

A MBP-FAAH fusion protein as a tool to produce human and rat fatty acid amide hydrolase: expression and pharmacological comparison

G. Labar^{1,2}, F. V. Vliet³, J. Wouters^{2,4}, and D. M. Lambert^{1,2}

¹ Unité de Chimie pharmaceutique et de Radiopharmacie, Ecole de Pharmacie, Faculté de Médecine, Université catholique de Louvain, Bruxelles, Belgium

² Drug Design and Discovery Center, Belgium

³ Institut de recherches microbiologiques Wiame, Bruxelles, Belgium

⁴ Laboratoire de Chimie biologique structurale, Faculté des Sciences, Facultés universitaires Notre-Dame de la Paix, Namur, Belgium

Received January 20, 2007

Accepted February 23, 2007

Published online May 4, 2007; © Springer-Verlag 2007

Summary. Fatty acid amide hydrolase (FAAH), a membrane-anchored enzyme responsible for the termination of endocannabinoid signalling, is an attractive target for treating conditions such as pain and anxiety. Inhibitors of the enzyme, optimized using rodent FAAH, are known but their pharmacology and medicinal chemistry properties on the human FAAH are missing. Therefore recombinant human enzyme would represent a powerful tool to evaluate new drug candidates. However, the production of high amounts of enzyme is hampered by the known refractiveness of FAAH to overexpression. Here, we report the successful overexpression of rat and human FAAH as a fusion to the *E. coli* maltose-binding protein, retaining catalytic properties of native FAAH. Several known FAAH inhibitors were tested and differences in their potencies toward the human and rat FAAH were found, underscoring the importance of using a human FAAH in the development of inhibitors.

Keywords: Fatty acid amide hydrolase – Endocannabinoid system – Protein overexpression – Fusion proteins – Inhibitors characterization – Maltose-binding protein

Introduction

In the early 90s, a major cellular signalling system involving endogenous lipids as molecular messengers was discovered. Two G protein-coupled receptors, the cannabinoid receptors CB₁ and CB₂ were the first components of this system to be cloned, in 1990 (Matsuda et al., 1990) and 1993 (Munro et al., 1993), respectively.

The CB₁ cannabinoid receptor, highly expressed in the CNS, is mainly involved in regulation of pain, feeding and locomotion, whereas the CB₂ cannabinoid receptor is primarily implicated in immunomodulation and inflammatory processes.

In 1994, anandamide (*N*-arachidonylethanolamine, AEA), was the first member of a family of endogenous lipids termed endocannabinoids to be identified. Other endocannabinoids – including 2-arachidonoyl glycerol, virodhamine and noladin ether – were soon isolated and characterized (see Lambert and Fowler, 2005 for a review).

To date, four enzymes responsible for the termination of endocannabinoid signal have been identified by molecular cloning. These include fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), monoacylglycerol lipase (MAGL) (Karlsson et al., 1997), as well as the recently cloned *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005) and fatty acid amide hydrolase-2 (FAAH-2) (Wei et al., 2006).

Several lines of evidence support the therapeutic interest of modulating FAAH activity. For example, using a model of FAAH^(-/-) mice, Cravatt's group pointed out the physiological relevance of FAAH in anandamide degradation and reported a phenotypic analgesia in mice lacking the enzyme (Cravatt et al., 2001; Lichtman et al., 2004b). This launched an effort towards a thorough molecular and pharmacological characterization of the protein, as well as the design of potent and selective FAAH inhibitors. An important breakthrough in the knowledge of FAAH was achieved with the elucidation of the tridimensional structure of the rat FAAH (Bracey et al., 2002). This structure unravelled how this 579 a.a. and 63 kDa

enzyme organizes itself as a dimeric protein, with two channels able to accommodate both the lipophilic nature of its substrate and the more hydrophilic ethanolamine leaving group. Moreover, two highly hydrophobic α -helices (α 18– α 19) constitute a lipophilic plateau, most likely anchoring the protein to cellular membranes (see McKinney and Cravatt, 2005 for a review on the fatty acid amide hydrolase).

