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Clarifying the Catalytic Roles of Conserved Residues in the Amidase Signature Family*

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Fatty acid amide hydrolase (FAAH) is a mammalian integral membrane enzyme responsible for the hydrolysis of a number of neuromodulatory fatty acid amides, including the endogenous cannabinoid anandamide and the sleep-inducing lipid oleamide. FAAH belongs to a large class of hydrolytic enzymes termed the "amidase signature family," whose members are defined by a conserved stretch of approximately 130 amino acids termed the "amidase signature sequence." Recently, site-directed mutagenesis studies of FAAH have targeted a limited number of conserved residues in the amidase signature sequence of the enzyme, identifying Ser-241 as the catalytic nucleophile and Lys-142 as an acid/base catalyst. The roles of several other conserved residues with potentially important and/or overlapping catalytic functions have not yet been examined. In this study, we have mutated all potentially catalytic residues in FAAH that are conserved among members of the amidase signature family, and have assessed their individual roles in catalysis through chemical labeling and kinetic methods. Several of these residues appear to serve primarily structural roles, as their mutation produced FAAH variants with considerable catalytic activity but reduced expression in prokaryotic and/or eukaryotic systems. In contrast, five mutations, K142A, S217A, S218A, S241A, and R243A, decreased the amidase activity of FAAH greater than 100-fold without detectably impacting the structural integrity of the enzyme. The pH rate profiles, amide/ester selectivities, and fluorophosphonate reactivities of these mutants revealed distinct catalytic roles for each residue. Of particular interest, one mutant, R243A, displayed uncompromised esterase activity but severely reduced amidase activity, indicating that the amidase and esterase efficiencies of FAAH can be functionally uncoupled. Collectively, these studies provide evidence that amidase signature enzymes represent a large class of serine-lysine catalytic dyad hydrolases whose evolutionary distribution rivals that of the catalytic triad superfamily.

The amidase signature (AS)[1] family was originally identified by primary structure analysis, which revealed a highly conserved serine- and glycine-rich sequence present in several amidases of bacterial and fungal origin (1, 2). The number of proteins containing the AS sequence has since greatly increased, and presently more than 80 known or predicted members of this family can be identified in public data bases. Proteins containing the AS sequence exist in archaea (3), eubacteria (1, 4–6), fungi (7), nematodes, plants, insects, birds (8), and mammals (9). The substrate specificities and biological functions of these enzymes vary widely, including carbon/nitrogen metabolism in fungi through the hydrolysis of acetamide (10), the generation of properly charged $tRNA^{Gln}$ in eubacteria through the transfer of ammonia from glutamine (11), and the degradation of neuromodulatory fatty acid amides in mammals (9). The evolutionary and functional breadth of the AS family highlights the importance of achieving a thorough understanding of its mechanistic and structural features. Toward this end, we have initiated a research program aimed at characterizing the structure and function of the AS enzyme fatty acid amide hydrolase (FAAH) (9, 12–14).

FAAH is a mammalian integral membrane enzyme responsible for the catabolism of the fatty acid amide family of endogenous signaling lipids (9). Representative fatty acid amides degraded by FAAH include the endocannabinoid anandamide (15) and the sleep-inducing lipid oleamide (16, 17). Fatty acid amides display an intriguing number of bioactivities in mammals, including the induction of sleep (16, 18, 19) and analgesia (20–22), indicating that FAAH may serve as an attractive target for pharmaceutical efforts aimed at influencing endogenous pain and/or sleep-wake systems (23, 24). In support of this notion, FAAH-resistant analogues of anandamide show enhanced pharmacological activity *in vivo* (20, 25).

Previous work in our laboratory has identified the catalytic nucleophile of FAAH as serine 241, one of three conserved serine residues in the AS sequence (13). Additionally, we have determined that FAAH does not utilize a histidine base for catalysis, indicating that AS enzymes employ a catalytic mechanism distinct from the Ser-His-Asp catalytic triad common to most serine hydrolases (26). Subsequent mutagenesis and kinetic efforts identified a conserved lysine residue, Lys-142, as a strong candidate for the catalytic base of FAAH responsible for nucleophile activation (14). Although the data obtained in these studies clearly supported central catalytic roles for Ser-241 and Lys-142, the abundance of conserved, potentially catalytic residues in the AS sequence raised the possibility that other residues might possess equally important and/or overlap-

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[1] The abbreviations used are: AS, amidase signature; FAAH, fatty acid amide hydrolase; OME, oleoyl methyl ester; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; FP-biotin, biotin-conjugated fluorophosphonate inhibitor.

ping catalytic functions. Indeed, a previous study on the *Rhodococcal* J1 amidase, a bacterial AS enzyme, had proposed that a conserved aspartic acid (Asp-237 in FAAH) acted as the catalytic base for this enzyme (27). In order to clarify the roles that conserved residues play in the hydrolytic mechanism of the AS family, we have examined the function of all potentially catalytic residues in FAAH. These residues were selected based on two criteria: 1) high conservation among AS enzymes, and 2) possession of a side chain capable of participating in acid/base catalysis and/or hydrogen bonding. The results of this investigation reveal that only a limited subset of conserved AS residues play catalytic roles, with several other conserved residues appearing to be primarily of structural importance.

EXPERIMENTAL PROCEDURES

Generation of FAAH Mutants—FAAH mutants were constructed in the prokaryotic expression vector pTrcHis A (Invitrogen) using the Quickchange procedure (Stratagene) (13). The N206A mutant was generated using overlap extension polymerase chain reaction (28, 29) with two complementary primers containing the desired mutation and two primers at the 5′ and 3′ ends of the FAAH cDNA. The mutants were subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen) using standard molecular biology procedures. The mutants were sequenced through their entire coding region and found to contain only the desired mutation.

