

Evidence for Distinct Roles in Catalysis for Residues of the Serine-Serine-Lysine Catalytic Triad of Fatty Acid Amide Hydrolase*[§]

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Fatty acid amide hydrolase (FAAH) is a mammalian amidase signature enzyme that inactivates neuromodulatory fatty acid amides, including the endogenous cannabinoid anandamide and the sleep-inducing substance oleamide. The recent determination of the three-dimensional structures of FAAH and two distantly related bacterial amidase signature enzymes indicates that these enzymes employ an unusual serine-serine-lysine triad for catalysis (Ser-241/Ser-217/Lys-142 in FAAH). Mutagenesis of each of the triad residues in FAAH has been shown to severely reduce amidase activity; however, how these residues contribute, both individually and in cooperation, to catalysis remains unclear. Here, through a combination of site-directed mutagenesis, enzyme kinetics, and chemical labeling experiments, we provide evidence that each FAAH triad residue plays a distinct role in catalysis. In particular, the mutation of Lys-142 to alanine indicates that this residue functions as both a base involved in the activation of the Ser-241 nucleophile and an acid that participates in the protonation of the substrate leaving group. This latter property appears to support the unusual ability of FAAH to hydrolyze amides and esters at equivalent rates. Interestingly, although structural evidence indicates that the impact of Lys-142 on catalysis probably occurs through the bridging Ser-217, the mutation of this latter residue to alanine impaired catalytic activity but left the amide/ester hydrolysis ratios of FAAH intact. Collectively, these findings suggest that FAAH possesses a specialized active site structure dedicated to a mechanism for competitive amide and ester hydrolysis where nucleophile attack and leaving group protonation occur in a coordinated manner dependent on Lys-142.

Fatty acid amide hydrolase (FAAH)¹ is an integral membrane enzyme that degrades members of the fatty acid amide class of neural signaling lipids, including the endogenous cannabinoid anandamide (1) and the sleep-inducing substance oleamide (2, 3). Studies of FAAH(–/–) mice have confirmed that

this enzyme is a key regulator of fatty acid amide signaling *in vivo* (4, 5). For example, FAAH(–/–) mice possess elevated endogenous brain levels of anandamide and related fatty acid amides that correlate with enhanced cannabinoid receptor 1-dependent analgesia in these animals (4). Likewise, FAAH inhibitors produce analgesic and anxiolytic effects in rodents (6). These findings suggest that FAAH may represent an attractive therapeutic target for the treatment of pain and related neural disorders. Toward this end, a deeper understanding of the catalytic mechanism of FAAH may assist in the design of specific inhibitors of this enzyme.

FAAH belongs to a diverse group of alkyl and aryl amidases known as the amidase signature (AS) family whose members are characterized by a conserved serine- and glycine-rich stretch of ~130 amino acids (7, 8). Proteins containing the AS sequence have been found in a broad range of organisms, including archaea (9), eubacteria (7, 10–12), fungi (13), nematodes, plants, insects, birds (14), and mammals (2, 3). Despite the evolutionary extent of AS enzymes, their catalytic mechanism has only recently been investigated. A series of mutagenesis and chemical labeling studies of FAAH has targeted residues conserved across the AS family and provided evidence that three of these residues, Ser-241, Lys-142, and Ser-217, are of primary importance for catalysis (15). In particular, these investigations have indicated roles for Ser-241 and Lys-142 as the catalytic nucleophile and a catalytic acid/base, respectively, with the latter residue contributing to the unusual ability of FAAH to hydrolyze structurally similar fatty acid amides and esters at equivalent rates (16, 17). Nonetheless, a more detailed understanding of the catalytic mechanism of FAAH has to date been limited by a lack of structural information for this enzyme and the AS family as a whole.

With the recently solved x-ray crystal structures of FAAH (18) and the distantly related bacterial AS enzymes malonamidase E2 (MAE2) (19) and peptide amidase (PAM) (20), the central catalytic residues of the AS family have been revealed to form a novel serine-serine-lysine catalytic triad (Ser-241, Ser-217, and Lys-142 in FAAH). This unusual arrangement of catalytic residues raises intriguing questions regarding the specific roles played by the bridging serine and lysine residues in hydrolysis. For example, based on the structures of MAE2 and PAM, models have been proposed for catalysis in which the bridging serine of the triad plays a primary role in both the base-catalyzed activation of the neighboring serine nucleophile and the acid-catalyzed protonation of the substrate-leaving group, whereas the lysine residue purportedly has a more supportive function mainly in the latter event (19, 20). Somewhat inconsistent with this mechanism, however, are previous mutagenesis analyses of FAAH that have indicated a more central participation of Lys-142 in catalysis (17). Here, through a combination of mutagenesis, substrate selectivity profiles, and chemical labeling experiments, we provide evidence that

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¹ The abbreviations used are: FAAH, fatty acid amide hydrolase; MAE2, malonamidase E2; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PAM, peptide amidase; OME, oleoyl methyl ester; FP, fluorophosphonate; AS, amidase signature; WT, wild type.

both Ser-217 and Lys-142 contribute to the base-catalyzed activation of the Ser-241 nucleophile of FAAH, whereas Lys-142 appears to play a uniquely important role in the acid-catalyzed protonation of the substrate-leaving group. This latter property of Lys-142, which is unaffected by mutation of Ser-217, may support the unusual ability of FAAH to hydrolyze fatty acid amides and esters at equivalent rates.

