Molecular Identification of a Functional Homologue of the Mammalian Fatty Acid Amide Hydrolase in Arabidopsis thaliana*

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N-Acylethanolamines (NAEs) are endogenous constituents of plant and animal tissues, and in vertebrates their hydrolysis terminates their participation as lipid mediators in the endocannabinoid signaling system. The membrane-bound enzyme responsible for NAE hydrolysis in mammals has been identified at the molecular level (designated fatty acid amide hydrolase, FAAH), and although an analogous enzyme activity was identified in microsomes of cotton seedlings, no molecular information is available for this enzyme in plants. Here we report the identification, the heterologous expression (in Escherichia coli), and the biochemical characterization of an Arabidopsis thaliana FAAH homologue. Candidate Arabidopsis DNA sequences containing a characteristic amide signature sequence (PS00571) were identified in plant genome data bases, and a cDNA was isolated by reverse transcriptase-PCR using Arabidopsis genome sequences to develop appropriate oligonucleotide primers. The cDNA was sequenced and predicted to encode a protein of 607 amino acids with 37% identity to rat FAAH within the amide signature domain (18% over the entire length). Residues determined to be important for FAAH catalysis were conserved between the Arabidopsis and rat protein sequences. In addition, a single transmembrane domain near the N terminus was predicted in the Arabidopsis protein sequence, similar to that of the rat FAAH protein. The putative plant FAAH cDNA was expressed as an epitope/His-tagged fusion protein in E. coli and solubilized from cell lysates in the nonionic detergent, dodecyl maltoside. Affinity-purified recombinant protein was indeed active in hydrolyzing a variety of naturally occurring N-acylphosphatidylethanolamines, a minor membrane lipid constituent of cellular membranes (1). In animal systems, anandamide (NAE 20:4) acts as an endogenous ligand for cannabinoid receptors and has varied physiological roles, such as the modulation of neurotransmission in the central nervous system (2). Anandamide also activates vanilloid receptors, functions as an endogenous analgesic (3), and appears to be involved in neuroprotection (4, 5). In other tissues, NAEs have been implicated in immunomodulation (6), synchronization of embryonic development (7), and induction of apoptosis (8). These endogenous bioactive molecules lose their signaling activity upon hydrolysis by fatty acid amide hydrolase (FAAH; see Ref. 9).

In plants NAEs are present in substantial amounts in desiccated seeds (–1 μg g−1 fresh weight), and their levels decline after a few hours of imbibition (10). Individual NAEs were identified predominantly as 12C, 16C, and 18C species with N-linoleoylthanolamine (NAE 18:2) generally being the most abundant. Like in animal cells, NAEs are derived from N-acylphosphatidylethanolamines (11, 12) by the action of a phospholipase D (13, 14). The occurrence of NAEs in seeds and their rapid depletion during seed imbibition to barely detectable levels in seedlings (12) suggests that these lipids may have a role in the regulation of seed germination and normal seedling development. In fact, recent experiments with Arabidopsis thaliana seedlings showed that when seeds were germinated and maintained on elevated levels (micromolar concentrations) of the naturally occurring NAE 12:0, seedling roots developed abnormally in a manner consistent with a disruption of both normal cell division and cellular expansion (15), signifying the importance of regulating NAE levels for normal plant growth and development.

The concept of NAEs acting as lipid mediators in plant systems is supported by an increasing amount of experimental evidence in vegetative tissues where normal levels of NAEs are quite low (low nanomolar concentrations). For example, NAE 14:0 appears to function as an endogenous modulator of pathogen elicitor signaling by plant cells (12, 16). NAE 14:0 levels, quantified by gas-liquid chromatography-mass spectrometry, increased 10–50-fold in leaves of tobacco plants that were treated for a few minutes with either xylanase or cryptogeen elicitor proteins (17). These “elicitor-activated” levels of NAE 14:0 (submicromolar concentrations) were sufficient to induce defense gene expression (e.g. phenylalanine ammonia-lyase and PAL2 transcript abundance) in tobacco plants within hours of treatment in a manner similar to but independent of

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AJ308736.

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§ The abbreviations used are: NAE, N-acyethanolamine; AS, amide signature; BioTrux, 2-[bis(2-hydroxyethyl)aminol]-2-hydroxymethylpropane-1,3-diol; CHAPS, 3-[(3-cholamidopropyl)dimethylammoniomol-
elicitors (17). Mammalian cannabinoid receptor antagonists at concentrations equimolar to NAE blocked the activation of defense gene expression induced by either NAEs or by fungal elicitor proteins (16). A membrane-associated protein was identified in leaves of tobacco (and other plant species), which specifically bound to [3H]NAE 14:0 with high affinity (K_d in the low nanomolar range). This NAE 14:0-binding protein was proposed to mediate the NAE activation of PAL2 expression in tobacco leaves, a conclusion that was based on the similarities between NAE binding properties in vitro and NAE-induced physiological responses in vivo, including experiments with cannabinoid receptor antagonists (16). Although some clear differences are evident between the emerging NAE signaling pathway in plants and the better characterized endocannabinoid signal pathway of animals, there appear to be some remarkable similarities in the formation and perception of bioactive acyl ethanolamides by these two diverse groups of multicellular organisms. Likewise, we propose that an FAAH-like mechanism operates in plants for NAE signal termination and overall regulation of NAE levels under various physiological conditions.