Expression and Analysis of FAAH Mutants—The FAAH mutants were expressed in the *Escherichia coli* strain BL21 and purified as described (12). All *E. coli* expression constructs contained a deletion of the N-terminal transmembrane domain of FAAH. Deletion of this region facilitated its purification from *E. coli* but had no effect on the enzymatic activity of FAAH (12). Importantly, the membrane-binding properties of FAAH were not affected by this deletion, and consequently, all purifications and enzyme reactions were performed in the presence of detergents.

Transient transfections of FAAH mutants into COS-7 cells were performed as detailed (9, 30). Cells were scraped in Dulbecco's modified Eagle's medium following transfection, rinsed twice with buffer 1 (12.5 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA), and resuspended in 200 μl of buffer 1. The cells were sonicated using a 50-watt tip sonicator and centrifuged in a table top ultracentrifuge at 100,000 $\times$ *g* for 1 h in a TLA-100 rotor (Beckman Instruments). The pellet from this spin was resuspended by sonication in 200 μl of buffer 2 (20 mM Hepes, pH 7.8, 150 mM NaCl, 10% glycerol, 1% Triton X-100), rocked for 1 h at 4 °C, and centrifuged at 100,000 $\times$ *g* for 1 h. The supernatant from this spin, constituting solubilized FAAH membrane extracts, was collected, and its protein concentration was determined using the D_c protein assay kit (Bio-Rad). Nearly all FAAH activity and immunoreactivity present in the crude COS-7 lysate resided in this soluble membrane extract. Samples could be frozen in buffer 2 at −80 °C indefinitely with no effect on enzyme activity. Western blots were performed using rabbit anti-FAAH antibodies (12) followed by horseradish peroxidase-coupled goat anti-rabbit antibodies. For FP-biotin studies, samples were treated with 2 μM FP-biotin for 30 min as described (31), followed by blotting using horseradish peroxidase-coupled avidin.

FAAH Activity Assays—All FAAH assays were performed as described using a TLC assay (12, 13). Briefly, [^{14}C]oleamide or oleoyl methyl ester (OME) was incubated with FAAH preparations, and aliquots were removed at various times and quenched with 0.2 N HCl. The substrate and product were extracted with ethyl acetate and separated on TLC plates using 50% ethyl acetate/hexanes. The radioactivity associated with substrate and product was then quantitated using a PhosphorImager (Packard Instrument Co.). Initial rates were determined during the linear phase of the reaction (up to approximately 20% conversion at 100 μM substrate). Errors presented with activity from COS-7 extracts indicate the standard deviation of the activity present in three independent transfections. Buffers used for standard assays and pH rate profile determinations were identical to those used previously (13). The k_{cat} values presented in the pH *versus* k_{cat} profiles of the S217A and S218A mutants were calculated based on the concentration of purified proteins measured by their UV absorbance at 280 nm as described previously (12). Solvent isotope effect studies were conducted in a buffer containing 50 mM Bis-Tris propane, 150 mM NaCl. The pD of the D_2O buffer was adjusted with a DCl solution prepared by saturating D_2O with gaseous HCl and was measured using a standard pH meter. The pD of the D_2O solution was calculated by adding 0.4 to the pH measured by the meter. Reactions were conducted in 95% D_2O. The ratio k^{H_2O}/k^{D_2O} was determined by measuring the rate of hydrolysis of saturating concentrations of oleamide or OME at pH (pD) values from 6.0 to 9.5. Maximal activity was obtained for FAAH between pH or pD 8.5 and 9.5 and the ratios of the activities in H_2O and D_2O at this plateau level are reported as k^{H_2O}/k^{D_2O}. The S217A mutant showed no pH or pD dependence and therefore the ratio k^{H_2O}/k^{D_2O} was pH-independent.

RESULTS

Sequence Alignments—Significant homology among AS enzymes is confined to a region corresponding to residues 134–257 of FAAH (referred to herein as the AS sequence). Comparison of the primary structures of 86 AS enzymes over this segment revealed 23 positions where residues were conserved in 75% or more of the aligned sequences (Fig. 1). Of these conserved positions, 11 amino acids in FAAH were selected for mutagenesis based on their potential ability to participate in acid/base chemistry and/or hydrogen bonding. The following mutants were generated: K142A, E143Q, D167A, N206A, S217A, S218A, D237A, D237N, D237E, S241A, R243A, R243K, K255A, and T257A.

Expression of FAAH Mutants—Initially, attempts were made to express the indicated mutants in *E. coli* using the pTrcHis vector, a system that provides high expression levels of FAAH protein with a His_6 tag for purification (12). In this system, only the K142A (14), S217A, S218A, and S241A (13) mutants were expressed at high enough levels to permit their purification. The properties of the purified S241A and K142A mutants have been characterized previously and indicate that these residues serve as the nucleophile and general base/acid catalyst of FAAH, respectively (13, 14). The purified S217A and S218A mutants were previously found to exhibit 2300- and 95-fold lower k_{cat} values than FAAH, respectively, with no changes in their K_m values for oleamide (13). The gel filtration migration profiles and circular dichroism spectra of the K142A, S217A, S218A, and S241A mutants were indistinguishable from those of FAAH, indicating that the observed catalytic deficits were not due to gross structural alterations (13, 14). All of the other FAAH mutants (E143Q, D167A, N206A, D237A, D237N, D237E, R243A, R243K, K255A, and T257A) were expressed mainly as inclusion bodies in *E. coli*, preventing a detailed analysis of their catalytic function in this system. Nonetheless, for many of the mutants (with the exception of D237N, D237A, R243A, and R243K), significant FAAH activity could be detected in crude *E. coli* lysates (data not shown).

