

An Odorant-binding Protein Facilitates Odorant Transfer from Air to Hydrophilic Surroundings in the Blowfly

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Abstract

Chemical sense-related lipophilic ligand-binding protein (CRLBP) is an insect odorant-binding protein (OBP) found abundantly in the taste and olfactory organs of the blowfly, *Phormia regina*. Through computational construction, a three-dimensional molecular model of a CRLBP indicated good fitting to a fluorescent ligand, 7-hydroxycoumarin (7-HC), in its ligand-binding pocket. By showing that the fluorescence of 7-HC bound to CRLBP migrated in a native electrophoresis gel, we confirmed that CRLBP formed a stable complex with 7-HC. In an odorant-binding experiment, 7-HC vapor odor was introduced by aeration to the aquatic solution containing CRLBP and its binding to CRLBP fluorospectrometrically quantified. Because olfactory organs as well as taste organs of flies respond to vapors, we suggest that CRLBP effectively transfers odorants from the air into aquatic surroundings by forming stable complexes with airborne molecules in both chemosensory organs.

Key words: binding assay, molecular modeling, odorant-binding protein, olfactory reception, perireceptor event

Introduction

Insect odorant-binding proteins (OBPs) are small, water-soluble proteins that are widely found in the olfactory systems of different species (Vogt, 1995; Pelosi, 1996; Vogt *et al.*, 1999). OBPs are involved in the first specific biochemical step of odor reception and are thought to carry lipophilic odorants to the olfactory receptor cells through hydrophilic surroundings (Vogt *et al.*, 1985; Vogt, 1995; Prestwich *et al.*, 1995; Pelosi, 1996; Steinbrecht, 1996, 1999; Ziegelberger, 1996; Kaissling, 1998). As molecular cloning or gene searching of insect OBPs progress, studies on functional structures correlating to their ligand-binding activities have been reported in several species (Campanacci *et al.*, 2002; Ban *et al.*, 2003). However, few direct experiments have clearly shown all three steps that are suggested to be involved in the process by which OBPs in the hydrophilic receptor lymph: (i) the catching of odorant molecules from the airspace through the pore(s) of sensilla; (ii) their transference to the lipophilic surface of the receptor cell membrane; and (iii) their passage to the receptor proteins. Previous studies have suggested that step (ii) is driven by the diffusion of odorant–OBP complexes, and

that step (iii) occurs by the conformational changes of OBPs near the receptor membrane (Sandler *et al.*, 2000). However, there is little experimental evidence reported for step (i). Therefore, in the present study we focus on this first step, and confirm that OBPs catch aerated odorants and introduce them into an aquatic solution by forming stable complexes.

The chemical sense-related lipophilic ligand-binding protein (CRLBP) used here is a unique OBP. It belongs to the insect OBP family, having 38% amino acid identity with a pheromone-binding-protein-related protein of *Drosophila melanogaster*, PBPRP-2 (Pikielny *et al.*, 1994; Hekmat-Scafe *et al.*, 2002). It was originally found as a major water-soluble protein in the taste sensilla of the blowfly, *Phormia regina* (Ozaki *et al.*, 1995; Ozaki and Tominaga, 1999). In a recent paper, Ozaki *et al.* (2003) indicated that CRLBP in the taste receptor lymph carries noxious components of an essential oil to stimulate the warning taste cell (traditionally called the ‘fifth cell’, after Dethier and Hanson, 1968) that may have been differentiated as a taste system for detecting and avoiding dangerous foods. This implies that even if flies possess

taste receptor cells having complete sets of receptor proteins (Clyne *et al.*, 2000; Scott *et al.*, 2001) and/or higher neuronal networks analyzing taste inputs (Mitchell and Itagaki, 1992; Stocker, 1994), they would be unable to obtain proper information for food selection without CRLBP. Thus, CRLBP, or more generally OBPs, appear to have a very important role in animal survival.

Further, the taste organ of the fly responds to some vapors with vigorous impulses from the fifth cell (Dethier, 1972). Hypothesizing that the fifth cell and olfactory receptor cells need the help of lipophilic ligand carriers to respond to vapors, so long as they are surrounded by hydrophilic receptor lymph, Ozaki *et al.* (1995) predicted the existence of CRLBP in the olfactory organ, but could not confirm it. Here, we determined that there is an OBP in the olfactory organ completely identical with labellar CRLBP, and showed that CRLBP mediates the transfer of vapor odor from air to the hydrophilic surroundings. This appears to occur in the olfactory and the taste organs in the fly so that it can taste vapor odors with the fifth cell.

