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(54) **Title:** FATTY ACID AMIDE HYDROLASE ASSAY

(57) **Abstract:** The invention provides methods for determining the activity of an ammoniagenerating enzyme and methods for identifying a compound capable of modulating the activity of such an enzyme.

FATTY ACID AMIDE HYDROLASE ASSAY

FIELD OF THE INVENTION

The invention relates to methods for determining the activity of an ammonia-generating enzyme and to their applications in identifying compounds that modulate the enzyme activities.

BACKGROUND OF THE INVENTION

Fatty acid amide hydrolase (FAAH), which may also be referred to as oleamide hydrolase and anandamide amidohydrolase, is an integral membrane enzyme. FAAH degrades fatty acid primary amides and ethanolamides, which are known to serve as endogenous signaling lipids. These include the endogenous cannabinoid anandamide and the seep-inducing oleamide. (M. P. Patricelli, et al., (1998) *Biochemistry* 37, 15177-15187; M. Maccarrone, et al., (1998). *J. Biol. Chem.* 273, 32332-32339). Although FAAH hydrolyzes a range of fatty acid amides (FAAs), FAAH appears to work most effectively on arachidonyl and oleyl substrates (B. F. Cravatt, et al., (1996) *Nature* 384, 83-87; and D. K. Giang, et al., (1997) *Proc. Natl. Acad. Sci. USA* 94, 2238-2242). Inhibitors of FAAH have been demonstrated to reduce pain, inflammation, and anxiety in animal models.

A number of assays for measuring FAAH activity have been reported. The majority of these assays utilize radiolabeled-substrates and thin-layer chromatography, activated charcoal or mass spectrometry to quantitate FAAH activity. In addition, a spectrophotometric assay using *p*-nitroanilide as a substrate and a fluorescence displacement assay have been reported. However, all of these assays have limitations such as low throughput, low sensitivity, or a requirement for radioactive material. An assay capable of efficiently, rapidly and accurately measuring FAAH activity and identifying compounds that inhibit or stimulate FAAH and that is also capable of high throughput would be beneficial.

All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method for measuring the activity of an ammonia-generating enzyme. The method involves two coupled reactions. One of the reactions is catalyzed by the ammonia-generating enzyme that generates ammonia as a product. The other reaction is a reductive amination reaction catalyzed by glutamate dehydrogenase that utilizes ammonia as a substrate. To carry out the method, a reaction mixture is provided that comprises (1) the ammonia-generating enzyme, (2) an ammonia-generating substrate for the ammonia-generating enzyme, (3) glutamate dehydrogenase, (4) a reductive amination substrate other than ammonia for the glutamate dehydrogenase, and (5) a reduced form of co-enzyme of glutamate dehydrogenase. The reaction mixture is incubated under conditions that allow for reactions catalyzed by the ammonia-generating enzyme and glutamate dehydrogenase. The activity of the ammonia-generating enzyme is measured by measuring the rate of the reductive amination catalyzed by the glutamate dehydrogenase. In one particular embodiment, the rate of the reductive amination catalyzed by the glutamate dehydrogenase is measured by measuring the consumption of the reduced form of the co-enzyme.

In another aspect, the invention provides a method for identifying a compound capable of modulating the activity of an ammonia-generating enzyme. The method employs the method for measuring the activity of an ammonia-generating enzyme provided by the present invention. To carry out the method, a test compound is added to the reaction mixture and the activity of the ammonia-generating enzyme is measured. In one embodiment, a method for identifying a compound capable of modulating the activity of fatty acid amide hydrolase is provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation of the effects of altering FAAH concentration in the presence of a constant concentration of oleamide.

Figure 2 is a graphical representation demonstrating that the rate of the GDH coupled FAAH reaction follows Michaelis-Menten kinetics.

Figure 3 is a graphical representation of a GDH-coupled FAAH assay showing that Compound A inhibits FAAH activity in a dose-responsive manner.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them.

The term “ammonia-generating enzyme” refers to any enzyme that catalyzes a reaction that yields ammonia as a product. The enzyme may be from any source, in any form, and in any purity, may be naturally occurring or recombinant, and may be a full-length enzyme or an enzymatically active truncated form thereof.

The term “ammonia-generating substrate” refers to a substrate upon which an ammonia-generating enzyme can act to produce ammonia as a product.

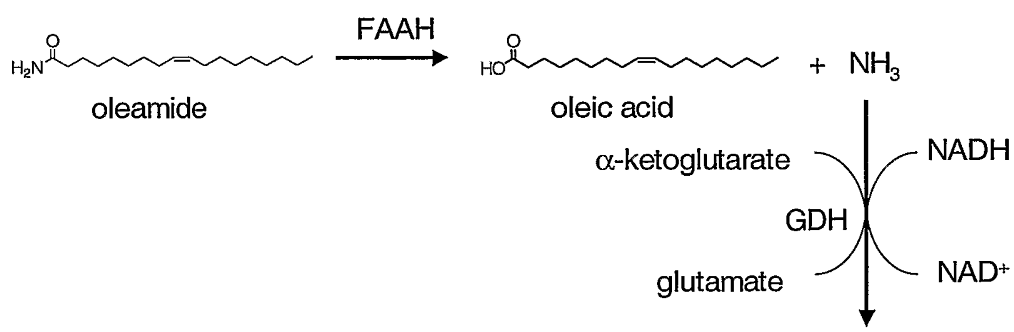
The term “glutamate dehydrogenase” refers to an enzyme that catalyzes the reductive amination of α-ketoglutarate to form glutamate. The enzyme may be from any source, in any form, and in any purity, may be naturally occurring or recombinant, and may be a full-length enzyme or an enzymatically active truncated form thereof.

