Closing the Gate to the Active Site

EFFECT OF THE INHIBITOR METHOXYARACHIDONYL FLUOROPHOSPHONATE ON THE CONFORMATION AND MEMBRANE BINDING OF FATTY ACID AMIDE HYDROLASE

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Giampiero Mei,*1 Almerinda Di Venere†, Valeria Gasperi*§, Eleonora Nicolai†, Kim R. Masuda†, Alessandro Finazzi-Agrò§, Benjamin F. Cravatt‖, and Mauro Maccarrone*‡,‡ From the 1INFN and 2Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", 00133 Rome, Italy, the 3Department of Biomedical Sciences, University of Teramo, 64100 Teramo, Italy, 4European Center for Brain Research/ Istituto di Ricovero e Cura a Carattere Scientifico S. Lucia Foundation, 00143 Rome, Italy, and the 5Departments of Cell Biology and Chemistry, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037

Fatty acid amide hydrolase (FAAH) is a dimeric, membrane-bound enzyme that degrades neuropeptidatory fatty acid amides and esters and is expressed in mammalian brain and peripheral tissues. The cleavage of ~30 amino acids from each subunit creates an FAAH variant that is soluble and homogeneously in detergent-containing buffers, opening the avenue to the in vitro mechanistic and structural studies. Here we have studied the stability of FAAH as a function of guanidinium hydrochloride concentration and of hydrostatic pressure. The unfolding transition was observed to be complex and required a fitting procedure based on a three-state process with a monomeric intermediate. The first transition was characterized by dimer dissociation, with a free energy change of ~11 kcal/mol that accounted for ~80% of the total stabilization energy. This process was also paralleled by a large change in the solvent-accessible surface area, because of the hydration occurring both at the dimeric interface and within the monomers. As a consequence, the isolated subunits were found to be much less stable (ΔG ~ 3 kcal/mol). The addition of methoxyarachidonyl fluorophosphonate, an irreversible inhibitor of FAAH activity, enhanced the stability of the dimer by ~2 kcal/mol, toward denaturant- and pressure-induced unfolding. FAAH inhibition by methoxyarachidonyl fluorophosphonate also reduced the ability of the protein to bind to the membranes. These findings suggest that local conformational changes at the level of the active site might induce a tighter interaction between the subunits of FAAH, affecting the enzymatic activity and the interaction with membranes.

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§ To whom correspondence may be addressed: Dept. of Experimental Medicine and Biochemical Sciences, University of Rome “Tor Vergata”, Via Montpellier 1, 00133 Rome, Italy. Tel.: 39-06-72596460; Fax: 39-06-72596468; E-mail: mei@med.uniroma2.it.

‖ To whom correspondence may be addressed: Dept. of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, I-64100 Teramo, Italy. Tel.: 39-0861-266875; Fax: 39-0861-412583; E-mail: mmaccarrone@unite.it.

The abbreviations used are: FAAH, fatty acid amide hydrolase; FAAAs, fatty acid amides; AS, amidase; signature; ΔΤM-FAAH, catalytically active fatty acid amide hydrolase mutant; MAFP, methoxyarachidonyl fluorophosphonate; GdmHCl, guanidinium hydrochloride; ASA, accessible surface area.
rounded by a large number (28) of α-helices (Fig. 1, top). The active site pocket is located in each subunit below the β-core, next to a large hydrophobic portion that allows the bottom of the enzyme to dip into the membrane lipid bilayer. Two tryptophans are buried within this functional area. The first residue, Trp531, is located at one end of the channel leading from the protein-membrane interface to the cytosolic port located at the opposite side. The second residue, Trp445, belongs to the other subunit and blocks another potential route for substrates. The position of the seven tryptophan residues present in each subunit is shown in Fig. 1, top, together with their respective solvent-exposed area (Fig. 1, bottom). On this basis, we attempted herein to investigate the structural features that determine FAAH activity and its membrane binding properties by spectroscopic techniques (i.e. circular dichroism, steady-state, and dynamic fluorescence) and by enzyme-linked immunosorbent assays of FAAH bound to synthetic vesicles. Reversible dissociation into stable monomers was obtained by high pressure. The data provide strong evidence that the protein quaternary structure is needed to achieve stability, by shielding from solvent not only the residues at the dimeric interface but also those buried inside each subunit. Not surprisingly, this folding strategy appears to be optimal to match the functional requirements of FAAH.