Materials and methods

Flies

The blowflies (*P. regina*) used here were originally donated by Professor Morita of Kyushu University. They were reared in our laboratory at $22 \pm 2^\circ\text{C}$ using Professor Morita's methods (personal communication), and fed with 0.1 M sucrose and water after emergence.

Preparation of CRLBP

CRLBP was purified from blowfly antennae according to the method of Ozaki *et al.* (1995). Antennae were isolated from heads and homogenized in a hand mortar with a small amount of liquid nitrogen for 20 min. The homogenate was mixed with sample buffer (4.75 mM sodium barbiturate-HCl, 10% glycerol, pH 6.8), centrifuged at 450 000 g for 1 h at 4°C and then the supernatant applied to native polyacrylamide gel electrophoresis.

The gel systems of Ornstein (1964) and Davis (1964) were used with some modifications: instead of Tris buffers, we used barbiturate buffers (stacking gel buffer: 9.3 mM sodium barbiturate-HCl, pH 6.8; resolving gel buffer: 91.1 mM sodium barbiturate-HCl buffer, pH 8.9; electrode buffer: 41.1 mM sodium barbiturate-glycine buffer, pH 8.3), and the stacking and resolving gels were composed of 4.5 and 13.5% acrylamide, respectively. Electrophoresis was carried out at a current of 30 mA for 3 h in a cold chamber at 4°C . CRLBP was collected by electrophoretic elution at 75 mA for 30 min (mini whole gel eluter, Bio-Rad Laboratories, USA) with an electrode buffer. The recovered CRLBP in an eluted sample was concentrated to 1 μM using a 10 000 NMWL filter (MILLIPORE ULTRAFREE). Approximately 660 μg CRLBP was purified from 8000 flies (the amount of protein

was determined with a Dc protein assay kit, based on Lowry method (Lowry *et al.*, 1951) (Bio-Rad Laboratories).

For comparative tests, bovine serum albumin (BSA) (Sigma, Germany) or β -lactoglobulin (BLG) (Nacalai Tesque, Kyoto, Japan) were used instead of CRLBP. These were prepared as a 1 μM solution in the same sodium barbiturate-HCl buffer containing 10% glycerol.

Molecular cloning and amino acid sequencing

Referring to the cDNA sequence of the CRLBP derived from the labellum (Hekmat-Safe *et al.*, 2002), we prepared forward primer, 5'-CGGGATCCGAACCTTACCAAGG-3', and reverse primer, 5'-CGACTTCTAAATTAAGTGACGTCAA-3'. For the polymerase chain reaction (PCR) template, single-stranded cDNAs were synthesized (using an RNA PCR Kit, Takara, Osaka, Japan) from poly(A)-rich RNA purified from antennae (using the PolyATtract System 1000, Promega). The PCR product made with primers 1 and 2 was used as the template for sequencing PCR (with a DNA Sequencing Kit, ABI) and the product was applied to the DNA sequencer (310NT Genetic Analyzer, ABI). Nucleotide sequencing was performed from both the 5' and 3' ends. Thus, the cDNA sequence was consistently obtained.

On the other hand, we directly determined the N-terminus amino acid sequence. Protein was separated from antennae extracts by native PAGE and blotted onto a PVDF membrane filter (Bio-Rad Laboratories). The excised filter containing the protein band under investigation, which was seen at the same migration position as the CRLBP derived from the labellum, was subjected to automatic Edman degradation in a protein sequencer (ABI, model 473A).

Computational modeling method

The crystal structure of the pheromone-binding protein (PBP) of *Bombyx mori* (PDB ID: 1DQE) was used for building the model of CRLBP. Sequence alignment and initial homology modeling were performed with the Homology module installed in Insight II (ver. 2000, Molecular Simulations Inc. San Diego, CA). An initial structure for the protein was optimized with molecular dynamics calculations at 298K by the cell multiple method, a distance-dependent dielectric constant, and a time step of 1 fs for 100 ps by sampling the conformation every 1 ps using Discover 3 (ver. 98.0, Molecular Simulations Inc.). One hundred conformations were minimized until the final root-mean-square deviation became less than 0.1 kcal/mol/Å. During the calculations, all of the C α atoms were fixed. The lowest energy conformation was selected for the substrate-docking study.