The term “reductive amination substrate” of glutamate dehydrogenase refers to a substrate, other than ammonia, upon which glutamate dehydrogenase can act in effecting a reductive amination.

The term “modulate,” “modulating,” or “modulation” means to change the activity of an ammonia-generating enzyme in catalyzing the generation of ammonia. Such changes include inhibition of the enzyme or activation of the enzyme, and can be total or partial.

The term “compound” as used herein refers to any composition of matter, which can be any synthetic or natural compound or composition, and can be organic and inorganic, including small molecules, peptides, proteins, sugars (mono- and polysaccharides), nucleic acids, fatty acids, and the like. The term "test compound" refers to any compound or a combination thereof that is being analyzed.

In one aspect, the present invention provides a method for measuring the activity of an ammonia-generating enzyme by quantitatively detecting the ammonia generated by the ammonia-generating enzyme using a coupled reductive amination reaction catalyzed by glutamate dehydrogenase. The term “coupled” means that the reductive amination reaction catalyzed by glutamate dehydrogenase is carried out simultaneously and in the same reaction with the reaction catalyzed by the ammonia-generating enzyme. The method comprises: (a) providing a reaction mixture comprising a predetermined amount of (1) the ammonia-generating enzyme, (2) an ammonia-generating substrate for the ammonia-generating enzyme, (3) glutamate dehydrogenase, (4) a reductive amination substrate other than ammonia for glutamate dehydrogenase, and (5) a reduced form of co-enzyme for glutamate dehydrogenase; (b) incubating the reaction mixture under conditions that allow for reactions catalyzed by the ammonia-generating enzyme and glutamate dehydrogenase; and (c) measuring the rate of the reductive amination catalyzed by glutamate dehydrogenase, wherein the rate of the reductive amination catalyzed by glutamate dehydrogenase is indicative of the activity of the ammonia-generating enzyme. This method provided by the present invention may be referred to hereinafter as “enzyme activity assay method.” An example of the reaction scheme utilized in the enzyme activity assay method of present invention is depicted below, wherein the ammonia-generating enzyme is fatty acid amide hydrolase (FAAH) and oleamide is used as the substrate for fatty acid amide hydrolase:



This method may be used to measure the activity of any ammonia-generating enzyme by using an appropriate substrate that yields ammonia as a product. One example of ammonia-generating enzyme is FAAH. Another example of ammonia-generating enzyme is peptidylarginine deaminase, which catalyzes the conversion of the carboxy-terminal Arg residues of various peptides to citrulline residues with the generation of ammonia. The optimal amount of the ammonia-generating enzyme that may be used in the reaction mixture may vary depending on a number of factors, such as the source or specific activity of the enzyme, and incubation conditions, and can be readily determined by a person skilled in the art. This method is particularly useful for measuring the activity of FAAH. The FAAH may be from any source, may be naturally occurring or recombinant, and may be a full-length enzyme or truncated form thereof. The optimal amount of the FAAH used in the reaction mixture may vary depending on a number of factors, such as the source or specific activity of the enzyme, and the substrate used, and can be readily determined by a person skilled in the art. Generally, the concentrations of the FAAH in the reaction mixture are greater than about 2 nM, and preferably greater than 10 nM.

The appropriate ammonia-generating substrate for a particular ammonia-generating enzyme, as well as its optimal amount to be used in the reaction mixture, can be readily selected by a person skilled in the art. Suitable ammonia-generating substrates for FAAH are fatty acid primary amides. In general, primary amides as suitable ammonia-generating substrates of FAAH have the general structure: NH_2-C(O)-R, where R is an alkyl chain that is optionally unsaturated, and may additionally be linear or branched as well as substituted or unsubstituted, and could contain saturated or unsaturated rings, wherein these rings could be fused or unfused, and contain heteroatoms. Where present, the unsaturated bonds of the fatty acid alkyl chain may have a cis configuration, such as in cis-9,10-octadecenomaide, cis-8,9-octadecenoamide, cis-11,12-octadecendoamide or cis-13, 14-docosenoamide. Examples of fatty acid primary amides that may be used in the FAAH activity assay method of the invention include oleamide, amides of myrtistic acid (tetradecanoic acid), palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid), caproic acid (hexanoic acid), caprylic acid (octanoic acid), capric acid (decanoic acid), lauric acid (dodecanoic acid), linoleic acid (octadecadienoic acid), linolenic acid (octadecatrienoic acid), arachidic acid (eicosanoic acid), arachidonic acid (eicosatetraenoic acid), behenic acid (docosamoic acid), and lignoceric acid (tetracosanoic acid). Still other fatty acid primary amides, such as the primary amides of undecanoic acid, oleic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, and 1-dodecylazacycloheptan-2-onerespectively. Fatty acid primary amides are widely available commercially and may be obtained from any source. The optimal amount of the fatty acid primary amides in the reaction mixture may vary depending on a number of factors, such as the specific fatty acid primary amide used or its purity, and can be readily determined by a person skilled in the art. The amount of the fatty acid primary amides in the reaction mixture is generally greater than about 10 μM and typically from about 20 to about 500 μM. In one embodiment, the amount of the fatty acid primary amides in the reaction mixture is about 50 μM.