EXPERIMENTAL PROCEDURES

Construction of FAAH Mutants—For the studies described below, rat FAAH mutants were constructed in the prokaryotic expression vector pTrcHisA (Invitrogen), which contains an N-terminal His₆ tag. Point mutants were generated using the QuikChange procedure (Stratagene). All of the enzymes contained an N-terminal truncation of amino acid residues 1–29, which constitute a predicted transmembrane domain. This deletion has been shown to have no effect on catalytic activity or membrane binding but does enhance expression and purification (21). For clarity, numbered residues refer to the full-length enzyme.

Expression and Purification of FAAH and Mutants—Enzymes were expressed in *Escherichia coli* strain BL21(DE3) and purified by sequential metal affinity, heparin-agarose, and gel-filtration chromatography as described previously (21). All of the enzymes yielded ~0.5 mg of protein/liter of culture volume.

Circular Dichroism Spectrometry—Protein samples at 0.5 mg/ml (7.75 μ M) in 10 mM Tris, pH 8.0, 100 mM NaCl, and 0.015% lauryldimethylamine oxide were measured by far-UV circular dichroism at 25 °C in a 0.1-cm cell on an Aviv stopped-flow CD spectrometer.

Synthesis of FAAH Substrates—[¹⁴C]Oleamide and [¹⁴C]oleoyl methyl ester (OME) were synthesized by reaction of [¹⁴C]oleoyl chloride with ammonium hydroxide and methanol, respectively, as described previously (2).

Enzyme Assays—Enzyme assays were performed by following the conversion of [¹⁴C]oleamide and [¹⁴C]OME to oleic acid using a thin layer chromatography (TLC) assay as described previously (21). Reactions were conducted in a buffer of 50 mM Bis-Tris propane, 50 mM CAPS, 50 mM citrate, 150 mM NaCl, and 0.05% Triton X-100. The pH of the buffer was adjusted using HCl or NaOH. Reactions were quenched with 0.5 N HCl at 4 time points. Oleic acid was separated from oleamide by TLC in 65% ethyl acetate, 35% hexanes and from OME by TLC in 20% ethyl acetate, 80% hexanes. The radioactive compounds were quantified using a Cyclone PhosphorImager (PerkinElmer Life Sciences). FAAH enzymes exhibited Michaelis-Menten kinetics, and apparent K_m and k_{cat} values were calculated from Lineweaver-Burk plots of four substrate concentrations run in triplicate. Additional assays conducted with 0.01 and 0.25% Triton X-100 afforded k_{cat} and K_m values for FAAH-catalyzed oleamide and OME hydrolysis equivalent to the values obtained with 0.05% Triton X-100, indicating that over this concentration range of detergent (0.01–0.25% Triton X-100), the substrate/detergent ratio did not significantly impact the measurement of the catalytic parameters of FAAH. Enzyme concentrations were estimated assuming an absorbance at 280 nm of 0.8 absorbance unit for a 1 mg/ml solution of FAAH (21).

Fluorophosphonate Labeling—Labeling reactions of 80 nM enzyme and 1 μ M fluorophosphonate-tetramethyl rhodamine (FP-rhodamine, Activx Biosciences) (22) were allowed to proceed at 25 °C for 5 and 20 min using the reaction buffer above and quenched with one volume of 2 \times SDS loading buffer. Control samples of wild type (WT) FAAH were labeled to completion and used as a reference for 100% reactivity. Quenched reactions were subsequently analyzed by SDS-PAGE with 1.2 pmol of protein/gel lane. The extent of FP-labeling was visualized in-gel using a Hitachi FMBio IIe flatbed laser-induced fluorescence scanner and quantified by measuring the integrated fluorescence band intensities. The rates of FP reactivity of FAAH variants were calculated using Equation 1,

$$E = E_0 e^{-k_{obs}t} \quad (\text{Eq. 1})$$

where E is the amount of unlabeled enzyme at time point t , E_0 is the total enzyme, and k_{obs} is the calculated rate of labeling. Values for FP-labeling are reported as the second order rate constant $k_{obs}/[I]$ where $[I]$ is the concentration of FP-rhodamine. For each mutant enzyme, similar $k_{obs}/[I]$ values were obtained at 5- and 20-min time points and these values were averaged from duplicate trials to provide the values reported in Table IV and Fig. 3. For WT-FAAH, $k_{obs}/[I]$ values were obtained from reactions with 8 nM enzyme and 100 nM FP-rhodamine, pH 7.0, measured at 15, 30, 45, and 60 s. These values were averaged to provide the $k_{obs}/[I]$ value reported in Table IV.