The substrate was roughly docked into the ligand-binding cleft and then the initial complex model was minimized. The whole structure of the complex model was optimized by the molecular dynamics/minimization procedure as described above without any structural constraints. The lowest-energy structure was selected as an energy-refined complex model.

7-HC binding experiment by native electrophoresis

The gel or buffer compositions were the same as those used for preparation of CRLBP, but the sample was a mixture of purified 4 μ g CRLBP and 1mM 7-HC solution in 40 μ l sample buffer. After electrophoresis, the gel was put on a UV box, and the fluorescent spot of the 7-HC was observed. Next, proteins on the gel were stained with Coomassie brilliant blue (CBB) to confirm co-migration of 7-HC and CRLBP.

7-HC vapor binding experiments

The fluorescent compound 7-HC (Nacalai Tesque, Kyoto, Japan), known as an attractive odorant for *D. melanogaster* (Yamada *et al.*, 2000) was used as the binding ligand in these experiments. The fluorescence spectrum of 7-HC was measured for its saturated solution in 4.75 mM sodium barbiturate-HCl buffer (pH 6.8) containing 10% glycerol. We placed the solution in a 1 cm light path fluorometer quartz cuvette and measured fluorescence spectra using a fluorescence spectrophotometer (F-4500, HITACHI, Japan) at a scan speed of 1200 nm/min and with a slit (EX/EM) of 5.0 nm/10.0 nm, in the measurement mode. When the 7-HC solution was excited at 325 nm, the emission maximum appeared at 455 nm; thus, in the following binding experiments, the fluorescence spectra of the tested solutions were measured at 455 nm with excitation at 325 nm.

Binding experiments were performed with the equipment illustrated in Figure 3A. By aeration with an air pump (Linicon LV-140, Medo/Nitto Kohki Co. Ltd, Japan), 7-HC vapor was continuously introduced into a quartz cuvette containing 200 μ l CRLBP solution for 15, 30 or 60 min. After the aeration for different periods, we monitored the

binding of 7-HC to CRLBP by measuring fluorescence spectra of the solution in the cuvette.

Results

Primary structure and three-dimensional molecular model of CRLBP

From the cDNA sequence obtained by PCR, we deduced the amino acid sequence of the antennal protein under investigation. Its full length of 125 amino acid residues was totally identical with the CRLBP derived from the labellum (S78710) (Ozaki *et al.*, 1995). We determined it had six conserved cysteine residues that form the three disulfide bonds essential for the functional three-dimensional structure. Moreover, the directly determined N-terminus 45 amino acid sequence was consistent with the deduced amino acid sequence.

Based on the primary structure, a three-dimensional model of CRLBP was constructed by referring to the three-dimensional model of PBP in *B. mori* (Sandler *et al.*, 2000). The optimized complex model of CRLBP shows that 7-HC had good contacts with the protein (Figure 1A). In particular, the hydroxyl and the lactone carbonyl groups of 7-HC form hydrogen bonds with Thr8 and Glu107, respectively (Figure 1B). In addition, the hydrophobic portion of the ligand is located at the hydrophobic interior of the binding pocket formed by Leu52, Phe55, Val57 and Phe117 (Figure 1B).

CRLBP as an odorant-carrier

The 7-HC-CRLBP complex should stably migrate in the receptor lymph and reach the receptor membrane surface.

