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Heterogeneity of Odorant-binding Proteins in the Antennae of *Bombyx mori*

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Abstract

Different odorant-binding proteins (OBPs) were isolated from total antennal homogenates of male and female *Bombyx mori*. Proteins were separated according to their isoelectric point by using preparative fast-flow isoelectrofocusing. Odorant-binding proteins were identified in immunoblots by antisera raised against the pheromone-binding protein (anti-PBP) and the general odorant-binding protein (anti-GOBP2) of *Antheraea polyphemus*. Four proteins cross-reacting with anti-PBP were detected in males and two in females, while three proteins cross-reacting with anti-GOBP2 were found in males and five in females. Both anti-PBP and anti-GOBP2 cross-reacting proteins had an apparent molecular weight of 15–16 kDa. In parallel, the same two antisera were used in immunocytochemical studies in order to determine the distribution of these proteins within the various subtypes of olfactory sensilla. The presence of multiple odorant-binding proteins within one moth species as well as their complex distribution pattern support the suggestion that soluble OBPs might have a function in odorant discrimination. **Chem. Senses 22: 503–515, 1997.**

Introduction

Soluble, low-molecular-weight proteins named odorant-binding proteins (OBPs) are involved in the perception of odorants in vertebrates and insects. In vertebrates they are secreted by different nasal glands and discharged into the nasal mucus (reviewed by Pelosi, 1994), while in insects they are homogeneously distributed in the sensillum lymph surrounding the dendrites of olfactory sensilla (reviewed by Pelosi and Maida, 1995). Insect OBPs are classified as pheromone-binding proteins (PBPs) and general odorant-binding proteins (GOBPs). Pheromone-sensitive long sensilla trichodea always express PBP (Steinbrecht *et al.*, 1992, 1995), whereas sensilla basiconica, which respond to plant odours and non-pheromonal compounds (Schneider *et al.*, 1964; Kafka, 1987), mainly express GOBP (Laue *et al.*, 1994; Steinbrecht *et al.*, 1995).

PBPs were identified by their ability to bind radiolabelled pheromones (Vogt and Riddiford, 1981; Vogt *et al.*, 1989; Maida *et al.*, 1993; Feixas *et al.*, 1995), by partial amino acid sequence homology (Vogt *et al.*, 1991a; Nagnan-Le Meillour *et al.*, 1996) or by molecular cloning approaches (Györgyi *et al.*, 1988; Raming *et al.*, 1989, 1990; Krieger *et al.*, 1991, 1993, 1996; Vogt *et al.*, 1991b). GOBPs were initially detected since they share some amino acid sequence homology with PBPs (Breer *et al.*, 1990). The term GOBP

was introduced by Vogt *et al.* (1991a), because GOBPs are highly conserved across species and expressed in both sexes, suggesting that they are not associated with intraspecific pheromone reception but rather with the perception of non-pheromonal, more general odorants. Moreover, GOBPs were subdivided into two subclasses, GOBP1 and GOBP2, based on amino acid sequence homology (Vogt *et al.*, 1991a). However, to date, no specific binding properties of GOBPs have been reported.

Proteins with characteristics of OBPs have also been identified in other orders of insects. In the antennae of *Drosophila* (Diptera) at least five OBPs have been identified by using molecular cloning approaches (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). In the fly *Phormia regina* Ozaki *et al.* (1995) detected an OBP-like protein (chemical-sense-related lipophilic-ligand-binding protein, CRLBP), which is expressed in taste sensilla, where it is most likely involved in the transduction of volatile compounds. OBPs have also been described for *Lygus lineolaris* (Heteroptera; Dickens *et al.*, 1995) and *Carausius morosus* (Phasmoptera; Tuccini *et al.*, 1996). Thus, odorant-binding proteins appear to be a general characteristic of insect olfactory organs.

