Biochemical and Biophysical Research Communications 446 (2014) 137–142

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

New fluorescent probes for ligand-binding assays of odorant-binding proteins

Rosa Mastrogiacomo [a], Immacolata Iovinella [a], Elio Napolitano [b,*]

[a] Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy
[b] Scuola Normale Superiore and Department of Pharmacy of the University of Pisa, Pisa, Italy

ARTICLE INFO

Article history:
Received 6 February 2014
Available online 22 February 2014

Keywords:
Ligand-binding assay
Fluorescent probe
Odorant-binding proteins
Naphtalene derivatives
Anthracene derivatives
Fluoranthene derivatives

ABSTRACT

Fluorescence-linked binding assays allow determination of dissociation constants at equilibrium and have recently become increasingly popular, thanks to their ease of operation. Currently used probes, such as 1-aminoanthracene and N-phenyl-1-naphthylamine, are excited and emit in the ultraviolet region, but alternative ligands operating in the visible spectrum would be highly desirable for applications in biosensing devices. Based on the two above structures, we have designed and synthesised six new fluorescent probes to be used in ligand-binding assays. The compounds are derivatives of naphatalene, anthracene and fluoranthene and present two aromatic moieties linked by an amine nitrogen. We have measured the emission spectra of the new probes and their binding to three odorant-binding proteins. The probes bind the tested proteins with different affinities, generally with dissociation constants about one order of magnitude lower than the parent compounds. The extended aromatic systems present in the new compounds produced a shift of both excitation and emission peaks at higher wavelength, close or within the visible spectrum, thus facilitating measurements in biosensors for odorants and small organic molecules using optical devices.

1. Introduction

Ligand-binding assays between proteins and small organic compounds are often performed using a fluorescent reporter and measuring the decrease in fluorescence produced by a competing ligand. This approach does not need any separation of bound from free ligand and therefore provides accurate data measured in conditions of equilibrium [1]. However, it requires the availability of a probe endowed with good affinity for the protein of interest, whose emission spectrum is significantly modified when the probe occupies the binding pocket of the protein, usually an environment more hydrophobic than the external buffer. Generally the fluorescence intensity increases significantly when the probe is bound to the protein and often a blue shift of the emission spectrum is also observed.

An inconvenience experienced with the use of fluorescent reporters is related to the fact that ligands structurally similar to the probe sometimes might act as better competitors. In such cases, what is measured is the ability to displace the fluorescent probe rather than the direct affinity to the protein. Another artefact is provided by the strong fluorescent signals observed in the presence of ligands capable of forming micelles, such as long-chain fatty acids. When this happens, the probe can bind inside the hydrophobic core of the micelle, emitting a signal similar to that produced in the binding pocket of a protein. Given these drawbacks, it would be advisable to perform the binding experiments with different probes.

Fluorescence methods have been extensively used in ligand-binding experiments with soluble proteins of chemical communication, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). OBP is the common name to designate two distinct classes of proteins in vertebrates and in insects, structurally different, but associated by a common function [2–4]. Vertebrates' OBPs belong to the lipocalin superfamily and present the typical β-barrel folding [5–8], while OBPs of insects are mainly constituted by α-helical domains arranged in compact structures [9,10]. CSPs, instead, are typical of insects and are also made of α-helical segments, but folded in a way different from that of OBPs [11,12]. All three classes of proteins present a hydrophobic cavity, capable of binding pheromones and general odorants [10,14,15]. Two fluorescent probes have been mostly used in ligand-binding experiments with these proteins: 1-aminoanthracene (1-AMA, **1**) and N-phenyl-1-naphthylamine (1-NPN, **2**). The first one generally exhibits better affinity for several OBPs of vertebrates, while the second proved to

* Corresponding author. Address: Department of Pharmacy, University of Pisa, Via Bonanno, 33, 56126 Pisa, Italy. Fax: +39 050 221 9660.
E-mail address: elio.napolitano@farm.unipi.it (E. Napolitano).

http://dx.doi.org/10.1016/j.bbrc.2014.02.067
0006-291X/

be a better ligand for insect OBPs and CSPs, as well as some vertebrate OBPs. Dissociation constants are in the micromolar range, between 1 and 5 μM in most cases [3].

