Revisiting the odorant-binding protein LUSH of *Drosophila melanogaster*: evidence for odour recognition and discrimination

Jing-Jiang Zhou[a], Guo-An Zhang[b], Wensheng Huang[c], Michael A. Birkett[a], Linda M. Field[a], John A. Pickett[a], Paolo Pelosi[d,*]

[a] *Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK*
[b] *College of Sciences and Biotechnology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China*
[c] *The GMOs Detection Laboratory, Animal and Plant Quarantine Institute, Beijing 100029, PR China*
[d] *Dipartimento di Chimica e Biotecnologie Agrarie, University of Pisa, Via S. Michele, 26124 Pisa, Italy*

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Abstract LUSH is a soluble odorant-binding protein of the fruit fly *Drosophila melanogaster*. Mutants not expressing this protein have been reported to lack the avoidance behaviour, exhibited by wild type flies, to high concentrations of ethanol. Very recently, the three-dimensional structure of LUSH complexed with short-chain alcohols has been resolved supporting a role for this protein in binding and detecting small alcohols. Here we report that LUSH does not bind ethanol and that wild type flies are in fact attracted by high concentrations of ethanol. We also report that LUSH binds some phthalates and that flies are repelled by such compounds. Finally, our fluorescence data, interpreted in the light of the three-dimensional structure of LUSH, indicate that the protein undergoes a major conformational change, similar to that reported for the pheromone-binding protein of *Bombyx mori*, but triggered, in our case, by ligand.

Key words: Odorant-binding protein; Chemical communication; Fluorescence binding assay; *N*-Phenylnaphthylamine; Phthalate; *Drosophila melanogaster*

1. Introduction

Two classes of soluble proteins of small size are involved in chemical communication in insects. Odorant-binding proteins (OBPs) contain about 130–150 amino acids and include members specific for pheromones and general odorants [1,2]. They are characterised by six conserved cysteines, paired in three interlocked disulphide bridges [3,4], and a compact structure, mainly constituted by α-helical domains, that defines an internal binding pocket [5,6]. Chemosensory proteins (CSPs) are slightly smaller than OBPs (100–120 residues) and present four conserved cysteines, linked in non-interlocked fashion [7], thus producing two small loops. CSPs are mainly folded, like OBPs, α-helical domains, but their three-dimensional structure presents unique characteristics [8]. Both OBPs and CSPs are highly concentrated in the lymph of chemosensilla and reversibly bind odorants and pheromones. The idea that both classes of protein might perform similar roles in insect chemoreception is further supported by the finding that antennae specifically express OBPs or CSPs, depending on the species.

Despite the wide and detailed information on the structure of these soluble proteins, as well as on their binding properties towards odours and pheromones, our understanding of their mode of action is still poor. The great diversity of these polypeptides and their high levels of expression certainly suggest that they play important roles for the survival of the individual or for the conservation of the species. Hypotheses have been proposed ranging from a passive role of carriers of chemical stimuli to and from olfactory receptors to more specific functions involving recognition of odours and pheromones [1,2,9].

The great number of both OBPs and CSPs expressed in the same species also points to a specific role of these proteins in odour discrimination. The *Drosophila* genome contains 51 genes encoding proteins of the OBP family [10] and several CSP genes, a number comparable to the 60 genes (including several pseudogenes) for olfactory receptors in the same species [11,12].

So far only two studies have provided some evidence that OBPs are required for a correct recognition of odours. The first reported that *Drosophila* mutants, carrying a deletion for a gene encoding an OBP (called LUSH by the authors) were abnormally attracted by high concentrations of ethanol, unlike wild type flies, which seemed to prefer ethanol at low concentrations. Towards many other odours, however, the mutants did not show any different behaviour from the wild type [13,14]. In the second paper, the observation that some colonies of fire ants *Solenopsis invicta* made several queens instead of a single one was related to the failed expression of a gene encoding a protein of the OBP family [15]. In this case, the OBP involved in this behaviour was extracted from the thorax. Therefore, rather than being involved in the olfactory recognition of chemical messengers, this protein could be required for the release of the appropriate chemical signal.