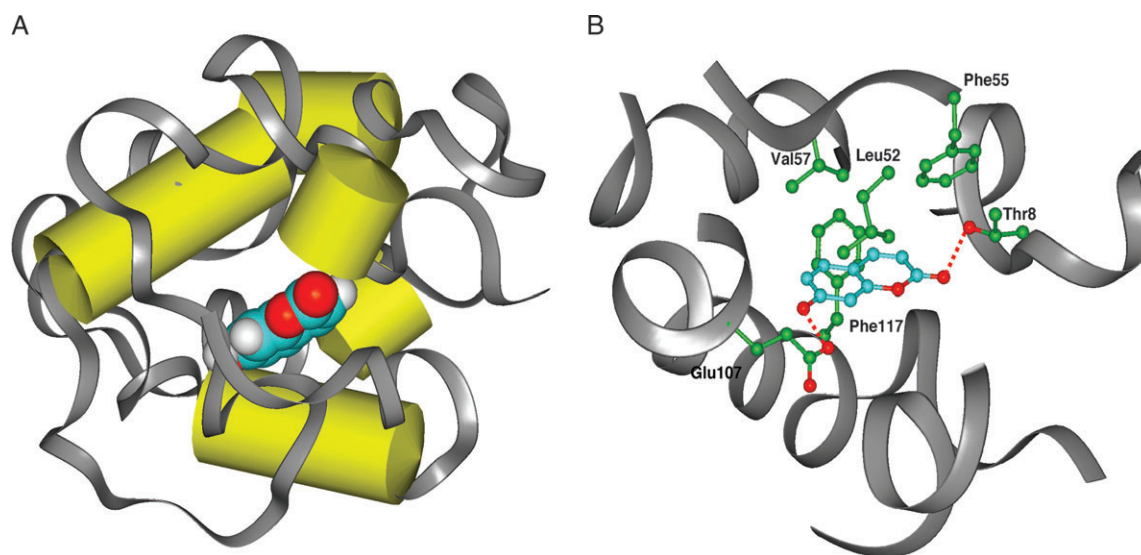


Figure 1 Three-dimensional molecular model of CRLBP. **(A)** Complex structure model. α -Helices are shown as yellow cylinders and the remaining parts are depicted as a gray ribbon. **(B)** The mode proposed for binding of 7-HC to CRLBP. Dotted lines indicate the hydrogen bonds between the 7-HC and the residues of CRLBP. A partial structure (10 Å from the ligand) of the protein is shown in gray ribbon. The ligand is colored sky blue for carbon and red for oxygen. Residues in the protein are colored green for carbon and red for oxygen. Hydrogen atoms are omitted for clarity.

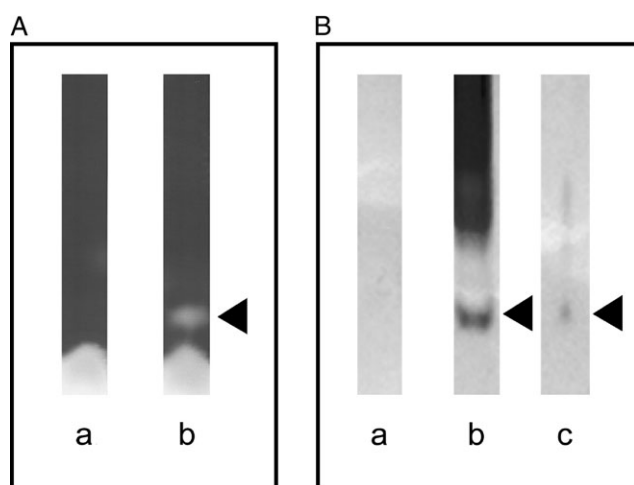


Figure 2 Fluorescence detection of 7-HC–CRLBP complex in the electrophoretic gel. **(A)** Fluorescence image of native-gel, to which 7-HC (a) and a mixture of 7-HC and water-soluble fraction of antennal extract (b) were applied, respectively. **(B)** Protein staining of the same gel, to which 7-HC (a), a mixture of 7-HC and water-soluble fraction of antennal extract (b) and purified CRLBP (c) were applied, respectively. The arrowhead in (A) indicates the migrating position of the 7-HC–CRLBP complex. The arrowheads in (B) indicate the migrating position of the CRLBP.

Considering this, we expected that the 7-HC–CRLBP complex could run stably in a native electrophoretic gel.

Figure 2 shows the result of native polyacrylamide gel electrophoresis (PAGE), in which 7-HC, the soluble protein fraction of antennal extract mixed with 7-HC and purified CRLBP were run in lanes a, b and c, respectively. After electrophoresis, gels were observed on the UV box to detect 7-HC (Figure 2A) and then stained with CBB for protein detection (Figure 2B). There are two 7-HC bands in Figure 2A: a bigger fluorescent band migrating at higher speed indicates an excess amount of 7-HC, which was not caught by CRLBP, and the smaller fluorescent band co-migrating with an acidic protein at lower migration speed (arrowheads in Figure 2A) indicates the 7-HC–CRLBP complex, its migrating position being the same as that of purified CRLBP (arrowheads in Figure 2B). This suggested that no other proteins in the antennal extract other than CRLBP function as a carrier for 7-HC.