In order to examine the catalytic properties of the large number of FAAH mutants that were ineffectively produced in *E. coli*, these variants were expressed in a eukaryotic system. cDNAs encoding FAAH mutants were transfected into COS-7 cells, resulting in significant levels of expression for all of the proteins examined with the exception of the D237A and R243K mutants (Fig. 2*A, upper panel*). The D237A and R243K mutants were expressed at greater than 20-fold lower levels than FAAH (data not shown), and their analysis was not pursued further. The N206A and D237N mutants were expressed approximately 5- and 10-fold worse than FAAH, respectively (Fig. 2*B, upper panel*), whereas all of the other mutants, K142A, E143Q, D167A, S217A, S218A, D237E, S241A, R243A, K255A, and T257A, expressed to levels at least 50% of that observed for FAAH. Thus, the expression of FAAH mutants in COS-7 cells provided a system where the majority of these variants could be directly compared. Importantly, the extremely low levels of endogenous FAAH activity in COS-7 cells permitted the kinetic analysis of transfected mutants with up to 300-fold reductions in catalytic activity.

Oleamide Hydrolase Activity of FAAH Mutants—The levels of oleamide hydrolase activity in solubilized membrane preparations from COS-7 cells transfected with various FAAH mu-

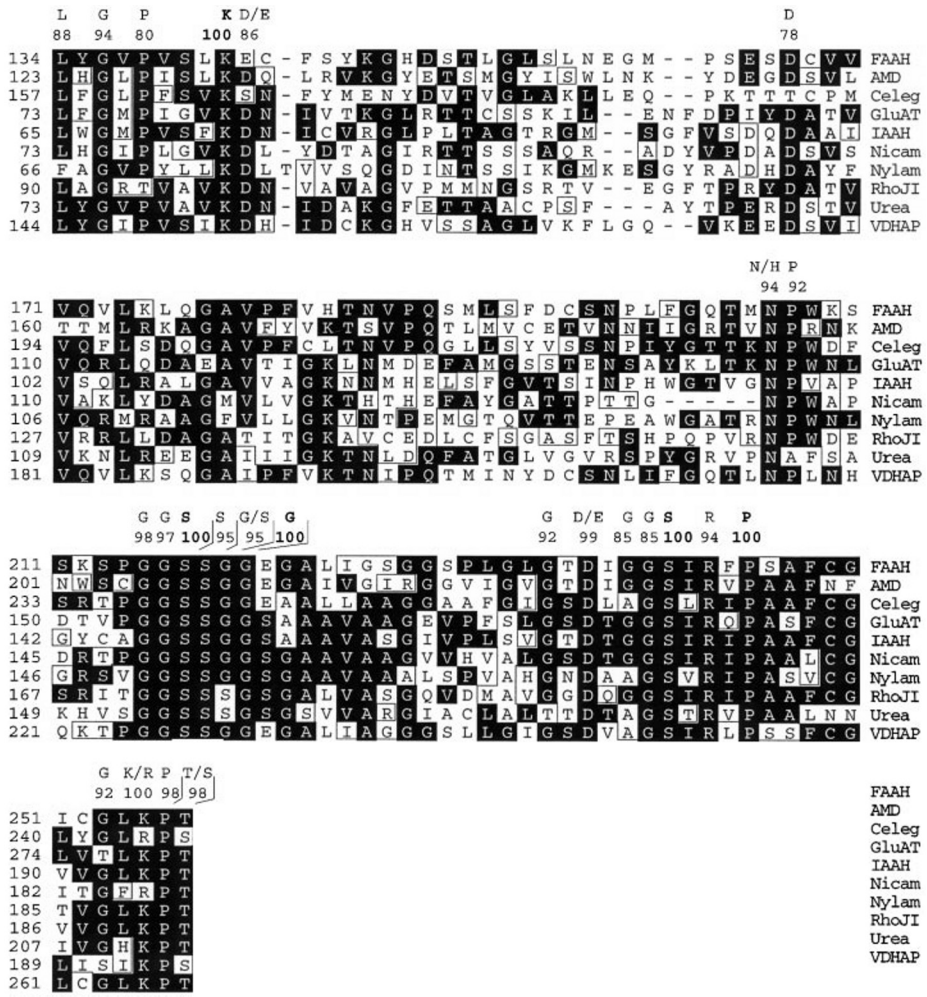

FIG. 1. **Sequence alignment of amidase signature enzymes.** Significant sequence homology can be found among AS enzymes over a region comprising residues 134–257 of FAAH. Consensus residues are shown *above* the alignment, and the percentage of residues matching the consensus from an alignment of 86 AS enzymes is indicated. *FAAH*, fatty acid amide hydrolase, *Rattus norvegicus* (accession number gi:1680722); *AMD*, acetamidase, *Emericella nidulans* (accession number gi:101782); *Celeg*, predicted amidase, *Caenorhabditis elegans* (accession number gi:6425411); *GluAT*, Glu-tRNA^{Gln} amidotransferase, *Bacillus subtilis* (accession number gi:2589195); *IAAH,* indoleacetamide hydrolase, *Pseudomonas syringae* (accession number gi:77820); *Nicam,* nicotinamidase, *Mycobacterium smegmatis* (accession number gi:3869278); *Nylam,* 6-aminohexanoate cyclic dimer hydrolase, *Flavobacterium* sp. (accession number gi:148711); *RhoJI,* JI amidase, *Rhodococcus rhodochrous* (accession number gi:563984); *Urea,* urea amidolyase, *Pichia jadini* (accession number gi:742250); *VDHAP,* vitamin D_3 hydroxylase-associated protein, *Gallus domesticus* (accession number gi:1079452).

tants are shown in Table I. The E143Q, D167A, N206A, D237E, K255A, and T257A mutants retained greater than 10% of the oleamide hydrolase activity of FAAH. When the relative expression levels of these mutants were taken into account, all of the enzymes with the exception of the K255A mutant displayed greater than 25% of wild type activity (the K255A mutant exhibited slightly less than 20% of wild type activity). The D237N mutant showed approximately 40-fold reduced activity, which in combination with its 10-fold lower expression indicated that this variant was at least 20% as active as wild type FAAH. In contrast to the significant catalytic activities displayed by the aforementioned FAAH mutants, the K142A, S217A, S218A, S241A, and R243A mutants all exhibited ≤1% of the oleamide hydrolase activity of FAAH. Thus, only five conserved AS residues (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243) were critical for the amidase activity of FAAH.