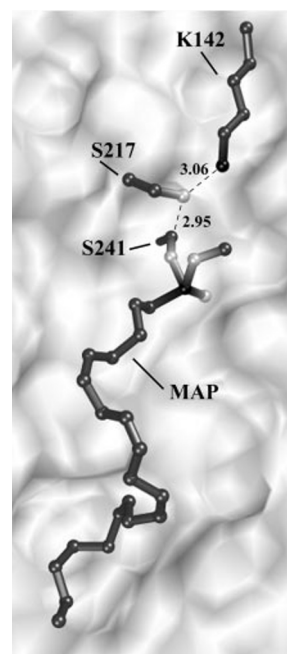


FIG. 1. The serine 241-serine 217-lysine 142 catalytic triad of FAAH. Shown is a portion of the FAAH active site with the Ser-241/Ser-217/Lys-142 catalytic triad highlighted. In this crystal structure, the Ser-241 nucleophile is covalently bound to the inhibitor methoxyarachidonyl phosphonate (MAP). Distances among Ser-241, Ser-217, and Lys-142 are shown in angstroms. The image was generated from the Protein Data Base file 1MT5 and rendered using Python-based Molecular Viewer (www.scripps.edu/~sanner/python/).

RESULTS

Generation and Purification of FAAH Variants with Mutations in the Serine-Serine-Lysine Catalytic Triad—Previous studies of FAAH have provided strong evidence that Ser-241 represents the catalytic nucleophile of the enzyme (16), a finding substantiated by the three-dimensional structure of FAAH where covalent modification of Ser-241 by a methoxyarachidonyl fluorophosphonate inhibitor was observed (18). The FAAH structure revealed that Ser-241 forms part of an unusual catalytic triad with Ser-217 and Lys-142 (Fig. 1), an active site architecture also observed in the structures of the bacterial AS enzymes MAE2 (19) and PAM (20). Given the complete conservation of this serine-serine-lysine triad among members of the AS family, a deeper investigation of the roles that its constituents play in catalysis is warranted. To examine the catalytic functions of Ser-217 and Lys-142, each residue was mutated to alanine and the resulting FAAH variants were expressed and purified as described previously (21). Additionally, the double mutant K142A/S217A was generated and purified. All of the mutant enzymes were properly folded based on gel-filtration profiles (data not shown) and far-UV circular dichroism spectra that matched those of WT-FAAH (Supplementary Fig. 1).

Comparative Analysis of the Amidase Activities of FAAH Mutants—FAAH mutants were analyzed using [¹⁴C]oleamide as a substrate following previously described methods (21). Consistent with past findings (16, 17), both the K142A and S217A FAAH mutants exhibited significant decreases in apparent k_{cat} values for oleamide hydrolysis relative to WT-FAAH at pH 9.0 (~40,000- and 3,000-fold, respectively) (Table I). The K142A/S217A double mutant displayed an even greater catalytic deficiency at this pH (~70,000-fold). In contrast to both the K142A and S217A mutants, which showed wild type apparent K_m values for oleamide, the K142A/S217A variant displayed a 3-fold increase in apparent K_m for oleamide, suggesting that the mutation of both Lys-142 and Ser-217 had a

TABLE I
Catalytic properties of FAAH mutants with oleamide

Enzyme	Catalytic triad			$k_{\text{cat}}^{a,b}$ s^{-1}	$K_m^{a,b}$ μM	$k_{\text{cat}} \text{ mutant}/k_{\text{cat}} \text{ WT}$	
	Ser	Ser	Lys			pH 9.0	pH 7.0
FAAH	+	+	+	14.4 ± 0.5^c	26 ± 1		
K142A	+	+	—	$(3.4 \pm 0.5) \times 10^{-4c}$	20 ± 5^d	2.4×10^{-5}	9.6×10^{-6}
S217A	+	—	+	$(4.3 \pm 0.1) \times 10^{-3c}$	15 ± 3^d	3.0×10^{-4}	5.0×10^{-4}
K142A/S217A	+	—	—	$(1.96 \pm 0.08) \times 10^{-4}$	85 ± 2	1.4×10^{-5}	

^a Determined at pH 9.0 unless otherwise noted.

^b FAAH is an integral membrane enzyme, and therefore assays are conducted in the presence of detergent. The reported k_{cat} and K_m values are thus apparent values.

^c Determined as previously described in Ref. 16 and 17 and found to agree with published results.