EXPERIMENTAL PROCEDURES

Materials, Antibodies, and Enzymes—MAFP was from Cayman Chemicals (Ann Arbor, MI). Guanidinium hydrochloride (GdmHCl) was from Roche Applied Science. Rabbit anti-FAAH polyclonal antibodies (15) were prepared by Primm S.r.l. (Milan, Italy), and goat anti-rabbit alkaline phosphatase conjugates (GAR-AP) were from Bio-Rad. The catalytically active mutant ΔTM-FAAH, still able to bind to membranes (14), was expressed in Escherichia coli with a hexahistidine tag and was extracted from cell lysates with lauryl-N-dimethylamino oxide (1). The solubilized protein was purified by a combination of metal affinity, heparin, and gel filtration chromatography (1), and the purified enzyme was used throughout the investigation. This mutant is referred to as “FAAH.” Enzyme activity was assayed by measuring the release of [14C]ethanolamine from [14C]anandamide (5 mCi/mmol; ARC, St. Louis, MO), using liquid scintillation counting (16).

Membrane Binding Assays—Negatively charged, unsaturated phosphatidylcholine liposomes were prepared in phosphate-buffered saline using the liposome kit (Sigma) as reported (17). The size of the vesicles was ~2 μm, as determined under the microscope by comparison with the size (10 ± 0.5 μm) of Mono Q beads (Amersham Biosciences). The binding of FAAH to liposomes was determined essentially as reported (17). Preliminary experiments showed that incubation of liposome suspensions corresponding to 1 μmol of phosphatidylcholine (50 μl) with 100 pmol of FAAH (corresponding to 2 μM) for 0–60 min at 25 °C yielded the highest amount of bound FAAH (i.e. 28 ± 3 pmol) after 30 min, without further increase in the following 30 min. Therefore, in the first set of

FIGURE 1. Top, ribbon model of the dimeric unit of FAAH (Protein Data Bank file 1MT5); shown in yellow is the β-core for both subunits and in blue the α-helices. The tryptophan residues side chains belonging to subunits A (left) and B (right) are colored in purple and red, respectively. Tryptophans and helices 18 and 19 on the B subunit are also labeled by their number. The MAFP molecule is reported in green. Bottom, accessible surface area for tryptophan residues.
experiments 2 μM FAAH was incubated for 30 min with liposome suspensions, before or after 30 min of incubation with various amounts of MAFP (0–100 μM range). In another set of experiments, 2 μM FAAH was incubated with 100 μM MAFP for different periods of time (0–60 min) and then with liposome suspensions for 30 min. The enzyme-liposome complexes were separated by flotation on a linear sucrose density gradient (18), and then an enzyme-linked immunosorbent assay was performed by coating the plate with enzyme-liposome complexes (50 μl/well). Specific anti-FAAH polyclonal antibodies (15), diluted 1:400, were used as first antibody, and goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad), diluted 1:2000, as second antibody. Color development of the alkaline phosphatase reaction was measured at 405 nm, using p-nitrophenyl phosphate as substrate. Controls included wells coated with nonimmune rabbit serum (Primm) or with different amounts of bovine serum albumin. The enzyme-linked immunosorbent assay test was calibrated using different amounts of FAAH (0–100 pmol/well), in the presence of liposomes (1 μmol of phosphatidylcholine/well). The absorbance values at 405 nm (A405) of unknown samples were within the linearity range of the calibration curve.

**Spectroscopic Assays**—Steady-state fluorescence spectra were recorded on a photon counting spectrometer (ISS, model K2, Champaign, IL). Dynamic fluorescence measurements were carried out using the phase-shift and demodulation technique (19). The light source of the dynamic fluorometer (ISS, model Koala, Champaign, IL) was a laser diode, whose emission wavelength was 405 nm, using p-nitrophenyl phosphate as substrate. Controls included wells coated with nonimmune rabbit serum (Primm) or with different amounts of bovine serum albumin. The enzyme-linked immunosorbent assay test was calibrated using different amounts of FAAH (0–100 pmol/well), in the presence of liposomes (1 μmol of phosphatidylcholine/well). The absorbance values at 405 nm (A405) of unknown samples were within the linearity range of the calibration curve.