Medicinal chemistry endeavours led to the discovery of several classes of inhibitors, including OL-135, an alpha-ketoheterocycle derivative (Boger et al., 2000, 2005), and URB597, a carbamate-type derivative (Kathuria et al., 2003; Tarzia et al., 2003). The inhibitors tested in vivo in rodent models showed analgesic and anxiolytic properties, further illustrating the therapeutic relevance of targeting this enzyme (Kathuria et al., 2003; Lichtman et al., 2004a).

Measurements of the potency of the new compounds were mainly conducted using rat brain membranes as enzyme source. However, given the potential of FAAH inhibitors in the treatment of several disorders such as anxiety and pain, efforts are aimed at obtaining potent and selective drug candidates directed against the human enzyme. The use of human brain homogenates as a source of human enzyme remains hardly conceivable, mainly for two reasons. First, the availability of the material is of critical concern and second, the heterogenous nature of the preparation would hamper the data interpretation since pharmacological and biochemical evidences suggest that endocannabinoids are hydrolyzed by additional enzymes.

Therefore the expression of a recombinant FAAH constitutes an answer to those concerns. Boger et al. reported the use of native and truncated (Δ 1–29) FAAH expressed in *E. coli*, based on a protocol published by Patricelli et al. (1998). However, fatty acid amide hydrolase, and more particularly the human enzyme, is known to be resistant to overexpression in heterologous hosts such as *E. coli*, resulting in very poor yields (Patricelli et al., 1998; Sipe et al., 2002).

In this context, the engineering of fusion proteins is a method widely used in order to obtain enhanced solubility and higher expression of the target proteins (Baneyx, 1999 and references therein).

We report here the heterologous expression of FAAH fused to the maltose-binding protein, as a powerful tool to obtain rat and human FAAH in high yields. This recombinant MBP-FAAH therefore constitutes a useful tool for the characterization of new enzyme inhibitors, as well as for the efficient comparison of inhibitors selectivity among species.

For example, URB597, a rat FAAH inhibitor with potent anxiolytic and analgesic activities, is shown here to be less effective than expected on the human enzyme.

Materials and methods

Expression plasmids

Rat FAAH cDNA (kindly donated by Prof. N. Ueda, Kagawa University, Japan) and human FAAH cDNA (kindly donated by Prof. B. F. Cravatt, Scripps Research Institute, USA) were introduced into pMAL-cRI vector (New England BioLabs) as an *EcoRI/SalI* fragment. pMAL-cRI vector introduces the maltose binding protein gene upstream of the inserted gene. Similarly to Patricelli et al. (1998), both FAAH genes encoded a truncated protein (Δ 1–29).

The 6-His MBP-FAAH construct was generated by PCR amplification and cloning in the pMAL-cRI vector. The Strep-tag MBP-FAAH construct was generated by inserting FAAH gene as an *EcoRI/XhoI* fragment into pASK43 plasmid, followed by subcloning in the pMAL-cRI vector as an *EcoRI/HindIII* fragment.

Protein expression

Expression of FAAH fused to MBP (MBP-FAAH) was carried out in the *E. coli* ER2508 strain (New England BioLabs). At an O.D. of $\sim$ 0.6, cultures were induced by the addition of IPTG (0.3 mM). After 2 h of incubation at 37 °C, cells were harvested by centrifugation, resuspended in buffer 1 (Tris 50 mM, NaCl 200 mM, DTT 10 mM pH 7.4) and lysed by sonication. An insoluble fraction containing FAAH activity was then isolated by centrifugation (10,000 g for 35 min, 4 °C). The MBP-FAAH present in the soluble fraction was submitted to further purification.

Purification of recombinant human MBP-FAAH

Amylose purification

The soluble fraction was loaded on an amylose resin chromatography column (New England BioLabs). After washing the column with buffer 1, the recombinant protein was eluted by addition of maltose 15 mM.

Ion metal affinity chromatography in native conditions

The sample (resuspended in buffer 1, without DTT) was loaded onto a nickel sepharose column. After washing, the recombinant protein was eluted using an imidazole gradient (0–200 mM) in buffer 2 (Tris 50 mM, NaCl 200 mM, pH 7.4).