^d Data are from Ref. 16 and 17.

modest effect on substrate binding. Both of the K142A and K142A/S217A mutants exhibited a strong pH dependence on catalysis (Fig. 2A), although their severely reduced activities were difficult to measure in the lower pH range (hydrolysis rates below $1 \times 10^{-5} \text{ s}^{-1}$ approached the sensitivity limit of the substrate assay). In contrast, the S217A mutant was found to display a relatively flat pH-rate profile for oleamide hydrolysis from pH 6.5 to 9.0 (Fig. 2A). The distinct pH-rate profiles of the FAAH mutants resulted in catalytic defects relative to WT-FAAH at pH 7.0 of 104,000- and 2,000-fold for the K142A and S217A variants, respectively.

Comparative Analysis of the Esterase Activities of FAAH Mutants—Typically, serine hydrolases hydrolyze ester substrates at much greater rates compared with structurally similar amides, reflecting the relative solvolytic potential of these compounds (23). FAAH represents a notable exception to this general principle as this enzyme has been shown to hydrolyze fatty acid amides and esters at equivalent rates by an acylation rate-limiting mechanism (17). Interestingly, this special property of FAAH depends on Lys-142 because a K142A mutant has been shown to strongly prefer OME over oleamide (17). Consistent with these findings, at pH 9.0 the K142A mutant exhibited only a 50-fold decrease in apparent k_{cat} for OME (Table 2), hydrolyzing this substrate at a 320-fold faster rate than oleamide (Table III). In contrast, the S217A mutant was found to hydrolyze OME at an ~ 5 -fold slower rate than oleamide (Table III). This preference for amide substrates over esters exhibited by the S217A mutant mirrored the substrate selectivity of WT-FAAH, which displayed a 2.5-fold greater activity with oleamide than OME (Table III). Thus, despite exhibiting considerable reductions in absolute catalytic activity with both oleamide and OME, the S217A mutant maintained wild type amide/ester hydrolysis ratios. The striking differences in the amide/ester hydrolysis ratios of the K142A and S217A mutants resulted in opposite relative substrate preferences for these enzymes with the former enzyme being a 120-fold better esterase but a 13-fold worse amidase than the latter enzyme at pH 9.0 (Tables I and II).

The K142A/S217A mutant exhibited a mixture of catalytic features that individually resembled either single mutant but collectively produced a more complex picture. For example, both the K142A/S217A and S217A mutants hydrolyzed OME at approximately 6000-fold slower rates than WT-FAAH at pH 9.0 (Table II). However, a steep pH dependence was observed for the esterase activity of the K142A/S217A mutant that resembled the pH-rate profile of the K142A mutant (Fig. 2). The catalytic activities of both the K142A and K142A/S217A mutants exhibited a linear dependence on solvent $[\text{OH}^-]$ with a slope of 0.7. In contrast, the S217A mutant showed a flattened pH-rate profile for OME hydrolysis similar to the pH-rate profile displayed by this enzyme for oleamide hydrolysis (Fig. 2B). The differences in the pH-rate profiles of the K142A/S217A and

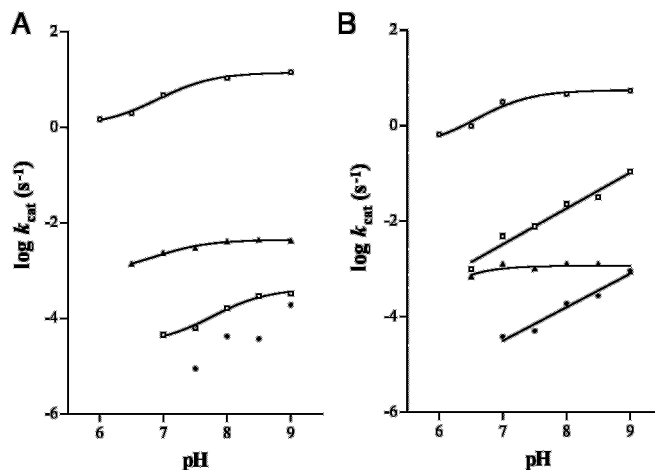


FIG. 2. pH versus $\log k_{\text{cat}}$ profiles. FAAH (open circles), K142A (open squares), S217A (closed triangles), and K142A/S217A (closed diamonds) enzymes are shown with either oleamide (A) or OME (B) as a substrate. All of the values are the average of triplicate measurements with $<20\%$ mean \pm S.D. Linear regression analysis was used to fit the K142A and K142A/S217A mutants with OME. All of the other traces were generated using a non-linear least-squares regression analysis (GraphPad Prism).

S217A mutants resulted in progressively slower relative rates of catalysis for the former enzyme as pH was lowered (Fig. 2A). Finally, the K142A/S217A mutant exhibited an ~ 5 -fold preference for OME over oleamide (Table III), thus displaying an amide/ester selectivity ratio that fell in between those displayed by either single mutant.