Monitoring the binding of OBPs and CSPs to different ligands through the use of fluorescent probes may turn-up useful in developing biosensors for odorant molecules. Both OBPs and CSPs, in fact, are excellent candidates for fabricating cheap and robust sensing elements, due to their ease of expression and exceptional stability to temperature, solvents and degrading agents [1,13,14,17]. Moreover, based on a wide and detailed knowledge of their three-dimensional structure, it is relatively easy to design mutants with improved affinity or better specificity for desired ligands [16]. Although OBPs have been used in sensing elements for gases and odours, none of such studies has adopted fluorescent measurements as a way of monitoring ligand binding [17]. However, optical methods offer several advantages, being simple, accurate, and sensitive. In this perspective, fluorescent ligands with both excitation and emission wavelengths in the visible spectrum would be much appreciated.

Here we report the design of new fluorescent probes, based on those currently used, and their affinities to three OBPs, a mutant of the pig OBP1, the PBP1 (pheromone-binding protein-1) of the silkmoth *Bombyx mori* and the OBP14 of the honeybee *Apis mellifera*.

2. Materials and methods

2.1. Materials

All enzymes were from New England Biolabs. Oligonucleotides were custom synthesized at Eurofins MWG GmbH, Ebersberg, Germany. All other chemicals were from Sigma–Aldrich, reagent grade.

2.2. Synthesis of the fluorescent probes

The preparation of the fluorescent probes was accomplished adopting general procedures described in the literature [18,19]. The synthetic details and their characterization, including NMR spectra, are reported as Supplementary information.

2.3. Preparation of the proteins

Recombinant pigOBP1-m2 and AmelOBP14 were prepared as previously reported [16,20]. For the synthesis of BmorPBP1 we amplified the gene received from Jurgen Krieger, University of Hohenheim, Germany, by PCR, using a forward primer containing an Nde I restriction site (5′-CATATGTCTCAAGAAGTCATGAA-3′) and a reverse primer containing a Bam HI site (5′-GGATCCTCAAACTTCAGCTAAAATTTC-3′). The crude PCR product was cloned in pGEM-T easy vector and then subcloned in pET15b (Novagen), using the two restriction sites. Protein expression and purification was accomplished as described for other OBPs [11].

2.4. Fluorescence measurements

Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25 °C in a right angle configuration, with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The protein was dissolved in 50 mM Tris–HCl buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

2.5. Ligand-binding experiments

The affinity of the fluorescent probes to each protein was measured by titrating a 2 μM solution of the protein with aliquots of 0.1 mM ligand in methanol to final concentrations of 0.1–2 μM. The probes excitation and emission wavelengths are reported in Table 1. Dissociation constants were evaluated using GraphPad Prism software. The affinity of other ligands was measured in competitive binding assays, where a solution of pigOBP1-m2 and 1-NNN (N-(1-Naphthyl)-1-aminonaphthalene, **4**) both at the concentration of 2 μM was titrated with 0.1 mM methanol solutions of each competitor to final concentrations of 0–2 μM. Dissociation constants of the competitors were calculated from the corresponding IC50 values (concentrations of ligand halving the initial fluorescence value of 1-NNN), using the equation: $K_D = [IC50]/1 + [1\text{-}NNN]/K1\text{-}NNN$, [1-NNN] being the free concentration of 1-NNN and K1-NNN being the dissociation constant of the complex protein/1-NNN.