The methods of the invention utilize glutamate dehydrogenase (GDH) in the GDH-coupled assay. Glutamate dehydrogenase may also be known by other names, such as glutamic dehydrogenase, glutamic acid dehydrogenase, L-glutamate dehydrogenase, L-glutamic acid dehydrogenase, NAD(P)-glutamate dehydrogenase, NAD(P)H-dependent glutamate dehydrogenase, and the like. The reductive amination by GDH requires ammonia, α-ketoglutarate and a reduced form of nicotinamide-adenine dinucleotide (NADH or NADPH) as co-enzyme. The reductive amination reaction catalyzed by glutamate dehydrogenase is illustrated below:

$$NH_3 + \alpha\text{-ketoglutarate} + NADH + H^+ \rightleftharpoons \text{glutamate} + NAD^+ + H_2O$$

In this reductive amination reaction, the amount of the ammonia consumed is in direct proportion to the amount of NADH consumed, or in direct proportion to the amount of glutamate produced or NADP produced. GDH that may be used in the enzyme activity assay method may be from any source, may be naturally occurring or recombinant, and may be a full-length enzyme or truncated forms thereof that are enzymatically active. This enzyme is commercially available. (Cat. 49392; Sigma-Aldrich Chemicals, St. Louis, MO). The amount of GDH in the reaction mixture should be sufficiently high in order for allowing for rapid and complete reaction of the ammonia that is generated by the ammonia-generating enzyme into glutamate. The amount of GDH in the reaction mixture is generally not lower than about 1 unit/ml, and is typically about 7 unit/ml or higher.

Any suitable reductive amination substrate of GDH may be used in the enzyme activity assay method of the present invention. Examples of suitable reductive amination substrate of GDH include α-ketoglutarate (also known as "alpha-ketoglutarate," "2-oxoglutarate," "α-oxoglutarate," or "2-oxopentanedioate) and 2-keto-6-hydroxyhexanoic acid. The amount of the reductive amination substrate of GDH in the reaction mixture should be sufficiently high in order for allowing for rapid and complete reaction of the ammonia that is generated by the ammonia-generating enzyme into glutamate. The optimal amount may vary depending on a number of factors, such as the activity of the ammonia-generating enzyme, the amount of NADH (NADPH), and the amount or activity of GDH used, and can be readily determined by a person skilled in the art. Where α-ketoglutarate is used as the substrate, its amount in the reaction mixture is generally not lower than 0.1 mM, and is typically from about 0.3 to about 10 mM. In one embodiment, the concentration of α-ketoglutarate in the reaction mixture is about 3 mM.

The reduced form of nicotinamide adenine dinucleotide used in the enzyme activity assay method of the present invention may be either NADH or NADPH. The amount of NADH or NADPH in the reaction mixture should be sufficiently high in order for allowing for rapid and complete reaction of the ammonia that is generated by the ammonia-generating enzyme into glutamate. The optimal amount may vary depending on a number of factors, such as the activity of the ammonia-generating enzyme, the amount of α-ketoglutarate, and the amount or activity of GDH used, and can be readily determined by a person skilled in the art. The amount of NADH or NADPH in the reaction mixture is generally from about 5 μM to about 1000 μM, preferably from about 50 μM to about 300 μM, and more preferably from about 100 μM to about 200 μM.

Other components may be optionally included in the reaction mixture in order to enhance the method of the invention. For examples, adenosine 5'-diphosphate (ADP) or guanosin 5'-diphosphate (GDP) may be included in the reaction mixture to enhance the activity of GDH. The ADP or GDP in the reaction mixture may be in any amount, but is generally greater than 20 μM. In one embodiment, ADP is present in the reaction mixture at about 2 mM. In addition, a detergent or other solubilizing agent may also be included in the reaction mixture to increase the solubility of an enzyme, substrate, or any other components in the reaction mixture. Examples of suitable detergent or solubilizing agent include Triton® X-100 (Sigma-Aldrich Chemicals, St. Louis, MO) and dimethyl sulfoxide (DMSO).

The reaction mixture is incubated under conditions that allow for the reactions catalyzed by the ammonia-generating enzyme and GDH to take place. Typically, the reaction mixture is incubated at relatively constant temperatures, usually between 15 °C and 50 °C, and more typically at a temperature of between about 20 °C and about 37 °C. The reaction mixture is generally maintained at a relatively constant pH that is optimal for the reactions, usually between about 4.0 and about 12. In one embodiment, the reaction mixture is maintained at a pH of from about 7.4 to about 10.5. The pH of the reaction mixture can be adjusted and maintained using a suitable buffer. Examples of suitable buffer include phosphate buffer, a TRIS buffer (Sigma-Aldrich Chemicals, St. Louis, MO), and a HEPES buffer (Sigma-Aldrich Chemicals, St. Louis, MO). The reaction mixture may be incubated for any duration, from a few minutes or shorter to a few hours or longer. An optimal duration may be determined based on a number of factors, such as the activity of the ammonia-generating enzyme or GDH, the temperature of incubation, the initial amount of the substrates in the reaction mixture, and so on, and can be readily determined by a person skilled in the art.

The rate of the reductive amination catalyzed by GDH may be determined by any suitable method known in the art. For example, the rate of the reductive amination may be determined by measuring one or more of the following parameters: (1) the consumption of the reductive amination substrate, (2) the consumption of the reduced form of nicotinamide adenine dinucleotide (NADH or NADPH), (3) the generation of oxidized nicotinamide adenine dinucleotide (NAD^+ or $NADP^+$), and (4) the generation of a reductive amination product. One particular method for determining the rate of the reductive amination catalyzed by GDH is to spectrophotometrically measure the consumption of the reduced nicotinamide adenine dinucleotide (NADH or NADPH) in the reaction mixture. It is known that NADH and NADPH each absorbs light strongly at wavelengths between about 290 nm and about 380 nm, while NAD^+, $NADP^+$, and other substrates or products of the reactions do not. Thus, as the NADH or NADPH in the reaction mixture is consumed (i.e., oxidized to NAD^+, $NADP^+$), the light absorbance of the reaction mixture at the above wavelengths decreases. The rate of the consumption of NADH or NADPH is in directly proportional to the absorbance decrease. As the rate of consumption of NADH or NADPH is also directly proportional to the rate of ammonia generation and, hence, the activity of the ammonia-generating enzyme, the rate of absorbance decrease at the above wavelengths is indicative of the activity of ammonia-generating enzyme, wherein a faster rate of absorbance decrease indicates a higher activity of the ammonia-generating enzyme, and vise versa.