CRLBP as a vapor trap

We introduced 7-HC vapor into the CRLBP solution by aeration and measured the fluorescence emission of the 7-HC trapped by CRLBP in a complex. Before aeration, the solvent buffer (4.75 mM sodium barbiturate–HCl buffer, pH 6.8, containing 10% glycerol) with or without CRLBP did not evoke any detectable fluorescence emission on excitation at 325 nm. As we continued aeration of the 7-HC vapor into the CRLBP solution for 15, 30 and 60 min, fluorescence emission subsequently increased (Figure 3C). The spectra curves had the maximum emission peaks at the same wave-

length as that of 7-HC itself (Figure 3B). When we introduced 7-HC vapor into the solvent without CRLBP for 15, 30 or 60 min and then excited the solution, weak fluorescence emissions were detected (Figure 3D). This is probably due to a small amount of 7-HC that dissolved in the aqueous solution. Therefore, it was suggested that 7-HC, which was introduced into the CRLBP solution as a vapor, was trapped in the aqueous environment mostly by forming a complex with CRLBP.

We also examined BSA and BLG, and found that neither BSA (Figure 3E) nor BLG bound 7-HC (Figure 3F) as effectively as did CRLBP (Figure 3C).

Discussion

To obtain chemosensory information, olfactory receptor neurons need to be open to the outside environment, but receptive membranes cannot be exposed directly to air as they have to be protected from drying or indiscriminate adsorption of lipophilic substances by a covering of receptor lymph. The dilemma for the receptor neurons in catching lipophilic odorants through hydrophilic surroundings is overcome by using OBPs as lipophilic substance carriers.

OBPs carry odorants in a shuttle system connecting two physico-chemical interfaces through the receptor lymph, catch odorants at the interface between the air and the receptor lymph, and then release them at the other interface between the receptor lymph and the receptor cell surface. Since Sandler *et al.* (2000) reported a three-dimensional molecular model of PBP (pheromone-specific OBP) in *B. mori*, conformational changes from the ligand-binding to the ligand-releasing form have been discussed in several OBPs (Deyu and Leal, 2002; Kruse *et al.*, 2003). Nevertheless, the odorant-trapping mechanism at the interface between air and the receptor lymph has not precisely elucidated. Our study using 7-HC vapor showed that an insect OBP, CRLBP, has a greater odorant-trapping ability than BSA or BLG (Figure 3). This suggests that the odorant-trapping process of insect OBPs is not passive like indiscriminate adsorption. It may occur in a ligand-selective manner depending on the fitness of the odorant molecules to the three-dimensional structure of the ligand-binding pocket in each OBP (Figure 1).

CRLBP is thought to be a member of the general odorant-binding proteins, which can bind a broad spectrum of odorants (Ozaki *et al.*, 1995), and it is probable that CRLBP does not have a very rigid ligand-binding pocket for a specific ligand but rather a flexible inside pocket that can fit various ligands. Moreover, the inside surface of its ligand-binding pocket is not as lipophilic as that of *B. mori* PBP. Thus, hydrogen bonds between the inside surface and the ligand might sometimes contribute to stable complex formation (Figure 1B).

To compare with mammalian lipocalin, we examined BLG, which has a β -barrel-type inside pocket like mammalian OBPs. BLG hardly solubilized 7-HC, and neither did BSA; this suggests that mammalian OBPs cannot replace