3. Results and discussion

3.1. Design and synthesis of the fluorescent probes

1-Aminoanthracene (**1**, Fig. 1) and N-phenyl-1-naphthylamine (**2**, Fig. 3) have been widely adopted as fluorescent ligands to probe a variety of odorant-binding and chemosensory proteins [3,13,21]. With the aim to obtain probes with improved characteristics, we have designed arylamines **3**–**8**, which can be envisaged as derived from parent compounds (**1**) and (**2**) by extending the size of one or both aryl portions (Fig. 1). In particular, increasing the number of carbon atoms was expected to improve the binding affinity, which is generally correlated with the lipophilicity, while the extension of the conjugation of the aromatic systems was expected to cause a general shift of absorptions and emissions to lower frequencies, hopefully in the visible region of the spectrum. A summary of the synthetic pathways adopted is presented in Fig. 1. Phenyl amines (**3**) and (**6**) were prepared by condensation of 1-aminoanthracene (**1**) and 3-aminofluoranthene (**13**), respectively, with 2-trimethylsilylphenyl triflate (**9**) in the presence of cesium fluoride [22]. 1-Naphthyl amines (**4** and **7**) and 2-naphthylamines (**5** and **8**) were prepared by direct condensation of the parent amine (1-naphthylamine or 3-aminofluoranthene) with 1-naphthol or 2-naphthol, with minor variants to reported methods for naphthylation of aromatic amines [18,23].

Table 1
Spectral characteristic of the new fluorescent probes and dissociation constants of their complexes with representative OBPs, measured with GraphPad Prism software.

Probe	Wavelength (nm)			K_D (μM) (SD)		
	Excitation	Em. buffer	Em. protein	pigOBP1-m1	BmorPBP1	AmelOBP14
PAA (**3**)	410	520	510	0.50 (0.09)	0.55 (0.077)	1.24 (0.15)
1-NNN (**4**)	345	420	412	0.26 (0.042)	0.58 (0.070)	0.13 (0.027)
2-NNN (**5**)	345	420	412	1.00 (0.50)	1.72 (0.37)	1.03 (0.085)
PAF (**6**)	315	525	515	0.17 (0.031)	–	1.09 (0.14)
1-NAF (**7**)	325	530	509	–	–	0.049 (0.004)
2-NAF (**8**)	325	535	510	–	–	0.068 (0.006)

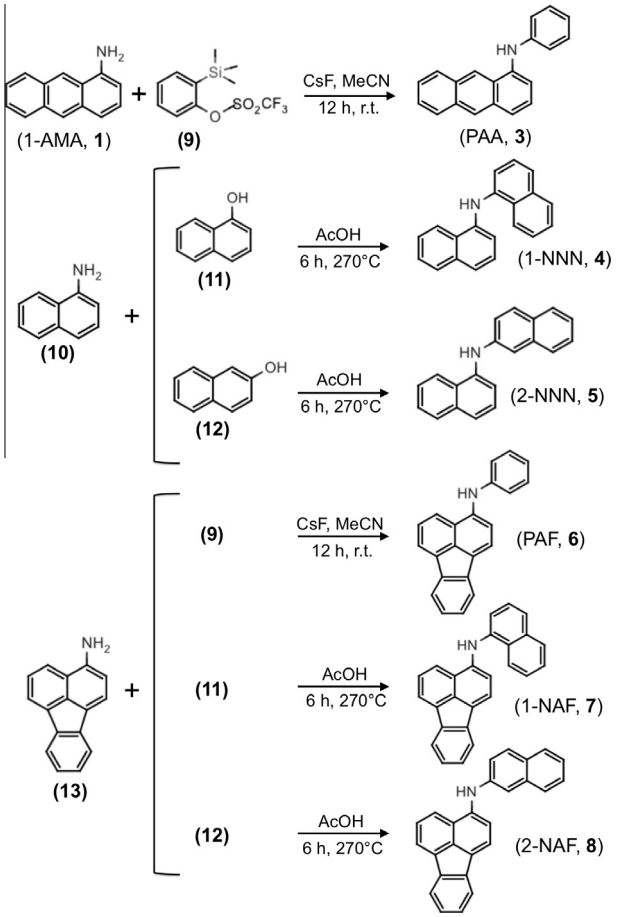

Fig. 1. Synthetic pathways used for the preparation of the new fluorescent probes **3–8.**