Typically, the light absorbance of the reaction mixture is measured at wavelengths between about 330 and about 370, and preferably at wavelengths of about 340 nm. The light absorbance can be measured readily by those skilled in the art using conventional spectrophotometric procedures. The measurements of the parameters of the reductive amination may be taken once at the end of the incubation period, at a plurality of time points during the incubation period, or continuously during the incubation period. The ammonia-generating enzyme activity can be quantitated according to methods known in art.

In another aspect, the present invention provides a method for identifying a compound capable of modulating the activity of an ammonia-generating enzyme, wherein the activity of the ammonia-generating enzyme is determined using the enzyme activity assay method described above. This method may be referred to hereinafter as "compound screening method" of the invention.

To identify a compound capable of modulating the activity of an ammonia-generating enzyme, a reaction mixture is incubated in the absence and presence of a test compound and the activity of the ammonia-generating enzyme is determined by enzyme activity assay method of the invention described above. Specifically, the method comprises: (a) providing a reaction mixture comprising a predetermined amount of (1) an ammonia-generating enzyme, (2) an ammonia-generating substrate for the ammonia-generating enzyme, (3) glutamate dehydrogenase, (4) a reductive amination substrate other than ammonia for glutamate dehydrogenase, and (5) a reduced form of co-enzyme for glutamate dehydrogenase; (b) incubating the reaction mixture in the absence and presence of a test compound and under conditions that allow for reactions catalyzed by the ammonia-generating enzyme and glutamate dehydrogenase; and (c) measuring the rate of the reductive amination catalyzed by glutamate dehydrogenase; wherein a difference in the rate of the reductive amination between the presence and absence of the test compound indicates that the test compound is capable of modulating the activity of the ammonia-generating enzyme. A test compound is identified as an inhibitor of the ammonia-generating enzyme if the rate of the reductive amination in the presence of the test compound is lower than that in the absence of the test compound. Conversely, a test compound is identified as an activator of ammonia-generating enzyme if the rate of the reductive amination in the presence of the test compound is higher than that in the absence of the test compound.

In one embodiment, there is provided a method for identifying a compound capable of modulating the activity of FAAH, which method comprises: (a) providing a reaction mixture comprising a predetermined amount of (1) FAAH, (2) a fatty acid primary amide as an ammonia-generating substrate for FAAH, (3) glutamate dehydrogenase, (4) α-ketoglutarate as a reductive amination substrate for glutamate dehydrogenase, (5) NADH or NADPH as co-enzyme for glutamate dehydrogenase; (b) incubating the reaction mixture in the absence and presence of a test compound under conditions that allow for reactions catalyzed by FAAH and glutamate dehydrogenase; and (c) measuring the rate of the reductive amination by measuring the consumption of the NADH or NADPH in the reaction mixture, wherein a consumption of the NADH or NADPH in the presence of the test compound that differs from that in the absence of the test compound indicates that test compound is capable of modulating the activity of FAAH. A test compound will be identified as an inhibitor of FAAH if the consumption of the NADH or NADPH in the presence of the test compound is lower than that in the absence of the test compound. Conversely, a test compound will be identified as an activator of FAAH if the consumption of the NADH or NADPH in the presence of the test compound is higher than that in the absence of the test. The FAAH may be from any source, may be naturally occurring or recombinant, and may be a full-length enzyme or enzymatically active, truncated form thereof. The optimal amount of the FAAH used in the reaction mixture may vary depending on a number of factors, such as the source or specific activity of the enzyme, and the substrate used, and can be readily determined by a person skilled in the art. Generally, the concentrations of the FAAH in the reaction mixture are greater than about 2 nM, and preferably greater than 10 nM. In one specific embodiment, oleamide is used as the fatty acid primary amide, NADH is used as the co-enzyme of GDH, and the consumption of NADH is determined by measuring the light absorbance of the reaction mixture at wavelengths of 340 nm.

The compound screening method of the present invention is useful for rapid screening of compounds capable of modulating the activity of an ammonia–generating enzyme, such as FAAH, using automated procedures. Such automated methods can be readily performed by using commercially available automated instrumentation and software and known automated observation and detection procedures. Multi-well formats are particularly attractive for high throughput and automated compound screening. Screening methods can be performed, for example, using a standard microplate well format. A microplate reader includes any device that is able to read a signal from a microplate (*e.g.*, 96 and 384-well plates). Such a signal may be detected spectrophotometrically, such as, for instance, reading the optical density of the NADH or NADPH absorbance at a wavelength of 340 nm. However, other detecting means may also be utilized, such as fluorometry (standard or time-resolved), luminometry, or photometry in either endpoint or kinetic assays. Using such techniques, a wide variety of compounds can be rapidly and efficiently screened for their respective effects on an ammonia-generating enzyme, such as FAAH. Sample handling and detection procedures can be automated using commercially available instrumentation and software systems for rapid, reproducible application of reagents, and automated screening of target compounds. To increase the throughput of a compound administration, currently available robotic systems such as the BioRobot 9600 from Quagen (Quagen, Inc. Valencia, CA), the Zymate from Zymark (Hopkinton, Mass) or the Biomek from Beckman Instruments (Fullerton, CA), most of which use the multi-well plate format, could be utilized.

EXAMPLES

The following examples relate to assays for measuring the activity of FAAH and their utility for identifying compounds capable of modulating the activity of FAAH. These examples are for illustrative purposes only and are not offered to limit the claimed invention. Various modifications or changes in light of these examples will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the claims.

