of the K142E mutant with various oleoyl substrates are presented in Table 3. In general, the k_{cat} values of this enzyme were 3−4 orders of magnitude lower than those of FAAH. Moreover, the K142E mutant's similar k_{cat} and low K_m values for oleamide, OMA, and OME suggested that the enzyme hydrolyzed these substrates in a deacylation rate-limiting manner. In support of this notion, relatively large quantities of an acyl-enzyme intermediate were isolated for this mutant enzyme from steady-state reactions with each substrate (Figure 2). In contrast, the levels of acyl-enzyme isolated for FAAH from steady-state reactions with oleamide and OME were significantly lower (Figure 2), indicating a predominantly acylation or mixed acylation/deacylation rate-limited reaction for the wild-type enzyme with these substrates.

The acylation rate constants (k_2) for the K142E mutant with oleamide, OME, OMA, and OpNA were determined by measuring the rate of presteady-state accumulation of ^{14}C-labeled acyl-enzyme in the presence of saturating concentrations of ^{14}C-labeled substrates (Figure 3 and Table 3). The

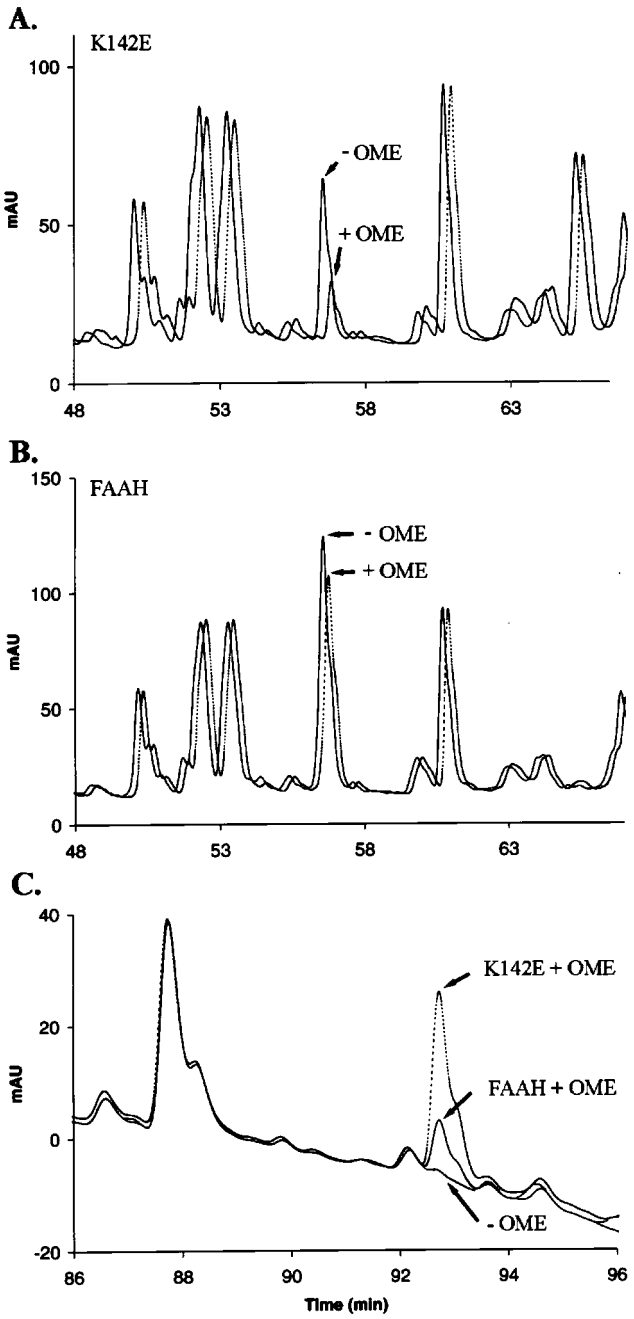

FIGURE 2: Identification of acyl-enzyme intermediates for FAAH and the K142E mutant. 75 μg of the K142E mutant (A, C) or FAAH (B, C) were incubated in the presence (dashed traces in A and B) or absence (solid lines in A and B) of 200 μM OME prior to digestion with trypsin and separation of the resulting peptides by HPLC. Traces in A and B were offset by 0.25 min for clarity. The peptide containing FAAH's serine nucleophile S241 (residues 213−243) eluted at 57 min (A, B) as judged by electrospray mass spectrometry (*15*). For the wild-type enzyme, this peak coeluted with a peptide containing residues 131−142, which was shifted to a later elution time for the K142E mutant. In the presence of OME, a significant reduction in the peak at 57 min was observed for both enzymes (dashed lines in A and B). This decrease correlated with the presence of a novel peak eluting at 93 min (C), which was identified by tandem electrospray mass spectrometry as residues 213−243 plus one bound acyl chain of OME on S241. For both FAAH and the K142E mutant, reactions with oleamide produced similar levels of acyl-enzyme to those found for reactions with OME.