**Equilibrium Unfolding Measurements**—Protein denaturation by guanidine hydrochloride (GdmHCl) was obtained after a 12-h incubation at 4 °C in the presence of different amounts of denaturant. Refolding of unfolded FAAH samples was achieved by diluting the denaturant with buffer. The transition was monitored by fluorescence and circular dichroism measurements. Data analysis was performed according to Scheme 1.

\[
\begin{align*}
N_2 & \leftrightarrow 2M \\
2U & \leftrightarrow 2M
\end{align*}
\]

**SCHEME 1**

where \(N, M, \) and \(U\) represent the native, intermediate, and unfolded protein species, and \(K_1\) and \(K_2\) are linked to the mole fraction of each species \(f_{N}, f_{M}, \) and \(f_{U}\) \((f_{N} + f_{M} + f_{U} = 1)\) according to Equation 1.

\[
\begin{align*}
f_u & = -K_1K_2(1 + K_2) + \frac{\sqrt{(K_1K_2)^2(1 + K_2)^2 + 4[N_0]K_1K_2^2}}{2[N_0]} \\
f_M & = \frac{f_U}{K_1} \\
f_N & = \frac{f_U[N_0]}{K_1K_2^2}
\end{align*}
\]

\[(\text{Eq. 1})\]

where \([N_0]\) represents the initial dimer concentration. The experimental data, \(Y\), have been fitted using the linear combination as shown in Equation 2.

\[
Y = f_NY_N + f_MY_M + f_UY_U
\]

**RESULTS**

**GdmHCl-induced Unfolding of FAAH**—The stability of FAAH has been studied by equilibrium unfolding measurements, following the change in intrinsic fluorescence. In particular, the red shift of the spectrum has been recorded as a function of GdmHCl concentration (Fig. 2). The transition from native to unfolded state is complex and suggests the presence of at least one intermediate species (Fig. 2). In order to ascertain whether dissociation into monomers was taking place, the measurements were performed at different protein concentrations. The data, reported in Fig. 2, demonstrate that the transition is concentration-dependent in the range 0–1 M GdmHCl.
Structural Properties of FAAH

consistently with the occurrence of a monomerization process. The data have therefore been fitted according to a two-step denaturation pathway, including a monomeric intermediate species (see "Experimental Procedures"). The best fitting parameters obtained with this model are shown in Table 1. The corresponding changes in the solvent-accessible surface area (ΔASA) have also been evaluated from the \( m_1 \) and \( m_2 \) values, according to Myers et al. (22) and are reported in Table 1. In a parallel experiment, circular dichroism spectra have been recorded in the peptide region (200–250 nm), in order to monitor the loss of secondary structure. The normalized signal intensity at 220 nm is reported in the inset of Fig. 2. The data have been fitted as above for the fluorescence measurements, and the results are reported in Table 1.

Pressure-induced Dissociation of FAAH—The existence of partially folded monomers along the unfolding landscape of FAAH allows the study of the oligomerization process in more detail, by means of high pressure denaturation measurements. The process is concentration-dependent (Fig. 3), and the analysis of the transition curves yields a free energy change similar to that obtained in the first GdmHCl-induced denaturation step (compare Tables 1 and 2). The volume change occurring during the dissociation process is in the range −120–130 ml/mol, in line with the values reported in the literature for other dimeric proteins (21). Using the shift of the midpoint dissociation curves reported in Fig. 3, it is possible to obtain an independent estimation of \( \Delta V \sim −120 \) ml/mol, as described under "Experimental Procedures."