EXAMPLE 1. Preparation of a Truncated Human FAAH

A truncated human FAAH (hFAAH) was prepared by the method described in this Example, and was used in the studies described in Examples 2-4 below. The amino acid sequence of this truncated human FAAH is shown in SEQ ID NO: 1, which comprises amino acids 32-579 of the full length human FAAH. The *E. coli* codon optimized DNA sequence encoding amino acids 32-579 of the full human FAAH is shown in SEQ ID No: 2.

A. Cloning and Construction of hFAAH Plasmids

Known molecular biological techniques are utilized to carry out the cloning and construction of constructs described herein. Such techniques are referred to, for example, in Davis et al., Basic Methods in Molecular Biology, Elsevir Sciences Publishing, Inc., New York, NY,1986; Hames et al., Nucleic Acid Hybridization, IL Press, 1985; Molecular Cloning, Sambrook et al., Current Protocols in Molecular Biology, Eds. Ausubel et al., John Wiley and Sons; Current Protocols in Human Genetics, Eds. Dracopoli et al., John Wiley and Sons; Current Protocols in Protein Science, Eds. John E. Coligan et al., John Wiley and Sons; and Current Protocols in Immunology, Eds. John E. Coligan et al., John Wiley and Sons).

pTrcHis A-hFAAH (encoding human (h) FAAH amino acids 30-579)

The following cDNAs were custom-synthesized and subcloned into a pUC119 vector (Blue Heron Biotechnology (Bothell, WA)). The *E. coli* codon optimization was carried out by using an optimization algorithm (Blue Heron Biotechnology (Bothell, WA)).

- pUC119-hFAAH (encoding hFAAH, amino acids 1-579): A cDNA containing the *E. coli* codon optimized DNA sequence of hFAAH (encoding amino acids 1-579) with a 5'-Xho I site, 3' stop codon , and 3'-EcoR I site. (SEQ ID NO: 3).

- pUC119-hFAAH (encoding hFAAH, amino acids 30-104): A cDNA containing the *E. coli* codon optimized DNA sequence of hFAAH (bp from 94 to 319 of SEQ ID NO: 3) with a 5'-Xho I site (SEQ ID NO: 4).

The pUC119-hFAAH (encoding hFAAH, amino acids 1-579) was digested with Xho I – EcoR I and the insert was subcloned into a Xho I – EcoR I-digested pTrcHis A vector (Invitrogen, Cat # V360-20) to generate pTrcHis A-hFAAH (encoding amino acids 1-579). The pTrcHis A-hFAAH vector was digested with Xho I-Hind III, and the resulting Xho I-Hind III (approximately 4.5 kb) and Hind III-Hind III (approximately 1.5 kb) pieces were ligated with the Xho I-Hind III fragment (225 bp) generated from digesting the pUC119-hFAAH (encoding amino acids 30-104) construct to generate an NH_2-terminally His-tagged pTrcHis A-hFAAH (encoding amino acids 30-579 of the full length hFAAH).

pET28a-hFAAH (encoding hFAAH, amino acids 32-579)

The human FAAH construct for subcloning into the prokaryotic expression vector pET28a(+) (Novagen, Catalog # 69864-3) was generated by PCR from the pTrcHis A-hFAAH (amino acids 30-579) construct using the following primers: sense primer, 5'-GGAATTCCATATGTCAGGTCGTCGTACCGCACGTG-3' (SEQ ID NO: 5) ; and antisense primer, 5'-CCGCTCGAGTTATGAGGATTGTTT TTCCGGAGTCAT-3' (SEQ ID NO: 6). The resulting PCR product was digested with Nde I-Xho I, and subcloned into a Nde I-Xho I –digested pET28a(+) vector to generate an NH_2-terminally His-tagged pET28a-hFAAH (encoding amino acids 32-579).

Summary of Human FAAH Constructs:

pTrcHis A-hFAAH encodes hFAAH, amino acids (30-579):

MGGS**HHHHHH**GMASMTGGQQMGRTLYDDDDKDRWGSELE----hFAAH

pET28a-hFAAH encodes hFAAH, amino acids (32-579):

MGSS**HHHHHH**SSGLVPRGSHM----hFAAH

"----" Does not represent amino acid positions. Each indicated hFAAH position is contiguous with the specifically indicated leading amino acid sequence comprising HHHHHH.

B. Expression and Purification of Truncated hFAAH

"RT" describes room temperature, which is typically 25°C ± 3°. The pET28a-hFAAH construct encoding amino acids corresponding to amino acids 32-579 of wild-type human FAAH was transformed into the *E. coli* BL21-AI strain. 1.2-liter cultures of the freshly transformed expressing strains were grown in SuperBroth media in the presence of 30 µg/ml kanamycin at 37 °C. At OD_{600} of approximately 0.12, the cultures were transferred to RT and induced at OD_{600} of 0.6 - 0.65 with 100 µM IPTG and 0.2% L-arabinose for 20 hours at RT. All operations below were at 4 °C unless otherwise noted. The cells were then harvested by centrifugation at 5000 x g. The cell pellets were washed twice by re-suspending in 700 ml of PBS and collected by centrifugation at 5000 x g. At this point, the cell paste is optionally frozen and stored at - 80 °C until needed. The cells were re-suspended in 60 ml of buffer A (20 mM Tris-HCl, pH 8.0/100 mM NaCl/1% Triton X-100) with stirring. After adding DNase and RNase (1 mg per 25 g *E. coli* pellet), the cell suspension was incubated for 1 hour at RT with mixing intermittently, cooled on ice for 10 min, and was sonicated with approximately 40-60 ten-second pulses. The resulting lysate was centrifuged at 10,000 x g for 35 minutes and the supernatant was loaded at 0.5 – 1 ml/min onto a 5 ml Ni-column (HiTrap chelating HP column from Amersham Biosciences (Cat # 17-0409-01) was charged with Ni according to the manufacturer's instructions) which has been equilibrated with buffer B (20 mM Tris-HCl, pH 8.0/300 mM NaCl/1% Triton X-100). The resulting flow-through was reloaded onto the column. The column was washed with 100 ml of buffer B and further washed in sequence with 50 ml of buffer B containing 10, 20, and 50 mM imidazole, respectively. The elution was performed in sequence with 25 ml of buffer B containing 100, 200, 400, and 700 mM imidazole, respectively. The majority of

FAAH was eluted with buffer B containing 100-200 mM imidazole. The eluted FAAH was dialyzed against buffer B overnight, frozen in liquid N_2, and stored at –80 °C.