3.2. Ligand-binding assays

To test the optical properties of the new probes, we used three structurally different OBPs, whose syntheses and properties had been previously described. The pig OBP1 is a lipocalin and presents a binding pocket which can accommodate terpenoids, such as citral and geraniol, medium chain aldehydes and also larger ligands, such as benzyl benzoate [1,24–26]. The three-dimensional structure**,** solved in complex with different odorants, indicates a relatively large binding site, where different ligands can sit assuming different orientations [26]. In this work, we have used a mutant of this OBP (pigOBP1-m2), where a phenylalanine residue (Phe88) present in the binding cavity had been replaced by a tryptophan, in order to improve the affinity of the protein for polyclyclic hydrocarbons [16]. The other two proteins are insect OBPs predominantly folded in α-helical regions, but differing in their number of cysteines. The *B. mori* PBP1 presents the classical motif of six cysteines paired into three interlocked disulfide bridges [27,28]. It was the first structure of an insect OBP to be solved [9], and the first where a major conformational change was observed, triggered by pH and binding of the specific pheromone [29,30]. The third protein, OBP14 of the honeybee, presents only 5 cysteines and two disulfide bridges, therefore is more flexible than other insect OBPs. Its structure has been solved both as apo-protein and in complex with several ligands [31].

Fig. S1 reports the emission spectra of the 6 new probes in plain buffer and in the presence of 2 μM protein. We can observe that, when bound to the protein, the emission wavelength undergoes only a minor blue shift, as compared to those reported for 1-AMA (**1**) and 1-NPN (**2**), but the intensity of the fluorescence is greatly increased, thus allowing reliable and accurate measurements. We therefore titrated the three proteins with the six probes to measure all relative dissociation constants. Fig. 2 reports the isotherms for each protein/probe pair for which we could observe significant affinity. The new fluorescent compounds generally appeared to be much stronger ligands for the three proteins, compared to the currently used probes 1-AMA (**1**) and 1-NPN (**2**), with dissociation constants at least one order of magnitute lower (Table 1). The better affinity values we measured can be partly justified by the increased hydrophobicity of the fluorescent ligands. The presence of aromatic residues in the binding cavities of all three proteins also contributes to stronger interactions with the polyaromatic structures of the probes (Fig. 2).

The three proteins also exhibited different behaviours with the six probes. In fact, while AmelOBP14 bound all of them, the pigOBP1-m2 showed affinity to only four of the new probes and the BmorPBP1 to only three of them. We can observe that the two largest molecules, the naphthyl derivatives of 3-aminofluoranthene (**7** and **8**) could only fit into the binding pocket of AmelOBP14, while the phenyl derivative (**6**) also bound pigOBP1-m2. What is special about AmelOBP14 is its higher flexibility, with respect to other insect OBPs, due to the presence of only two of the three disulfide bridges. The pig OBP, on the other hand, being a lipocalin with a single disulfide bridge, is more likely to swell and thus accept larger ligands, compared to insect OBPs.

On the basis of previous structural evidence that 1-NPN indeed binds inside the hydrophobic pockets of OBPs [31], we can reasonably assume that our new probes fit in the same way into the proteins. We have verified this hypothesis by measuring quenching of intrinsic fluorescence of tryptophan by the probes, limited to the pig OBP1-m2 (Fig. 2). The reduction of tryptophan fluorescence is probe-concentration dependent and comparable in intensity to the values we had previously measured with 1-AMA and polycyclic hydrocarbons [16].

Although apparently these probes bind OBPs with good affinities, we discovered strange behaviours when looking at the low concentration regions of the binding curves. In fact, if we assume that the molar emission value of the probe when bound to the protein is the same across the entire concentration range, then we end up with the unacceptable result that a concentration of the probe as low as 0.1–0.2 μM is able to saturate 1 μM of the protein. The conclusion is that our first assumption is not valid and that different phenomena may occur at low concentrations of the probe.