Recently, depletion of NAEs during seed imbibition/germination was determined to occur via two metabolic pathways: one lipoxygenase-mediated for the formation of NAE oxylipins from NAE 18:2, and one amidase-mediated for hydrolysis of saturated and unsaturated NAEs (18). Hydrolysis of NAEs was reconstituted and characterized in microsomes of cottonseeds and appeared to be catalyzed by an enzyme similar to the FAAH of mammalian species (18). Here we report the identification of a plant orthologue of mammalian FAAH by bioinformatic approaches, isolation of its cDNA sequence, expression of this cDNA in Escherichia coli, and identification of the protein product as an NAE amidohydrolase. These results support our previous studies on the metabolism of NAEs in plant tissues and for the first time provide molecular evidence for a conserved pathway in both plants and animals for the hydrolysis of NAEs. Moreover, the results of this research now provide a means to manipulate the levels of endogenous NAEs in plants to evaluate the physiological role(s) of these bioactive lipids.

**EXPERIMENTAL PROCEDURES**

**Materials—**[1-14C]Arachidonic acid was purchased from PerkinElmer Life Sciences, and [1-14C]lauric acid was from American Biosciences. [1-14C]Myristic acid, arachidonic acid, lauric acid, linoleic acid, myristic acid, anandamide, ethanolamine, phenylmethylsulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma. [1-14C]Linoleic, [1-14C]palmitic acids, and [1,2-14C]ethanolamine were purchased from PerkinElmer Life Sciences. Ceramide was from Avanti Polar Lipids (Alabaster, AL), and 2-arachidonyl glycerol was from Cayman Chemical (Ann Arbor, MI). Methyl arachidonyl fluorophosphonate (MAFP) was from Tocris Cookson, Inc. (Ellisville, MO), n-dodecyl β-D-maltoside (DDM) was from Calbiochem, and Silica Gel G (60 Å) coated glass plates for thin layer chromatography (20 × 20 cm, 0.25 mm thickness) were from Whatman. Specific types of N-[1-14C]acyl ethanolamines (and nonradiolabeled NAEs) were synthesized from ethanolamine and the respective 1-14C-labeled fatty acids (and non-radiolabeled FFAs) by first producing the fatty acid chloride (19) and purified by TLC as described elsewhere (18).

**Bioinformatics and cDNA Isolation—**BLAST searches (blast, wustl.edu) in various data bases were done using the amide signature (AS) consensus block embedded in rat FAAH (blacks.fhcrc.org). DNA sequences containing a characteristic AS sequence (PS00571) were identified in the A. thaliana genome data base annotated by the Institute for Genomic Research (TIGR) and available at www.tigr.org, and one candidate Arabidopsis FAAH orthologue, AT5g64440, was selected for further analyses. This selection was based largely on results from sequence analysis tools and data base comparisons including BLAST and cDART (www.ncbi.nlm.nih.gov), ProDom (20), Prosite (21), TM-HMM transmembrane and topology predictor (22, 23), and pSORT (24).

Sequence alignments and some sequence analyses were made with DNAsis software (Hitachi).

Sequence-specific primers were designed within the 5′- and 3′-UTR regions of At5g64440 based on predicted exon sequences and were used for reverse transcriptase–RT-PCR (forward, 5′-CATTCAATTC-CCAACAGTCTACCCGG-3′ and reverse, 5′-GTCGAGTGAAGAATTTC-CAACACGG-3′). The template for RT-PCR was total RNA extracted from the leaves of mature Arabidopsis plants using Trizol reagent (Invitrogen). Fresh leaf tissue (100 mg) was harvested, ground to a fine powder in liquid nitrogen, and combined with 2 ml of Trizol reagent for DNA isolation according to the manufacturer’s instructions. For RT-PCR, the first strand cDNA synthesis from total RNA was carried out at 50 °C for 30 min and incubated for 4 min at 94 °C before the targeted amplification of the At5g64440 mRNA by Platinum Taq (RT-PCR mixture; Invitrogen) was achieved through 25 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min followed by a final polymerization step at 72 °C for 7 min. The RT-PCR product was gel-purified and ligated into pTrcHis for nucleotide sequencing. DNA cloning of both strands (complete 2 × each strand) verified the identity of the cDNA as the At5g64440 gene product, and the complete cDNA sequence was deposited in GenBankTM.