The LUSH mutant therefore would represent the only example of a protein of the OBP family involved in the perception of a specific odour. Very recently the three-dimensional structure of LUSH was resolved by X-ray crystallography and shown to contain a putative binding pocket for small alcohols

*Corresponding author. Fax: (39)-050-571564.
E-mail address: ppelosi@agr.unipi.it (P. Pelosi).

Abbreviations: 1-NPN, *N*-phenylnaphthylamine; OBP, odorant-binding protein; CSP, chemosensory protein

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[16]. On this basis we decided to express LUSH and investigate the affinity to known odorant molecules.

2. Materials and methods

2.1. Protein synthesis and purification

The LUSH protein of *D. melanogaster* was obtained by bacterial expression, using the vector pET22b (Novagen), which yields most of the protein in the periplasmic space in its soluble form. This method produced the polypeptide in high yield (20 mg/l of culture, half of which was present in its soluble form), without any added residue with respect to the native protein. The recombinant LUSH was purified by a combination of gel filtration and anion exchange chromatography, along with a protocol used for several proteins of the same class [7].

2.2. Ligands

All ligands were commercially available. Most were purchased from Aldrich (Dorset, UK). These include 1-hexanol, 2-acetylthiophene, phenylacetonitrile, isovaleric acid, cyclohexanol, *iso*-amyl alcohol, 2-phenylethanol, phenylacetaldehyde, butylbutyrate, hexylbutyrate, hexyl acetate, *iso*-amyl acetate, acetoin, 2,3-butanediol, ethyl acetate, diphenyl phthalate, dibutyl phthalate, 2,6-di-*tert*-butyl-4-methylphenol, *cis*-jasmone, (β)-caryophyllene oxide, (1*R*,5*S*)-myrtenal. Oleoamide was obtained from Sigma (Dorset, UK). Di-(2-ethylhexyl) phthalate was purchased from Fisons and dimethyl phthalate from Hopkin and William Ltd. *N*-Phenyl-1-naphthylamine (1-NPN) was from Sigma.

2.3. Fluorescence measurements

Emission fluorescence spectra were recorded on a luminescence spectrometer LS50B (Perkin-Elmer) 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 buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

2.4. Intrinsic fluorescence

The tryptophan intrinsic fluorescence was measured on a 2 μM solution of the protein, using an excitation wavelength of 295 nm and recording the emission spectrum between 300 and 360 nm. Quenching of intrinsic fluorescence was measured in the same condition and in the presence of ligands at concentrations of 2–16 μM.

2.5. Binding assays

To measure the affinity of the fluorescent ligand 1-NPN to LUSH, a 2 μM solution of protein in 50 mM Tris, pH 7.4 was titrated with aliquots of 1 mM methanol solutions of the ligand to final concentrations of 1–16 μM. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 μM concentration and concentrations of 1–16 μM for each competitor.

2.6. Coupled gas chromatography-mass spectrometry (GC/MS)

A capillary GC column (50 m × 0.32 mm ID HP-1) fitted with an on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, UK). Ionisation was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°C/min to 250°C.

2.7. Data analysis

To determine binding constants, the intensity values corresponding to the maximum of fluorescence emission were plotted against free ligand concentrations. Bound ligand was evaluated from the values of fluorescence intensity assuming the protein was 100% active, with a stoichiometry of 1:1 protein:ligand at saturation. The curves were linearised using Scatchard plots. Dissociation constants of the competitors were calculated from the corresponding IC_{50} values, using the equation: $K_D = [IC_{50}]/(1+[1\text{-NPN}]/K_{1\text{-NPN}})$, [1-NPN] being the free concentration of 1-NPN and $K_{1\text{-NPN}}$ being the dissociation constant of the complex OBP/1-NPN.