The ~100-fold catalytic deficiency displayed by the S218A mutant was similar to the 95-fold reduction in activity observed for the *E. coli*-derived form of this enzyme (13). Considering that the K142A, S217A, and S241A mutants all exhibited greater than 1000-fold reductions in k_{cat} relative to FAAH when purified from *E. coli* (13, 14), their activities were predictably below the background levels of endogenous FAAH activity in COS-7 cells (0.2 nmol/min/mg). The R243A mutant exhibited more than a 200-fold decrease in oleamide hydrolase activity that, when evaluated in the context of its slightly lower expression, corresponded to at least a 100-fold decrease in amidase activity.

Fluorophosphonate Reactivity of FAAH Mutants—The ability of FAAH mutants to react with a biotin-conjugated fluorophosphonate inhibitor (FP-biotin) (31) was examined. Greatly reduced rates of labeling with fluorophosphonates are typically caused by mutations that 1) decrease the strength of the serine nucleophile of a hydrolase (32) and/or 2) disrupt residues involved in transition state stabilization, such as those composing the oxyanion hole of a hydrolase (33, 34). All of the FAAH mutants that displayed at least 20% wild type activity also exhibited near wild type levels of FP-biotin reactivity (Fig. 2, *A* and *B, lower panels*). In contrast, the K142A, S217A, and S241A mutants showed dramatically decreased reactivities with FP-biotin (Fig. 2*A, lower panel*), consistent with their compromised catalytic activities. Interestingly, the S218A and R243A mutants, despite their severely reduced amidase activities, reacted with FP-biotin to a similar extent as FAAH (Fig. 2, *A* and *B, lower panels*). More detailed kinetic analyses revealed that the S218A and R243A mutants displayed wild type rates of reactivity with FP-biotin (data not shown), indicating that these residues do not participate in the activation of the nucleophile of FAAH. The loss of fluorophosphonate reactivity exhibited by the S241A and K142A mutants agrees with data from previous studies and supports proposed roles for these residues as the nucleophile and catalytic base/acid of FAAH, respectively (13, 14). In contrast, the high catalytic activity and fluorophosphonate reactivity of the D237N mutant (Fig. 2*B, lower panel*) argue against a previously suggested role for this residue as a catalytic base involved in nucleophile activation (27).

Esterase Activities of FAAH Mutants—FAAH is unusual among serine hydrolases in that the enzyme degrades structurally similar amides and esters at equivalent rates (14). The

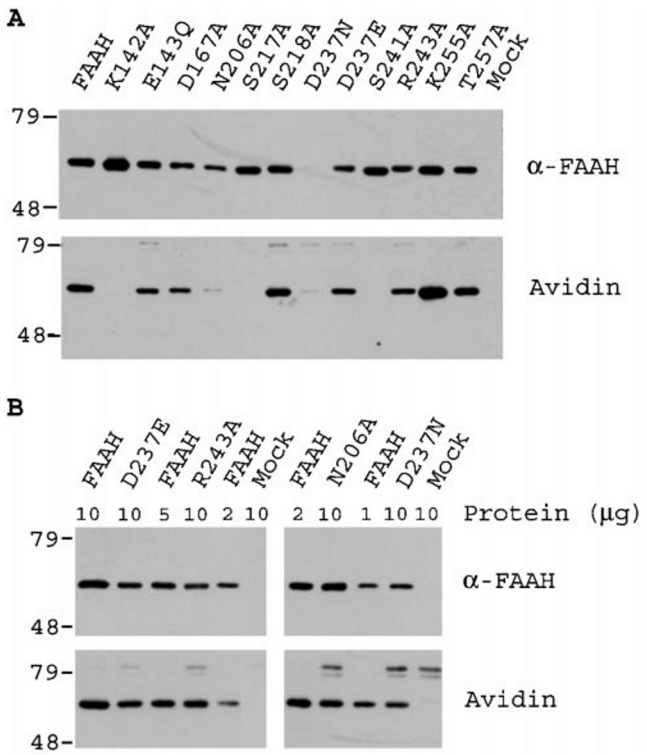

FIG. 2. **Expression of FAAH mutants in COS-7 cells.** The indicated FAAH mutants were expressed in COS-7 cells, and membrane extracts were isolated as described under "Experimental Procedures." All protein samples were treated with 2 μM FP-biotin for 30 min prior to quenching with 2× SDS loading buffer. *A,* 10 μg of each protein extract were loaded on a reducing SDS-PAGE gel and protein was detected by blotting with either anti-FAAH antibodies (*upper panel*) or avidin-horseradish peroxidase (*lower panel*). In general the relative amount of labeling with FP-biotin as judged by the avidin-horseradish peroxidase signal was consistent with the amount of FAAH immunoreactivity. However, the K142A, S217A, and S241A mutants exhibited no detectable labeling with FP-biotin despite wild type levels of FAAH immunoreactivity. *B,* the FAAH and avidin signals from varying amounts of FAAH protein extract (1–10 μg) were compared with those from 10 μg of protein extract of the D237E, R243A, N206A, and D237N mutants. Please note that the *left* and *right panels* represent different exposure times. The amount of FAAH immunoreactivity observed for 10 μg of the D237E and R243A mutant protein extracts (*right panel*) was similar to that for 5 μg of FAAH extract, indicating a 2-fold decrease in expression for these two mutants. The N206A and D237N mutants exhibited approximately 5- and 10-fold lower expression levels than FAAH, respectively. For the N206A, D237E, D237N, and R243A mutants, the relative amount of FAAH immunoreactivity correlated well with the amount of FP-biotin signal as judged by avidin detection, suggesting that these proteins label with FP-biotin to near wild type levels.

