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Fatty Acid Amide Hydrolase Substrate Specificity

Dale L. Boger,[a,c,*] Robert A. Fecik,[a,c] Jean E. Patterson,[a,c] Hiroshi Miyauchi,[a,c] Matthew P. Patricelli[b,c] and Benjamin F. Cravatt[b,c]

[a]*Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[b]*Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[c]*The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

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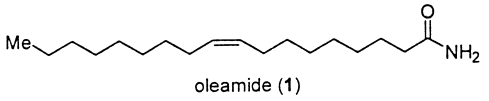
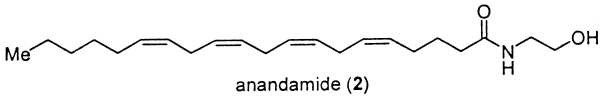
Abstract—Fatty acid amide hydrolase (FAAH), also referred to as oleamide hydrolase and anandamide amidohydrolase, is a serine hydrolase responsible for the degradation of endogenous oleamide and anandamide, fatty acid amides that function as chemical messengers. FAAH hydrolyzes a range of fatty acid amides, and the present study examines the relative rates of hydrolysis of a variety of natural and unnatural fatty acid primary amide substrates using pure recombinant rat FAAH.

Oleamide[1] (**1**) and anandamide[2] are the archetypal members of a growing class of fatty acid amides that function as chemical messengers. Oleamide induces physiological sleep in animals[3] and analgesic and cannabinoid-like behavioral responses in mice albeit without binding to cannabinoid receptors.[4,5] It has been found to modulate serotonin receptors,[4,6–9] benzodiazepine-sensitive $GABA_A$ receptors,[10,11] and antagonize glial gap junction cell communication.[12,13] Anandamide, an endogenous ligand for the cannabinoid receptors (CB1),[2] also activates the vanilloid (capsaicin) receptor[14,15] (VR1) and it has been implicated as an endogenous analgesic.[16] The regulation of oleamide and anandamide is therefore of important therapeutic potential in the management of sleep disorders and pain.



oleamide (**1**)



anandamide (**2**)

Endogenous oleamide and anandamide are hydrolyzed and inactivated by fatty acid amide hydrolase (FAAH),[17,18] a serine hydrolase distributed in the CNS.[19] This suggests that FAAH is important in the regulation of the activity of fatty acid amides at their sites of action. The enzyme has been shown to hydrolyze fatty acid amides and esters at near equivalent rates,[20] to accept a range of variations in the amine of the amide,[21] but no systematic study of its fatty acid acyl chain dependence has been disclosed.[17,18,22] In efforts to define additional potential endogenous substrates for FAAH, including those presently unrecognized, and to establish substrate structural features that contribute to FAAH hydrolysis that would be useful in inhibitor or agonist design, herein we establish the substrate specificity of the purified recombinant rat enzyme towards a variety of natural and unnatural fatty acid primary amide substrates. In the present study we determined the relative rates of hydrolysis of 29 primary amide substrates containing modifications in the acyl chain.

The rat and human enzymes are 82% homologous, share the identical conserved signature amidase consensus sequence and Src homology 3 (SH3) binding domain, are single copy genes presumably serving identical purposes in rat and man, and exhibit identical relative and absolute inhibition by an extensive series of inhibitors.[18,23] Therefore, it is reasonable to expect the human enzyme to exhibit an analogous, if not identical, substrate specificity. Early and more limited studies comparing the selectivities bear out this expectation but were conducted enlisting crude preparations of microsomal rat FAAH[17] and extracts of COS cell-transfected human FAAH[18] that makes it unlikely, but does not preclude, the participation of other enzymes in the

*Corresponding author. Tel.: +1-858-784-7522; fax: +1-858-784-7550; e-mail: boger@scripps.edu

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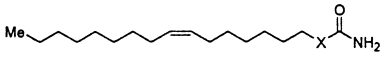
measured hydrolysis reactions. In order to insure that the comparisons made herein reflect only hydrolysis by FAAH, the studies were conducted with solubilized, pure recombinant rat FAAH free of other enzymatic activities.

The rate of hydrolysis for each compound relative to oleamide is listed in Table 1. To measure the relative rates of hydrolysis, an ethanolic solution of each compound (7.5 mM, 10 μL) was added to a solution of FAAH (8 μL, 0.51 ng/μL in 20 mM Hepes (pH 7.8), 150 mM NaCl, 10% glycerol, 1% Triton X-100)[20] in a pH 9 buffer (Tris/EDTA, 382 μL) at 25 °C. Such a high concentration (187 μM) of substrate is well above the K_m of oleamide (5–10). Given the structural similarities in the substrates examined, this concentration might be expected, but was not confirmed in each case, to ensure V_{max} conditions allowing direct comparison of substrate rates of hydrolysis under steady-state conditions. A 50 μL aliquot of the reaction mixture was taken at appropriate time points, and quenched with 1 M HCl (600 μL). This was extracted with EtOAc (3×600 μL), concentrated under a N_2 stream, and the residue dissolved in EtOAc (25 μL). A sample of this solution (1 μL) was analyzed by GC versus an internal standard.

