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Full Mass Spectrometric Characterization of Human Monoacylglycerol Lipase Generated by Large-Scale Expression and Single-Step Purification

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Abstract

The serine hydrolase monoacylglycerol lipase (MGL) modulates endocannabinoid signaling *in vivo* by inactivating 2-arachidonoylglycerol (2-AG), the main endogenous agonist for central CB1 and peripheral CB2 cannabinoid receptors. To characterize this key endocannabinoid enzyme by mass spectrometry-based proteomics, we first overexpressed recombinant hexa-histidine-tagged human MGL (hMGL) in *Escherichia coli* and purified it in a single chromatographic step with high yield (≈ 30 mg/L). With 2-AG as substrate, hMGL displayed an apparent $V_{\max}$ of 25 $\mu\text{mol}/(\mu\text{g min})$ and K_m of 19.7 μM , an affinity for 2-AG similar to that of native rat-brain MGL (rMGL) ($K_m = 33.6 \mu\text{M}$). hMGL also demonstrated a comparable affinity ($K_m \approx 8\text{--}9 \mu\text{M}$) for the novel fluorogenic substrate, arachidonoyl, 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE), in a sensitive, high-throughput fluorometric MGL assay. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) unequivocally demonstrated the mass (34 126 Da) and purity of this hMGL preparation. After in-solution tryptic digestion, hMGL full proteomic characterization was carried out, which showed (1) an absence of intramolecular disulfide bridges in the functional, recombinant enzyme and (2) the post-translational removal of the enzyme's N-terminal methionine. Availability of sufficient quantities of pure, well-characterized hMGL will enable further molecular and structural profiling of this key endocannabinoid-system enzyme.

Keywords

Monoacylglycerol lipase; MGL; 2-Arachidonoyl glycerol; 2-AG; Endocannabinoids; Fatty acid amide hydrolase; FAAH; Cannabinoid receptors; Protein expression; *E. coli*; Mass spectrometry; Fluorogenic substrate; Post-translational processing; Signal transduction; Proteomic analysis; MALDI-TOF MS

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Introduction

The endocannabinoid signaling system is composed of two main cannabinoid receptors (CB1 and CB2), their principal endogenous ligands [the endocannabinoids *N*-arachidonylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG)], and endocannabinoid biosynthesizing and metabolizing enzymes. In mammals, signaling through the endocannabinoid system elicits central and peripheral effects that influence diverse processes including feeding behavior, substrate metabolism, and substance-abuse motivational reward.¹ Two enzymes that degrade endocannabinoids, fatty acid amide hydrolase (FAAH)² and monoacylglycerol lipase (MGL),³ are mainly responsible for reduction of endocannabinoid-system activity and its consequent (patho)physiological effects. Although FAAH may inactivate 2-AG, its main biological substrate is anandamide, whereas MGL exclusively hydrolyzes 2-AG to stoichiometric amounts of arachidonic acid (AA) and glycerol *in vivo*.⁴ Unlike anandamide, 2-AG acts as a full agonist at both CB1 and CB2 cannabinoid receptors. The amounts of tissue 2-AG are much higher than those of anandamide, making MGL a particularly critical modulator of endocannabinoid transmission.⁵ Indeed, inhibition of MGL gene expression acutely and markedly increases cellular steady-state 2-AG level.⁶

Several pharmacological considerations mandate increased knowledge of MGL. Endocannabinoid-system hypoactivity has been implicated in pain, anxiety, and stress-related responses.¹ There is a great deal of interest in the design and profiling of MGL inhibitors for their therapeutic potential as novel analgesics and anxiolytics.^{1,7,8} Potentially, inhibition of MGL represents a more selective pharmacotherapeutic approach for activating the endocannabinoid system as compared to the use of cannabinoid-receptor agonists, which can also evoke unwanted CB1 receptor-mediated motor and psychotropic side effects unless peripherally directed.⁹ High-affinity inhibitors selective for MGL (vs FAAH or the cannabinoid receptors) are currently lacking for two main reasons. MGL inhibitor assays routinely rely upon the use of crude rat-brain extracts or cell lysates, the biochemical heterogeneity of which complicates *in vitro* profiling of exogenous agents as MGL inhibitors.¹⁰ Furthermore, molecular characterization of MGL is very limited for lack of an accurate crystal structure. Partial three-dimensional homology models for MGL based on sequence alignments and the crystal structures of bromoperoxidase A2 from *Streptomyces aureofaciens* and chloroperoxidase L from *Streptomyces lividans*^{11–13} are provisional and cannot substitute for experimentally determined enzymatic, proteomic, and structural information.