equivalent k_{cat} and k_{cat}/K_m values for the amide and ester substrates of FAAH are not due to a common rate-limiting deacylation step but rather to similar acylation rates for these substrates (14). The *E. coli*-derived K142A mutant was previously found to lose this atypical behavior, reacting with esters more than 500-fold faster than amides (14). Consistent with this finding, the esterase activity of the K142A mutant from COS-7 cells was compromised only 30-fold (at pH 9.0) despite a complete loss of detectable amidase activity. The hydrolysis rates for OME were determined for the S217A, S218A, S241A, and R243A mutants in order to evaluate whether any of these mutations also altered the unusual amide/ester reactivity of FAAH (Table II). The S217A and S241A mutants exhibited negligible esterase activity, whereas the S218A mutant displayed similarly compromised esterase and amidase activities relative to those of FAAH. Although both the amidase and esterase activities of the S217A mutant were below the background activity in COS-7 cells, we observed similar amidase and esterase activities for this mutant when purified from *E. coli* (data not shown). The amidase and esterase activities of the S241A mutant were below detection for both COS-7- and *E. coli*-derived forms of the protein.

TABLE I
Oleamide hydrolysis rates of FAAH mutants expressed in COS-7 cells

Enzyme	*V*	Relative activity
	nmol/min/mg	%
FAAH	200 ± 20	100
K142A	0[a]	<0.1
E143Q	120 ± 15	60
D167A	95 ± 10	48
N206A	21 ± 5	11
S217A	0[a]	<0.1
S218A	2.0 ± 0.4	1.0
D237E	23 ± 2	12
D237N	4.5 ± 0.5	2.3
S241A	0[a]	<0.1
R243A	0.73 ± 0.07	0.3
K255A	34 ± 6	17
T257A	130 ± 30	65

[a] $V \leq 0.2 \pm 0.03$ nmol/min/mg, the activity of extracts from cells transfected with empty vector.

TABLE II
Oleoyl methyl ester hydrolysis rates of FAAH mutants expressed in COS-7 cells

Enzyme	*V*	Relative activity
	nmol/min/mg	%
FAAH	180 ± 19	100
K142A	5	3
S217A	0[a]	<0.3
S218A	6 ± 1	3
S241A	0[a]	<0.3
R243A	43 ± 6	24
K142A/R243A	0[a]	<0.3

[a] $V \leq 0.6 \pm 0.1$ nmol/min/mg, the activity of extracts from cells transfected with empty vector.

Interestingly, the R243A mutant hydrolyzed OME at only a 4-fold slower rate than FAAH (Table II), contrasting sharply with its ~300-fold reduced amidase activity (Table I). Considering the approximately 2-fold reduced expression of this mutant relative to FAAH, its esterase activity approached wild type levels at pH 9.0. The R243A mutant also exhibited 1) a K_m value for OME, 16 ± 4 μM, similar to that of FAAH (34 ± 8 μM) and 2) wild type rates of reactivity with FP-biotin (see above). These data indicate that the R243A mutant is a properly folded protein with an active site structure that is largely intact.

Given that both the R243A and K142A mutants displayed altered amide/ester selectivities, we considered the possibility that these residues might depend on one another for proper function. For example, Arg-243 could reduce the pK_a of Lys-142, allowing this lysine residue to serve as a general acid/base catalyst at physiological pH. In order to test such a postulate, a mutant was constructed in which both Lys-142 and Arg-243 were mutated to alanine (K142A/R243A). The K142A/R243A mutant possessed significantly lower esterase activity than the less active of the two single mutants, K142A, suggesting that the effect of the R243A mutant on amide/ester selectivity was not occurring through modification of the properties of Lys-142.

pH Rate Profiles of FAAH Mutants—We have previously found that for oleamide, the k_{cat} and k_{cat}/K_m values of FAAH depend on an ionizable base with an apparent pK_a of 7.9, whereas K_m is pH-independent (13). However, the pH rate profile of FAAH is unusual in that the slope of k_{cat} *versus* pH is <1 even well below the apparent pK_a determined in the fit.