The coincidence within the experimental error of this \( \Delta V \) value with those reported in Table 2 demonstrates that the equilibrium follows a stochastic behavior, unlike the dissociation process observed for larger structures and aggregates (21). A comparison between the effects of low GdmHCl concentrations (\( \sim 0.6 \) m) and high pressure (\( \sim 2500 \) bars) on the protein fluorescence is shown in Fig. 4. The two spectra are superimposable, suggesting that a similar intermediate species is produced, independently of the method of perturbation used. In both cases, the broader profile obtained indicates the solvation of some tryptophan residues as a consequence of dissociation. From the crystallographic analysis (Fig. 1), it is tempting to speculate that this effect is due only to Trp\(^{245}\) and Trp\(^{356}\), which are located at the dimeric interface. The spectrum of the fully unfolded enzyme (in 3 M GdmHCl) supports this hypothesis because it exhibits more drastic changes, such as a more red-shifted position and the appearance of a shoulder at 320 nm. The latter effect is likely because of the contribution of tyrosines, which generally occurs at \( \sim 305 \) nm (19) and becomes detectable only in the unfolded protein. In fact, in this case the nonradiative energy transfer to tryptophan is negligible because of the mean larger distances among donors and acceptors.

MAFP Reduces the Binding of FAAH to Synthetic Vesicles—MAFP is an irreversible inhibitor of FAAH that has recently allowed crystallization and three-dimensional analysis of the enzyme at 2.8 Å resolution (1). Before studying the influence of MAFP on protein structure and stability, we have checked whether it was able to modify the interaction of FAAH with membranes. In the first set of experiments, 2 \( \mu \)M FAAH was preincubated with different concentrations of MAFP for 30 min and then with liposome suspensions for a further 30 min. The FAAH inhibitor was able to reduce dose-dependently the amount of FAAH bound to artificial membranes, with a significant effect at 10 \( \mu \)M MAFP and a maximum effect at 100 \( \mu \)M MAFP, corresponding to FAAH/MAFP molar ratios of 1:5 and 1:50, respectively (Fig. 5A). Under the latter conditions, only \( \sim 40\% \) of FAAH was bound to liposomes compared with controls (\( \sim 12 \pm 2 \) vs. \( 28 \pm 3 \) pmol). Instead, the addition of MAFP after incubation of FAAH with liposomes under the same experimental conditions did not significantly affect enzyme binding to the vesicles (Fig. 5A). In another set of experiments, 2 \( \mu \)M FAAH was preincubated with 100 \( \mu \)M MAFP for different periods of time (0–60 min) and then with liposomes for 30 min. These experiments showed that already after 5 min

![FIGURE 2. Steady-state fluorescence dependence on GdmHCl concentration.](image)

**TABLE 1**

Thermodynamic parameters characterizing the unfolding of FAAH

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \Delta G_{1}^{m} ) kcal/mol</th>
<th>( \Delta G_{2}^{m} ) kcal/mol</th>
<th>( m_1^{b} )</th>
<th>( m_2^{b} )</th>
<th>( \Delta ASA_1^{c} ) pmol</th>
<th>( \Delta ASA_2^{c} ) pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 ( \mu )M FAAH (fluorescence)</td>
<td>12.9 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>7.0 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>14,000 ± 200</td>
<td>6100 ± 400</td>
</tr>
<tr>
<td>0.02 ( \mu )M FAAH (fluorescence)</td>
<td>11.7 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td>7.2 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>14,400 ± 400</td>
<td>7000 ± 1000</td>
</tr>
<tr>
<td>0.2 ( \mu )M FAAH (CD)</td>
<td>12.2 ± 0.7</td>
<td>2.7 ± 0.4</td>
<td>6.1 ± 0.7</td>
<td>2.0 ± 0.3</td>
<td>12,000 ± 2000</td>
<td>5000 ± 1000</td>
</tr>
<tr>
<td>0.02 ( \mu )M FAAH (CD)</td>
<td>12.4 ± 0.7</td>
<td>2.7 ± 0.5</td>
<td>6.0 ± 0.5</td>
<td>2.0 ± 0.1</td>
<td>12,000 ± 1100</td>
<td>5000 ± 4000</td>
</tr>
<tr>
<td>0.2 ( \mu )M FAAH + 4 ( \mu )M MAFP (fluorescence)</td>
<td>14.5 ± 0.5</td>
<td>3.1 ± 0.1</td>
<td>6.5 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>12,800 ± 400</td>
<td>3800 ± 4000</td>
</tr>
</tbody>
</table>