Expression of functional mouse MGL in insect cells has been reported, albeit with moderate yield (≈ 3 mg enzyme/L culture).¹⁴ Given documented interspecies differences in the biochemistry, pharmacology, tissue-distribution, and phylogeny of endocannabinoid-system protein components,^{15–17} a well-characterized preparation of purified human MGL appears critical to obtaining detailed knowledge of the enzyme's structure sufficient to design and profile potent, selective MGL inhibitors as potential therapeutics. To address this need, we have developed an MGL expression system in *Escherichia coli* that generates substantial amounts of recombinant hexa-histidine-tagged human MGL (hMGL) in high yield. Enzymatic characterization demonstrates hMGL's hydrolysis of and high affinity for both 2-AG and arachidonoyl, 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE), a novel fluorogenic model substrate. Complete characterization of hMGL by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provided unequivocal documentation of its purity, molecular mass, intramolecular sulfhydryl profile, and post-translational processing.

Experimental Section

Materials

Standard laboratory chemicals and buffers were from Sigma Chemical Co. (St. Louis, MO) and Fisher Chemical (Pittsburgh, PA), if not otherwise specified. AHMMCE was synthesized and purified at the Center for Drug Discovery, Northeastern University, from commercial reagents by a synthetic route to be detailed elsewhere. Crude rat-brain MGL (rMGL) was prepared as described.¹⁸ Kits for protein determination by the Bradford dye-binding method were from Bio-Rad Laboratories (Hercules, CA).

Vector Construction

A full-length cDNA clone of the human MGL transcript variant 1 (gi: 51242951) was obtained from OriGene Technologies (Rockville, MD). The coding part of the MGL DNA sequence (except the translation initiation codon ATG) was amplified by a polymerase chain reaction (PCR) using forward AACACGTGCCAGAGGAAAGTTCCC and reverse AA-GAGCTCAGGGTGGGGACGCAG primers containing *Pml*I and *Sac*I restriction enzyme recognition sites, respectively. iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA) was used in the PCR amplification with 33 cycles, each consisting of denaturation at 94 °C for 10 s, annealing at 55 °C for 33 s, and extension at 72 °C for 1 min. The PCR product and pET45b vector were digested with *Pml*I and *Sac*I restriction enzymes, and the vector was dephosphorylated with CIP followed by in-gel purification using the MinElute Gel Extraction Kit (Qiagen Corp., Valencia, CA). The fragment was inserted into the vector directly downstream after the 6 histidine and valine codons, generating the construct pET45His6hMGL for expression of hMGL containing an N-terminal His6-tag. GC10 *E. coli* cells were used for DNA transformation and plasmid propagation. Mini- and midi-scale plasmid DNA preparations were performed using a GenElute Plasmid Miniprep Kit (Sigma) and PureYield Plasmid Midiprep System (Promega, Madison, WI), respectively. In-frame vector-fragment junctions and the coding sequence of the recombinant gene were confirmed by sequencing. The pET45His6hMGL construct was transformed into BL21 (DE3) and Origami (DE3) *E. coli* expression strains (Novagen, Madison, WI).

Analytical Screening for hMGL Expression

An *E. coli* colony containing pET45His6hMGL plasmid was inoculated into 2 mL of Luria broth (Sigma) supplemented with ampicillin (100 µg/mL) and grown overnight at 37 °C with shaking (250 rpm). The next morning, 0.2 mL of this culture was inoculated into 20 mL of fresh Luria broth-ampicillin medium and incubated under the same conditions. When culture turbidity reached an OD₆₀₀ of 0.5–0.7, expression from the T7 promoter was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) (Sigma). At each hour during the first 6 h of induction and, finally, at 19 h post-induction, a 1-mL culture sample was collected. Cells were harvested by centrifugation at 5000g for 10 min at 4 °C and washed with phosphate-buffered saline (PBS). Soluble and inclusion-body subfractions were prepared using B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). In brief, *E. coli* cells (5–10 mg) were collected from 1 mL of culture by centrifugation at 7000g for 5 min and resuspended in 150 µL of B-PER reagent. The suspension was centrifuged at 15 000g for 5 min, and the supernatant containing soluble protein was recovered. The resulting pellet was resuspended in 150 µL of B-PER reagent followed by addition of 3 µL of lysozyme solution (10 µg/µL) (Sigma) and 500 µL of 1:10-diluted B-PER reagent. Inclusion bodies were collected by centrifugation at 15 000g for 10 min. Fractions were processed for evaluation of hMGL expression over time by SDS-PAGE and immunodetection (below).