**Protein Expression—**For protein expression, oligonucleotide primers (forward, 5′-ATGCGTAGATCAGCCGTAAGATG-3′ and reverse, 5′-GTTGGTATCCATCATATGCGATACG-3′) were designed to amplify only the open reading frame of the above At5g64440 cDNA, and PCR conditions were as above, except that a 10- to 1 ratio of polymers (Taq-to-Pfu; Invitrogen) was used for amplification, and the template was the RT-PCR product (GenBankTM AM308736) in pTrcHis. The open reading frame-PCR product was gel-purified as above and cloned into expression vector pTrcHis and protein expression constructs were introduced into chromosomally competent E. coli TOP10 cells as host. Transformed colonies were selected with correct in-frame fusion and cDNA sequence by sequencing of plasmid DNA over the vector insert junctions and by sequencing the inserts completely on both strands.

Selected transformed cell lines were grown in LB medium without glucose to an A_600 of 0.6 to 0.7 and induced with 1 mM IPTG for 4 h. Pelleted cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.2 mM DDM) at a ratio of 1-10^5 (E. coli cells-to-DDM molecules; assuming 0.1 A_600 = 10^5 cells/ml, see Ref. 25). After incubation on ice for 30 min, resuspended cells were sonicated on ice with six 10-s bursts at high intensity with a 10-s cooling ice bath) period between each burst. The selection of DDM as the detergent, and determination of optimal DDM concentration and content ratio was based on empirical comparisons for recovery of solubilized active enzyme with the highest specific activity. By the same criteria, DDM was determined to be better for solubilizing active enzyme than either Triton X-100 or CHAPS.

**Solubilization and N2O Affinity Purification—**Traditionally, cultured cells (50 ml) were pelleted, resuspended in 8 ml of native binding buffer (50 mM NaPO4 buffer, pH 8.0, and 0.5 M NaCl) with 8 mg of lysozyme (Sigma) and 0.2 mM DDM (final), incubated on ice for 30 min, and disrupted by sonication as above. The crude lysate was centrifuged at 105,000 × g for 1 h in a Sorvall Discovery 90 model ultracentrifuge (Beckman Ti45 rotor). The supernatant was combined with ProBond resin, precharged with N2O, and gently agitated for 60 min to keep the resin suspended in the lysate supernatant. The resin with adsorbed protein was settled, and the supernatant was aspirated off. The resin was washed 4 times to remove nonspecific proteins, and the adsorbed proteins were eluted with imidazole-containing buffer. Eluted proteins were concentrated, and imidazole was removed with 50 ml Tris-HCl, pH 8.0, 100 mM NaCl, and 0.2 mM DDM by dialysis using Centricron YM-30 (Millipore, Bedford, MA) devices. Affinity-purified proteins were stored at −80 °C in 10% glycerol and were stable for more than 2 months.

**Gel Electrophoresis and Western Blotting—**Protein samples were diluted in 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, and separated on 8% acrylamide/amine/SDS gels (Bio-Rad) at 35 mA for 30 min and 60 mA for 60 min. For Western blot analysis, separated proteins were electrophoretically transferred to polyvinylidene fluoride (0.2 μm, Bio-Rad) membrane in a Semidry Trans-Blot apparatus (Bio-Rad) for 30 min at constant 14 V. Recombinant proteins expressed as c-Myc-epitope fusions were localized with a 1:50000 dilution of 1-Myc antibodies (Abcam, Cambridge, MA) monoclonal, (Invitrogen), and detected by chemiluminescence (Bio-Rad substrate solutions) following incubation with 1-2500 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad).

**NAE Amidohydrolase Assays—**NAE substrates were synthesized
and purified, and enzyme assays were conducted as described previously (18) with a few modifications. Generally the enzyme source was incubated with 100 μM [14C]NAE (20,000 dpm) in 50 mM BisTris buffer, pH 9.0, for 90 min to survey for NAE amidohydrolase activity (18). Enzyme activity was examined for time, temperature, protein, and substrate concentration dependence. For enzyme characterization, reactions were initiated with 1 μM of affinity-purified protein and incubated at 30°C with shaking for 30 min. Assays of lysates of E. coli cells expressing rat FAAH (WT-FAAH; Ref. 26) served as a comparison of NAE amidohydrolase activity, whereas non-transformed cell lysates or cell lysates with the Arabidopsis cDNA cloned in reverse orientation with respect to the lacZ promoter served as negative controls for activity assays. Enzyme assays were terminated by the addition of boiling isopropanol alcohol (70°C), and lipids were extracted into chloroform. Lipid products were separated by TLC, and the distribution of radioactivity was evaluated by radiometric scanning (18). Activity was calculated based on the radiospecific activity of [14C]-labeled substrate. A general serine hydrolase inhibitor, phenylmethylsulfonyl fluoride (PMSF), and an irreversible active site-directed FAAH inhibitor, MAFP, were used to probe the sensitivity of recombinant Arabidopsis NAE amidohydrolase activity. Inhibitors were added from stock solutions dissolved in isopropanol alcohol for PMSF or Me2SO for MAFP, and activity was adjusted for minimal solvent effects where necessary based on assays in the presence of the appropriate amount of solvent alone. Protein content was determined by Coomassie Blue dye binding using bovine serum albumin as the protein standard (27).