Until now, only one PBP and two GOBPs have been described for the silkmoth *Bombyx mori*. The PBP was identified by binding affinity to tritiated bombykol, the main component of the sex-pheromone blend, and by cross-reactivity with an anti-PBP antiserum raised against the PBP of *Antheraea polyphemus* (Maida *et al.*, 1993). Both GOBPs were detected by amino acid sequence homology to PBP and their presence in both sexes (Vogt *et al.*, 1991a). Recently, the full amino acid sequences of one PBP and two GOBPs in *B. mori* were reported (Krieger *et al.*, 1996).

The main unsolved question concerning insect OBPs is their function. Until recently, it was thought that odorant-binding proteins are involved in solubilization, transport and/or deactivation of odorants (reviewed by Kaissling, 1996; Ziegelberger, 1996). Functional studies on the PBP of *A. polyphemus* supported the hypothesis that a complex of PBP and pheromone acts on the receptor cell membrane rather than the pheromone alone (Van den Berg and Ziegelberger, 1991; Ziegelberger, 1995). Upon pheromone stimulation of isolated sensory hairs of olfactory sensilla, a redox shift of the PBP from a reduced to an oxidized form, by forming a disulphide bridge, was observed (Ziegelberger, 1995). This shift only occurred in the presence of receptor cell membrane, suggesting that receptor activation might be associated with PBP oxidation, leading to a rapid deactivation of the pheromone–PBP complex. In addition to the transporter and deactivator function, a participation of OBPs in odour discrimination was suggested (Krieger *et al.*, 1991; Vogt *et al.*, 1991a). Recently, a different affinity to two distinct pheromone components has been reported for the two recombinant PBPs of *Antheraea pernyi* (Du and Prestwich, 1995), demon- strating that a certain odorant specificity is encoded in the two pheromone-binding proteins.

Since OBP heterogeneity could be a potential for different odour specificities, we searched for OBP-like proteins in antennal homogenates of *B. mori* males and females by a biochemical procedure which separates the proteins at a high resolution. We first fractionated proteins according to their isoelectric point (pI; resolution: 0.05–0. 1 units of pH), then separated them by native polyacrylamide gel electrophoresis and finally identified odorant-binding proteins by using two antisera raised against the PBP and the GOBP2 of *A. polyphemus*. The same antisera were used in immunocytochemical experiments at the electron microscope level, in order to study the distribution of odorant-binding proteins among olfactory sensilla on the antenna of male and female *B. mori*.

Materials and methods

Animals

Cocoons from *B. mori* were kindly supplied by the Istituto Sperimentale per la Zoologia Agraria, Padova (Italy). Pupae were separated according to their sex and stored in small boxes until emergence. The imagines were kept at 10°C. Their antennae were used 3–4 days after emergence for biochemical procedures and up to 30 days after emergence for immunocytochemistry.

Antisera

The anti-PBP and anti-GOBP2 antisera were raised by immunizing rabbits with purified PBP or GOBP2 of *A. polyphemus*, as described by Steinbrecht *et al.* (1992, 1995). Both antisera cross-react with respective OBPs of *B. mori* (Maida *et al.*, 1993; Steinbrecht *et al.*, 1995).

Preparation of antennal extracts

Whole antennae of male and female *B. mori* were homogenized on ice for 20 min with a home-made motor-driven homogenizer in 20 mM Tris–HCl buffer (pH

7.3) in the presence of 1 mM of phenylmethylsulphonyl fluoride and 2 mg/ml leupeptin, as protease inhibitors. Homogenates were centrifuged at 3000 *r.p.m.* for 15 min in order to obtain cuticle-free supernatants which were centrifuged again at 10 000 *r.p.m.* for 15 min prior to use in further experiments. All reagents were purchased from Sigma, unless stated otherwise.