Preparative hMGL Purification

A single *E. coli* BL21 (DE3) colony containing the pET45His6hMGL plasmid was inoculated into 12 mL of Luria broth-ampicillin medium and grown overnight with shaking (250 rpm) at 37 °C. The next morning, 10 mL of this culture was inoculated into 1 L of fresh Luria broth-ampicillin and allowed to grow until culture turbidity reached an OD₆₀₀ of 0.6–0.8, at which time expression from the T7 promoter was induced by adding isopropyl- β -D-thiogalactopyranoside (1 mM). After 5 h induction, the cells were harvested by centrifugation at 5000g for 10 min at 4 °C, washed with PBS, and held at –80 °C. Five grams (wet-weight) of cells were resuspended in 50 mL of lysis buffer (100 mM NaCl, 50 mM Tris, pH 8.0, and Triton X-100 (0.5%)) supplemented with lysozyme (0.2 mg/mL) and DNase I (25 μ g/mL) and disrupted on ice by three, 1-min sonication cycles, each consisting of 1-s sonication bursts at 50 W power separated from each other by a 2-s interval (Vibra-Cell 500W, Sonics, Newtown, CT). The cell lysate was centrifuged at 10 000g for 30 min at 4 °C. To isolate hMGL by immobilized metal affinity chromatography (IMAC), the supernatant was incubated with 1.0 mL (bed volume) of pre-equilibrated BD Talon metal-affinity resin (Clontech, Mountain View, CA) for 1 h at room temperature with gentle agitation. The suspension was then transferred to a gravity-flow column and allowed to settle. The resin was washed with 15 mL of lysis buffer, then 15 mL of lysis buffer containing 0.1% Triton X-100 and 10 mM imidazole. His6-tagged protein was eluted with 4 mL of lysis buffer containing 0.1% Triton X-100 and 200 mM imidazole and analyzed by SDS-PAGE.

SDS-PAGE and Western Blotting

Protein samples and molecular mass markers (Bio-Rad) were denatured at 70 °C for 5 min in Laemmli buffer containing 5% β -mercaptoethanol and resolved on 10% SDS-PAGE gels. Gels were either stained using Commassie blue (Bio-Rad) or transferred to polyvinylidene fluoride membranes for immunodetection according to the QIAexpress Detection and Assay Handbook using a 1:10 000 dilution of anti-5His-horseradish peroxidase antibody (Qiagen). Protein bands were visualized using the ECL Western Blotting Analysis System (GE Healthcare, Piscataway, NJ). A FluorChem Imaging System (Alpha Innotech Corp., San Leandro, CA) was used to photograph developed gels and blots.

MGL Assay

Prior to enzyme assay, the purified hMGL was desalted with a Zeba spin-column (Pierce) and 25 mM Tris-HCl, pH 7.4, containing 5 mM MgCl₂ and 2 mM EDTA (TME buffer). MGL activity was assessed by two methods. In the first, hydrolysis of 2-AG to AA was quantified by HPLC.¹⁸ Briefly, 2-AG at varying concentrations from 12.7 to 400 μ M and hMGL (0.5–2.0 ng) in TME buffer (150 μ L) were incubated at 37 °C. Reaction samples (50 μ L) were taken immediately at the start of the incubation and after 20 min, diluted 1:4 with acetonitrile, and centrifuged at 20 000g for 5 min at room temperature. A 10- μ L aliquot of supernatant was injected onto the HPLC, with chromatographic conditions previously detailed.¹⁸ In an 8-min HPLC run, 2-AG eluted at 3.0 min, and AA at 6.0 min, allowing the reaction to be followed by either substrate (2-AG) turnover or product (AA) formation. Analytes were quantified with external standards.

Alternatively, a new fluorometric assay (to be detailed elsewhere) was developed in a 96-well format by which MGL activity was monitored as the hydrolysis of the reporter model substrate AHMMCE to coumarin fluorophore. In brief, known amounts of hMGL were incubated with various concentrations of AHMMCE for up to 120 min at room temperature ($\approx$ 22 °C), during which fluorescence readings at 360 nm/460 nm ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$) were taken every 15 min using a Synergy HT Plate Reader (Bio-Tek). Under these incubation conditions, negligible spontaneous AHMMCE hydrolysis was observed (data not shown),

Relative fluorescence units were converted to the amount of coumarin formed based upon a standard curve of coumarin fluorescence.