EXAMPLE 2. FAAH Dose-Response Curve at 100 µM Oleamide

To determine whether the assay provides for quantitative detection of FAAH activity, the assay was carried out with increasing concentrations of FAAH. The reactions were carried out in 96-well clear polystyrene plates. The reaction mixture (250 µl) contained 50 mM Tris-HCl, pH 8.0, 100 µM oleamide, 150 µM NADH, 3 mM α-ketoglutarate, 2 mM ADP, 6.0 unit/ml GDH, 0.1% Triton® X-100, and the indicated volumes of approximately 150 nM FAAH. The reactions were incubated at 37 °C and the absorbance at 340 nm was collected over a period of 30 min with readings taken in 10-second intervals using a SpectraMax Microplate Spectrophotometer® (Molecular Devices, Palo Alto, CA) equipped with Softmax Pro® software (Molecular Devices, Palo Alto, CA). As shown in Figure 1, the rate of the reaction increases with increasing concentration of FAAH.

EXAMPLE 3. FAAH Initial Velocity Dependence on Oleamide Concentration

An assay was carried out to determine whether the GDH-coupled FAAH assay is compatible with enzyme kinetics and to demonstrate that FAAH follows typical Michaelis-Menten enzyme kinetics. The reactions were carried out in 96-well clear polystyrene plates. The reaction mixture (250 µl) contained 50 mM Tris-HCl, pH 8.0, 150 µM NADH, 3 mM α-ketoglutarate, 2 mM ADP, 12 unit/ml GDH, 0.1% Triton® X-100, the indicated concentrations of oleamide, and approximately 10 nM FAAH. The reactions were incubated at 37 °C and the data were collected as described in Example 1. Oleamide concentrations are plotted on the x axes and the initial rates are plotted on the y axes. The data were fit to the Michaelis-Menten equation. As shown in Fig. 2, this GDH-coupled FAAH assay allows measuring kinetic constants of FAAH. This data also demonstrates that FAAH follows a typical Michaelis-Menten kinetics. The Km value, which is oleamide substrate concentration at which the reaction rate is half of its maximal value, was determined to be 19.8 μM.

EXAMPLE 4. Measurement of FAAH Inhibition by Compound A.

To further demonstrate the utility of the assay, the inhibitory effects of 1-oxazolo[4,5-b]pyridin-2-yl-5-phenyl-pentan-1-one ((PNAS, (2000) vol. 97, No. 10:p5042), hereinafter, Compound A) on FAAH activity were examined using the GDH-coupled FAAH assay. The reactions were carried out in 96-well clear polystyrene plates. The reaction mixture (200 μl) contained 50 mM NaPi, pH 7.4, 50 μM oleamide, 150 μM NADH, 3 mM α-ketoglutarate, 2 mM ADP, 1 mM ethylenediaminetetraacetic acid (EDTA), 12 unit/ml GDH, 0.1% Triton X-100®, the concentrations of Compound A indicated on Figure 3, and approximately 10 nM FAAH. Oleamide (500 μM) dissolved in 25% DMSO and 25% EtOH was used as a stock solution. Compound A stock solutions, dissolved in 50% DMSO, were used. The final concentrations of DMSO and EtOH were each 7.5%. The reactions were incubated at 37 °C and the data were collected as described in Example 1. The results shown in Figure 3 demonstrate that Compound A inhibits FAAH in a dose-responsive manner.

SEQUENCE LISTING GUIDE

SEQ ID NO: 1 - Amino acids 32-579 of full length homo sapien FAAH.

SEQ ID NO: 2 - Nucleotide sequence encoding amino acids 32 to 579 of full length homo sapien FAAH, optimized for expression in *E.coli.*

SEQ ID NO: 3 - The DNA sequence of the *E. coli* codon optimized sequence of hFAAH (amino acids 1-579) with a 5'-Xho I site, 3' stop codon, and 3'-EcoR I site.

SEQ ID NO: 4 - The DNA sequence from bp 94 to bp 319 of SEQ ID NO: 3 with a 5'-Xho I site.

SEQ ID NO: 5 and 6 – Primers.

SEQUENCE LISTING

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Ahn, Kyunghye

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CLAIMS

What is claimed is:

1. A method for measuring the activity of an ammonia-generating enzyme, comprising the steps of:

(a) providing a reaction mixture comprising a predetermined amount of:

(1) the ammonia-generating enzyme,

(2) an ammonia-generating substrate for the ammonia-generating enzyme,

(3) glutamate dehydrogenase,

(4) a reductive amination substrate for glutamate dehydrogenase, and

(5) a reduced form of co-enzyme for glutamate dehydrogenase;

(b) incubating the reaction mixture under conditions that allow for reactions catalyzed by the ammonia-generating enzyme and glutamate dehydrogenase; and

(c) measuring the rate of the reductive amination catalyzed by glutamate dehydrogenase, wherein the rate of the reductive amination catalyzed by glutamate dehydrogenase is indicative of the activity of the ammonia-generating enzyme.