2.8. Behaviour experiments

Behaviour of flies towards volatile chemicals was measured basically adopting the protocol used by Kim et al. [13]. Accordingly, traps were assembled by inserting the end of yellow tips (previously shortened to make openings wide enough for a fly to pass) into the cut bottom of 0.5 ml Eppendorf tubes. About 50 μl of food (mashed rotten apple) or melted agarose, previously mixed with the appropriate amount of odorant, were put in the caps of the tubes. To a 10 cm Petri dish, containing two traps with the two stimuli to be compared, 10 flies were added, which had been starved for 10 h. After 7 h in the dark, flies in each trap were counted. Each experiment was replicated 10 times and the data averaged. The data were subjected to *t*-test with 95% confidence limit, using GenStat (VSN International, Hemel Hempstead, UK).

3. Results and discussion

LUSH was expressed in a bacterial system, using standard protocols. A first attempt, using the plasmid pET5b, produced the protein in good yields (20 mg/l of culture), but completely as inclusion bodies. The protein could be solubilised by boiling with dithiothreitol, using a protocol already successfully adopted for other proteins of the same class [17]. By air oxidation, the protein was correctly refolded and regained its binding properties. However, a better alternative proved to be the use of the plasmid pET22b, which directs the protein to the periplasmic space. This system provided about half of the LUSH protein in soluble form, without any modification with respect to the natural mature protein. The protein was easily purified by a combination of gel filtration on Sephacryl-100 and anion exchange chromatography on DE-52 and used for binding assays.

The fluorescent probe 1-NPN, which was successfully used with other OBPs both of vertebrates and of insects, reversibly binds LUSH with a dissociation constant of 1.5 μM. The intrinsic fluorescence of the tryptophan contained in the protein is not affected by the presence of 1-NPN or other ligands in the binding cavity, suggesting that the only tryptophan residue of the protein is not close to the ligand. In fact, the only tryptophan in LUSH is the last but one residue, and in the three-dimensional structure of LUSH it is reported to be buried inside the binding cavity. Therefore, we would expect quenching of its fluorescence when a ligand enters the binding pocket, as observed with other proteins of the same class [21,22], unless this residue, together with the C-terminal region, is pushed out of the cavity by the entering ligand. In fact, in this hypothetical conformation, the tryptophan would be exposed to solvent molecules, as when inside the protein, therefore no energy transfer would occur.

The ability of other ligands to displace 1-NPN from the complex was measured in competitive binding assays. Potential ligands included several volatiles reported to elicit electrophysiological responses from the antennae of *Drosophila* [18–20] and some structurally related compounds (listed in the legend of Fig. 1). None of them was able to significantly displace 1-NPN from the complex, when used at concentrations up to 16 μM. Ethanol was equally ineffective, even at concentrations as high as 15 mM.

Since 1-NPN was the only compound for which we could measure any binding, we searched for potential ligands among aromatic compounds structurally related to this fluorescent probe. We therefore decided to test some phthalate esters. These chemicals, which are used as plasticisers in certain plastics, are often found as contaminants of organic solvents and could have been responsible for the previously reported aversion behaviour to small alcohols [13].