All MGL assays were performed in triplicate for each substrate concentration, and protein was determined with the Bradford dye-binding microassay (Bio-Rad). Michaelis–Menten kinetic parameters were derived with Prism software, Version 4 (GraphPad, San Diego, CA).

MALDI-TOF MS Analysis

A Bio-Spin 6 column (Bio-Rad) with 50 mM ammonium bicarbonate buffer, pH 8.0, containing 0.02% 5-cyclohexyl-1-pentyl- β -D-maltoside was used for desalting purified hMGL. An aliquot of intact, desalted hMGL was analyzed by MALDI-TOF to determine its molecular weight. The remainder of the sample, prior to analysis by MALDI-TOF MS, was either directly incubated overnight with MS-grade trypsin (“Trypsin Gold,” Promega) or subjected to either alkylation or reduction and alkylation using iodoacetamide (IAM) and dithiothreitol (DTT) before trypsin digestion.¹⁹ Intact or digested hMGL samples were co-crystallized with α -cyano-4-hydroxycinnamic acid matrix by spotting 0.5 μ L of sample and 0.5 μ L of 5 mg/mL matrix prepared in 60% acetonitrile/ 0.1% trifluoroacetic acid in water onto an Opti-TOF 384-well plate insert. After crystallization, the dried spot was washed with 5 μ L of 0.1% aqueous trifluoroacetic acid for rapid desalting. In those cases where salt and detergent suppressed signal, the digest was first diluted 10-fold with matrix solution followed by spotting 1 μ L for analysis. Spectra of hMGL trypsin digests were acquired on a 4800 MalDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) fitted with a 200-Hz solid state UV laser (wavelength 355 nm) in reflectron mode. Intact hMGL spectra were acquired in the linear mode. Data were accumulated from several positions within each sample well to determine the ions present. All MS spectra of the tryptic digests were externally calibrated using a mixture of peptide standards [des-Arg1-bradykinin at MH^+ 904.4681, angiotensin I at MH^+ 1296.6853, Glu-fibrino peptide at MH^+ 1570.6774, ACTH (clip 1–17) at MH^+ 2093.0867, ACTH (clip 18–39) at MH^+ 2465.1989 and ACTH (clip 7–38) at MH^+ 3657.9294]. Intact hMGL spectra were externally calibrated using the Invitromass HMW calibrant (Invitrogen, Carlsbad, CA). MS/MS spectra were acquired on select ions of interest under the following conditions: precursor isolation set to resolution of 200, collision energy of 2kV, CID cell pressure of 2×10^{-5} torr, air as collision gas. The instrument was first calibrated in MS/MS mode using five daughter ions (at m/z 175.119, 684.346, 813.389, 1056.475 and 1441.634) generated from the fragmentation of Glu-fibrino peptide (MH^+ 1570.6774). Data were accumulated until spectra were of optimal quality and then analyzed by comparing the monoisotopic peaks with the theoretical molecular weights corresponding to the expected peptide digestion products. The maximum allowable error was 100 ppm. Theoretical molecular weights of expected peptides after digestion were calculated using MS-digest (UCSF MS Facility, San Francisco, CA) and FindPept and FindMod tools (ExPASy Server, Swiss Institute of Bioinformatics, Geneva, Switzerland).

Results and Discussion

Large-Scale Expression, Purification, and Enzymatic Profiling of hMGL

Potent and highly selective MGL inhibitors are lacking. Yet, such agents are of great interest as potential therapeutics, especially for suppressing inflammatory pain through a peripheral analgesic mechanism devoid of unwanted central nervous system psychoactivity.^{7–9} Facile production of a substantial quantity of well-characterized, catalytically competent MGL—particularly the human homologue as pharmaceutical target—is a critical prerequisite for addressing this therapeutic need and satisfying the protein requirements for crystallization and nuclear magnetic resonance spectroscopy. These considerations led us to pursue human

MGL overexpression in a prokaryotic system, which generally offers greater ease and economy of protein generation than a eukaryotic system. We incorporated a His6-tag into the N-terminus of the protein to facilitate its isolation using IMAC²⁰ and its immunodetection. From preliminary experiments comparing BL21 (DE3) and Origami (DE3) *E. coli* expression strains, the BL21 strain was selected for large-scale hMGL production due to the ~2-fold greater yield of functional enzyme obtained relative to the Origami strain. Since increasing amounts of soluble hMGL were lost intracellularly to inclusion bodies over extended induction times, 4–5 h was considered the optimal expression period for catalytically active hMGL. Although 0.5% Triton X-100 inhibited hMGL by ~50% (data not shown), the presence of Triton during cell lysis and enzyme purification was deemed necessary to obviate nonspecific hMGL adhesion to glass and plasticware and the resulting, extensive hMGL loss. Although detergents other than Triton (e.g., CHAPS) also facilitated IMAC purification (data not shown), no systematic detergent survey was performed. With this optimized large-scale expression and purification protocol, up to 30 mg of functional hMGL could be reliably obtained from 1 L of cell culture.