A reasonable hypothesis is that some of the probes might bind two molecules of protein, acting as bridges. This is suggested by the fact that in most cases the new probes can be seen as two aromatic moieties, linked by an NH group, each able to bind an OBP. We have tried to verify such hypothesis by analysing a mixture of OBP (specifically the AmelOBP14) and one of the new probes (1-NNN, **4**) or 1-NPN (**2**) as control, by gel filtration, in order to detect formation of aggregates. After separation on a Superose-12 column, the fractions were analysed by SDS–PAGE and fluorescence intensity was measured at 410 and 407 nm in the samples containing 1-NNN (**4**) or 1-NPN (**2**), respectively. The results are reported in Fig. 3. We can first observe that the absorbance at 280 nm shows a major peak corresponding to fraction 20, where most of the protein is eluted, as visualised by SDS–PAGE (inset). This elution volume, when compared with a calibration curve, suggests a molecular weight of around 28 kDa. Such value corresponds to about twice the calculated molecular weight of the protein, strongly indicating the presence of a dimer. This finding is in contrast with the report that dimers were not observed in the crystal, nor predicted as stable species using several tools [31]. In any case, the presence of weaker peaks at lower elution volumes (12 and 16 mL) indicates that higher aggregation states of the protein could

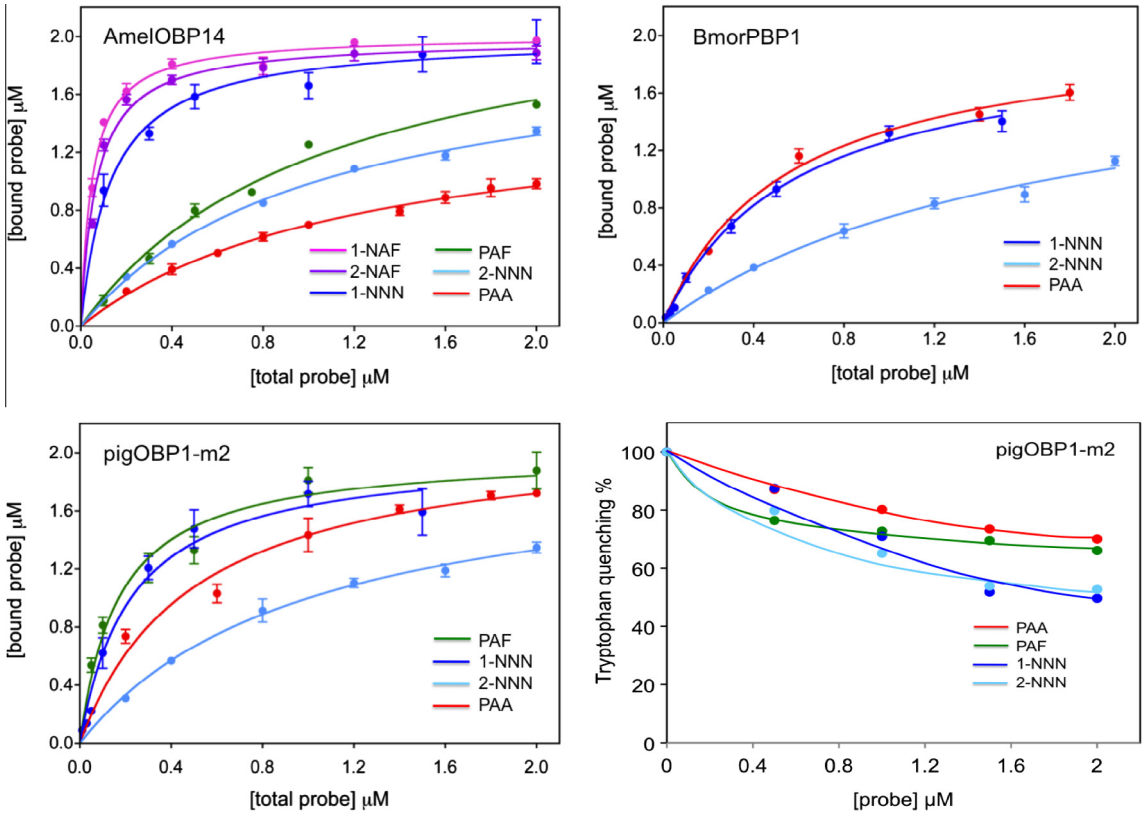

Fig. 2. Upper and left lower panels. Binding of new probes to a mutant of pig OBP1 (pigOBP1-m2), *Bombyx mori* PBP1 (BmorPBP1) and honeybee OBP14 (AmelOBP14). Solutions of the protein in 50 mM Tris–HCl buffer, pH 7.4, at the concentration of 2 µM were titrated with aliquots of 0.1 mM solutions of the probe in methanol to the indicated final concentrations. Right lower panel. Tryptophan intrinsic fluorescence quenching following bindings of four of the new probes to pigOBP1-m2. All four probes reduce the fluorescence of tryptophan in a concentration-dependent fashion, indicating that they bind in the core of the protein.

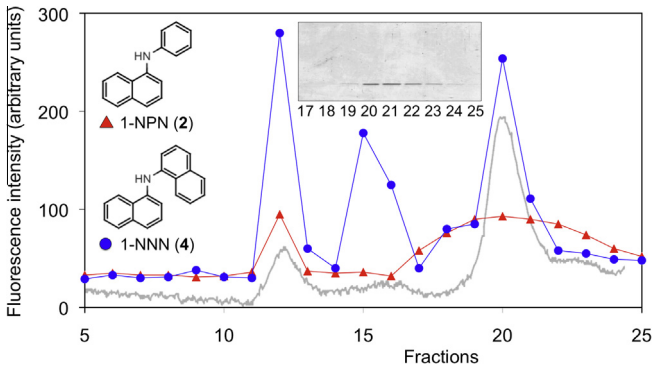