Purification of human MBP-FAAH in denaturing conditions

After the centrifugation step, the insoluble fraction was washed two times in buffer 3 (urea 2 M, Tris 20 mM, NaCl 500 mM, triton 2%, pH 8). The insoluble proteins were then solubilized in buffer 4 (guanidine 6 M, Tris 20 mM, NaCl 500 mM, imidazole 5 mM, β -mercaptoethanol 2 mM, pH 8). After centrifugation, the denatured proteins were submitted to ion metal affinity chromatography, and eluted using an imidazole gradient (0–500 mM).

Purification on streptactin resin

Soluble fraction (in Tris 20 mM, NaCl 200 mM, DTT 10 mM, CHAPS 0.5%, pH 8.3) was loaded on a streptactin resin chromatography column (IBA protein tools). After washing, recombinant protein was eluted by addition of 2.5 mM desthiobiotin.

Fractions purity was assessed by standard polyacrylamide gels electrophoresis, followed by Coomassie blue or silver staining. For Western blot studies, proteins were electrophoresed on acrylamide gels (Nupage 4–12% in Bis-Tris buffer, Invitrogen) and transferred on nitrocellulose membranes. Immunodetection was then carried out using anti-MBP (New England Biolabs) or anti-FAAH antibodies (Cayman).

Enzyme assay

Fatty acid amide hydrolase activity was measured using a method adapted from Omeir et al. (1995). Briefly, radiolabeled [^3H]-anandamide (American radiolabeled Chemicals, final concentration 2 μM , 60 Ci/mol) was incubated for 10 min at 37 °C in the presence of recombinant MBP-FAAH protein in 200 μl of reaction volume (assay buffer: Tris-HCl 10 mM, EDTA 1 mM, BSA 0.1% (w/v), pH 7.6). Anandamide hydrolysis was stopped by the addition of 400 μl of a cold methanol/chloroform (1–1) mixture. After centrifugation at 700 g for 5 min, radioactivity in the upper aqueous phase was measured by liquid scintillation.

To measure the inhibitors potency, compounds were diluted in the adequate solvent (DMSO, ethanol, or methyl acetate). The IC_{50} were determined and the K_i were calculated using the formula: $K_i = \text{IC}_{50} / (1 + [\text{AEA}] / K_m)$. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma, arachidonyl trifluoromethyl ketone (ATFMK) from Tocris, methyl arachidonyl fluorophosphonate (MAFP), cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597) and phenyl hexanoyl oxazolopyridine (CAY10402) from Cayman Chemical, and cyclohexylcarbamic acid biphenyl-4-yl ester (compound **26** from Tarzia et al., named "Tar26" herein) was synthesized following Tarzia et al. (2003) procedure.

Where specified, rat brain homogenates were used to compare anandamide hydrolase activity. Briefly, male Wistar rats (250–300 g) were purchased from IFFA-CREDO (Les Oncins, France). Following decapitation, brains were carefully dissected on ice. All the manipulations were performed at 0–4 °C. Tissues were homogenized in 20 mM HEPES, 1 mM MgCl_2 , pH 7.0 using a potter and subsequently centrifuged for 20 min at 36,000 g. The pellet was resuspended in the same buffer and centrifuged again for 20 min at 36,000 g. The latter operation was performed twice. The resulting pellet was stored at –80 °C in a conservation buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl_2 , pH 7.6). All experiments on animals were approved by the institutional ethics committee, and the housing conditions were as specified by the Belgian Law of November 14, 1993, on the protection of laboratory animals (agreement no. LA 1230315).

Results and discussion

Overexpression of fatty acid amide hydrolase as a fusion to maltose-binding protein

Fatty acid amide hydrolase belongs to the recently discovered endocannabinoid system, and the therapeutical potential of this membrane-bound protein is currently explored, notably since its inhibition was reported to induce analgesia and anxiolysis in rodents (Kathuria et al., 2003). Development of new drugs targeting more efficiently the human enzyme requires the production of a recombinant human FAAH protein. However, as it might be seen when considering the expression yield of the native protein in *E. coli*, FAAH is refractory to overexpression. Indeed, human enzyme has been reported to be even more difficult to produce and purify than the rat

FAAH (Patricelli et al., 1998; Sipe et al., 2002). Beside this, methods commonly used to improve the expression of proteins in *E. coli* include the coexpression of chaperones, the use of modified *E. coli* strains encoding tRNA's for rare codons and the engineering of fusion proteins.