Comparative Analysis of the FP Reactivities of FAAH Mutants—Assuming that the K142A and S217A mutants, similar to WT-FAAH (17), both hydrolyze amide and ester substrates through an acylation rate-limiting mechanism (a premise that is supported by the wild type apparent K_m values displayed by these enzymes for oleamide and OME), the reduced catalytic activities of these mutants suggest roles for each residue in nucleophile activation, leaving group protonation, or both. To distinguish the impact of mutation of Lys-142 and Ser-217 on nucleophile activation separate from leaving group protonation, the rates of reactivity of each mutant enzyme with a fluorophosphonate (FP-rhodamine) (22) were measured. Reduced rates of labeling of serine hydrolases by FPs are typically caused by mutations that 1) decrease the strength of the serine nucleophile (15, 24) and/or 2) disrupt residues involved in transition state stabilization, such as those composing the oxyanion hole (25, 26). Based on the FAAH crystal structure, neither Lys-142 nor Ser-217 appears to participate in the oxyanion hole and, therefore, changes in the FP-labeling rates of the K142A, S217A, and K142/S217A mutants were interpreted to reflect primarily alterations in the strength of the Ser-241 nucleo-

TABLE II
Catalytic properties of FAAH mutants with oleoyl methyl ester

Enzyme	Catalytic triad			$k_{\text{cat}}^{a,b}$	$K_m^{a,b}$	$k_{\text{cat}} \text{ mutant}/k_{\text{cat}} \text{ WT}$	
	Ser	Ser	Lys			pH 9.0	pH 7.0
				s^{-1}	μM		
FAAH	+	+	+	5.5 ± 0.6^c	21 ± 3^d		
K142A	+	+	—	0.110 ± 0.004^c	12 ± 3^d	2.0×10^{-2}	1.7×10^{-3}
S217A	+	—	+	$(9.0 \pm 0.6) \times 10^{-4}$	14 ± 3	1.6×10^{-4}	4.1×10^{-4}
K142A/S217A	+	—	—	$(9.4 \pm 0.5) \times 10^{-4}$	57 ± 11	1.7×10^{-4}	1.2×10^{-5}

^a Determined at pH 9.0 unless otherwise noted.

^b FAAH is an integral membrane enzyme, and therefore assays are conducted in the presence of detergent. The reported k_{cat} and K_m values are thus apparent values.

^c Determined as previously described in Ref. 16 and found to agree with published results.

^d Data are from Ref. 16.

TABLE III
Relative substrate selectivities of FAAH mutants at pH 9.0

	FAAH	K142A	S217A	K142A/ S217A
$k_{\text{cat}}^{\text{OME}}/k_{\text{cat}}^{\text{oleamide}}$	0.38	320	0.21	4.8

phile. A direct comparison of the FP reactivity rates of WT-FAAH and FAAH mutants was made at pH 7.0, because above this pH value, the rate of labeling of WT-FAAH was too fast to measure. At pH 7.0, the K142A and S217A mutants exhibited 6,100- and 2,900-fold reductions in FP reactivity rates, respectively, relative to WT-FAAH (Table IV). The K142A/S217A variant showed an even greater loss of FP reactivity (70,000-fold relative to WT-FAAH) that nonetheless appeared specific as the rate of FP labeling of this double mutant was still over an order of magnitude faster than the background labeling of the S241A mutant (Table IV). Interestingly, as the pH of the reaction was raised, the FP reactivity of the K142A mutant increased to a greater extent than the S217A mutant (Fig. 3). Thus, by pH 8.5, the K142A mutant exhibited a 2-fold greater $k_{\text{obs}}/[I]$ value than the S217A mutant, which represented a 4-fold overall shift in relative FP reactivity compared with the rates observed at pH 7.0. Collectively, these findings indicate that at physiological pH, the nucleophilicity of Ser-241 depends to a similar extent on Lys-142 and Ser-217, with the role of the former residue being better compensated for by increasing concentrations of solvent hydroxide.

DISCUSSION

The recent determination of the three-dimensional structure of FAAH has demonstrated that this enzyme possesses a serine nucleophile (Ser-241) that forms an unusual catalytic triad with a second serine residue (Ser-217) and a lysine residue (Lys-142) (18). This organization of catalytic residues has also been observed in the structures of the distantly related bacterial AS enzymes, MAE2 (19) and PAM (20). These findings, in combination with the complete conservation of Ser-241, Ser-217, and Lys-142 among the >80 AS enzymes identified to date argue that each of these residues plays an important role in catalysis. Nonetheless, many questions remain regarding the AS family serine-serine-lysine catalytic triad. For example, how does the Ser-217/Lys-142 portion of the FAAH triad promote the two key steps of acylation, namely the base-catalyzed activation of the Ser-241 nucleophile and the acid-catalyzed protonation of the substrate-leaving group? Additionally, how do these residues function either individually or in concert to impart upon FAAH its unusual ability to hydrolyze amides and esters at equivalent rates? Finally, do all of the AS enzymes invoke the same catalytic mechanism, or alternatively, might these enzymes exhibit differences in how they hydrolyze their respective substrates? To begin to address these important

issues, we have conducted a comparative analysis of the roles played by Ser-217 and Lys-142 in catalysis using a combination of mutagenesis, substrate selectivity profiles, and chemical-labeling experiments.