RESULTS

Tentative Identification of Arabidopsis NAE Amidohydrolase—In animal tissues, fatty acid amide hydrolase (EC 3.5.1.4), a member of the amidease signature (AS) family (28, 29), hydrolyzes NAEs to produce FFA and ethanolamine (30). A similar enzymatic activity was characterized previously in cottonseed microsomes (18). Mammalian FAAH enzymes have a conserved stretch of ~130 amino acids (31) containing a Ser/ Ser/Lys catalytic triad (32). The predicted amidease structure has a central conserved motif of GGSS/G/A/S/G (33), and a somewhat longer stretch of amino acids G(GA/S)(G/G)/(GS/SA)/(GSVV)/(X)/(LIVM)/(GSA/V)/(I)(LIVM)/(GS)/G(X)(6)/(GSAT)(X)/(GA)(X/D)E/(X)/(GA)(XS)/(LIVM)/(X)/U/(GSAC) is present in all enzymes of the amidease class (PS00457). Two serine residues at 217 and 241, highly conserved in the AS sequence, were found essential for enzymatic activity of recombinant rat FAAH (34). Mutation of either one of the residues into alanine caused complete loss of activity of the enzyme (34, 35). Also, mutation of serine 218 into alanine caused marked loss of activity (35). Taking these conserved residues in the AS consensus sequence into consideration, several putative plant orthologues were identified computationally. BLAST searches2 in various data bases using the AS consensus block embedded in rat FAAH (blocks.there.org) identified one A. thaliana gene (At5g64440) that was selected for further characterization (Fig. 1).

The structure and organization of the At5g64440 gene is relatively complex with 21 exons including 5'-UTR (untranslated region) and 3'-UTR (Fig. 1A). The predicted gene is 4689 nucleotides in length and encodes a predicted protein of 607 amino acids with a molecular mass of 66.1 kDa. Based on the presence of the conserved residues characteristic of the canonical AS sequence, this gene seemed likely to encode an Arabidopsis NAE amidohydrolase. To assess if this gene was expressed and to isolate a full-length cDNA for functional studies, oligonucleotide primers were designed within the 5'- and 3'-UTR, and a cDNA fragment was amplified by RT-PCR from Arabidopsis leaf RNA (Fig. 1B). The RT-PCR product was sequenced and found to be 99.9% identical with the corresponding TC139316 annotated at TIGR. The protein domain prediction tools, ProDom (20), identified six domain families in the Arabidopsis protein, five of which were also found in rat FAAH (Fig. 1C). A single putative transmembrane segment was identified near the N terminus (TMHMM, Refs. 22 and 23) similar to the predicted topological organization in rat FAAH.

Alignment of the deduced amino acid sequences from the Arabidopsis NAE amidohydrolase cDNA and the rat FAAH (28) showed only 18.5% identity over the entire length. Alignment within the AS sequence of 125 amino acids (Fig. 2A, underlined residues) showed 37% identity with five residues determined to be important for catalysis to be absolutely conserved (Ref. 31; Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243, denoted by arrows; Fig. 2A). Comparison of the 47-amino acid motif within the AS showed the Arabidopsis protein had close to 60% identity with FAAHs from several mammalian species (Fig. 2B). Organization of predicted secondary structure within this Arabidopsis and rat FAAH AS motif was similar (Fig. 2C), and the structure of the rat enzyme has been confirmed by x-ray crystallography (36). In addition, this putative A. thaliana NAE amidohydrolase and rat FAAH have similar predicted molecular weights (~66 kDa), similar predicted topologies (single transmembrane segment near the N terminus with the C terminus facing the cytosol, via TMHMM transmembrane and topology predictor; Refs. 22 and 23), and similar predicted subcellular locations (secretory pathway, pSORT; Ref. 24). Although the Arabidopsis NAE amidohydrolase shared several domains with glutamyl-tRNA amidotransferases (Fig. 1C), these amino acid-modifying enzymes are localized to chloroplasts in higher plants, and there is no indication of stromal targeting sequences at the N terminus of the At5g64440 gene product.