In this context, several studies have highlighted the advantages of fusing target proteins to other ones, especially the maltose-binding protein. For instance, *Plasmodium falciparum* falcipain-2 (Goh et al., 2003), *Pseudomonas aeruginosa* Outer Membrane Protein OprD (Epp et al., 2001), alanine carrier protein (Kanamori et al., 1999) and several GPCRs (Grissamer et al., 1993; Stanasila et al., 1999) have been successfully expressed by fusion to MBP. Two hypotheses explaining the role of MBP in successful overexpression involve the enhanced solubility of the passenger protein when fused to MBP (Fox et al., 2003), as well as the ability of MBP to act as, or to recruit, chaperones in the vicinity of the growing protein, thus helping the proper folding of the peptide (Baneyx, 1999; Kapust and Waugh, 1999).

We therefore applied these strategies in order to reach high levels of expression of the fatty acid amide hydrolase.

We produced in high yield fatty acid amide hydrolase as a fusion to maltose binding protein (MBP-FAAH) in *E. coli* ER2508, a strain lacking the maltose-binding protein gene (Fig. 1). Typical yields were of tens of milligrams by liter of culture medium. This should be compared with the 1–1.5 mg/l of medium obtained by

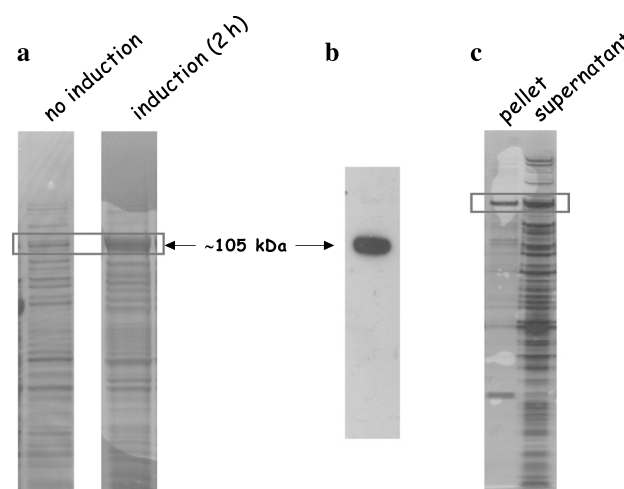


Fig. 1. Expression of MBP-FAAH in *E. coli*. Electrophoresis of soluble *E. coli* proteins via (a) Coomassie blue staining, (b) Western blot using anti-FAAH antibody, before and following IPTG induction (2 h–37 °C). The arrow indicates the band corresponding to MBP-FAAH (105 kDa). c Silver staining: pellet and supernatant fractions after centrifugation (10,000 g) of the crude lysate

Patricelli et al. (1998). As can be seen in Fig. 1b, an antibody raised against the C-terminal part of the fatty acid amide hydrolase only recognizes the overexpressed protein with the expected molecular weight of ~105 kDa.

In order to ensure that fusing FAAH to maltose binding protein does not result in an abolishment of the hydrolase activity, we compared the activities of MBP-FAAH and anandamide hydrolase from a rat brain homogenate. Kinetic parameters were found similar for the enzymes from both sources, with K_m values of $5.31 \pm 1.0 \mu\text{M}$ and $3.68 \pm 0.64 \mu\text{M}$ for rat MBP-FAAH and anandamide hydrolase from rat brain homogenates, respectively.

It is interesting to note that the MBP-FAAH protein was found both in the supernatant and in the pellet fraction following centrifugation (Fig. 1c). Actually, the recombinant protein found in the insoluble fraction constitutes an enriched source of nearly pure enzyme, suitable for enzymatic studies and inhibitors evaluation.

MBP-FAAH purification

In an attempt to assess if the overexpressed human MBP-FAAH could be amenable to structural studies purposes, the soluble fraction was submitted to purification.

First, the affinity of the MBP moiety for amylose was exploited in a chromatography column. This resulted in an enriched MBP-FAAH fraction (Fig. 2a).