Comparison of the Kinetic Properties of the K142A and S217A FAAH Mutants—The general defects in amidase and esterase activity observed for the S217A and K142A FAAH mutants support central roles in catalysis for both residues. Interestingly, however, the relative impact of each mutation on the amidase and esterase activities of FAAH was quite different. For example, at physiological pH, the S217A mutant exhibited nearly equivalent deficiencies for oleamide and OME hydrolysis (2,000- and 2,500-fold, respectively), whereas the K142A variant showed a much greater reduction in oleamide hydrolysis (104,000-fold) than OME hydrolysis (600-fold) (Tables I and II). The esterase activity of the K142A mutant showed very strong pH dependence (Fig. 2), suggesting that solvent hydroxide was capable of partially substituting for the loss of this lysine residue. This compensatory action of hydroxide resulted in the K142A mutant becoming a fairly efficient esterase at higher pH values (2% WT activity at pH 9.0). In contrast, the K142A mutant displayed a more modest increase in amidase activity at higher pH values, possibly reflecting the absolute requirement for protonation of the amine-leaving group of amide substrates. Unlike the K142A mutant, the S217A variant exhibited little pH dependence with either amide or ester substrates (Fig. 2). The flattened pH-rate profiles of the S217A mutant do not appear to reflect a physical rate-limiting step because this enzyme has been shown to exhibit a significant solvent deuterium isotope effect for oleamide hydrolysis (1.8-fold), indicating that a proton-transfer event probably occurs in the rate-limiting step (15). Although it remains unclear why the S217A mutant fails to show pH dependence, this property may reflect the competing effects of pH on different steps in the acylation reaction. For example, as pH is raised, solvent-driven, base-catalyzed activation of Ser-241 may be accelerated in the S217A mutant; however, this effect could be counterbalanced by a reduction in Lys-142-directed protonation of the substrate leaving group. For such a model to be correct, Lys-142 would have to exhibit an altered pK_a value so that the protonation state of this residue was affected in the range of pH 7.0–9.0. Notably, the K142A/S217A double mutant exhibited a strong pH dependence for OME hydrolysis that mirrored the behavior of the K142A mutant (Fig. 2). These data are consistent with a model where the flattened pH-rate profiles of the S217A mutant are at least in part dependent on Lys-142.

Evidence That Both Lys-142 and Ser-217 Are Involved in Activation of the Ser-241 Nucleophile of FAAH—Several properties of the K142A mutant suggest that this residue participates in the base-catalyzed activation of the Ser-241 nucleophile. For example, the strong pH dependence of the K142A

TABLE IV
FP reactivity of FAAH mutants at pH 7.0

	FAAH	K142A	S217A	K142A/ S217A	S241A
$k_{\text{obs}}/[\text{I}]$ ($\text{M}^{-1} \text{s}^{-1}$)	$(1.4 \pm 0.6) \times 10^5$	23 ± 7	49 ± 12	2 ± 1	0.15 ± 0.11