Functional Identification of Arabidopsis NAE Amidohydrolase—The Arabidopsis putative NAE amidohydrolase was cloned into pTrcHis and pTrcHis2 for expression in E. coli of N-terminal and C-terminal epitope- and polyhistidine-tagged fusion proteins, respectively. E. coli lysates were surveyed for expression of enzyme activity using [14C]NAE 18:2 (radiolabeled on the carbonyl carbon) as substrate. Representative chromatograms shown in Fig. 3 indicate that, like the recombinant rat FAAH (expressed in the same vector), the recombinant Arabidopsis protein effectively hydrolyzed [1-14C]NAE 18:2 to [1,14C]FFA 18:2. As a control, E. coli expressing the Arabidopsis cDNA in reverse orientation showed no hydrolytic activity (Fig. 3). In these preliminary experiments with crude E. coli lysates, the Arabidopsis NAE amidohydrolase activity was linear up to 60 min at protein amounts up to 50 μg. The enzyme was optimally active at alkaline pH (e.g. pH 8–9) and was inactivated by treatment at 100°C for 10 min. The Arabidopsis NAE amidohydrolase did not hydrolyze ceramide, nor did ceramide influence NAE hydrolysis (not shown). The Arabidopsis NAE amidohydrolase did not catalyze the reverse reaction of NAE hydrolysis (formation of NAE) under any conditions tested (not shown). Higher activity was reproducibly recovered in cells expressing C-terminal fusions, compared with cells expressing N-terminal fusions. Similar to reports for the rat protein (26), the recombinant Arabidopsis NAE amidohydrolase was mostly associated with E. coli membranes.

Affinity Purification of Recombinant Enzyme—The Arabidopsis NAE amidohydrolase, expressed as a C-terminal fusion protein, was solubilized in DDM and subjected to native Ni2+-affinity purification, SDS-PAGE, Western blot analyses, and enzyme activity assays (Fig. 4). A protein of ~70 kDa was enriched under native conditions by Ni2+-affinity purification and was detected by the c-Myc antibody (Fig. 4, A and B, arrows, Rec. protein lanes). Likewise, NAE amidohydrolase activity was enriched in this native affinity-purified protein fraction (Fig. 4C) by ~375-fold, relative to the DDM-solubilized supernatant (Supt) fraction. More stringent denaturing condi-

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2 W. Gish, blast.wustl.edu.
Fig. 1. A, the structure and organization of the Arabidopsis NAE amidohydrolase genomic sequence (TIGR/TAIR ID At5g44440). This gene is 4689 bp in length, and the predicted protein is 607 amino acids in length with a molecular mass of 66.1 kDa and pI of 6.44. There are 21 exons including 5'-UTR and 3'-UTR (www.tigr.org). The boxes represent exons, and bars between exons represent introns. The light shaded boxes are UTRs. B, schematic structure of the CDNA corresponding to At5g44440. Sequence-specific RT PCR primers were designed based on the genomic sequence A. thaliana (54) annotated at TIGR. The arrows denote the position of primers in the 5'- and 3'-UTR. RT-PCR was performed with total RNA extracted from the Arabidopsis leaves, and the nucleotide sequence of the isolated cDNA was deposited in GenBank™ (AY308736) and was 99.9% identical to coding region of TC139316 (arabidopsis.org). C, schematic of domain organization of predicted Arabidopsis NAE amidohydrolase protein. Various domains identified in other proteins with ProDom (20) are depicted above the diagram of the polypeptide. These domains are also found in rat FAAH except the one denoted by an asterisk. Domains are organized to scale and domain family identifiers (ProDom) are provided. PD038838, from amino acid 271–407, is found in 167 other proteins including a glutamyl-tRNA amidotransferase subunit A from Methanococcus jannaschii (Swiss-Prot Q58560). PD001110, from amino acid (aa) 138–276, is found in 121 other proteins including a predicted glutamyl-tRNA amidotransferase subunit A from Aeropyrum pernix (Swiss-Prot Q8F780). PD002554, from amino acid 477–575, is found in 173 other proteins including an unknown, predicted amidase from Homo sapiens (Swiss-Prot Q5NV19). PD030412, from amino acid 197–253, is found in 64 other proteins including a predicted glutamyl-tRNA amidotransferase subunit A from Mesorhizobium loti (Swiss-Prot Q9BF76). PD578534, from amino acid 298–358, is found in 36 other proteins including a FAAH from Rattus norvegicus (Swiss-Prot P97612). PD001217, from amino acid 60–88, is found in 234 other proteins including a predicted dipeptide binding/transporter protein from Yersinia pestis (Swiss-Prot Q82A19). P500571 (PROSITE dictionary) denotes the amidase consensus sequence motif of G/GA/S/G(S/G)X(GS/GS/VY/X(LM)VM/GS/X6(GSAT) X(GAI/DE)X(GA/S/LVM)R(P/GSAC) present in all proteins of the amidase class (29, 55–59). A single predicted transmembrane-spanning region shaced near the N terminus (TMHM, Refs. 22 and 23) and the amidase signature sequence (Ref. 31; see Fig. 2A) used to conduct the original search for homologues are also diagrammed.