A second fusion protein, bearing a 6-his tag at C-term of the protein (MBP-FAAH-his), was engineered in order to enhance the purification. Unfortunately, the affinity of

MBP-FAAH-his for Ni^{++} or Co^{++} ion metals was very low and no major purity improvement was obtained.

Different resins, including hydrophobic and anion- or cation-exchange resins, were then tested. The most promising results were obtained using an anion exchange chromatography as first step of the purification scheme. Flow-through from this column, containing FAAH activity, was subsequently loaded on an amylose column. This strategy led to a relative good level of purity, but unfortunately some impurities (with a molecular weight between 50 and 100 kDa) were still present in the sample.

In an effort to understand the difficulties of purifying MBP-FAAH and to gain information concerning the nature of the contaminants, a Western Blot using antibodies raised against the MBP moiety was performed. All the impurities present in the sample were detected by the anti-MBP antibody. Furthermore, it could be stated that these impurities only occur during the overexpression process (Fig. 3a). Therefore, we postulate that high expression level of MBP-FAAH in *E. coli* is accompanied by the co-expression of a series of by-products of the protein (Fig. 3), that could not be separated from the complete MBP-FAAH.

If this assumption is correct, the use of a C-term tag should allow to overcome the problem. However, as stated above, C-term 6-his tag did not result in any efficient binding of the protein. Therefore, we tried a C-term Strep-tag, which allowed us to reach 90% of purity in one step, using a streptactin resin. As it can be seen in Fig. 2c, some expression by-products still remain in the eluted fraction, to a much lower extent though compared to the

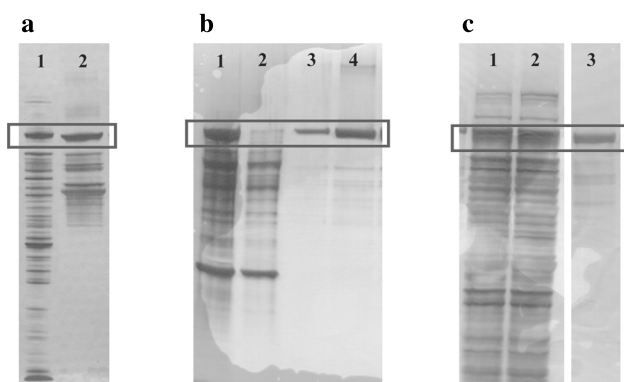


Fig. 2. Chromatographic purification of MBP-FAAH. **a** Purification of MBP-FAAH on an amylose resin: 1 supernatant fraction; 2 elution from amylose column (maltose 15 mM). **b** Ion metal affinity chromatography in denaturing conditions: 1 insoluble *E. coli* protein fraction in guanidine 6 M; 2 flow-through fraction; 3–4 elution from nickel resin. **c** Purification of MBP-FAAH on a streptactin column: 1 supernatant fraction; 2 flow-through fraction; 3 elution from streptactin column

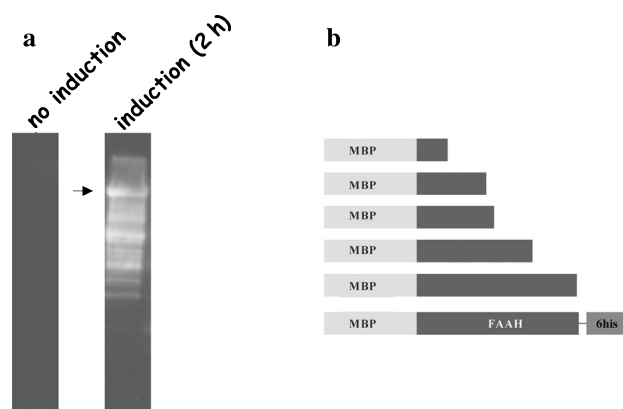


Fig. 3. MBP-FAAH is co-expressed with lower molecular weight products in *E. coli*. **a** Electrophoresis of soluble *E. coli* proteins followed by anti-FAAH antibody revelation, before and following IPTG induction (2 h–37 °C). **b** Hypothesis of MBP-FAAH and MBP-FAAH by-products co-expression

protein purified using amylose affinity chromatography. The difficulty to eliminate these impurities most probably reflects secondary interactions between some hydrophobic portions of the protein, resulting in soluble aggregates. Therefore, this method does not seem to be applicable to crystallization of human FAAH, as these non specific hydrophobic interactions lead to a non homogenous preparation. In addition, inefficient binding to the streptactin resin results in a loss of MBP-FAAH protein during the purification procedure. Nevertheless, using this method, the yield of purification was of about 5 mg/l of culture medium.