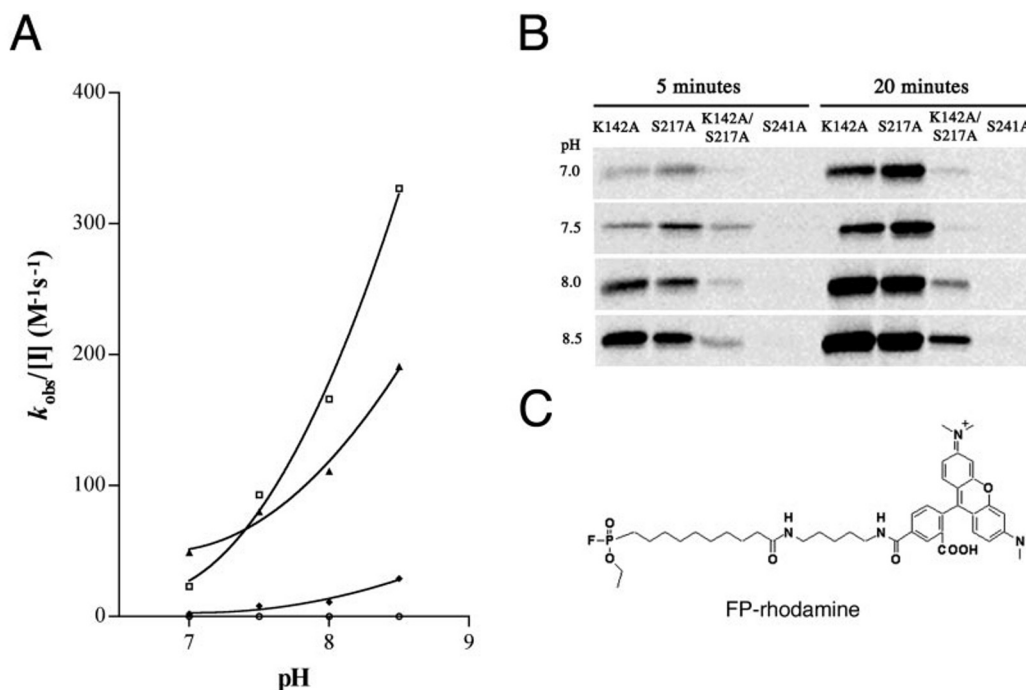


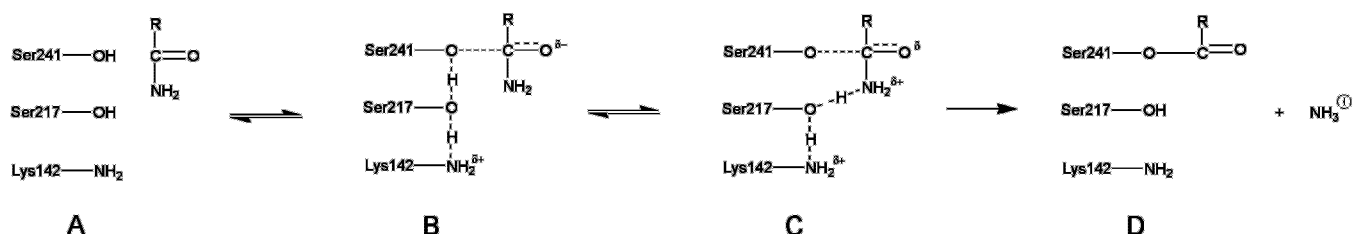
FIG. 3. **FP-rhodamine labeling of FAAH mutants.** A, pH versus $k_{\text{obs}}/[\text{I}]$ profiles for K142A (open squares), S217A (closed triangles), K142A/S217A (closed diamonds), and S241A (open circles) mutant enzymes with FP-rhodamine as the labeling reagent. Values are the average of quadruplicate measurements. B, representative data from labeling reactions of FAAH mutants with FP-rhodamine. C, structure of FP-rhodamine (21). Non-linear least-squares regression analysis was used to fit all of the data traces (GraphPad Prism).

mutant resembles the behavior of serine protease mutants that lack their respective catalytic histidine bases (27–29). The greatly reduced FP reactivity of the K142A mutant also implicates this residue in nucleophile activation. The special properties of FPs (high electrophilicity, excellent leaving group) make these reagents useful mechanistic probes of nucleophile strength for members of the serine hydrolase family (15, 17, 24). At physiological pH, the K142A mutant was found to exhibit a 6,100-fold reduction in FP-labeling rate compared with WT-FAAH (Table IV). A similar defect was observed in the S217A mutant (2,900-fold), whereas the FP reactivity of K142A/S217A double mutant was compromised to an even greater extent (70,000-fold). Collectively, these results indicate that both Lys-142 and Ser-217 participate in the activation of the Ser-241 nucleophile of FAAH, possibly through a mechanism outlined in Scheme 1.

In the proposed catalytic mechanism shown in Scheme 1, an uncharged Lys-142 initiates catalysis by accepting a proton from Ser-217, which in turn deprotonates the Ser-241 nucleophile to facilitate attack on the substrate carbonyl. This route for formation of the tetrahedral intermediate contrasts with the mechanism proposed previously for the bacterial amidases MAE2 (19) and PAM (20). Based on structural data, the lysine residue of the catalytic triad in these enzymes has been suggested to exist primarily in a protonated or charged state, thus restricting the function of this residue to the acid-catalyzed, leaving group protonation step of acylation. Consequently, the bridging serine is proposed to act independently of the lysine residue as the catalytic base involved in nucleophile activation. Although biochemical data for or against this proposed model for bacterial AS enzyme catalysis remain sparse (neither PAM

nor MAE2 has been subjected to extensive mutagenesis or kinetic analysis), it is interesting to note that PAM shows very weak reactivity with FPs and related nucleophile-directed labeling reagents (20). Indeed, in the structure of PAM, the aldehyde inhibitor chymostatin is bound to the enzyme active site but does not form a covalent adduct with the serine nucleophile. These findings suggest that the PAM nucleophile exists in a deactivated state, a feature that contrasts sharply with the serine nucleophile of FAAH, which exhibits rapid rates of labeling by FPs ($1.4 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7.0) (Table IV). Collectively, these data suggest that individual AS enzymes may operate through different mechanisms, possibly depending on the initial protonation state of the lysine residue of the triad. For AS enzymes similar to PAM, a protonated lysine would be unable to participate as a base involved in nucleophile activation, resulting in a correspondingly weak serine nucleophile; however, for AS enzymes similar to FAAH, an unprotonated lysine would increase the basic character of the bridging serine residue, in turn leading to a strengthening of the serine nucleophile. Finally, it is worth noting that the K142A mutant showed a stronger pH dependence for FP reactivity than the S217A mutant (Fig. 3), a finding that paralleled the more dramatic pH dependence observed for the esterase activity of the former enzyme (Fig. 2). These data indicate that alkaline pH is more capable of compensating for the loss of Lys-142 than for Ser-217 and suggest further that, in the case of the K142A mutant, solvent hydroxide may activate Ser-217 to initiate the acylation reaction.