SDS-PAGE followed by either Coomassie staining or Western blot analysis with anti-5-His-HRP antibody detection of various subfractions from *E. coli* lysates revealed that our single-step, IAMC-based purification protocol affords a single protein band under reducing conditions, suggesting purity (Figure 1). The protein's molecular mass, ~35 kDa as estimated relative to protein standards, corresponds well to the hMGL calculated molecular mass of 34 123 Da and the reported molecular masses of purified, His6-tagged recombinant mouse MGL (~36.9 kDa),¹⁴ rat brain MGL (33.4 kDa),²¹ and a monoacylglycerol-hydrolyzing enzyme from rat adipose tissue (~33 kDa).²²

The kinetic parameters of the purified protein's MGL activity were next characterized with native substrate, 2-AG. Recombinant hMGL hydrolyzed 2-AG with an apparent K_m of 19.7 μM and V_{max} of 25.1 $\mu\text{mol}/(\mu\text{g min})$ (Table 1). The K_m of recombinant hMGL compares very favorably with the K_m of 33.6 μM for crude rMGL determined by us in parallel assays and also with the 10.0 μM K_m for recombinant rat MGL, as reported.²³ However, rMGL evidence a considerably lower V_{max} (0.37 $\mu\text{mol}/(\text{mg min})$) than that of purified hMGL. This difference likely reflects the high content of extraneous tissue protein in the crude rMGL preparation, for partially purified MGL from porcine brain hydrolyzes 2-AG with a reported V_{max} of 3.5 $\mu\text{mol}/(\text{mg min})$.²⁴

hMGL showed good affinity for the novel, model fluorogenic reporter AHMMCE developed in our laboratory with a K_m of 8.8 μM , only ~2-fold less than that for native substrate, 2-AG (Table 1). The hMGL V_{max} of 0.55 $\mu\text{mol}/(\mu\text{g min})$ for AHMMCE is, however, markedly (~45-fold) lower than that for 2-AG. The comparative, substrate-related differences noted in hMGL kinetic parameters between 2-AG and AHMMCE are similar to other successful applications of model fluorogenic substrates, including those used to monitor FAAH activity.^{25,26} Fluorogenic reporters may have turnover rates orders of magnitude below those of the corresponding natural substrates, even if both have comparable enzyme affinities.^{25,27} Nonetheless, the inherently high sensitivity of fluorometric analysis enables facile monitoring of catalysis.^{25–27}

Complete hMGL Characterization by MALDI-TOF MS

In the linear mode, MALDI-TOF MS analysis of native hMGL demonstrated a single charged protein species of average mass 34 126 (Figure 2A), consonant with its calculated molecular mass of 34 123 Da. The absence of any significant peaks in the MALDI-TOF mass spectrum not directly assigned to hMGL also demonstrates unequivocally the homogeneity of the hMGL isolated from our expression system.

Purified hMGL was further characterized after in-solution trypsin digestion by peptide fingerprinting and tandem MALDI-TOF/TOF MS (Figure 2B; Table 2). The tryptic fragments identified by MALDI MS in Figure 2B correspond to the theoretical fragments obtained from the *in silico* tryptic digestion of the hMGL sequence shown in Figure 3. As shown in Table 2, the complete sequence of hMGL can be confirmed unambiguously using high-confidence mass matching, with less than 47 ppm error between exact and experimental masses. Together, the intact mass and peptide fingerprint results accurately and confidently identify the purified recombinant protein as hMGL.