Free-flow isoelectrofocusing

Antennal homogenates of ~2000 antennae of male or female *B. mori* were analysed by continuous flow high efficiency isoelectrofocusing (CHIEF Octopus; Dr. Weber GmbH, München, Germany), using an Ampholine gradient from pH 4 to 7 (0.5% Servalyte; Serva, Heidelberg, Germany) with 100 mM phosphoric acid as the anodal stabilizing medium and 50 mM NaOH as the cathodal stabilizing medium. Hydroxypropylmethylcellulose (HPMC, 0.2%) was added as a protein stabilizer. The whole separation was carried out at 4°C (1350 V, 30 mA, 50 W). The Servalyte solution sandwiched between the anodal and cathodal stabilizing media built up a pH gradient in the lower part of the separation chamber as soon as high voltage was supplied to the electrodes. The proteins, simultaneously supplied via a separate inlet, were separated by the pH gradient formed, according to the differences of their pIs. From each separation procedure 96 fractions (4 ml each) were collected. Their pH was measured with a pH electrode (M3-Ingold). Finally, individual fractions were analysed by electrophoresis (10 µl of each fraction per electrophoretic separation).

Before each CHIEF fractionation, the OCTOPUS device was scrutinized for separation quality by using a mixture of protein standards, which only slightly differed in their pI (~0.1–0.2 units of pH). Even when we analysed a high concentration of this protein mixture, single proteins were fractionated in 1–2 fractions without overlapping each other.

Analytical isoelectrofocusing

Analytical isoelectrofocusing (IEF) was carried out under native conditions in 5% polyacrylamide gels and a gradient of Ampholines (pH 4–6 Servalyte, Serva) using a Mini IEF Cell (Mod. 111; Bio-Rad). Proteins were visualized by using the Bio-Rad silver-stain procedure.

SDS and native PAGE

Electrophoresis under denaturating conditions was performed in 15% polyacrylamide gels with 1% sodium dodecyl sulphate (SDS–PAGE) using a Bio-Rad Mini-Protean II apparatus and a discontinous buffer system according to Laemmli (1970). Native electrophoresis was performed under the same conditions, but without SDS (native PAGE). Proteins were stained with Coomassie brilliant blue R250 or silver-stain.

Immunoblotting

After electrophoretic separation, proteins were electrotransferred onto nitrocellulose membranes (NCs), according to the semi-dry blotting procedure of Kyhse-Anderson (1984). First, NCs were blocked with 1% bovine serum albumine in phosphate-buffered saline and 0.05% Tween 20 for 1 h at room temperature (Batteiger *et al.*, 1982), then incubated overnight with the anti-PBP or anti-GOBP2 antiserum at a dilution of 1:500. Bound antibodies were detected by goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Bio-Rad; dilution 1:2000) and 4-chloro-1-naphthol as substrate.

Fine-structure immunolabelling

Tissue processing and post-embedding immunolabelling was done as described by Steinbrecht *et al.* (1995). Briefly, antennae were first cryofixed in super-cooled liquid propane (–180°C) and then submitted to freeze-substitution (starting temperature –90°C). Acetone (Roth, Karlsrühe, Germany) with 1–3% glutaraldehyde (Taab, Aldermaston, UK) or with 2% osmium tetroxide (Roth; at –70°C changed for pure acetone) served as substitution media. After controlled warming to room temperature over 5 days, antennae were embedded in LR White resin (hard grade; The London Resin Co., London, UK). Polymerization was carried out at 60°C for 2 days. Ultrathin sections were cut with a Reichert Ultracut or OMU II ultramicrotome and collected on Formvar-coated grids. Immunolabelling of sections with anti-PBP and anti-GOBP2 antisera (dilutions of 1:3000 to 1:30 000) was done by a two-step indirect method, using anti-rabbit immunoglobulin G coupled to 10 nm colloidal gold as secondary antibody (Amersham). Silver intensification according to Danscher (1981) enlarged the gold colloids for a better visualization. Labelled sections were examined with a Zeiss EM 10A electron microscope. A sensillum was considered as specifically labelled if the labelling density of the outer sensillum-lymph was significantly higher than background labelling of haemolymph, which is an analogous structure to the sensillum lymph and