Diphenyl phthalate and dibutyl phthalate proved the most

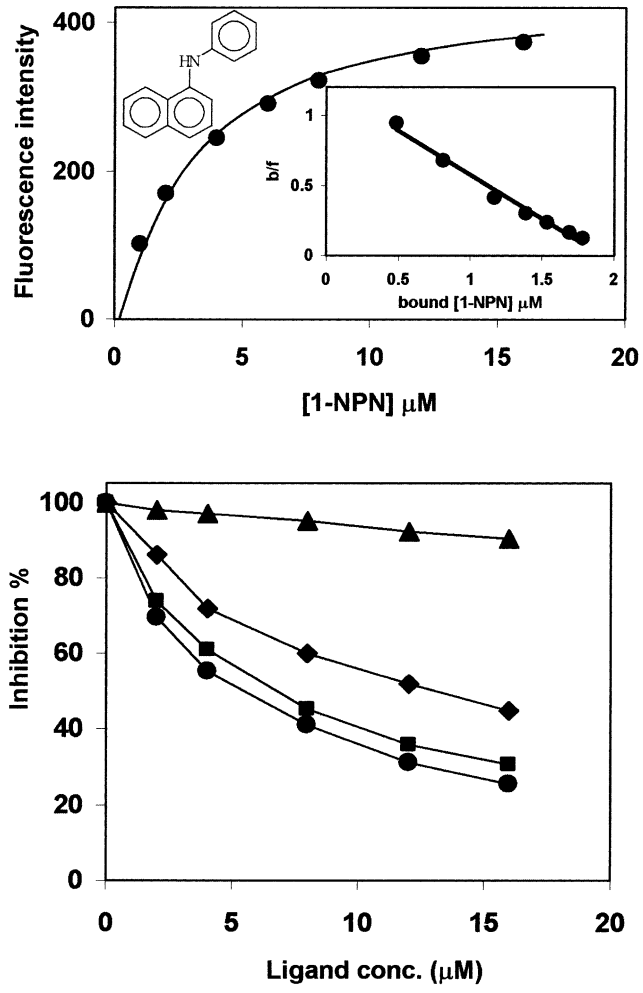

Fig. 1. Upper panel: Binding of 1-NPN to recombinant LUSH. The protein (2 μM in Tris buffer) was titrated with increasing amounts of 1-NPN in methanol to final concentrations of 1–16 μM. The binding curve and the Scatchard plot (inset) show the presence of a single saturable binding site, with a dissociation constant of 1.5 μM and without cooperativity effects. Lower panel: Binding of phthalates to recombinant LUSH. The curves show displacement of the fluorescent probe 1-NPN by dimethyl phthalate (triangles), dibutyl phthalate (circles), diphenyl phthalate (squares) and di-(2-ethylhexyl) phthalate (diamonds). The protein and the fluorescent probe were both incubated at the concentration of 2 μM and the mixture was titrated with methanol solutions of the competitors to final concentrations of 2–16 μM. The following compounds, known to elicit electrophysiological responses from *Drosophila* antennae, did not appreciably displace 1-NPN at concentrations up to 16 μM: ethanol (up to 15 mM), 1-hexanol, phenylacetonitrile, 2-acetylthiophene, isovaleric acid, cyclohexanol, *iso*-amyl alcohol, 2-phenylethanol, phenylacetaldehyde, oleoamide, butylbutyrate, hexylbutyrate, hexyl acetate, *iso*-amyl acetate, acetoin, 2,3-butanediol, ethyl acetate, di-*tert*-butylhydroxytoluene, *cis*-jasmone, (β)-caryophyllene, (1*R*,5*S*)-myrtenal.

efficient in displacing 1-NPN from its complex with LUSH, with apparent dissociation constants of 4.4 and 5.1 μM, respectively. Both shorter chain derivatives, such as dimethyl phthalate, and longer chain compounds, such as di(2-ethylhexyl) phthalate, proved to be weaker ligands, the first not showing appreciable binding, the second with a dissociation constant of 10.7 μM.

We then decided to study the behaviour of wild type *D. melanogaster* to different concentrations of ethanol as well as to dibutyl phthalate, which proved to be the best ligand of LUSH. We adopted the same protocol described by Kim et al. [13], as reported in Section 2. Initially we tested the flies with traps containing food (mashed rotten apple) mixed with ethanol at final concentrations of 1% and 50%. The flies clearly preferred the higher ethanol concentration (Fig. 2). To avoid effects of possible contaminants, we verified the absence of organic compounds, particularly of phthalates, in the sample of ethanol by GC/MS. As in our conditions no peak of contaminants was detectable, we estimated that the concentration of any of such compounds, if present, would be lower than 10 ppm.

Similar experiments were performed with pairs of traps containing dibutyl phthalate at the final concentration of 1 mM, diluted in mashed apples or in 50% ethanol in agarose. In each case the same diluent (mashed apple or 50% ethanol in agarose) was used in the control trap. The flies showed a statistically significant ($P < 0.001$) behaviour of avoidance to dibutyl phthalate, regardless of the medium used.