Gene expression led to purification of the recombinant protein to homogeneity (single 70-kDa band on gel), but also inactivated the enzyme irreversibly (not shown). Hence we proceeded with the biochemical characterization of the active enzyme preparation, affinity-purified to near-homogeneity.

**Biochemical Characterization**—Recombinant NAE amidohydrolase activity was evaluated by incubating affinity-purified NAE amidohydrolase with [1-^14^C]NAE 20:4, [1-^14^C]NAE 18:2, [1-^14^C]NAE 16:0, [1-^14^C]NAE 14:0, or [1-^14^C]NAE 12:0 and measuring the rate of conversion to their respective [1-^14^C]PFA products. NAE amidohydrolase exhibited saturation kinetics with respect to all NAE substrates tested including those identified in plant tissues and those not found in plant tissues. The enzyme exhibited typical Michaelis-Menten kinetics when initial velocity measurements were made at increasing substrate concentrations (Fig. 5), and parameters calculated from these plots are summarized in Table 1. The relative apparent $K_m$ values of the Arabidopsis enzyme varied by a factor of about 4
FIG. 3. Representative radiochromatograms of NAE amidohydrolase activity assays surveyed in *E. coli* harboring expression plasmids. Lysates from cells expressing recombinant rat FAAH and human FAAH A, full-length alignment of Arabidopsis NAE amidohydrolyase amino acid sequence (GenBank™ accession number AY280736) with rat FAAH (GenBank™ accession number U72497) (28). These proteins are members of the AS sequence-containing superfamily that includes amidase or amidohydrolase (EC 3.5) enzymes involved in the reduction of organic nitrogen compounds and ammonia production (31, 33). The AS region is underlined and consists of about 125 amino acids. There is 18.5% identity between the Arabidopsis protein and rat FAAH when compared over the entire length of the proteins, whereas there is 37% identity within the AS. Conserved residues essential for rat FAAH activity (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243) are indicated with arrows. B, alignment of more conserved AS sequence motif (30) for the enzymes that hydrolyze NAE; mouse (GenBank™ accession number U82535) (53), porcine (GenBank™ accession number AB007320) (60), rat (GenBank™ accession number U72497) (28), and human (GenBank™ accession number U82535) (53). Identical residues within this motif between the plant and animal FAAHs are highlighted in black boxes. Within this motif there is 55–60% identity between the Arabidopsis and mammalian FAAH enzymes. C, secondary structure prediction (PSIPRED), 61, 62) of the AS (C, coil; H, helix; E, strand) is depicted above the rat and Arabidopsis (A) AS sequence motifs. Residues with similar secondary structure are highlighted in black boxes, illustrating the high degree of similarity within the active site (or AS sequence in NAE amidohydrolase A). Structural analysis has been confirmed for rat FAAH by x-ray crystallography (36) and suggests a functional link between these AS and Arabidopsis motif sequences despite limited primary amino acid sequence identity.

FIG. 2. Comparative alignment of Arabidopsis NAE amidohydrolase amino acid sequence (GenBank™ accession number AY172070) with human FAAH. A, full-length alignment of Arabidopsis NAE amidohydrolase amino acid sequence (GenBank™ accession number AY280736) with rat FAAH (GenBank™ accession number U72497) (28). These proteins are members of the AS sequence-containing superfamily that includes amidase or amidohydrolase (EC 3.5) enzymes involved in the reduction of organic nitrogen compounds and ammonia production (31, 33). The AS region is underlined and consists of about 125 amino acids. There is 18.5% identity between the Arabidopsis protein and rat FAAH when compared over the entire length of the proteins, whereas there is 37% identity within the AS. Conserved residues essential for rat FAAH activity (Lys-142, Ser-217, Ser-218, Ser-241, and Arg-243) are indicated with arrows. B, alignment of more conserved AS sequence motif (30) for the enzymes that hydrolyze NAE; mouse (GenBank™ accession number U82535) (53), porcine (GenBank™ accession number AB007320) (60), rat (GenBank™ accession number U72497) (28), and human (GenBank™ accession number U82535) (53). Identical residues within this motif between the plant and animal FAAHs are highlighted in black boxes. Within this motif there is 55–60% identity between the Arabidopsis and mammalian FAAH enzymes. C, secondary structure prediction (PSIPRED), 61, 62) of the AS (C, coil; H, helix; E, strand) is depicted above the rat and Arabidopsis (A) AS sequence motifs. Residues with similar secondary structure are highlighted in black boxes, illustrating the high degree of similarity within the active site (or AS sequence in NAE amidohydrolase A). Structural analysis has been confirmed for rat FAAH by x-ray crystallography (36) and suggests a functional link between these AS and Arabidopsis motif sequences despite limited primary amino acid sequence identity.
noted for both enzymes in terms of the lower $k_{cat}/K_m$ for NAE 16:0 than for NAE 20:4, despite the much higher levels of endogenous NAE 16:0 content in both systems. These parameters together suggest that the Arabidopsis recombinant enzyme recognizes a wide range of NAE types, similar to the situation with mammalian FAAH, and highlights the caution of over-interpreting in vitro kinetics data because the best substrate in these studies for the Arabidopsis enzyme, NAE 16:0, has not been detected in plants (12).