acylation rates for the K142E variant with these substrates were 100−600 times slower than their respective hydrolysis

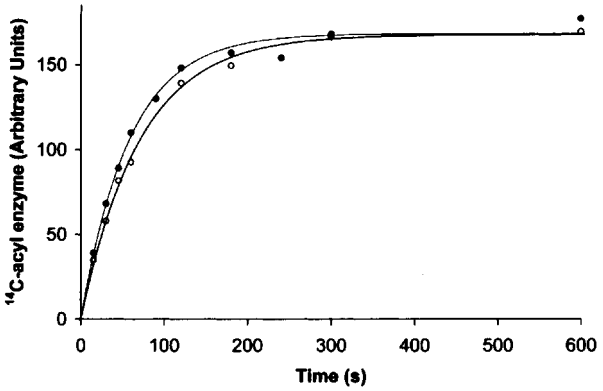

FIGURE 3: Kinetics of acyl-enzyme accumulation for the K142E mutant with oleamide and OME. The K142E mutant was incubated with [^{14}C]oleamide (open circles) or [^{14}C]OME (closed circles). Acylated protein was separated from unbound substrate by SDS−PAGE and transferred to a PVDF membrane. [^{14}C]acyl-enzyme was quantified by phosphorimaging. The acylation rate constants (k_2) were determined by nonlinear least-squares regression.

rates with FAAH. Interestingly however, the K142E mutant was acylated by OME and oleamide at nearly equivalent rates that in turn exceeded the acylation rate of OpNA by approximately 50-fold. Thus, the unusual acylation specificity of FAAH was retained in the K142E variant despite this mutant's significantly reduced catalytic efficiency.

The pH dependence of k_2 for the K142E mutant showed little change from pH 7−9 and decreased below pH 7 (Figure 1A). A similar pH dependence was observed for k_{cat} (data not shown) and the enzyme's reactivity with FP−biotin (Figure 1B). These results indicated that for the K142E mutant both acylation and deacylation were governed by a residue with a pK_a < 6, consistent with a role for glutamate 142 as the catalytic base in this enzyme. Notably, the FP−biotin reactivity of the K142E mutant was comparable to that of the K142A variant at pH 9.0 ($k_{obs}/[I]_{K142E} = 68 \pm 10$ M^{-1} s^{-1} versus $k_{obs}/[I]_{K142A} = 64 \pm 10$ M^{-1} s^{-1}), implying that at this pH the nucleophile strengths of these enzymes were attenuated to similar degrees. Considering further that only the K142E mutant retained FAAH's ability to normalize the acylation rates of amides and esters, these data highlight that the unusual substrate selectivity of FAAH does not correlate with the extent of base-catalyzed activation of the enzyme's nucleophile. Finally, a mutant FAAH in which lysine 142 was replaced with glutamine (K142Q) was generated to control for possible steric effects of glutamate 142's side chain on the kinetic properties of the K142E mutant. The K142Q variant behaved indistinguishably from the K142A enzyme, indicating that the preservation of FAAH's acylation specificity in the K142E mutant was due primarily to the ability of glutamate 142 to participate in proton-transfer events.

DISCUSSION

Collectively, the kinetic properties of the K142A and K142E mutants clearly support that lysine 142 functions as a general base in FAAH-catalyzed hydrolytic reactions. The greatly reduced amidase activity and FP−biotin reactivity as well as the altered pH−rate profile of the K142A mutant are properties similar to those displayed by serine protease

mutants lacking their respective catalytic histidine bases (*19*, *23*−*25*). Moreover, the K142E mutant exhibited pH dependencies for k_{cat}, k_2, and FP−biotin reactivity that were consistent with a basic residue involved in catalysis with a pK_a < 6, likely attributable to the introduced glutamate serving as FAAH's catalytic base. Still, the dramatically different substrate selectivities displayed by the K142A and K142E mutants suggested that lysine 142 might also serve an additional role(s) in FAAH's catalytic cycle. For example, the enhanced relative reactivity of the K142A enzyme with substrates containing less basic leaving groups (OME and OpNA) could be attributed to the absence of a general acid component of catalysis critical for leaving group protonation. The relative importance of general acid-catalyzed leaving group protonation for amide and ester hydrolysis has been emphasized previously by Fersht through semiempirical calculations of the rates of amide and ester alcoholysis (*26*). While esters were hydrolyzed much more rapidly than amides by alcoholate anions, the rates of neutral alcohol hydrolysis of amides and esters were nearly identical, due in principle to the ability of the neutral alcohol to transfer its proton directly to the leaving group during the hydrolytic reaction. The relative acylation rates of FAAH and the K142E mutant, oleamide ≥ OME > OpNA, are consistent with a strong degree of general acid-catalyzed leaving group protonation. The ability to substitute lysine 142 with glutamate, but not alanine or glutamine, and maintain FAAH's acylation specificity, independent of the degree of serine nucleophile activation, strongly supports a function for this lysine residue as a general acid catalyst involved in leaving group protonation.