These behaviour results and the binding affinity of LUSH to some phthalates provide a new interpretation of the phenomenon previously observed [13]. The original hypothesis stated that LUSH could modify the perception of ethanol at high concentrations. This requires some specific binding of ethanol to LUSH, which in our study we failed to detect. It can be argued that a second binding site could be present on the protein, where ethanol could bind, without affecting the binding of 1-NPN in the other pocket. This hypothesis, however, is not supported by the recently published X-ray structure, which showed the presence of a single binding site in LUSH [16], in agreement with previous reports for other in-

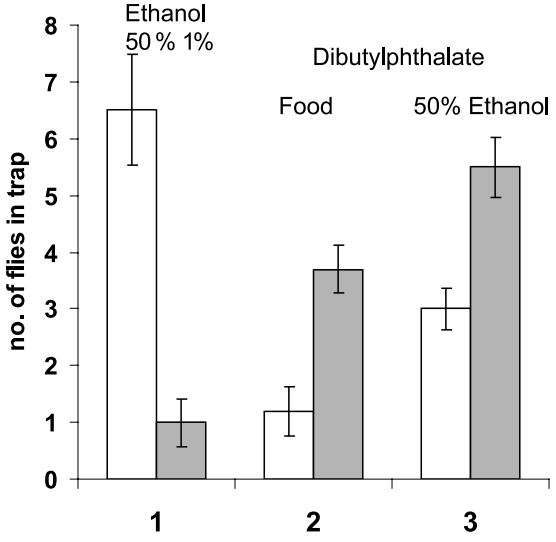

Fig. 2. Behavioural responses of wild type (Canton-S) *D. melanogaster* to pure ethanol (where absence of phthalates had been verified by GC/MS) at concentrations of 1% and 50% (**1**) and to dibutylphthalate at 1 mM concentrations diluted in mashed apple (**2**) or in 50% ethanol in agarose (**3**). Tests were performed using the traps described in Section 2, with 10 flies per experiment and 10 replicates (total of 100 flies per odorant). Bars indicate S.E.M. Filled columns: flies in traps with 1% ethanol (**1**) or without dibutyl phthalate (**2**, **3**); empty columns: flies in traps with 50% ethanol (**1**) or with dibutyl phthalate (**2**, **3**).

sect OBPs [5,6]. Moreover, the behaviour experiments performed with pure ethanol clearly show that this compound is a potent attractant for wild type flies at high concentrations (Fig. 2).

Our reinterpretation of the results of Kim and Smith [13,14], assuming that LUSH is specific for aromatic compounds structurally similar to phthalates, rather than for ethanol, provides the first and only evidence that insects use OBPs as tools to distinguish different odorants.

This finding also suggests that binding experiments could be used as a first screening to indicate which OBP gene should be deleted or inactivated in order to get mutants with desired types of anosmia.

In the light of these results, we conclude that the avoidance previously reported for wild type flies could be due to impurities, such as phthalates or structurally related compounds, present as contaminants in the ethanol used. Furthermore, the loss of avoidance in the mutants would result from the inability to detect large aromatic compounds. This conclusion provides new and increased interest for the previously published data as LUSH would be directly and strictly required for the perception of an odorant, rather than being involved only in modulating the response to ethanol. The recently published structure of LUSH with a molecule of butanol in its binding pocket does not in our opinion show a true binding role of this protein for small alcohols.

The C-terminus of LUSH, which contains the only tryptophan residue of the protein, is located inside the core of the protein. When we investigated the effect of our ligands on the tryptophan fluorescence, we were unable to measure any quenching up to ligand concentrations of 16 μM. This could indicate that when the ligands (1-NPN or phthalate) enter the binding site, the C-terminus of the protein is displaced and the tryptophan residue moves outside the core of the protein. Such a major conformational change has been observed with the pheromone-binding protein of *Bombyx mori*, as an effect of a pH increase from 4.5 to 6.5. In our case, the effect would occur at pH 7 and, most interestingly, as a consequence of ligand binding. This hypothesis, if verified in the structure of LUSH complexed with a ligand, would represent the first example of a major conformational change of an OBP related to substrate binding and have interesting implications for the mode of action of these proteins and their interactions with membrane-bound receptors.

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