Two different mechanism-based inhibitors of mammalian FAAH were tested for potency on the hydrolysis of [1-14C]NAE 18:2 by this novel plant NAE amidohydrolase (Table II). PMSF, a nonspecific irreversible serine hydrolase inhibitor that inhibits NAE hydrolysis by mammalian FAAH at low mM concentrations (38), was only modestly effective on the Arabidopsis enzyme (inhibited by 44% at 10 mM). However, MAFP, the irreversible active-site targeted inhibitor of rat FAAH (36), completely eliminated NAE hydrolysis by the Arabidopsis enzyme at 10 mM. Overall, our biochemical results strongly support the identification of At5g64440 as a functional homologue of the mammalian FAAH.

**DISCUSSION**

The results presented here predict and functionally confirm that the Arabidopsis gene AT5g64440 (Fig. 1) encodes a homologue of the mammalian FAAH. Although there was limited primary amino acid sequence identity over the length of the Arabidopsis protein compared with the rat protein (18%), there was substantially higher similarity within the amidase catalytic domain both at the primary (37–60% depending on the lengths compared) and secondary structural levels (Fig. 2). Expression of the Arabidopsis cDNA in *E. coli* indicated that the Arabidopsis protein product was capable of hydrolyzing a wide range of NAE substrates to free fatty acids (Figs. 3–5 and Table I), a feature also of the mammalian enzyme (30, 39). Kinetic parameters summarized in Table I indicated that the plant enzyme has similar affinities for NAE substrates as the FAAH from several mammalian species (28, 39–43). Moreover, the inhibition of the Arabidopsis NAE amidohydrolase by
TABLE I
Summary of apparent kinetic parameters of the affinity-purified recombinant A. thaliana NAE amidohydrolase
Parameters were estimated by fitting the data in Fig. 5 to the Michaelis-Menten equation (Prism software, version 3.0, GraphPad).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAE 20:4</td>
<td>$13.6 \times 10^6$</td>
<td>17.9</td>
<td>0.33</td>
<td>$2.4 \times 10^6$</td>
</tr>
<tr>
<td>NAE 18:2</td>
<td>$26.2 \times 10^6$</td>
<td>14.1</td>
<td>0.26</td>
<td>$9.9 \times 10^5$</td>
</tr>
<tr>
<td>NAE 14:0</td>
<td>$50.8 \times 10^6$</td>
<td>12.1</td>
<td>0.22</td>
<td>$4.3 \times 10^5$</td>
</tr>
<tr>
<td>NAE 12:0</td>
<td>$37.0 \times 10^6$</td>
<td>9.1</td>
<td>0.17</td>
<td>$4.6 \times 10^4$</td>
</tr>
<tr>
<td>NAE 10:0</td>
<td>$17.6 \times 10^6$</td>
<td>13.9</td>
<td>0.26</td>
<td>$1.5 \times 10^4$</td>
</tr>
</tbody>
</table>

TABLE II
The effects of two mechanism-based inhibitors of mammalian FAAH on the hydrolysis of $1^3$CNAE 18:2 by the affinity-purified recombinant Arabidopsis enzyme
Assays were conducted for 30 min at 30 °C in the absence or presence of increasing concentrations of phenylmethylsulfonyl fluoride (PMSF) or methylarachidonyl fluorophosphonate (MAFP). The amount of $[1^3$C]FFA 18:2 formed was quantified by radiometric scanning following TLC separation of reactions products. The data are means ± S.D. of three replicates and are representative of two experiments.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Specific activity</th>
<th>Relative inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol h$^{-1}$ mg$^{-1}$ protein</td>
<td>%</td>
</tr>
<tr>
<td>PMSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM</td>
<td>10.56 ± 0.29</td>
<td>0</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>11.34 ± 0.55</td>
<td>7</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>9.06 ± 1.86</td>
<td>14</td>
</tr>
<tr>
<td>1 mM</td>
<td>7.89 ± 0.37</td>
<td>25</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>6.72 ± 0.70</td>
<td>36</td>
</tr>
<tr>
<td>10 mM</td>
<td>5.96 ± 0.43</td>
<td>44</td>
</tr>
<tr>
<td>MAFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM</td>
<td>10.46 ± 0.32</td>
<td>0</td>
</tr>
<tr>
<td>0.1 nM</td>
<td>9.69 ± 0.89</td>
<td>7</td>
</tr>
<tr>
<td>1 nM</td>
<td>6.52 ± 0.56</td>
<td>46</td>
</tr>
<tr>
<td>10 nM</td>
<td>0.00 ± 0.00</td>
<td>100</td>
</tr>
</tbody>
</table>

MAFP (Table II), the active site-directed irreversible inhibitor of rat FAAH (36, 44), strongly suggests a conserved enzyme mechanism between the plant and animal NAE amidasomes supporting the predictions from sequence/domain comparisons.