Table 1. Relative rates of rat FAAH hydrolysis for **1**–**32**

Compound	Relative rate of hydrolysis[a]
Oleamide (18:1(Δ^9), **1**)	1.00
Anandamide (**2**)	1.68
Dodecanoamide (12:0, **3**)	0.74
Myristoamide (14:0, **4**)	0.83
Palmitoamide (16:0, **5**)	0.72
Stearamide (18:0, **6**)	0.69
Myristoleamide (14:1(Δ^9), **7**)	0.86
Palmitoleamide (16:1(Δ^9), **8**)	0.79
6*Z*-Octadecenamide (18:1(Δ^6), **9**)	0.91
7*Z*-Octadecenamide (18:1(Δ^7), **10**)	1.09
9*E*-Octadecenamide (18:1 (Δ^{9E}), **11**)	0.52
12*Z*-Octadecenamide (18:1(Δ^{12}), **12**)	0.92
13*Z*-Octadecenamide (18:1(Δ^{13}), **13**)	0.82
15*Z*-Octadecenamide (18:1(Δ^{15}), **14**)	0.90
5*Z*-Eicosenamide (20:1(Δ^5), **15**)	1.16
8*Z*-Eicosenamide (20:1(Δ^8), **16**)	1.12
11*Z*-Eicosenamide (20:1(Δ^{11}), **17**)	1.05
13*Z*-Eicosenamide (20:1(Δ^{13}), **18**)	1.03
Erucamide (22:1(Δ^{13}), **19**)	0.83
Nervonamide (24:1(Δ^{15}), **20**)	0.82
Linoleamide (18:2($\Delta^{9,12}$), **21**)	1.04
Linoelaidamide (18:2($\Delta^{9E,12E}$), **22**)	0.54
11,14-Eicosadienamide (20:2($\Delta^{11,14}$), **23**)	1.27
α-Linolenamide (18:3($\Delta^{9,12,15}$), **24**)	1.38
8,11,14-Eicosatrienamide (20:3($\Delta^{8,11,14}$), **25**)	1.38
11,14,17-Eicosatrienamide (20:3($\Delta^{11,14,17}$), **26**)	1.40
Arachidonamide (20:4($\Delta^{5,8,11,14}$), **27**)	3.11
4,7,10,13,16,19-Docosohexenamide, **28**)	0.82
	
X = $CH(CH_3)$, 2-methyloleamide (**29**)	0.07 (0.09)[b]
X = $CH(CH_3)_2$, 2,2-dimethyloleamide (**30**)	0.03 (0.01)[b]
X = O (**31**)	0.1[b]
X = NH (**32**)	< 0.001[b]

[a]Rate = 2.148 nmol/min/4.08 ng FAAH for oleamide under the defined reaction conditions (0.526 nmol/min/ng FAAH). Other initial rates (≤20% substrate hydrolysis) are reported relative to this rate of hydrolysis for oleamide: standard deviation ± 5–9%, average ± 7%.
[b]Rat microsomal FAAH, data taken from ref 8.

Initial rates of hydrolysis were established by plotting the amount of substrate consumed (nmol) versus time (min) and enlisted time points constituting ≤20% substrate hydrolysis.

A Hewlett-Packard 3396 Series II integrator and Hewlett-Packard 5890 Series II GC fitted with a 15 m×0.53 mm×0.5 μm NUKOL™ capillary column (Supelco) was used for GC analysis with the aid of a FID detector. The instrument settings used were: temperature, 200 °C (isothermal); initial time, 1.00 min; injection port, 250 °C; detector, 275 °C; carrier gas, He; flow rate, 22.8 mL/min. Linoleic acid or eicosanoic acid was used as an internal standard.