Evidence That Lys-142 Plays a Key Role in Protonation of the Substrate-leaving Group, Which in Turn Sets the Amide/Ester Selectivity Ratios of FAAH—Independent of pH, the K142A



SCHEME 1. **Proposed mechanism for the acylation step of amide and ester hydrolysis catalyzed by FAAH (shown for amides).** Lys-142, initially in a deprotonated state (A), abstracts a proton from Ser-217, which in turn abstracts a proton from the Ser-241 nucleophile (B). Attack of the nucleophile on the substrate carbonyl is proposed to occur in a coupled manner with proton donation from Ser-217 to the nitrogen atom of the amide substrate (C). This latter step requires the coincidental donation of a proton from Lys-142 to Ser-217, resulting in the formation of an acyl enzyme intermediate where both Lys-142 and Ser-217 have returned to their initial protonation states (D). In this mechanism, because nucleophilic attack and leaving group protonation take place nearly simultaneously, the acylation rates of amide and ester substrates would be normalized.

mutant was found to exhibit >100-fold lower rates of hydrolysis with oleamide than OME, a substrate selectivity profile that differed dramatically from WT-FAAH, which exhibited a slight preference for oleamide over OME (Tables I and II and Fig. 2). The greatly diminished amidase activity of the K142A mutant suggests that this lysine residue is important for protonating the substrate-leaving group. Indeed, disruption of this step of the acylation reaction may be expected to selectively affect amidase activity over esterase activity because amines are much poorer leaving groups than alkoxy substituents. Based on the structures of FAAH and the related AS enzymes MAE2 and PAM, the impact of Lys-142 on leaving group protonation, as well as on nucleophile strength, would be predicted to occur indirectly via the action of this residue on the bridging Ser-217 of the triad (Scheme 1). In all of these structures, the lysine residue of the triad is too far away (>4.5 Å) to make direct contacts with either the serine nucleophile or the predicted position of the substrate-leaving group. Thus, it was surprising to find that the S217A mutant, despite displaying defects in both catalytic activity and nucleophile strength, exhibited wild type amide/ester hydrolysis ratios. Although it remains unclear how the S217A mutant maintains a preference for amide over ester substrates, we speculate that water may substitute for the absence of Ser-217 in this enzyme. If so, then the bridging water molecule would appear more capable of transmitting the effects of Lys-142 on leaving group protonation than nucleophile activation. Regardless, the observation that in the absence of Ser-217 FAAH still hydrolyzes amides at a greater rate than esters reveals that for this enzyme substrate selectivity is not coupled to catalytic power. Consistent with this notion, a K142E mutant has been found to display equivalent rates of acylation with both oleamide and OME, despite showing greatly diminished catalytic activity with each substrate (17). Collectively, these findings suggest that FAAH possesses a specialized active site structure dedicated to normalizing the acylation rates of amide and ester substrates. Because disruption of this property of FAAH appears to require conversion of Lys-142 to a residue that is incapable of transferring a proton, we hypothesize that FAAH achieves its unusual ability to competitively hydrolyze amides and esters at equivalent rates by forcing protonation of the leaving group early in the transition state of acylation concomitant with nucleophilic attack on the substrate carbonyl (Scheme 1). As previously predicted by Fersht (23), a mechanism where acid-catalyzed leaving group protonation is tightly coupled to base-catalyzed nucleophile activation could result in nearly identical rates of hydrolysis for amide and ester substrates. Indeed, Komiyama and Bender (30) predicted such a mechanism for amide hydrolysis by serine proteases. However, an alternative mechanism of hydrolysis involving two discrete proton transfer events was proposed for ester substrates to account for their preferential hydrolysis by

these enzymes. Thus, what may be special about FAAH is not how the enzyme hydrolyzes amides but rather that it steers esters through the same reaction pathway.

Conclusions and Physiological Implications—It is worth considering why FAAH may have evolved a catalytic mechanism that achieves the competitive hydrolysis of amide and ester substrates. *In vivo*, FAAH must negotiate the binding and hydrolysis of its fatty acid amide substrates in a background of high concentrations of endogenous fatty acid esters (e.g. monoacylglycerols). If FAAH had invoked a typical serine protease-like mechanism and hydrolyzed ester substrates in a deacylation rate-limiting manner with acylation rates that exceeded those of amides by 2–3 orders of magnitude (31–34), the enzyme might have been saturated by lipid esters and failed to function as an amidase *in vivo*. Thus, we speculate that FAAH has evolved a mechanism that accomplishes the competitive degradation of amide and ester substrates to allow the enzyme to control the magnitude and duration of signals communicated by endogenous fatty acid amides within a complex milieu of structurally similar lipid natural products.

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