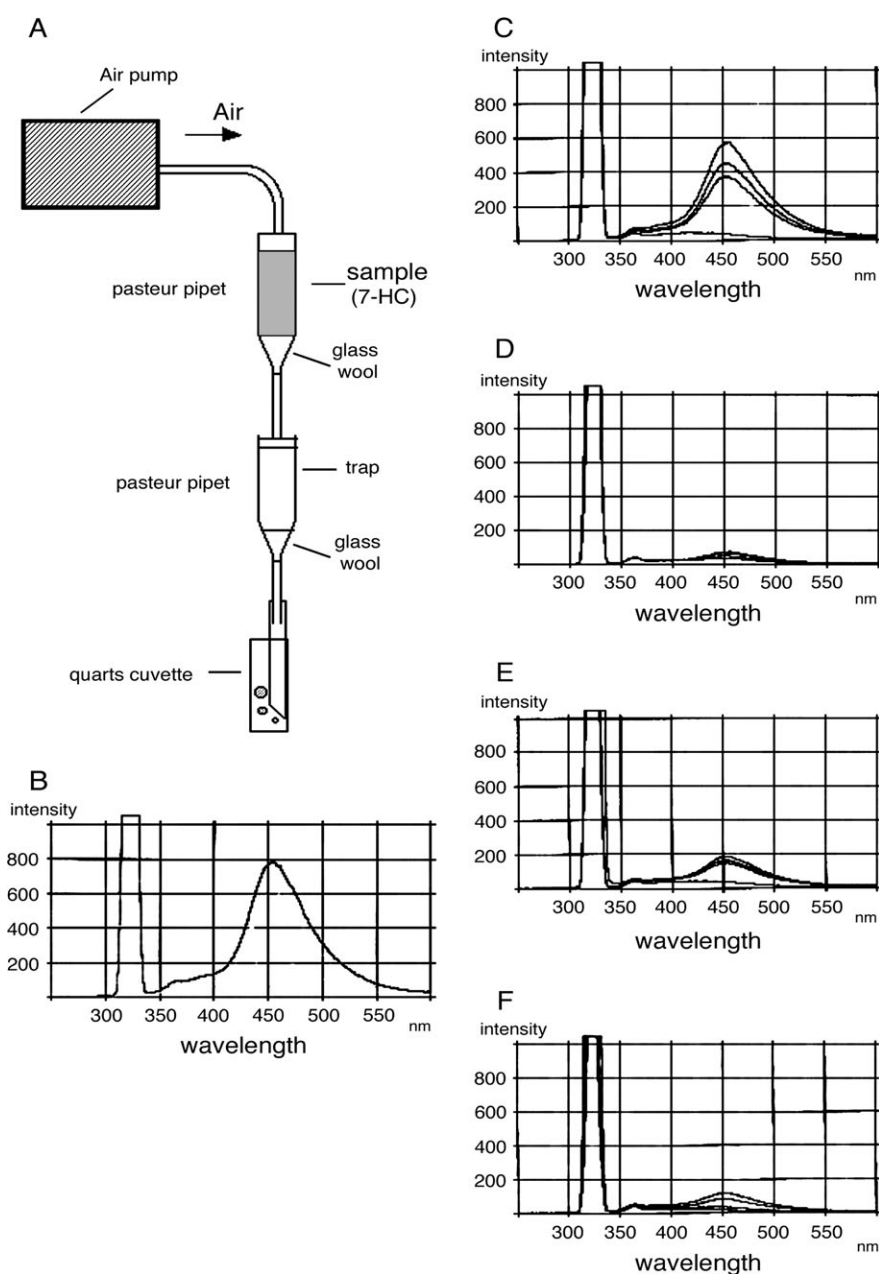


Figure 3 Fluorescence measurement of 7-HC-CRLBP complex formation by aeration. **(A)** Apparatus for fluorescence measurement of aquatic sample solutions in a sodium barbiturate-HCl buffer, to which 7-HC vapor was introduced by aeration. **(B)** Fluorescence emission spectrum of 7-hydroxycoumarin in a sodium barbiturate-HCl buffer. **(C, D)** Fluorescence emission spectra of CRLBP and the plane buffer solution, to which 7-hydroxycoumarin vapor was introduced for 15, 30 and 60 min. **(E, F)** Fluorescence emission spectra of the BSA and BLG solutions, to which 7-hydroxycoumarin vapor was introduced for 15, 30 and 60 min. Every solution was excited at 325 nm.

CRLBP (Figure 3). Mammalian OBP development may have diverged from that of insect OBPs, so their respective structural designs are very different. Although insect and mammalian OBPs may have similar roles as odorant carriers in their chemosensory systems, mammalian and insect OBP families could have different ligand-binding spectra and would not be compatible with each other.

In mammalian olfactory receptor neurons, most electrophysiological experiments have ignored OBPs or shuttle sys-

tems for odorants. Xu *et al.* (2005) indicated that in insects an OBP (LUSH) is required for activation of pheromone-sensitive neurons. Moreover, Van den Berg and Ziegelberger (1991) demonstrated that electrophysiological responses from the pheromone-sensitive sensillum in a moth were suppressed when intrinsic PBP was removed from the receptor lymph. Pophof (2002) has recently shown the contribution of OBPs to receptor specificity in lepidoptera. Further, Ozaki *et al.* (1995) suggested that the electrophysiological response

of the fifth cell of the taste sensillum in the fly was suppressed when intrinsic CRLBP was blocked by anti-CRLBP antiserum. Considering our results together with these reports, the contribution of OBPs can be seen to be indispensable to the various shuttle systems carrying lipophilic stimulants in insect chemosensory organs.

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