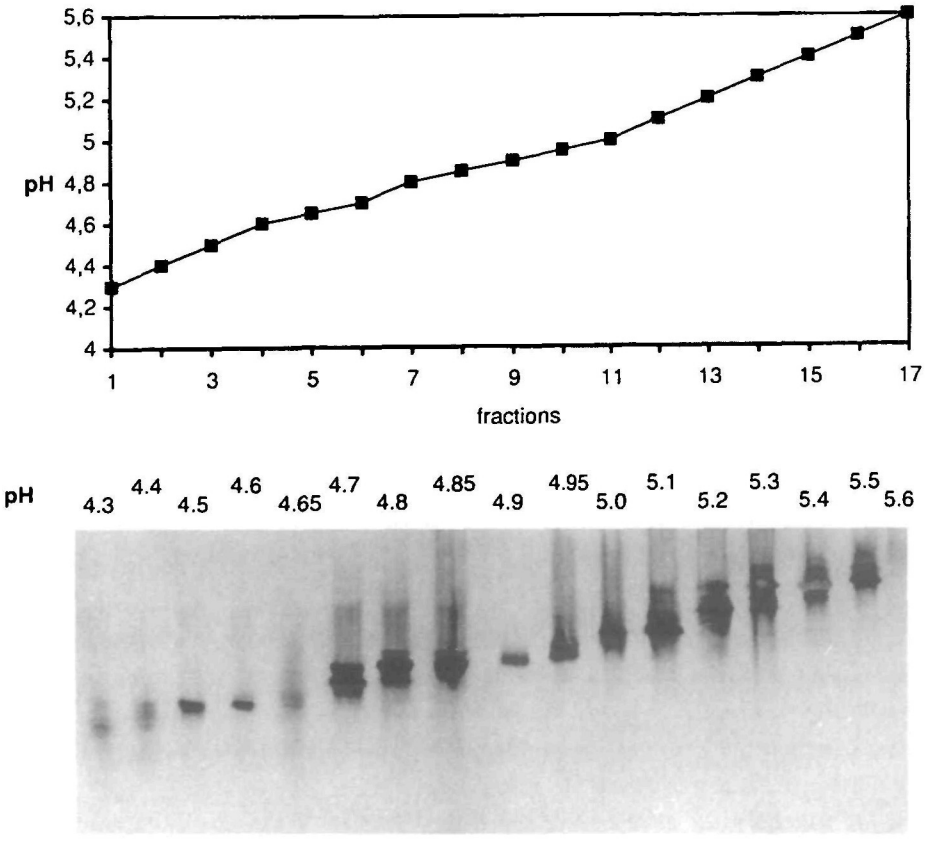

Figure 1 Top: pH profile of consecutive fractions from antennal homogenates of male *B. mori* after CHIEF fractionation. The pH in each fraction was measured with a pH electrode. Bottom: analytical IEF of the same fractions on a 5% native gel using an Ampholine gradient (pH 4–6). Bands were stained by the Bio-Rad silver staining procedure.

known to be devoid of PBP and GOBP2. Incubations with preimmune sera allowed for an additional control of labelling specificity.

Results

Protein fractionation and identification strategies

Proteins from antennal homogenates of male and female *B. mori* were fractionated using CHIEF with an Ampholine gradient of pH 4–7. This technique permitted a separation of proteins with a pI differing by 0.05–0.1 units (Figure 1, top). The analytical IEF of consecutive fractions (between pH 4.3 and 5.6) of male antennae showed the differences in pI of the proteins present in each fraction (Figure 1, bottom).

To identify odorant-binding proteins, CHIEF fractions were analysed by native PAGE and immunoblotting using the two antisera raised against the PBP and the GOBP2 of *A. polyphemus*. No sign of cross-reactivity, either of the anti-PBP antiserum with GOBP2s or of the anti-GOBP2 antiserum with PBPs, was observed (Figures 2–4). Immunoblotting after SDS–PAGE of total homogenates revealed no heterogeneity of PBPs and GOBPs, because all OBPs have similar molecular weight and migrate as a single band (see total homogenates in Figure 3). Separation of total antennal homogenates by analytical native PAGE is only possible with small amounts of antennal homogenates, permitting the detection of only the most abundant OBPs in immunoblots and therefore limiting the utility of this technique in estimating OBP diversity.