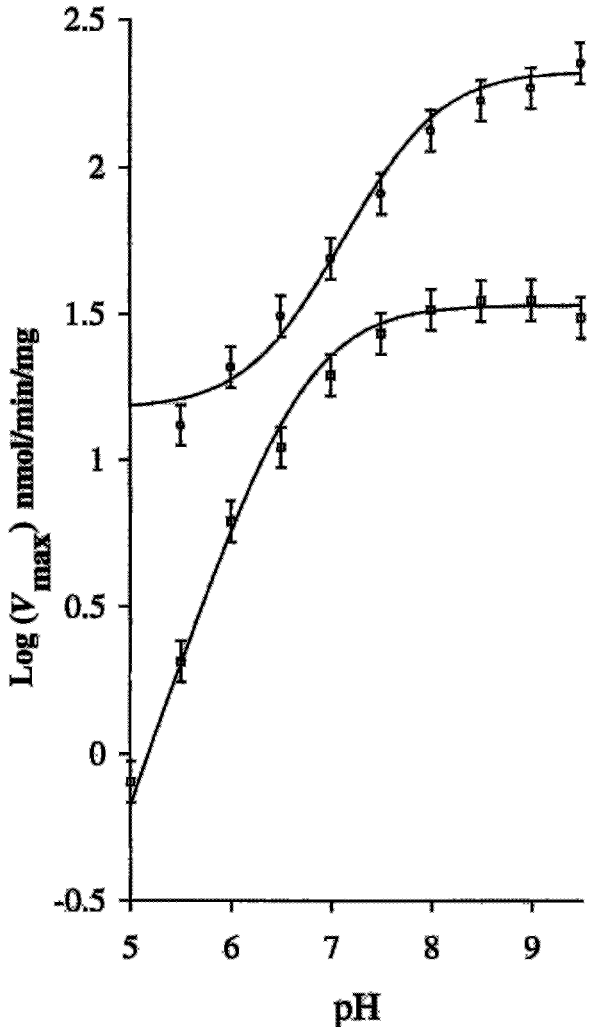

FIG. 3. **pH *versus* V_{max} profiles for OME hydrolysis by FAAH and the R243A mutant from transfected COS-7 cells.** The pH dependences of V_{max} for OME hydrolysis by FAAH (*open circles*) and the R243A (*open squares*) mutant are shown with the curve fits obtained by non-linear least squares regression. The curve fit for FAAH indicates a catalytic base with a pK_a of 7.9 and assumes a lower activity limit at low pH that is 15-fold lower than the maximal activity. The curve fit for the R243A mutant indicates a catalytic base with a pK_a of 6.8 with no lower activity limit.

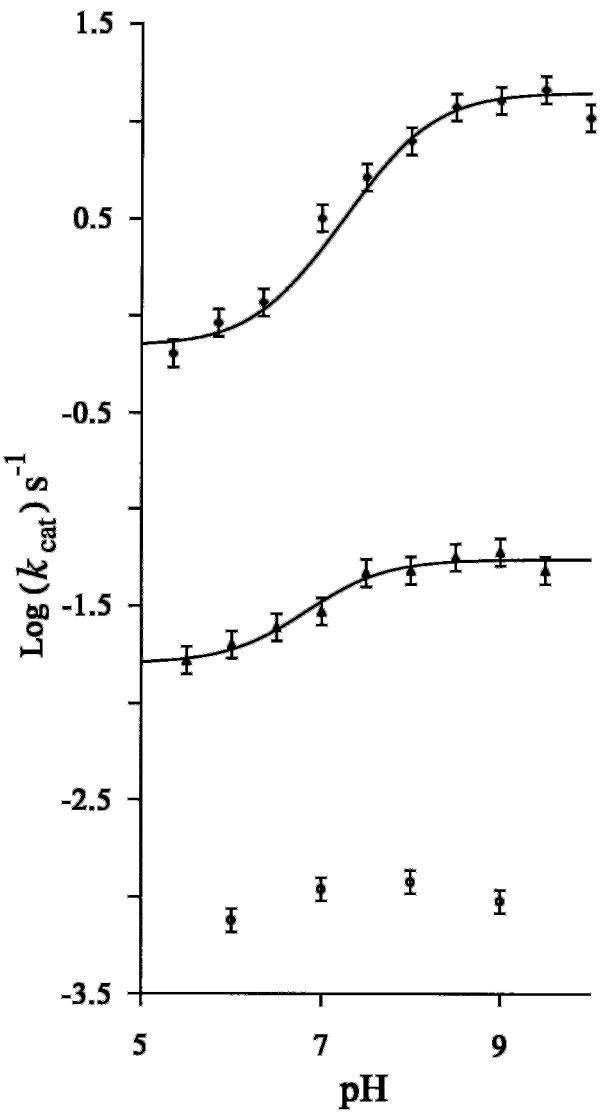

FIG. 4. **pH *versus* k_{cat} profiles for oleamide hydrolysis by FAAH, the S217A mutant, and the S218A mutant.** The pH dependences of k_{cat} for oleamide hydrolysis by FAAH (*solid diamonds*), the S218A mutant (*solid triangles*), and the S217A mutant (*open circles*) are shown. Curve fits were obtained by non-linear least squares regression and indicate a basic residue involved in catalysis with a pH of 7.9 (FAAH) or 7.1 (S218A). The lower activity limits for FAAH and the S218A mutants were 20- and 3.5-fold below the maximal activity, respectively. The S217A mutant exhibited less than a 30% change in activity between pH 6.0 and 9.0, and no ionizable residue pK_a values could be predicted from the profile.

Therefore, to obtain the indicated pK_a value, a curve fit was employed that assumed a non-zero activity for the enzyme with its catalytic base in the protonated state. The value of this lower activity limit was 15–20-fold below the maximal activity exhibited by FAAH. Even with this parameter, the curve fit was not ideal, and due to the instability of FAAH below pH 5, it has not been possible to directly confirm the existence of a lower activity limit.

We have previously shown that the K142A mutant exhibits a log-linear dependence of k_{cat} on pH with a slope of 0.9, consistent with a function for Lys-142 as a general base catalyst (14). The activity of the S241A mutant was not detectable with either OME or oleamide and therefore could not be analyzed for pH dependence. The pH rate profile of the R243A mutant was determined for the enzyme from solubilized COS-7 microsomes using saturating concentrations of OME (*open squares*, Fig. 3). The pH rate profile of FAAH with OME was also determined (*open circles*, Fig. 3) and did not differ significantly from the pH *versus* k_{cat} profile determined for the purified enzyme with oleamide (*solid diamonds*, Fig. 4). In contrast to the unusual pH rate profile of FAAH, the R243A mutant exhibited an ideal single ionizable residue pH rate profile dependent on an apparent catalytic base with a pK_a of 6.8. Notably, due to the differences in their respective pH rate profile shapes, the activity of the R243A mutant was essentially identical to that of FAAH at pH 7.0 (taking into account the approximately 2-fold lower expression of the mutant).