The sulfhydryl profile of MGL has been a particular focus of modeling studies. Six cysteine residues are present in rat MGL (accession NP 612511), whereas four cysteine residues are found in human MGL (accession NP 009214). Inhibition of rMGL by sulfhydryl-specific reagents has implicated cysteine(s) in MGL activity, at least two of which have been modeled in close proximity to the lipophilic substrate binding pocket.¹² Consequently, we explored the presence or absence of disulfides within hMGL. Table 3 lists the cysteine-containing peptides identified by MALDI-TOF MS of native (i.e., trypsin-treated), alkylated (IAM/trypsin-treated), and reduced/alkylated (DTT/IAM/trypsin-treated) hMGL. The peptides identified under the alkylated and reduced/alkylated conditions do not differ in sequence from the peptides identified under native conditions. The only mass difference corresponds to the addition of the acetamide tag. The presence of disulfide bonds would covalently link two cysteines via their sulfhydryl side chains to yield a single peptide with a mass corresponding to the sum of the two individual tryptic peptides. Neither the native nor the alkylated digestions produced any peptides that were not present in the reduced/alkylated sample. The absence of any peptide mass changes between the alkylated and the reduced/alkylated samples indicates that no disulfide bonds were present. Although awaiting experimental confirmation, modeling of critical cysteine residues into MGL^{12,13} is consistent with our conclusion that catalytically active hMGL lacks disulfide bonds.

Tandem MS analysis also unambiguously identified a post-translational hMGL modification. After trypsin digestion, hMGL should theoretically produce the N-terminal, His6-tagged fragment MAHHHHHHVPEESSPR (1924.9 Da), but we have not observed it in any of our trypsin digests (Figure 3). Rather, an ion with m/z 1793.8 was invariably detected and identified by tandem MS-MS analysis as the peptide AHHHHHHVPEESSPR (Figure 2, C,D). It appears, therefore, that removal of the N-terminal Met (131.2 Da) from hMGL by methionyl aminopeptidase occurs post-translationally, a modification favored when the penultimate amino acid is alanine.²⁸

As this work was being prepared for publication, Labar et al.²⁹ reported expression in *E. coli* of human MGL fused to an N-terminal His6-tag and a C-terminal Strep-tag. Since the primary aim of that study was to characterize potential MGL inhibitors, enzyme purity was inferred solely from SDS-PAGE analysis after a two-step MGL enrichment protocol dependent upon the dual tags. This more complicated protocol likely contributes to the markedly lower reported yield (5–10 mg/L) of functional human MGL than we have achieved (30 mg/L). The sensitivity of 2-oleoylglycerol hydrolysis by that MGL preparation to disulfide compounds interacting with cysteine residues²⁹ is congruent with our direct proteomic demonstration that multiple free sulfhydryl groups exist in hMGL.

Conclusion

To help meet the requirements for human MGL by efforts aimed at discovering selective MGL inhibitors and elucidating the structure of this important enzyme, hMGL was expressed at a high level in *E. coli* and purified in a single step using IMAC. Functional characterization of the hMGL enzyme demonstrated that its affinities (K_m) for both native

substrate (2-AG) and a novel fluorogenic reporter, AHMMCE, were comparably high with acceptable turnover rates ($V_{\max}$). The affinity of hMGL for 2-AG was similar to that of the crude rat-brain enzyme. Proteomic data obtained through a full MS characterization established that our large-scale expression system and isolation protocol generated unequivocally pure hMGL, making this the first report on the functional characteristics and proteomics of purified hMGL. The carbamidomethylation patterns of cysteine-containing peptides identified in trypsin digests of native, alkylated, and reduced/alkylated hMGL indicate that functional enzyme does not contain disulfide bridges, suggesting the importance of free sulfhydryls to functional hMGL. MALDI TOF-MS analysis of hMGL tryptic peptides demonstrated the removal of N-terminal methionine as a post-translational modification. Our *E. coli* expression system offers a high-yield source of functional hMGL whose purity makes it a unique asset for crystallization, structural analyses, and (in combination with a novel fluorogenic reporter) identification of potential pharmacotherapeutics that modulate hMGL activity.

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Abbreviations

AA	arachidonic acid
2-AG	2-arachidonoyl-glycerol
AHMMCE	arachidonoyl, 7-hydroxy-6-methoxy-4-methylcoumarin ester
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
DTT	dithiothreitol
FAAH	fatty acid amide hydrolase
His6	hexa-histidine tag
HPLC	high-pressure liquid chromatography
hMGL	recombinant hexa-histidine-tagged human monoacylglycerol lipase
IAM	iodoacetamide
IMAC	immobilized metal affinity chromatography
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MGL	monoacylglycerol lipase
<i>m/z</i>	mass-to-charge ratio
rMGL	crude rat-brain monoacylglycerol lipase
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
TME	25 mM Tris-HCl, pH 7.4, 5 mM MgCl ₂ , 2 mM EDTA

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Figure 1.

Coomassie-stained SDS-PAGE profiling (A) and Western blot immunodetection (B) of hMGL purification by IMAC. Total (lane 1) and soluble (lane 2) protein from *E. coli* cells expressing hMGL; proteins unbound to IMAC resin (lane 3); proteins washed from IMAC resin by lysis (lane 4) and lysis-10 mM imidazole (lane 5) buffers; final elution from IMAC resin using lysis-200 mM imidazole buffer (lane 6). Detection was performed according to the procedures described in the Experimental Section.