Fig. 3. Gel filtration of complexes between AmelOBP14 and 1-NPN of 1-NNN. Aliquots of the protein, alone or complexed with 1-NPN or 1-NNN were chromatographed through a Superose-12 gel filtration column (0.5 × 20 cm). Protein elution was monitored by recording absorbance a 280 nm (grey continuous line) and SDS–PAGE on the collected fractions of 1 mL (inset). The elution of the probes was also monitored by measuring the relative fluorescence on the single fractions. With 1-NNN (blue dots), unlike 1-NPN (red triangles), strong fluorescence is associated with two minor protein peaks, likely corresponding to oligomeric aggregates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

be present in solution. Strong fluorescence is associated with these peaks, more intense with 1-NNN (**4**) than with 1-NPN (**2**), although we could not clearly visualise a protein band in the SDS–PAGE analysis (not shown). It is noteworthy to observe that the strong fluorescence peak, present in fractions 15 and 16, is visible only with 1-NNN (**4**) and is associated with minor amounts of the protein. All together, we can conclude that: (a) the protein can exist in at least three forms, which can be visualised as oligomeric aggregates, all binding strongly the larger fluorescent probe 1-NNN (**4**), but only very weakly 1-NPN (**2**); (b) 1-NNN (**4**) and likely the other large probes, when bound to these aggregates, produce a much stronger fluorescence than when bound to the main form.

Therefore, protein aggregation could provide a reasonable model for the unusual behaviour observed when titrating the proteins with the large new probes. A strong fluorescence yield is recorded at the beginning of the curve, corresponding to the binding of the probe to oligomeric forms; then, when the probe binds the main form of the protein (dimer or monomer), the yield is much lower and the curve becomes flatter. It is obvious that in such circumstances we cannot use the value of fluorescence at infinite concentration of probe to calculate the molar yield and evaluate the concentration of bound probe in the first part of the curve. Therefore, the binding curve and the calculated dissociation constants cannot be regarded as description of a simple equilibrium between protein, ligand and complex.

Nevertheless, when measuring binding affinities of ligands in competitive binding experiments we can ignore, in most cases, these complex phenomena occurring at very low concentrations of the probe. In fact, competition of a ligand generally is measured at concentrations comparable to that of the protein. In such conditions, we can assume that interactions of the probe with more than one molecule of protein is largely replaced by conventional binding between ligand and protein with a 1:1 stoichiometry.