In order to determine the pH rate profiles of the S217A and S218A mutants, whose activities in COS-7 extracts were extremely low or below detection, these enzymes were purified from *E. coli* as described previously (13). Both the S217A (*open circles*, Fig. 4) and S218A (*solid triangles,* Fig. 4) mutants exhibited much weaker pH dependences than FAAH. The pH rate profile of the S218A mutant was qualitatively similar to FAAH, showing dependence on an apparent base with a pK_a of 7.1. However, this mutant exhibited only a 3.5-fold reduction in activity from pH 8.0 to 5.5, compared with the 20-fold reduction

in activity observed for FAAH over this same pH range. The S217A mutant exhibited an essentially flat pH rate profile with less than a 30% change in k_{cat} over the pH range 6.0–9.0. Thus the pH rate profiles of the mutants, K142A, R243A, S217A, and S218A, were all readily distinguishable from one another and deviated significantly from the pH rate profile of wild type FAAH.

Deuterium Isotope Effects—The lack of pH dependence exhibited by the S217A mutant suggested that the rate-limiting step for oleamide hydrolysis by this enzyme may not involve a proton transfer or depend on an ionizable residue. Enzymatic reactions that exhibit no pH dependence sometimes operate by a mechanism in which a physical step is rate-limiting, such as a conformational change in enzyme structure. Further evidence that a reaction proceeds with a non-chemical rate-limiting step can be obtained by determining the solvent deuterium isotope effect for the reaction. The absence of a solvent deuterium isotope effect indicates that there is no proton transfer occurring in the transition state of the reaction and is consistent with a non-chemical rate-limiting step. One example of an enzyme that exhibits such behavior is prolyl oligopeptidase, a serine hydrolase that shows no solvent deuterium isotope effect and, like FAAH, hydrolyzes amides and esters at similar rates (35).

The solvent deuterium isotope effects on the k_{cat} values for oleamide hydrolysis were determined to be 2.9 and 1.8 for FAAH and the S217A mutant, respectively. The K_m values of FAAH for oleamide in D_2O and H_2O were equivalent, suggesting that the decreased activity of the enzyme in D_2O was not due to a loss of substrate binding or structural integrity. Additionally, the k^{H_2O}/k^{D_2O} values were similar for FAAH-catalyzed oleamide and OME hydrolysis. These solvent deuterium isotope effects indicate that a proton transfer is likely occurring in the rate-limiting step(s) of the reactions catalyzed by both FAAH and the S217A mutant.

DISCUSSION

Prior to this investigation, a comprehensive analysis of the function of conserved residues in the AS sequence had not been conducted. Although several reports have described the mutagenesis of a select number of conserved AS residues in either FAAH (13, 14, 36, 37) or the *Rhodococcal* J1 amidase (27), these efforts have produced contradictory proposals regarding the nature of the core catalytic components of the AS family. In the present investigation, all of the conserved AS residues capable of either acid/base chemistry and/or hydrogen bonding were mutated in FAAH, and the resulting mutants were analyzed in a single expression system. The results of this study clarify the respective catalytic importance of conserved AS residues and provide strong support for the classification of AS enzymes as a family of serine-lysine catalytic dyad hydrolases.

Only five of the conserved residues in the AS sequence, Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243, were critical for the amidase activity of FAAH. The reduced expression levels of folded protein for other FAAH mutants when produced in *E. coli*, as well as the poor expression of the D237A, D237N, and N206A mutants in COS-7 cells, indicate that several of the conserved residues of the AS sequence are likely structurally important rather than catalytically important. These results, when coupled with the observation that a large number of conserved glycine and proline residues are found in the AS sequence, suggest that this region encodes both a catalytic and structural domain. It is interesting to note that with the exception of Arg-243, the residues most critical for catalysis (Lys-142, Ser-217, Ser-218, and Ser-241) were also the only residues that could be mutated to yield proteins that expressed well in *E. coli*. Thus, there appears to be strikingly little overlap between the catalytic and structural components of the AS sequence.

In all cases, FAAH mutants with high catalytic activity showed fluorophosphonate reactivities comparable with that of the wild type enzyme. Interestingly, however, the converse was not necessarily observed, as the S218A and R243A mutants exhibited greatly reduced amidase activity but wild type fluorophosphonate reactivity. These data indicate that although efficient catalysis was strongly dependent on a functional Ser-241 nucleophile, an effective nucleophile did not in turn guarantee high amidase activity. Although the precise catalytic functions played by Ser-218 and Arg-243 are currently unclear, these residues would be predicted to impact the activity of FAAH through a mechanism independent of the nucleophilicity of Ser-241. Potential roles in facilitating leaving group protonation and/or transition state stabilization could satisfy such a criterion.

Our results do not support a previous proposal that Asp-237 (or its analogous residue in the *Rhodococcal* JI amidase) serves as a catalytic base for the activation of the serine nucleophile of the AS family (27). Mutation of Asp-237 to asparagine or glutamate produced enzymes with only modest decreases in catalytic activity (2–4-fold) and reactivities with FP-biotin comparable to that of FAAH. These properties are inconsistent with an important catalytic function for this residue. Considering further the poor expression of the D237N and D237A mutants, a structural role for this residue seems more likely.

Given the greatly reduced fluorophosphonate reactivity displayed by the S217A mutant, it is tempting to speculate that this residue may be involved in nucleophile activation. However, it is important to stress that the reactivity of serine hydrolases with fluorophosphonate inhibitors not only depends on nucleophile strength but also, in some cases, on transition state binding residues (*e.g.* oxyanion hole residues) (33, 34). Although such functions can potentially be distinguished by separately determining the binding and reactivity components of the second order rate constant for fluorophosphonate reactivity ($k_{obs}/[I]$) (33, 34), this type of analysis has not been possible in our system due to the low solubility and weak binding of FP-biotin to FAAH. Regardless, the S217A mutant does react with ethoxy oleyl fluorophosphonate at serine 241 (13), clearly demonstrating that Ser-217 is not the nucleophile of FAAH. Additionally, we have previously shown that the S217A mutant purified from *E. coli* possesses a 2300-fold lower k_{cat} than FAAH and an unaltered K_m value for oleamide (13). Collectively, such kinetic data, when coupled with the total conservation of Ser-217 among AS enzymes, support a central role for this residue in the catalytic mechanism for the AS family, possibly as a participant in nucleophile activation or as an oxyanion hole constituent.