Figure 2. MALDI-TOF MS analysis of native (A) and trypsin-digested (B), purified hMGL. In panel B, (*) designates the fragments that match with the hMGL tryptic peptides, and (#) designates the peptide subjected to tandem MS/MS analysis, which is displayed in panel C and identified as the N-terminal peptide AHHHHHHVPEESSPR. Expected *b*- and *y*-ions of precursor ion 1793.84 are given in panel D.

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      10      20      30      40      50      60
(M) AHHHHHHVPE ESSPRRT PQS IPYQDLPHLV NADGQYLFCR YWKPTGTPKA LIFVSHGAGE

      70      80      90     100     110     120
HSGRYEELAR MLMGLDLLVF AHDHVGHGQS EGERMVVSDF HVFVRDVLQH VDSMQKDYPG

     130     140     150     160     170     180
LPVFLLGHSM GGAIAILTAA ERPGHFAGMV LISPLVLANP ESATTFKVLA AKVLNLVLPN

     190     200     210     220     230     240
LSLGPIDSSV LSRNKTEVDI YNSDPLICRA GLKVCFGIQL LNAVSRVERA LPKLTVPFLL

     250     260     270     280     290     300
LQGSADRLD SKGAYLLMEL AKSQDKTLKI YEGAYHVLHK ELPEVTNSVF HEINMWVSQR

     310
TATAGTASPP

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Figure 3.

Amino acid sequence of recombinant hexa-histidine-tagged human MGL (hMGL). The cysteines and the amino acids of the catalytic triad are underlined and highlighted in green and pink, respectively. The N-terminal methionine (M) was removed *in vivo* post-translationally by methionyl aminopeptidase.

Table 1

Apparent Kinetic Parameters of Crude Rat-Brain MGL (rMGL) and Recombinant Hexa-histidine-tagged Human MGL (hMGL) Overexpressed in *E. coli* with the Natural Substrate 2-AG or the Model Fluorogenic Reporter Substrate, AHMMCE

substrates	K_m		V_{max}	
	rMGL (μM)	hMGL (μM)	rMGL ($\mu\text{mol}/(\text{mg min})$)	hMGL ($\mu\text{mol}/(\mu\text{g min})$)
2-AG	33.6	19.7	0.37	25.1
AHMMCE	n.d. ^a	8.8	n.d. ^a	0.55

^a n.d., not determined.

Table 2
MALDI TOF MS Fingerprinting of Recombinant Hexa-histidine-tagged Human MGL (hMGL) Digested with Trypsin