* Free energy change in absence of denaturant.
* Cooperative index of each transition (see "Experimental Procedures").
* Change in the solvent-accessible surface area upon denaturation.
MAFP reduced to ~40% of the controls FAAH binding to liposomes, without any further reduction in the following 55 min (Fig. 5B). On the other hand, 10 μM MAFP added to 2 μM FAAH for 5 min, before or after incubation with liposomes for 30 min, fully and irreversibly inhibited enzyme activity, which decreased from 820 ± 50 nM/min of controls to undetectable levels in MAFP-treated samples. Taken together, these data suggest that MAFP rapidly (within 5 min) modifies the surface of FAAH, thus reducing its membrane binding ability; however, the MAFP-induced modification does not occur when FAAH is already bound to artificial membranes.

**MAFP Enhances the Stability of FAAH**—The observation that MAFP-treated FAAH binds to a lesser extent with synthetic membranes suggests that MAFP induces conformational changes to the enzyme. We have therefore re-investigated the fluorescence properties of FAAH as a function of GdmHCl and pressure in the presence of MAFP. As shown in Figs. 2 and 3, the transition curves are shifted toward higher denaturant concentrations and pressure values, respectively, and the best fits of the data yielded a larger $\Delta G$, as reported in Tables 1 and 2. Instead, the stability of the monomeric species ($G_2$) was not affected by MAFP (Table 1). The smaller $m_1$ and $m_2$ values obtained in the presence of MAFP (Table 1) suggest that a stiffer hydrophobic region is formed around the ligand-binding site. This effect is in line with the slight difference observed in the fluorescence spectrum, both at high denaturant concentrations and at high pressure. In particular, the asymmetric center of mass values at 3 M GdmHCl or 2300 bars, with or without MAFP, were less than 2 nm apart (Figs. 2 and 3), a finding that is diagnostic of a reduced solvation of the tryptophan residues in the presence of the ligand.

**Effects of MAFP Binding on FAAH Dynamic Fluorescence**—The effects of MAFP binding to FAAH have been monitored by binding of FAAH was assayed as follows: a, by incubating the enzyme with MAFP for 30 min at the indicated molar ratios before (white bars) or after (dark bars) the addition of liposomes for a further 30 min; b, preincubating 2 μM FAAH with 100 μM MAFP for different periods of time and then adding liposomes for 30 min. For details see “Experimental Procedures.” In both panels 100% = 28 ± 3 pmol FAAH, and * denotes $p < 0.01$ and ** denotes $p < 0.05$ versus controls.

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta G$ a</th>
<th>$\Delta V$ b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 μM FAAH</td>
<td>12.2 ± 0.2</td>
<td>119 ± 9</td>
</tr>
<tr>
<td>0.02 μM FAAH + 4 μM MAFP</td>
<td>11.6 ± 0.1</td>
<td>130 ± 7</td>
</tr>
</tbody>
</table>

a $\Delta G$ indicates free energy change.
b $\Delta V$ indicates volume change.

FIGURE 3. Effects of high pressure on the FAAH spectral center of mass in the case of a diluted (2.1 x 10^{-6} m) and concentrated (2.1 x 10^{-7} m) protein sample (open and filled circles, respectively). The triangles represent the concentrated sample in the presence of ~20 excess of MAFP. The solid lines correspond to the best fit obtained (Table 2).

FIGURE 4. Steady-state emission spectra of FAAH at 1 bar (solid line) and 2400 bars (dotted). The spectra in the presence of 0.6 M (filled diamonds) and 3 M GdmHCl (dashed line) at atmospheric pressure are also reported for comparison. a.u., absorbance units.

FIGURE 5. Effect of MAFP on the binding of FAAH to liposomes. Binding of FAAH was assayed as follows: a, by incubating the enzyme with MAFP for 30 min at the indicated molar ratios before (white bars) or after (dark bars) the addition of liposomes for a further 30 min; b, preincubating 2 μM FAAH with 100 μM MAFP for different periods of time and then adding liposomes for 30 min. For details see “Experimental Procedures.” In both panels 100% = 28 ± 3 pmol FAAH, and * denotes $p < 0.01$ and ** denotes $p < 0.05$ versus controls.
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Circular dichroism, steady-state, and dynamic fluorescence measurements. The CD spectra in the peptidic region (Fig. 6a) show a small change ($\approx -5\%$) at 222 nm suggesting that minor changes are induced at the secondary structure level. A more significant effect ($\approx -9\%$) is observed in the fluorescence emission spectrum (Fig. 6b), indicating that some modifications of the enzyme tertiary structure take place upon the addition of MAFP. This effect has been studied in more detail, by assessing the dependence of the FAAH fluorescence on MAFP concentration and excitation wavelength.

