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

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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

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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)