position	observed MW	expected MW	error ppm	peptide sequence	modification
1-15	1793.837	1793.833	-2.400	(-) AHHHHHHVPESSPR (R)	
16-40	2988.515	2988.478	-12.200	(R) RTPQSIPYQDLPHLVNADGQYLF $\overline{\text{C}}$ R (Y)	CAM
17-40	2775.377	2775.356	-7.600	(R) TPQSIPYQDLPHLVNADGQYLF $\overline{\text{C}}$ R (Y)	
17-40	2832.394	2832.377	-5.800	(R) TPQSIPYQDLPHLVNADGQYLF $\overline{\text{C}}$ R (Y)	CAM
41-49	1077.561	1077.573	10.800	(R) YWKPTGT $\overline{\text{P}}$ K (A)	
50-64	1537.786	1537.787	0.600	(K) ALIFVSHGAGEHSGR (Y)	
50-70	2299.174	2299.158	-7.000	(K) ALIFVSHGAGEHSGRYEELAR (M)	
71-94	2648.304	2648.296	-12.500	(R) MLMGLDLLVFAHDHVGHCQSEGER (M)	
95-105	1335.683	1335.688	3.500	(R) MVVSDFHVFVR (D)	
95-116	2648.304	2648.296	-3.000	(R) MVVSDFHVFVRDVLQHVDSMQK (D)	2 MSO
117-167	5247.900	5247.774	-24.000	(K) DYPGLPVFLGHSMGGAI $\overline{\text{A}}$ ILTA $\overline{\text{A}}$ ERPGHFAGMVLISPLVLANPESATTFK (V)	
117-167	5263.521	5263.769	47.000	(K) DYPGLPVFLGHSMGGAI $\overline{\text{A}}$ ILTA $\overline{\text{A}}$ ERPGHFAGMVLISPLVLANPESATTFK (V)	MSO
168-209	4549.582	4549.506	-16.600	(K) VLA $\overline{\text{A}}$ KVLNLVLPNLSLGPIDSSVLSRNKTEVDIYNSDPLI $\overline{\text{C}}$ R (A)	
173-193	2206.292	2206.280	-5.200	(K) VLNLVLPNLSLGPIDSSVLSR (N)	
194-209	1879.929	1879.922	-3.800	(R) NKTEVDIYNSDPLI $\overline{\text{C}}$ R (A)	
194-209	1936.929	1936.943	7.300	(R) NKTEVDIYNSDPLI $\overline{\text{C}}$ R (A)	CAM
194-229	4148.007	4148.174	40.300	(R) NKTEVDIYNSDPLI $\overline{\text{C}}$ RAGLKVCFGIQLLNAVSRVER (A)	2 CAM
196-209	1637.772	1637.784	7.200	(K) TEVDIYNSDPLI $\overline{\text{C}}$ R (A)	
196-209	1694.815	1694.805	-5.600	(K) TEVDIYNSDPLI $\overline{\text{C}}$ R (A)	CAM
196-213	2064.069	2064.043	-12.600	(K) TEVDIYNSDPLI $\overline{\text{C}}$ RAGLK (V)	CAM
196-229	3792.123	3791.993	-34.100	(K) TEVDIYNSDPLI $\overline{\text{C}}$ RAGLKVCFGIQLLNAVSRVER (A)	
210-226	1845.978	1846.037	31.700	(R) AGLKVCFGIQLLNAVSR (V)	CAM
210-229	2230.171	2230.249	34.800	(R) AGLKVCFGIQLLNAVSRVER (A)	CAM
210-233	2639.411	2639.518	40.400	(R) AGLKVCFGIQLLNAVSRVERALPK (L)	
214-226	1419.775	1419.778	1.800	(K) VCFGIQLLNAVSR (V)	
214-226	1476.787	1476.799	8.100	(K) VCFGIQLLNAVSR (V)	CAM
227-262	3975.303	3975.181	-30.700	(R) VERALPKLTVPFLLQGSADRLCD $\overline{\text{S}}$ KGAYLLMELAK (S)	MSO
234-247	1529.870	1529.869	-0.900	(K) LTVPFLLQGSADR (L)	
234-252	2076.087	2076.116	13.800	(K) LTVPFLLQGSADRLCD $\overline{\text{S}}$ K (G)	

position	observed MW	expected MW	error ppm	peptide sequence	modification
234–252	2133.063	2133.137	34.700	(K) LTVPFLLLLQGSADRI $\bar{C}$ DSK (G)	CAM
248–280	3765.919	3765.971	13.600	(R) LCDSKGAYLLMELAKSQDKTLKIYEGAYHVLHK (E)	
253–262	1108.595	1108.607	10.800	(K) GAYLLMELAK (S)	
270–280	1329.686	1329.695	6.700	(K) IYEGAYHVLHK (E)	
270–300	3725.689	3725.853	44.000	(K) IYEGAYHVLHKELPEVTNSVFHEINMWVSQR (T)	
281–300	2415.194	2415.176	7.400	(K) ELPEVTNSVFHEINMWVSQR (T)	
281–310	3269.600	3269.589	–3.200	(K) ELPEVTNSVFHEINMWVSQRTATAGTASPP (–)	

Table 3

The Cysteine Containing Fragments Detected in Trypsin Digest of Recombinant Hexa-histidine-tagged Human MGL (hMGL)

position	hMGL/trypsin	hMGL/IAM/trypsin ^a	hMGL/DTT/IAM/trypsin ^a
17–40	(R) TPQSIPYQDLPHLVNADGQYLFCR (Y)	(R) TPQSIPYQDLPHLVNADGQYLFC*R (Y)	(R) TPQSIPYQDLPHLVNADGQYLFC*R (Y)
194–209	(R) NKTEVDIYNSDPLICR (A)	(R) NKTEVDIYNSDPLIC*R (A)	(R) NKTEVDIYNSDPLIC*R (A)
214–226	(K) VCFGIQLLNAVSR (V)	(K) VC*FGIQLLNAVSR (V)	(K) VC*FGIQLLNAVSR (V)
234–252	(K) LTVPFLLLQGSADRLCDISK (G)	(K) LTVPFLLLQGSADRLC*DISK (G)	(K) LTVPFLLLQGSADRLC*DISK (G)

^a C* corresponded to carbamidomethylated cysteine.