Noteworthy, the alternative strategies we used to enhance the expression level of FAAH in *E. coli* did not succeed. For example, the use of *E. coli* Rosetta strain, a strain known to give an answer to the rare codons problem, and of five different strains co-producing the chaperones DnaK, DnaJ, GrpE, GroES and GroEL (alone or in association) did neither enhance the expression level of native FAAH nor improve the MBP-FAAH by-products coexpression problem (data not shown).

Inhibitor selectivity between rat and human FAAH

The second aim of this overexpression strategy was to provide an abundant and reliable source of enzyme in order to set up several enzymology studies. For instance, the high yield of bacterial expression of FAAH using fusion to maltose-binding protein allows to screen for differences in FAAH inhibitors efficiencies or to compare inhibitor selectivity among species. Human and rat enzymes share 82% amino acid identity (Giang and Cravatt, 1997). Analysis of the tridimensional FAAH structure reveals that some of the differences in amino acids are found in the active site of the enzyme and therefore could result in changes in inhibitor recognition.

Along this line, a series of six well known FAAH inhibitors – four irreversible inhibitors (i.e. URB597,

“Tar26”, MAFP and PMSF) and two reversible compounds (i.e. ATFMK and CAY10402) – were studied for their ability to inhibit the rat and human enzymes. Thus the IC₅₀ were determined using both rFAAH-MBP and hFAAH-MBP and the K_i values were calculated.

In five cases, human recombinant FAAH seems to be more prone to inhibition than the rat protein, as summarized in Table 1. Indeed, the $K_i^{\text{rat}}/K_i^{\text{human}}$ ratio ranged from 1.85 for Tar26 to 6.75 for PMSF. However, in the case of URB597, it appears that the rat enzyme is more potently inhibited than the human one ($K_i^{\text{rat}}/K_i^{\text{human}} = 0.53$). Actually, despite the 500 fold difference between URB597 and “Tar26” IC₅₀ values on rat brain homogenates reported by Piomelli’s group (Tarzia et al., 2003; Kathuria et al., 2003), in our hands the difference between these compounds in the same preparation reached only 4. Moreover, if we compare the inhibitor potency for URB597 and “Tar26” on rat and human enzymes, we observe that this ratio of 4 is verified for the rat enzyme (4.14), and felt to 1.18 in the case of the human FAAH.

If we compare it to other compounds, URB597 is ~2 and ~80 times more potent than ATFMK and PMSF, respectively, on the rat enzyme. On the human FAAH however, URB597 is only 6 times better than PMSF and 2 times less potent than ATFMK.

These values suggest that URB597, despite being a potent inhibitor of rat FAAH and showing interesting anxiolytic and analgesic properties in rats, loses part of its potency when applied to human enzyme. Given that human and rat enzymes present 18% differences in amino acids, we can hypothesize that an interaction between rat FAAH and URB597 is lost with the human enzyme.

Conclusions

In this study, different strategies in order to enhance the heterologous expression of both rat and human fatty acid amide hydrolase in *E. coli* were applied. The most successful involved the expression of rat and human fatty acid amide hydrolase fused to maltose binding protein.

This strategy led to the expression of ~50 mg/l of medium, 50 times higher compared to the native enzyme. This recombinant engineered protein is active and displays the same Michaelis constant (K_m) as the anandamide hydrolase activity from rat brain homogenates, the commonly used FAAH source (Tarzia et al., 2003; Omeir et al., 1995).