We suggest the annotation of "glutamyl-tRNA amidotransferase similarity" now accompanying this At5g64440 gene be modified to include the new functional information herein regarding NAE hydrolysis. Most amidases, including the mammalian FAAHs, carry the glutamyl-tRNA amidotransferase similarity annotation due to the presence of sequence similarity within the amidase signature domain, often as the only identifiable domain within this family of proteins. Based on our functional studies, it is unlikely that this At5g64440 gene product functions as a glutamyl-tRNA amidotransferase, and this activity has not been attributed to membrane-bound mammalian FAAH enzymes. Additionally, in plants these glutamyl-tRNA amidotransferases are localized in the stroma of chloroplasts as soluble, oligomeric complexes of multiple subunits (45, 46). In fact, nuclear-encoded, chloroplast-localized orthologues of the glutamyl-tRNA amidotransferase subunits have been cloned from Arabidopsis and expressed/characterized by the Soll group (GenBank™ accession numbers, AF241841, AF240465, AF239836, and AF224745), and these proteins share less than 24% amino acid sequence identity with the At5g64440 NAE amidohydrolase. Thus there is a need to clarify the descriptive annotation within these amidase protein subfamilies.

The signal-mediated activation of NAE metabolism constitutes a major regulatory feature of the endocannabinoid signaling system in animal tissues through the rapid generation and timely degradation of bioactive acylethanolamines (4, 9, 28, 47, 48). Although a principal role for NAE 20:4 as an endogenous ligand for cannabinoid receptors has emerged as a paradigm for endocannabinoid signaling (2, 38), other types of NAEs as well as other fatty acid derivatives likely interact with this pathway directly or indirectly to modulate a variety of physiological functions in vertebrates (49–52). An increasingly detailed understanding of the degradation of these bioactive NAEs by FAAH has pointed to this metabolic step as a key regulator of NAE levels and hence NAE function in vivo (9, 29, 30). Recent major advances in the understanding of FAAH function in mammals at the structural level (36), mechanistic level (32), and the physiological level (9) have been made possible only through the cloning, expression, and manipulation of the cDNA/gene encoding FAAH (53). We anticipate the identification of this plant cDNA will facilitate a similar increased appreciation for this lipid pathway in plant physiology.

Research in the last decade has made it apparent that NAE metabolism occurs in plants by pathways analogous to those in vertebrates and invertebrates (12, 18), pointing to the possibility that these lipids may be part of an evolutionarily conserved mechanism for the regulation of physiology in multicellular organisms. Two physiological situations in plant systems have been identified in which the endogenous levels of NAEs are transiently modulated. The first is in the perception of fungal elicitors by plant cells, wherein the levels of endogenous NAE 14:0 were elevated 10–50-fold in leaves of tobacco plants following elicitation (17). In other work, NAEs (mostly C12, C16, and C18 types) were quantified in desiccated seeds of higher plants but were metabolized rapidly during the first few hours of seed imbibition/germination (10), in part by an amidohydrolase-mediated pathway (18), suggesting that the transient changes in NAE content may play a role in seed germination. In fact, Arabidopsis seedlings germinated and grown in the presence of exogenous NAE exhibited dramatically altered developmental organization of root tissues (15).

With evidence of conserved enzymatic machinery in plants for the formation and degradation of NAEs, and the potent biological effects caused by altered exogenous NAE levels, it is now important to begin to address NAE function in plants by forward and reverse genetics approaches. The identification of an Arabidopsis cDNA clone encoding a functional NAE amidohydrolase re-enforces the similarity between plants and animals in terms of NAE metabolism, but more importantly provides a tool for the future manipulation of NAE levels in plants as a means to understand the physiological role(s) of these bioactive lipids in the plant kingdom.

Acknowledgments—We thank Drs. John Ohlrogge and Fred Beisson, Michigan State University, for sharing their catalogue of Arabidopsis lipid genes in advance of its publication. Dr. Cecilia Hillard, Medical College of Wisconsin, with permission of Dr. Benjamin Cravatt, Scripps Research Institute, kindly provided the cloned rat WT-FAAH and ΔTM-FAAH in pTrcHis2 for our studies.

REFERENCES