Fractionation of antennal proteins by CHIEF prior to separation in native PAGE resulted in the identification of several anti-OBP cross-reacting proteins in both sexes. The criteria which we adopted to distinguish different cross-reacting OBPs were: (i) the pI; (ii) the mobility in native PAGE; and (iii) the presence of at least one non-cross-reacting fraction between immunoreactive bands of the same mobility. The most intensely cross-reacting band among bands of the same mobility in consecutive fractions, i.e. of the same protein, was used to attribute a pI

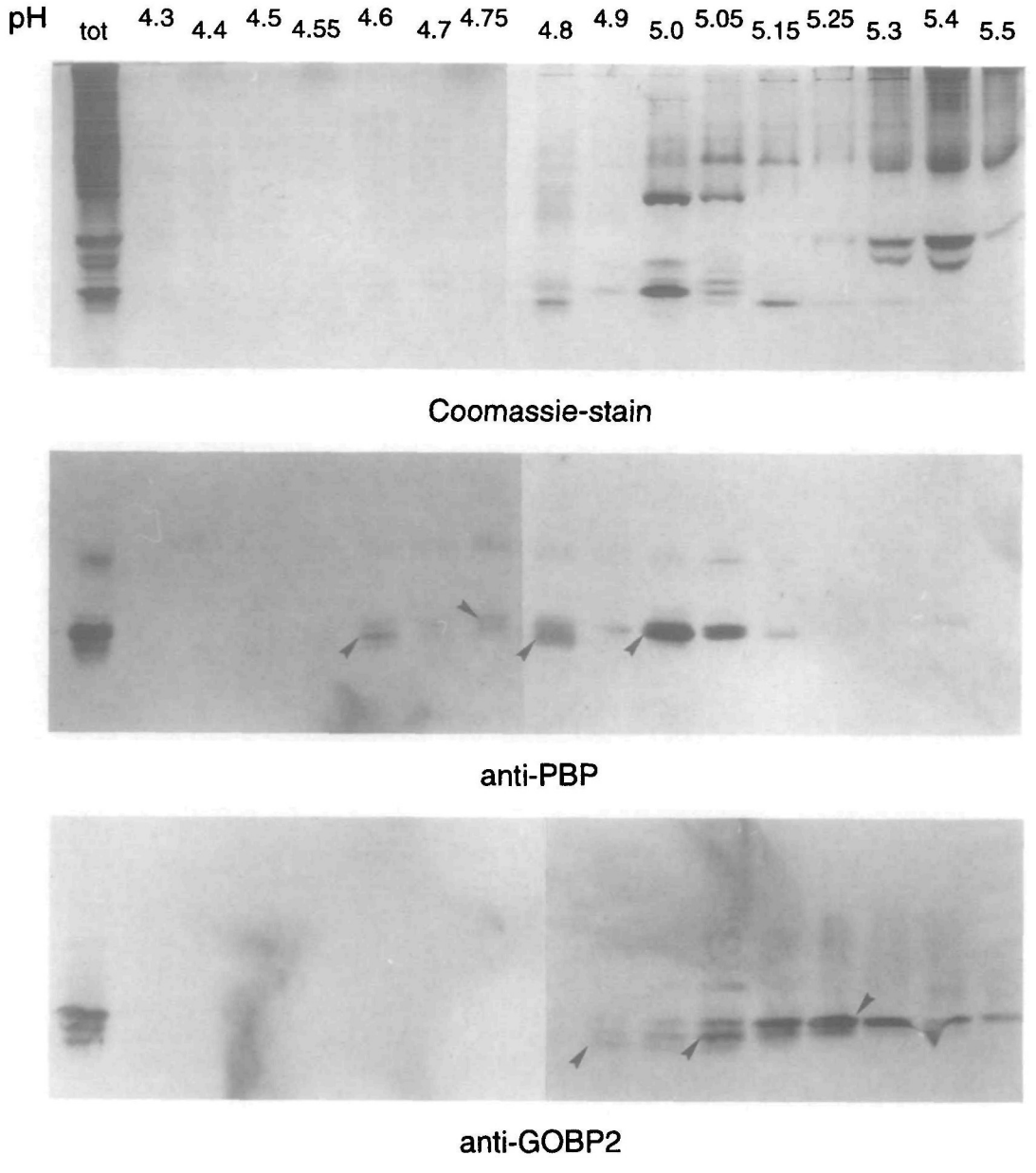

Figure 2 Top: CHIEF fractions of *B. mori* male antennae analysed in 15% native PAGE. Protein patterns were visualized by Coomassie stain. Middle: immunoblotting with the anti-PBP antiserum (dilution 1:1000). Bottom: immunoblotting with the anti-GOBP2 antiserum (dilution 1:1000). tot represents the total antennal homogenate (equivalent to four antennae) before CHIEF fractionation. Arrows indicate the different PBPs and GOBPs at the pH where they show the maximum level of cross-reactivity. The anti-PBP immunoreactive proteins at pH 4.6 and 4.75 are only faintly stained with Coomassie (top), indicating their presence in smaller amounts.

to a protein. The aim of this study was not to determine exactly the number of OBPs, but rather to show their heterogeneity.

Identification of PBPs and GOBP2s

The electrophoretic analysis of consecutive CHIEF fractions from male and female antennal homogenates was performed under native (Figures 2 and 4) and denaturating (Figure 3) conditions. In each of the Figures 2–4 the protein pattern after Coomassie staining (top), and the cross-reactivity with the anti-PBP (middle) and with the anti-GOBP2 antiserum (bottom) are shown.

Proteins recognized by the anti-PBP antiserum in immunoblots after native PAGE had a pI between 4.6 and 5.2. In males, PBPs were identified at a pH of 4.6 (one band), 4.75 (one band), 4.8 (one band) and 5.0 (one band) (Figure 2). In most of the cross-reacting fractions and in total homogenates an additional band was detected, which