It is interesting to note that the acylation rates of OME and OpNA were 5- and 50-fold slower, respectively, with the K142E mutant than with the K142A variant at pH 9.0, despite the two enzymes possessing apparently similar nucleophile strengths. Additionally, FAAH hydrolyzed OME only 20-fold faster than the K142A variant at pH 9.0, even though the mutant enzyme displayed a greater than 1000-fold reduction in its relative reactivity with FP−biotin. These results suggest that the presence of a general acid catalyst may negatively impact the acylation rates of more activated substrates such as OME and OpNA. One possible explanation for this behavior is that FAAH forces its substrates through a reaction pathway in which the leaving amine or alcohol must be partially protonated in the transition state. Such an effect could be achieved through a required coupling of nucleophile deprotonation and leaving group protonation, similar to a mechanism proposed by Komiyama and Bender for the hydrolysis of unactivated amides by serine proteases (*27*). However, central to Komiyama and Bender's proposal is the premise that serine proteases hydrolyze their ester and anilide substrates through an alternative mechanism involving two discrete proton transfer steps, one for formation and one for breakdown of the tetrahedral intermediate, respectively. Thus, FAAH's special substrate selectivity could originate from the enzyme steering esters and anilides through the same reaction pathway as amides. Independent of the mechanism employed, FAAH's ability to react with amides and esters at equivalent rates likely requires substantial contributions from both structural and catalytic residues. Indeed, the surprising observation that lysine 142 can be replaced with glutamate without altering the enzyme's substrate selectivity perhaps argues that FAAH has evolved an active site that is structurally predisposed for competitive reactivity with amides and esters. Moreover, the inability of the K142E mutant to match FAAH in terms of absolute acylation/hydrolysis rates reveals that FAAH's catalytic efficiency and catalytic selectivity depend on distinguishable functions of the same residue, with the former relying on a strong catalytic base and the latter requiring coupled acid−base catalysis.

The relative reactivity of amides and esters has been the subject of numerous chemical and enzymatic investigations. Serine proteases typically react with esters at much greater rates than amides, reflecting the relative solvolytic potential of these compounds. Surprisingly however, the substrate selectivity of FAAH does not conform to this general principle. Instead, FAAH normalizes the acylation and hydrolytic rates of its fatty acid amide and ester substrates, prompting the question: what purpose might this unusual characteristic of the enzyme serve in vivo? On this note, FAAH has recently been shown to catabolize a second endogenous cannabinomimetic lipid, 2-arachidonoyl glycerol (2-AG), at rates comparable to those of fatty acid amides (*28*−*30*). Interestingly, 2-AG and related mono-acyl glycerols have been found at equal to or greater concentrations than fatty acid amides in vivo (*31*−*33*), indicating that if FAAH possessed a serine protease-like catalytic mechanism, the enzyme might encounter difficulty accessing its endogenous amide substrates in the presence of these fatty acid esters. For example, most serine proteases hydrolyze specific ester substrates in a deacylation rate-limiting manner with acylation rates that exceed those of amides by at least 2−3 orders of magnitude (*18*−*22*). The accumulation of acyl-enzyme for such ester substrates reduces their K_m values below K_s by a factor of $k_3/(k_2 + k_3)$. If FAAH exhibited a similar preference for esters over amides, the endogenous levels of mono-acyl glycerols would likely saturate the enzyme, rendering it ineffective against its slower fatty acid amide substrates (bearing in turn higher K_m values). Accordingly, FAAH has evolved an alternative catalytic mechanism that forces the competitive degradation of amides and esters, and it is this form of "directed nonselectivity" that may empower the enzyme to function in vivo as both a fatty acid amidase and esterase. Indeed, FAAH's ability to hydrolyze both amide and ester endocannabinoids at similar rates may facilitate the coordinated control of the levels of these signaling molecules in vivo. In contrast to FAAH, serine proteases rarely encounter endogenous ester versions of their polypeptide substrates, and thus few selective pressures would have been placed on these enzymes to evolve a mechanism that directs the hydrolysis of amides and esters at competitive rates.

Finally, the absolute conservation of lysine 142 and serine 241 among amidase signature enzymes supports the notion that these residues function as the catalytic base/acid and nucleophile, respectively, for the entire enzyme family. Hence, it will be of great interest to discern whether FAAH's special catalytic features prove unique to this enzyme or, alternatively, are elements inherent to the amidase signature family as a whole. Regardless, the characterization of FAAH as a novel type of serine amidase, distinct in mechanism from proteases, strengthens prospects for the development of chemical inhibitors that selectively target this enzyme in vivo.

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