2. The method according to claim 1, wherein the reduced form of co-enzyme for glutamate dehydrogenase is selected from NADH or NADPH.

3. The method according to claim 2, wherein the reductive amination substrate for glutamate dehydrogenase is α-ketoglutarate.

4. The method according to claim 2, wherein the rate of the reductive amination catalyzed by the glutamate dehydrogenase is measured by measuring the consumption of the reduced form of the co-enzyme.

5. The method according to claim 4, wherein the consumption of the reduced form of the co-enzyme is measured spectrophotometrically at a wavelength between about 290 nm and about 380 nm.

6. The method according to claims 5, wherein the ammonia-generating enzyme is fatty acid amide hydrolase.

7. A method for identifying a compound capable of modulating the activity of an ammonia-generating enzyme, comprising the steps of:

(a) providing a reaction mixture comprising a predetermined amount of:

(1) the ammonia-generating enzyme,

(2) an ammonia-generating substrate for the ammonia-generating enzyme,

(3) glutamate dehydrogenase,

(4) a reductive amination substrate for glutamate dehydrogenase, and

(5) a reduced form of co-enzyme for glutamate dehydrogenase;

(b) incubating the reaction mixture in the absence and presence of a test compound and under conditions that allow for reactions catalyzed by the ammonia-generating enzyme and glutamate dehydrogenase; and

(c) measuring the rate of the reductive amination catalyzed by the glutamate dehydrogenase; wherein a difference in the rate of the reductive amination between the presence and absence of the test compound indicates that the test compound is capable of modulating the activity of the ammonia-generating enzyme.

8. The method according to claim 7, wherein the reduced form of co-enzyme for glutamate dehydrogenase is selected from NADH or NADPH.

9. The method according to claim 8, wherein the reductive amination substrate for glutamate dehydrogenase is α-ketoglutarate.

10. The method according to claim 8, wherein the rate of the reductive amination catalyzed by the glutamate dehydrogenase is measured by measuring the consumption of the reduced form of the co-enzyme.

11. The method according to claim 10, wherein the consumption of the reduced form of the co-enzyme is measured spectrophotometrically at a wavelength between about 290 nm and about 380 nm.

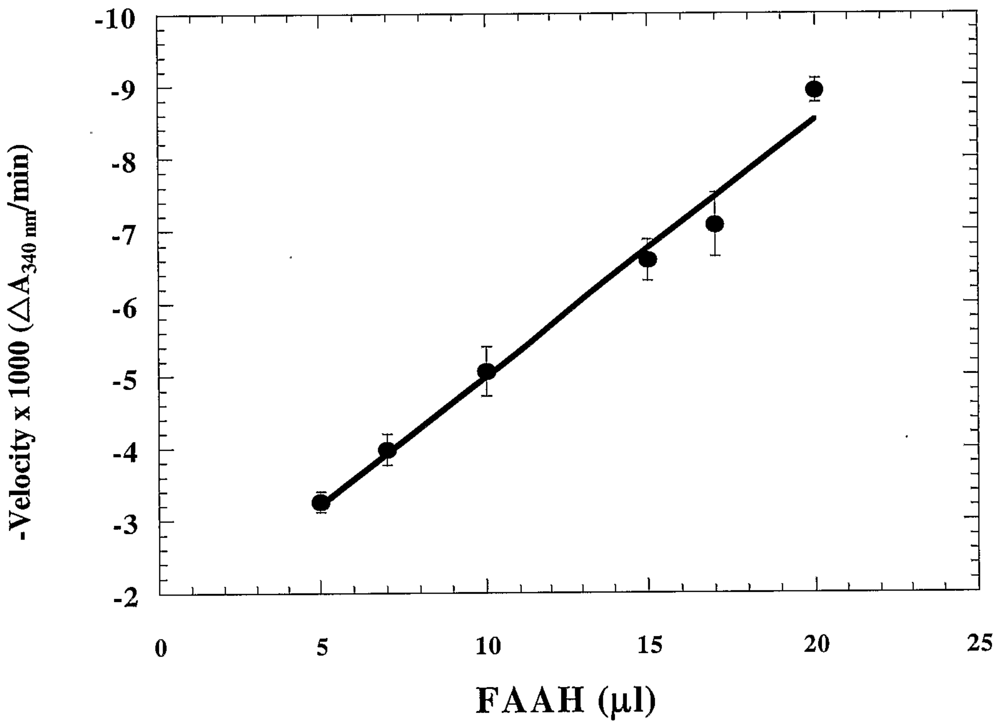
12. The method according to claim 11, wherein the ammonia-generating enzyme is fatty acid amide hydrolase.

13. The method according to claim of 12, wherein the reduced form of co-enzyme for glutamate dehydrogenase is NADH.

14. The method according to claim of 13, wherein the consumption of the reduced form of co-enzyme is measured spectrophotometrically at a wavelength of about 340 nm.

15. The method according to claim 14, wherein the ammonia-generating substrate is oleamide.

Figure 1.