To verify this hypothesis, we have measured replacement of the probe 1-NNN (**4**) in the core of pigOBP1-m2 by compounds previously reported [25,26] as good ligands (benzyl benzoate, citralva, decanal) or poor ligands (ethyl benzoate and 2-phenylethanol). The results are reported in Fig. 4 and show good agreement with

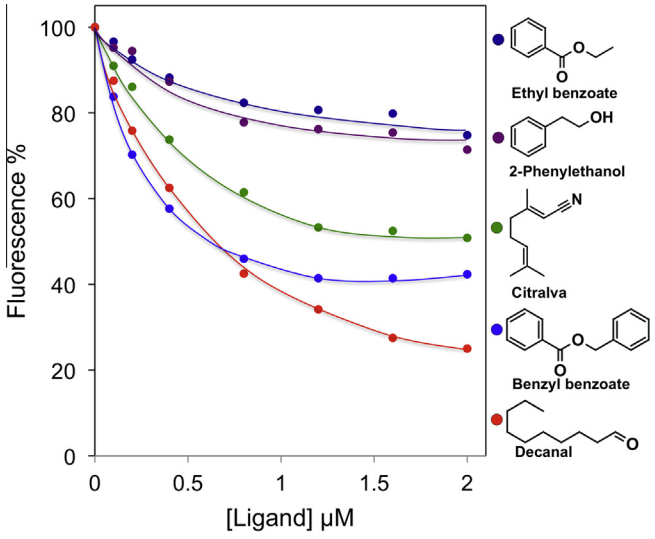

Fig. 4. Affinity of different ligands to pigOBP1-m2, measured in competitive binding assays. Using 1-NNN as the fluorescent reporter, we obtained results similar to those measured with other techniques and previously reported in the literature [25,26]. Ethyl benzoate and 2-phenylethanol, reported as poor ligands, fail to appreciably displace the fluorescent probe from the complex, while strong ligands citralva, benzyl benzoate and decanal show apparent dissociation constants of 0.38, 0.11 and 0.12 μM, respectively, in this experiment.

the data previously measured with other techniques. In fact, ethyl benzoate and 2-phenylethanol, two among reported as poor ligands, fail to appreciably displace the fluorescent probe from the complex, while strong ligands citralva, benzyl benzoate and decanal show apparent dissociation constants of 0.38, 0.11 and 0.12 μM, respectively, in this experiment.

The new probes that we have designed and synthesised provide alternatives to those currently used, specifically for ligand-binding studies with OBPs and other binding proteins. They also present characteristics that can be of advantage in some situations.

First, their excitation and emission wavelengths are higher than those of other probes, such as 1-AMA (**1**) and 1-NPN (**2**), close or within the visible range. This characteristic allows the use of simpler and cheaper materials to monitor the optical phenomena, a fact particularly interesting when analysing large number of samples on plates as part of throughput screenings, as well as designing biosensors for odours monitoring based on OBPs and fluorescence measurements.

The novel probes, being larger than 1-AMA (**1**) and 1-NPN (**2**), represent new tools for investigating the binding properties of proteins with large cavities or flexible enough to accommodate more than one molecule of ligand. This is the case of the CSP (Chemosensory protein) of *Mamestra brassicae*, that can fit three molecules of 12-bromo-1-dodecanol in its binding pocket [14].

A detailed study of the interactions between these large probes (particularly the fluoranthene derivatives) and flexible proteins is beyond the aims of the present report and requires the resolution of three-dimensional structures of the relative complexes.

Acknowledgments

We thank Paolo Pelosi, University of Pisa, for helpful discussion and suggestions and for revising our manuscript. We are grateful to Jurgen Krieger of the University of Hohenheim, Germany, for a gift of the plasmid containing the gene encoding BmorPBP1. E.N. thanks the Department of Pharmacy, University of Pisa, for hosting this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.067.

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