Interestingly, after expression in *E. coli* and centrifugation of the bacterial lysate, MBP-FAAH protein and activity localize in the insoluble, as well as in the soluble

Table 1. Inhibition potencies for anandamide hydrolase activity on rat and human MBP-FAAH fusion proteins. Potencies are expressed as p*K_i* values (p*K_i* = -log *K_i*). Values are the mean ± SEM of at least two experiments done in duplicate

	Rat	Human
PMSF	5.81 ± 0.04	6.64 ± 0.03
ATFMK	7.41 ± 0.02	7.91 ± 0.04
MAFP	8.69 ± 0.04	9.48 ± 0.09
CAY10402	8.92 ± 0.03	9.30 ± 0.05
“Tar26”	7.08 ± 0.03	7.35 ± 0.05
URB597	7.70 ± 0.05	7.42 ± 0.04

fractions. Both can thus be used as source of FAAH activity, since we did not observed differences between the enzymatic properties of these two fractions (data not shown). The fact that MBP-FAAH activity is notably found in the pellet after centrifugation could reflect the hydrophobic nature of the fatty acid amide hydrolase (Bracey et al., 2004).

Use of an antibody raised against the C-term part of the fusion protein (i.e. FAAH) only revealed one band, at the expected molecular weight of ~105 kDa. On the contrary, an antibody raised against the N-term MBP highlights a series of proteins, whose molecular weights range between ~50 and ~100 kDa. Moreover, these contaminants are not present before induction (Fig. 3). Therefore, we postulated that the high level of expression in *E. coli* is accompanied by the production of several by-products, probably resulting from a premature stop in transcription or translation. In agreement with this hypothesis, the use of a C-term his-tag for purification (in denaturing conditions) resulted in a pure protein, ruling out a possible role of proteolysis (Fig. 2b). Due to the poor affinity of the his-tag protein in native conditions, we generated a fusion protein carrying a C-term Strep-tag. This small tag allowed us to purify the MBP-FAAH to a good extent (90%) in one step. However, this protein fraction does not seem to be suitable for crystallographic purposes, since some products of lower molecular weight, whose elimination is problematic, still remain in the preparation.

The high level of expression of fatty acid amide hydrolase in fusion with MBP, besides further illustrating the interest of engineering target proteins refractory to heterologous expression, permits the massive production of pure FAAH activity and therefore allows for the easy onset of different kinetics and inhibitors characterization studies.

For instance, in this study, we investigated the recognition profile of a series of six reversible and irreversible inhibitors on rat and human FAAH recombinant proteins.

Among these compounds URB597, reported to have strong anxiolytic and analgesic properties in rat through inhibition of FAAH (Tarzia et al., 2003), proved to be less potent on the human enzyme as compared to the rat one. This once again illustrates the caution needed when extrapolating inhibitors potencies from species to species, and strengthens the need to design new compounds targeting the human enzyme.

Acknowledgments

The authors would like to greatly thank Professor B. F. Cravatt and Professor N. Ueda for providing FAAH encoding plasmids. We also thank Professor V. Stalon, C. Tricot and Y. Oudjama (Institut de recherches

microbiologiques JM Wiame, Belgium) and Dr. G. G. Muccioli (Pharmacology Department, University of Washington, USA) for very helpful discussion on protein expression and purification. This work was supported by the FRFC (FNRS) no. 2.4654.06 grant.