In examining the relative hydrolysis rates of compounds **1**–**32** (Table 1), it is apparent that FAAH accommodates a wide variety of fatty acid primary amide substrates. There is little variation in hydrolysis rates between fatty acid amides of similar chain lengths and degrees of unsaturation, but the rate of hydrolysis increases with greater degrees of unsaturation. In addition, unsaturation closer to the amide seems to increase the rate of hydrolysis (e.g. **15**–**18**) while that at the more distal sites appears to have little or no impact. Notably, arachidonamide (**27**) is hydrolyzed 3 times faster than oleamide. Consistent with past observations made with crude enzyme preparations, arachidonamide (**27**) is hydrolyzed 2 times faster than anandamide (**2**) confirming that primary amides are better FAAH substrates than the corresponding ethanolamides.[21] In addition and consistent with preceding studies, oleamide is hydrolyzed slower than anandamide (ca. 1.7×). However, the distinctions are small and do not alter the conclusions that both are superb substrates or that arachidonyl substrates are hydrolyzed faster than the corresponding oleoyl substrates (**1** versus **27**, ca. 3×). Removal of the unsaturation (**6** versus **1**, 0.7×), or even more significant, incorporation of a *trans* versus *cis* double bond substantially slows the rate of hydrolysis and this latter effect was greater than its removal (cf. **1** versus **6** versus **11**).[17,18] Even more significant, alkyl substitution at the α-position of the amide or incorporation of a chain heteroatom effectively renders the substrate resistant to hydrolysis (see compounds **29**–**32**).[8] These results are in agreement with the general trends observed with rat microsomal crude preparations of FAAH,[17,18,21,22] and those deduced from inhibitor studies where C1–C12 and π-unsaturation at the oleamide $\Delta^{9,10}$ or arachidonamide $\Delta^{8,9}/\Delta^{11,12}$ positions enhance inhibitor potency and presumably contribute to substrate binding.[23–25]

Discussion

Although no recent systematic search for endogenous fatty acid primary amides has been conducted, palmitoamide (16:0, **5**), palmitoleamide (16:1, **8**), oleamide (18:1, **1**), and linoleamide (18:2, **21**) have been detected in human plasma[26] with oleamide being the most abundant. Erucamide (22:1, **19**) has been identified as an endogenous angiogenic factor stimulating new blood

vessel formation.[27] The results detailed herein illustrate that all would be candidate substrates for FAAH, regulating the endogenous concentration of this entire class of potential signaling molecules.

The results of the studies detailed herein can be summarized as follows:

1. Fatty acid primary amides are among the best class of substrates for FAAH and are hydrolyzed faster than the corresponding ethanolamides (**27** versus **2**, ca. 2×).
2. Essentially all unsaturated fatty acid primary amides containing *cis* double bonds are hydrolyzed effectively by FAAH suggesting a general mechanism for regulation and deactivation of an entire class of signaling molecules.
3. Long chain saturated fatty acid amides are hydrolyzed slower than the corresponding *Z* unsaturated fatty acid amides and the rate of hydrolysis increases incrementally with increases in the degree of unsaturation.
4. Introduction of *Z* unsaturation at or near the oleamide $\Delta^{9,10}$ or arachidonyl $\Delta^{8,9}/\Delta^{11,12}$ positions enhances the rate of hydrolysis more effectively than at more distal sites. This is consistent with proposals that the substrates adopt a bent,[24] but not hairpin,[21] bound conformation with only C1–C12 contributing to substrate binding.[23,24]
5. Introduction of *E* versus *Z* unsaturation diminishes the rate of hydrolysis (**11** versus **1**, **22** versus **21**) resulting in a two-fold reduction in the rate of hydrolysis under our assay conditions and this effect is larger than that resulting from simply removing the double bond (**1** versus **6** versus **11**).
6. Introduction of one or two methyl groups at the C2 position provides more stable analogues attributable to increased steric hindrance around the carbonyl group.[8] The addition of each methyl group increases stability roughly 10-fold with **30** being nearly 100-fold more stable than oleamide.
7. Introduction of a heteroatom α to the carbonyl substantially increases stability ($NH > O > CH_2$)[8] attributable to electronic stabilization of the reacting carbonyl. The primary urethane **32** was at least 1000× more stable than oleamide showing no evidence of FAAH hydrolysis.

The present study demonstrates that pure recombinant rat FAAH is an enzyme of broad substrate specificity and is capable of hydrolyzing a wide array of unsaturated, and to a lesser extent saturated, fatty acid primary amides. However, when substituted adjacent to the amide carbonyl, the substrates can be made sterically or electronically resistant to hydrolysis. In addition to defining the structural features favorable for fatty acid amide hydrolysis by FAAH and identifying additional potential endogenous substrates, this information should also be of use in the design of oleamide and anandamide analogues resistant to enzymatic degradation. When the results of this survey of the fatty acid acyl chain are combined with those that have examined variations in the amide amine[21] including esters,[28,29] they define a large class of potential endogenous molecules as candidate substrates subject to FAAH regulation.

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