Based on the kinetic properties, substrate selectivities, and chemical reactivities of the S241A and K142A mutants expressed in *E. coli*, we previously proposed that FAAH utilizes Ser-241 as a catalytic nucleophile and Lys-142 as a catalytic base for nucleophile activation (13, 14). The results presented here fortify these conclusions, providing compelling support that Lys-142 acts as the catalytic base for FAAH in a role analogous to histidine in the catalytic triad of traditional serine hydrolases (38, 39). Most importantly, Lys-142 is the only conserved ionizable residue among AS enzymes that can be mutated to yield an enzyme with extreme reductions in both hydrolase activity and fluorophosphonate reactivity. The absence of any other ionizable residue that affects nucleophile strength suggests that the Ser-Lys dyad of FAAH is not modified by an additional ionizable residue analogous to the aspartic acid of the catalytic triad (32, 38).

Why FAAH displays such an unusually flat pH rate profile is currently unclear. One other serine hydrolase, carboxypeptidase Y, has been found to exhibit a weak pH dependence of activity that plateaus at acidic pH (40). Interestingly, like FAAH, carboxypeptidase Y is capable of reacting with amide and ester substrates at similar rates, provided they contain a C-terminal carboxyl moiety (40, 41). The modest pH dependence of carboxypeptidase Y has been proposed to reflect a mechanism in which the proton from the serine nucleophile of the enzyme is transferred to the leaving group of the substrate concomitant with nucleophile attack (40). In this mechanism, the protonated histidine could support activity by giving a proton to the leaving group while simultaneously removing a proton from the attacking nucleophile. We have postulated a similar mechanism for FAAH to explain the equivalent acylation rates of its amide and ester substrates (14). This mechanism was based on experimental evidence that the similarity of the amide and ester hydrolysis rates of FAAH depends on the presence of a residue that can both receive a proton from the serine nucleophile of the enzyme and donate a proton to the leaving group of the substrate in a concerted or coupled manner (14).

The catalytic mechanisms described above for carboxypeptidase Y and FAAH are essentially equivalent but were initially proposed to explain two different catalytic properties: the weak pH dependence of carboxypeptidase Y and the similar amide/ester acylation rates of FAAH. It is therefore possible that a catalytic mechanism in which nucleophile attack and leaving group protonation are strongly coupled is responsible for both of the unusual properties shared by these two enzymes (*i.e.* flattened pH rate profiles and comparable amide/ester hydrolytic efficiencies).

The pH rate profiles of the FAAH mutants investigated in this study follow an interesting trend that supports the aforementioned mechanism. For example, the two mutants that exhibit comparable amide/ester hydrolysis rates, S217A and S218A, also show exaggeratedly flat pH rate profiles, whereas the R243A mutant, which hydrolyzes esters much faster than amides, shows a steep pH dependence in the acidic range. The pH dependence previously reported for the K142A mutant, a FAAH variant that also hydrolyzes esters much faster than amides, is also very steep throughout its catalytically active range (14). Thus the ability of FAAH to react with amides and esters at comparable rates seems tightly correlated with a modest pH dependence on catalysis, particularly at acidic pH.

The presence of a highly conserved residue, Arg-243, in the AS sequence that is necessary for amidase activity but dispensable for esterase activity suggests that the AS family represents a pure class of amidases rather than a collection of general hydrolytic enzymes (including both amidases and esterases). To date (with the notable exception discussed below), the biological substrates of all known AS enzymes are amides (1, 4–7, 11). This feature distinguishes the AS family from the superfamily of Ser-His-Asp catalytic triad hydrolases, which contains both amidases and esterases (26). In general, esterases utilizing the catalytic triad are very poor amidases (42, 43), whereas the amidases (proteases) are exceptional esterases, hydrolyzing esters much faster than even their biological amide substrates (32, 44, 45). Whether the low esterase activity exhibited by FAAH is the result of selective pressure on the enzyme to reduce esterolytic activity or is an inherent outcome of the special catalytic mechanism used by the AS family remains to be determined. On this note, the ability to normalize the efficiency of amide and ester hydrolysis may be important for the function of FAAH *in vivo*. Presently, at least two FAAH substrates, the amide anandamide (9, 46) and the ester 2-arachidonoylglycerol (47, 48), are thought to be involved in endogenous cannabinoid signaling. The similar reactivity of FAAH with these substrates may serve to coordinate the levels of these signaling molecules *in vivo* and/or prevent inefficient amide hydrolysis due to competition from inherently more reactive ester substrates (14). Interestingly, one product of the current investigation is a FAAH mutant, R243A, that is an efficient fatty acid esterase but a severely defective fatty acid amidase. The introduction of this mutant into cell or organismal systems lacking FAAH may provide a means to distinguish the relative importance of the amidase and esterase activities of this enzyme *in vivo*.

Serine hydrolases are one of the most widely distributed and thoroughly studied enzyme families. The vast majority of serine hydrolases thus far identified, including most serine proteases, lipases, and esterases, contain the classical Ser-His-Asp catalytic triad. More recently, a select set of distinct serine hydrolases have been characterized including the Ser-Lys dyad proteases of eubacteria (49–53) and a Ser-Asp-containing lipase, Ca^{2+}-dependent phospholipase A_2 (54). However, these alternative serine hydrolase types are limited to a small subset of enzymes or organisms. In contrast, the AS enzymes represent a large family of non-classical serine hydrolases whose evolutionary distribution rivals that of the catalytic triad superfamily.

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