References

- Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol* 10: 411–421
- Boger DL, Sato H, Lerner AE, Hedrick MP, Fecik RA, Miyauchi H, Wilkie GD, Austin BJ, Patricelli MP, Cravatt BF (2000) Exceptionally potent inhibitors of fatty acid amide hydrolase: the enzyme responsible for degradation of endogenous oleamide and anandamide. *Proc Natl Acad Sci USA* 97: 5044–5049
- Boger DL, Miyauchi H, Du W, Hardouin C, Fecik RA, Cheng H, Hwang I, Hedrick MP, Leung D, Acevedo O, Guimaraes CR, Jorgensen WL, Cravatt BF (2005) Discovery of a potent, selective, and efficacious class of reversible alpha-ketoheterocycle inhibitors of fatty acid amide hydrolase effective as analgesics. *J Med Chem* 48: 1849–1856
- Bracey MH, Hanson MA, Masuda KR, Stevens RC, Cravatt BF (2002) Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science* 298: 1793–1796
- Bracey MH, Cravatt BF, Stevens RC (2004) Structural commonalities among integral membrane enzymes. *FEBS Lett* 567: 159–165
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384: 83–87
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* 98: 9371–9376
- Epp SF, Pechere J, Kok M (2001) Raising antibodies against OprD, an outer membrane protein of *Pseudomonas aeruginosa* using translational fusions to MalE. *J Microbiol Methods* 46: 1–8
- Fox JD, Routzahn KM, Bucher MH, Waugh DS (2003) Maltodextrin-binding proteins from diverse bacteria and archaea are potent solubility enhancers. *FEBS Lett* 537: 53–57
- Giang DK, Cravatt BF (1997) Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc Natl Acad Sci USA* 94: 2238–2242
- Goh LL, Loke P, Singh M, Sim TS (2003) Soluble expression of a functionally active *Plasmodium falciparum* falcipain-2 fused to maltose-binding protein in *Escherichia coli*. *Protein Expr Purif* 32: 194–201
- Grishammer R, Duckworth R, Henderson R (1993) Expression of a rat neurotensin receptor in *Escherichia coli*. *Biochem J* 295: 571–576
- Kanamori M, Kamata H, Yagisawa H, Hirata H (1999) Overexpression of the alanine carrier protein gene from thermophilic bacterium PS3 in *Escherichia coli*. *J Biochem (Tokyo)* 125: 454–459
- Kapust RB, Waugh DS (1999) *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci* 8: 1668–1674
- Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C (1997) cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. *J Biol Chem* 272: 27218–27223
- Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* 9: 76–81
- Lambert DM, Fowler CJ (2005) The endocannabinoid system: drug targets, lead compounds, and potential therapeutic applications. *J Med Chem* 48: 5059–5087
- Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL, Cravatt BF (2004a) Reversible inhibitors of fatty acid

- amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J Pharmacol Exp Ther* 311: 441–448
- Lichtman AH, Shelton CC, Advani T, Cravatt BF (2004b) Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* 109: 319–327
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346: 561–564
- McKinney MK, Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem* 74: 411–432
- Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365: 61–65
- Omeir RL, Chin S, Hong Y, Ahern DG, Deutsch DG (1995) Arachidonoyl ethanolamide-[1,2-¹⁴C] as a substrate for anandamide amidase. *Life Sci* 56: 1999–2005
- Patricelli MP, Lashuel HA, Giang DK, Kelly JW, Cravatt BF (1998) Comparative characterization of a wild type and transmembrane domain-deleted fatty acid amide hydrolase: identification of the transmembrane domain as a site for oligomerization. *Biochemistry* 37: 15177–15187
- Sipe JC, Chiang K, Gerber AL, Beutler E, Cravatt BF (2002) A missense mutation in human fatty acid amide hydrolase associated with problem drug use. *Proc Natl Acad Sci USA* 99: 8394–8399
- Stanasila L, Massotte D, Kieffer BL, Pattus F (1999) Expression of delta, kappa and mu human opioid receptors in *Escherichia coli* and reconstitution of the high-affinity state for agonist with heterotrimeric G proteins. *Eur J Biochem* 260: 430–438
- Tarzia G, Duranti A, Tontini A, Piersanti G, Mor M, Rivara S, Plazzi PV, Park C, Kathuria S, Piomelli D (2003) Design, synthesis, and structure-activity relationships of alkylcarbamate acid aryl esters, a new class of fatty acid amide hydrolase inhibitors. *J Med Chem* 46: 2352–2360
- Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, Ueda N (2005) Molecular characterization of N-acyl ethanolamine-hydrolyzing acid amidase, a novel member of the cholesteryl glycerophosphorylcholine hydrolase family with structural and functional similarity to acid ceramidase. *J Biol Chem* 280: 11082–11092
- Wei BQ, Mikkelsen TS, McKinney MK, Lander ES, Cravatt BF (2006) A second fatty acid amide hydrolase with variable distribution among placental mammals. *J Biol Chem* 281: 36569–36578

Authors' address: Didier M. Lambert, Unité de Chimie pharmaceutique et de Radiopharmacie, Université catholique de Louvain, Avenue E. Mounier 73.40, 1200 Bruxelles, Belgique,
Fax: +3227647363, E-mail: lambert@cmfa.ucl.ac.be