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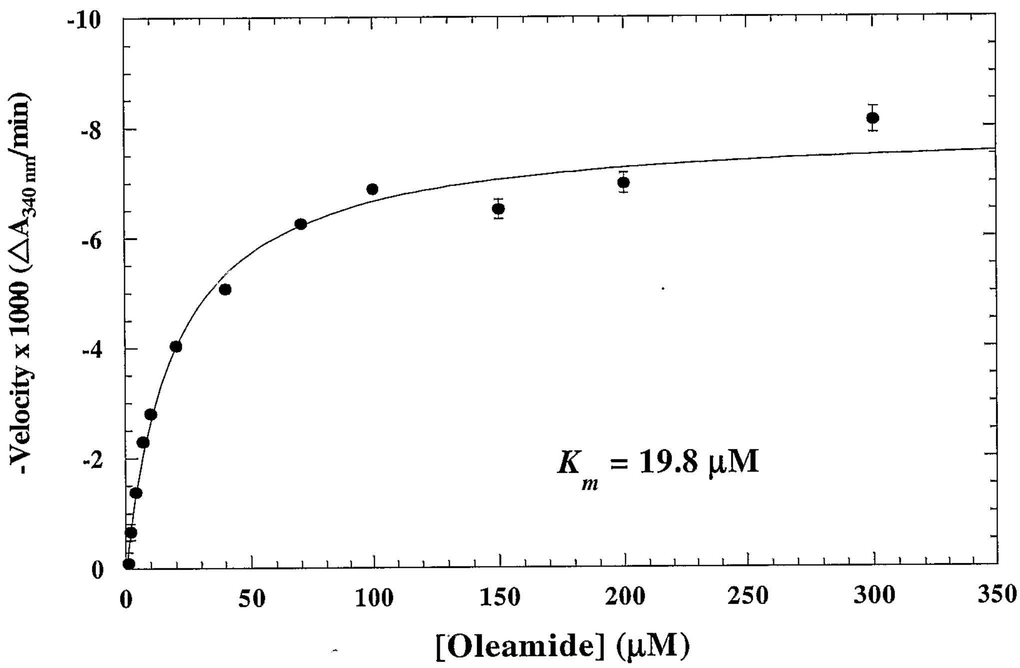
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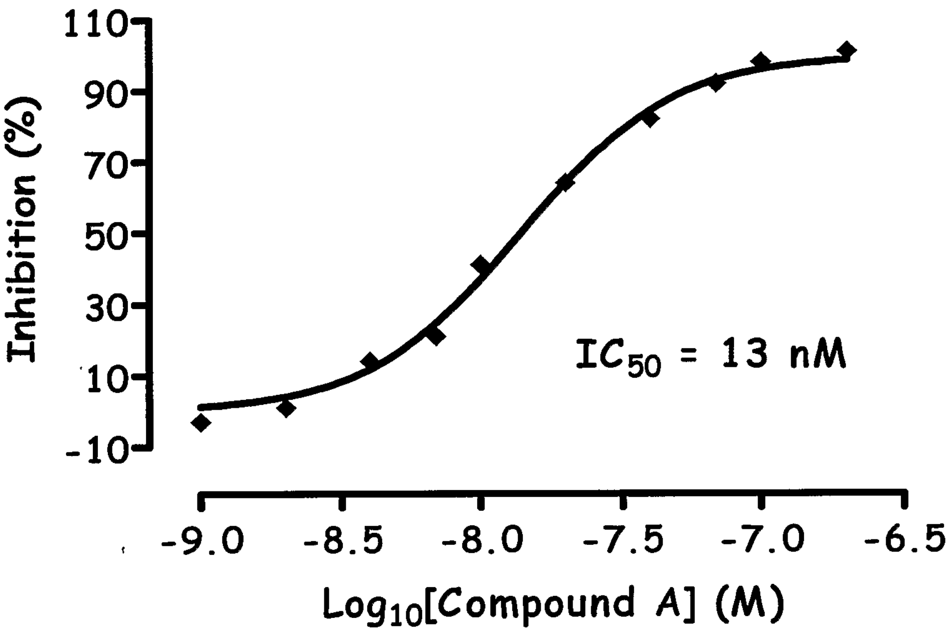
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/000251

A. CLASSIFICATION OF SUBJECT MATTER
C12Q1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LABAHN J ET AL: "An Alternative Mechanism for Amidase Signature Enzymes" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 322, no. 5, 4 October 2002 (2002-10-04), pages 1053-1064, XP004449794 ISSN: 0022-2836 the whole document In particular: p. 1053, Abstract; p. 1054, col. 1, paragraphs 2-3; p. 1056, col. 2, paragraph 2 - p. 1059, col. 2, paragraph 1; p. 1062, col. 2, paragraph 1 (enzyme assay) ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2006	18/04/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Angioni, C

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/000251

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 135 092 A (YAMASA SHOYU KABUSHIKI KAISHA; RIKAGAKU KENKYUSHO) 27 March 1985 (1985-03-27) the whole document In particular: p. 2, l. 22 - p. 3, l. 30; p. 5, l. 6-15; claims 1-5	1-15
A	US 5 198 335 A (SEKIKAWA ET AL) 30 March 1993 (1993-03-30) the whole document In particular: col. 1, l. 10-34; col. 8, l. 60 - col. 9, l. 7	1-15
A	EP 0 287 112 A (FUJI PHOTO FILM CO., LTD) 19 October 1988 (1988-10-19) the whole document	1-15
A	EP 0 365 158 A (TOYO JOZO KABUSHIKI KAISHA; ASAHI KASEI KOGYO KABUSHIKI KAISHA) 25 April 1990 (1990-04-25) the whole document	1-15
A	MACCARRONE MAURO ET AL: "Anandamide hydrolysis by human cells in culture and brain" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 48, 27 November 1998 (1998-11-27), pages 32332-32339, XP002374759 ISSN: 0021-9258 cited in the application the whole document	1-15
T	DE BANK P A ET AL: "A spectrophotometric assay for fatty acid amide hydrolase suitable for high-throughput screening" BIOCHEMICAL PHARMACOLOGY, PERGAMON, OXFORD, GB, vol. 69, no. 8, 15 April 2005 (2005-04-15), pages 1187-1193, XP004807865 ISSN: 0006-2952 the whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2006/000251

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0135092	A	27-03-1985	AU	565122	B2	03-09-1987
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