The fluorescence decay of FAAH, measured by phase and modulation techniques (19), is rather heterogeneous and is characterized by a double, continuous distribution of lifetimes (Fig. 7a). Such a complex decay depends on the presence of both solvent-exposed and fully buried tryptophan residues (Fig. 1) that give different contributions (i.e. at longer and shorter wavelengths, respectively) to the total fluorescence signal. This is confirmed by the biphasic trend of the fluorescence spectrum position at increasing excitation wavelengths; the center of mass is in fact shifted from $\approx 345$ to 360 nm, as a consequence of the photoselection of the more red-emitting species at longer excitation wavelengths (Fig. 7b). This effect is considerably reduced in the presence of MAFP (Fig. 7b), indicating that some partially exposed tryptophan residues became shielded upon ligand binding and were embedded in a more hydrophobic environment. This hypothesis would imply a reduced heterogeneity in the fluorescence dynamics, which has been indeed observed by measuring the lifetimes as a function of MAFP concentration (Fig. 7a).

**DISCUSSION**

**FAAH Stability Is Strongly Dependent on Its Quaternary Structure**—Dimeric proteins represent an effective paradigm of the hierarchical organization that characterizes the complexity of biological macromolecules. For instance, their quaternary structure can be regarded as the simplest case of protein-protein interaction. Moreover, proteins can gain new structural and functional properties upon association of subunits, which are often unstable when separated (23, 24). Fully folded monomeric intermediates are in fact an exception rather than a rule; in most cases they are only partially folded prior to association, resembling the so-called molten globule state, because of their loose tertiary structure. Thus, a possible rationale for subunit association into dimeric structures may reside in the enhanced stability gained by a better shielding of the hydrophobic regions from the solvent molecules. In line with this, the unfolding experiments indicate that most of the free energy change occurs during the first transition, i.e. when the two subunits are taken apart (Tables 1 and 2). This process is also accompanied by a considerable change in the accessible surface area, as deduced from the $m_1$ values reported in Table 1. It is noteworthy that such a change ($\Delta$ASA $\approx 14,000$ Å$^2$) is 3–4 times larger than that estimated from the crystallographic data (1) if only a dissociation into fully unfolded monomers had taken place (i.e. $1600$ Å$^2 \times 2 = 3200$ Å$^2$). Thus, it can be inferred that the protein quaternary structure protects not only the residues at the dimeric interface from solvation but also a part of those buried inside each subunit. This hypothesis is in line with the
loss (~15%) of the CD signal at 222 nm (Fig. 2, inset), diagnostic of a conformational change possibly associated with a partial loosening also of the protein secondary structure. According to high pressure measurements, the substantial volume decrease at 2300 bars (Table 2) would also point to the same direction, i.e. to water penetration into the protein core upon dissociation. Pressure is known to physically force the solvent to fill cavities inside a protein matrix, thus perturbing the native conformation. In fact, negative ΔV values such as that calculated here for FAAH have been found in the vast majority of pressure-induced dissociation and unfolding studies (21, 25).

The mutual protection from solvent molecules upon subunit association appears to be a well designed folding strategy to match the functional requirements of FAAH. Indeed the enzyme has to deal with hydrophobic substrates requiring an appropriate environment to be bound and processed. Such an environment, which includes a series of channels interconnected through the protein structure (1, 5), is probably the “Achilles’ heel” of the monomers, being more easily flooded by water when the two subunits are taken apart.

*Insights into Structural Changes Induced by MAFP Binding—* As shown in Figs. 2, 3, and 5 and in Tables 1 and 2, the binding of MAFP by FAAH has two important consequences as follows: (i) ~40% decrease in the ability of the protein to stick to the liposomes; (ii) ~12% increase in the stabilization of free energy. These features suggest that some kind of conformational change is occurring upon MAFP binding, which involves the protein domain that interacts with the surface of lipid bilayers. Previous studies have demonstrated that the trimmed enzyme (ΔTM-FAAH) is still active and able to bind to membranes, despite the lack of the 1–29 amino acid trans-membrane tail (14). The putative region of ΔTM-FAAH still interacting with the lipid leaflets has been identified to be the α-helices 18 and 19, located in the lower part of the protein structure (Fig. 1a), quite close to the substrate entrance gate to the active site from the membrane side (1). The CD spectra with or without MAFP (Fig. 6a) indicate that MAFP binding does not produce major modifications in the protein secondary structure, whereas significant effects are seen in the FAAH tertiary structure, as shown by steady-state (Figs. 6b and 7b) and dynamic fluorescence (Fig. 7a). The data demonstrate that MAFP binding effects are greater at longer excitation wavelengths, i.e. where the contribution of the “blue emitting” species (phenylalanines, tyrosines, and fully buried tryptophans) is negligible because of photoselectection. Looking at the location of the seven tryptophans per subunit of FAAH (Fig. 1a and b), it is evident that five of them belong to two quite distinct classes, i.e. solvent-exposed (Trp411, Trp508, and Trp570) and fully buried (Trp445 and Trp556) and Trp531) are instead only partially buried, the first one within the dimeric interface, and the second one between the active site and the membrane binding domain. The latter seems to be the best candidate to probe changes in the FAAH fluorescence dynamics upon MAFP binding, because of its peculiar location. In fact, the heterogeneity of the fast emission decay (Fig. 7a) is dramatically affected by binding of the inhibitor, which suggests an increased rigidity in the domain surrounding Trp531. In addition, Fig. 8 shows that the dynamics of this residue is constrained by the presence of the MAFP molecule. Also phenylalanine 432 cooperates with MAFP in the immobilization of the tryptophan 531 side chain. The lack of local mobility of Trp531 is in line with the reduced relaxation at λex ~300 nm, as reported in Fig. 7. The occurrence of larger structural changes upon MAFP binding may not be excluded, even if they cannot be detected by tryptophan fluorescence. As a matter of fact, these structural changes could be relevant, as demonstrated by the loss of the membrane binding ability (Fig. 5). In the same line, a tighter structure of the region around Trp531 might also indirectly affect the residues at the protein surface and, most
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notably, arginine 486 and aspartic acid 403 (Fig. 8). These two residues face the lipid bilayer when FAAH is bound to membranes and delineate the internal channel that crosses each subunit and contains the active site. Furthermore, it is important to note that the above-mentioned phenylalanine 432 belongs to one of the two α-helices (α19) which constitute the FAAH-membrane binding cap. It is therefore tempting to speculate that even a small displacement occurring in this portion of the protein might weaken the interaction with liposomes (Fig. 5).

The correlation between the presence of MAFP and the enhanced protein stability is more difficult to explain. One possibility is that upon ligand binding the two subunits can penetrate each other a little further, so that dissociation is more enhanced protein stability is more difficult to explain. One possibility is that upon ligand binding the two subunits can penetrate each other a little further, so that dissociation is more difficult (Tables 1 and 2 and Figs. 2 and 3). This hypothesis is also supported by the behavior of thermophilic enzymes. Their increased rigidity protects against unfolding and preserves the catalytically active structure at elevated temperature (26).

Quite interestingly, one peculiar FAAH region that can be involved in this process is the area around Trp445. This residue in fact protruding in each partner monomer, making the sur-

involved in this process is the area around Trp445. This residue is in fact protruding in each partner monomer, making the sur-

rounding patch particularly rich in contacts between two monomers. The proximity of Trp445 to the ligand-binding site of the partner chain makes this hypothesis extremely attractive, because it could provide new clues on the structure-to-function relationship of FAAH and, in particular, on the possible link between its quaternary interactions and the mechanism of substrate binding and release.

In conclusion, this is the first detailed investigation of the stability of FAAH under chemical or physical denaturation. The present findings suggest that local conformational changes at or near the active site might help to tighten the interaction between the two subunits of FAAH, affecting not only its activity but also its interaction with biological membranes.

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