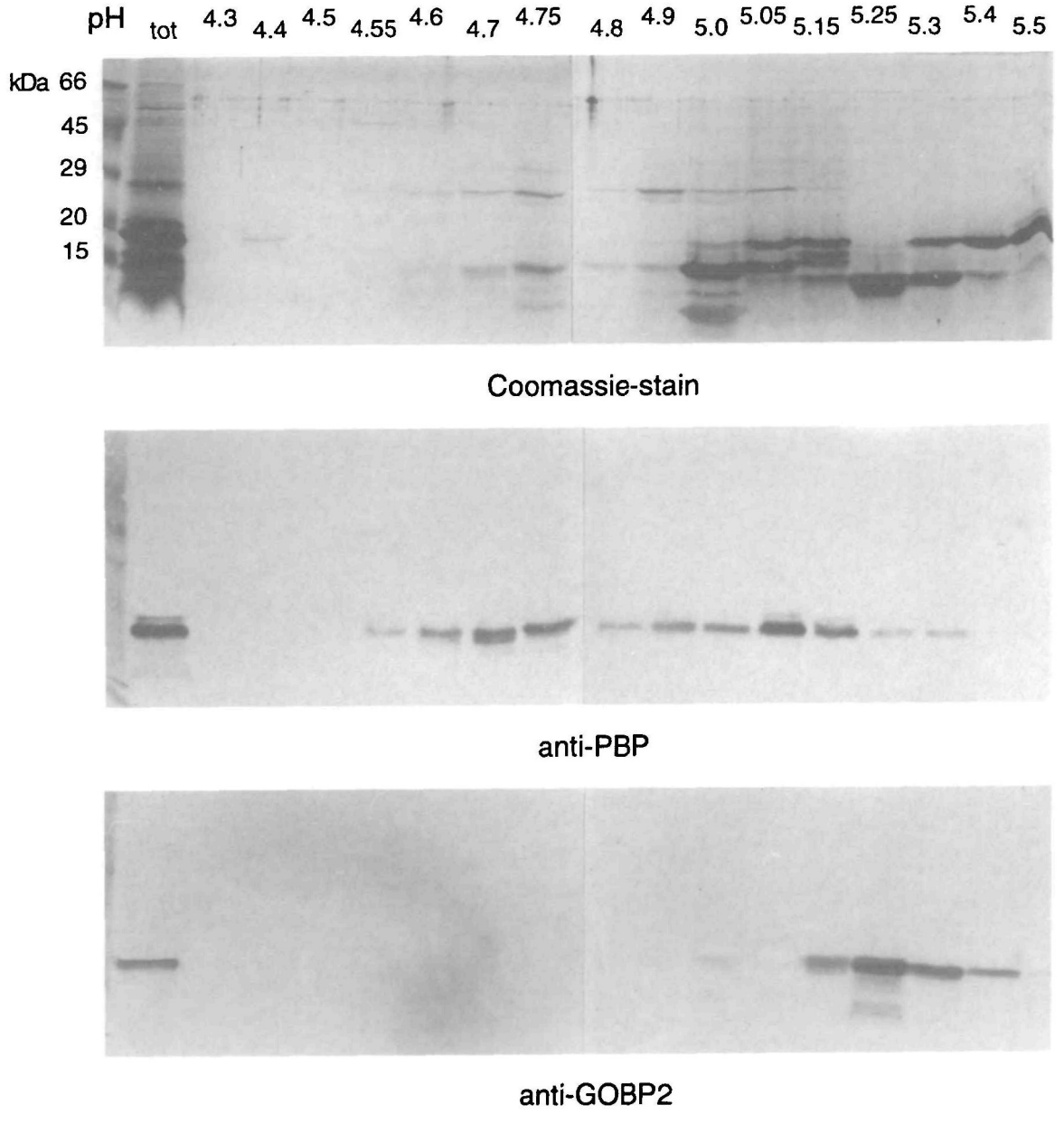

Figure 3 Top: CHIEF fractions of *B. mori* male antennae analysed in 15% SDS–PAGE. Proteins were stained with Coomassie. Molecular weight standards as indicated. Middle: immunoblotting with the anti-PBP antiserum (dilution 1:1000). Bottom: immunoblotting with the anti-GOBP2 antiserum (dilution 1:1000). tot represents the total antennal homogenate.

migrated only through the upper third of the native gel. These upper bands showed nearly the same relative mobility in the different fractions as the lower ones (see Figure 2, middle). Therefore, they most probably represent PBP dimers rather than additional PBPs.

Estimations from Coomassie staining (Figure 2, top) revealed that ~1 μg/antenna of the PBP pI 4.8 and ~2 μg/antenna of the PBP pI 5.0 could be recovered, while the PBP pIs 4.6 and 4.75 were present in significantly smaller amounts. In contrast to separation by native PAGE, PBPs, as well as GOBP2s, migrated only as a single band of 15–16 kDa in a given fraction after separation by SDS–PAGE (compare Figure 2 with Figure 3). In female antennae, only two anti-PBP immunoreactive proteins were labelled in immunoblots after native PAGE, one at a pH of 4.7 and one at 5.2 (Figure 4).

As with PBPs, sex differences were observed with respect to proteins that cross-reacted with the anti-GOBP2 antiserum. In males, anti-GOBP2 cross-reacting proteins were detected at pH 4.9 (one band), 5.05 (one band) and 5.25 (one band) (Figure 2, bottom). Again these proteins migrate, under denaturating condititons (SDS–PAGE), as

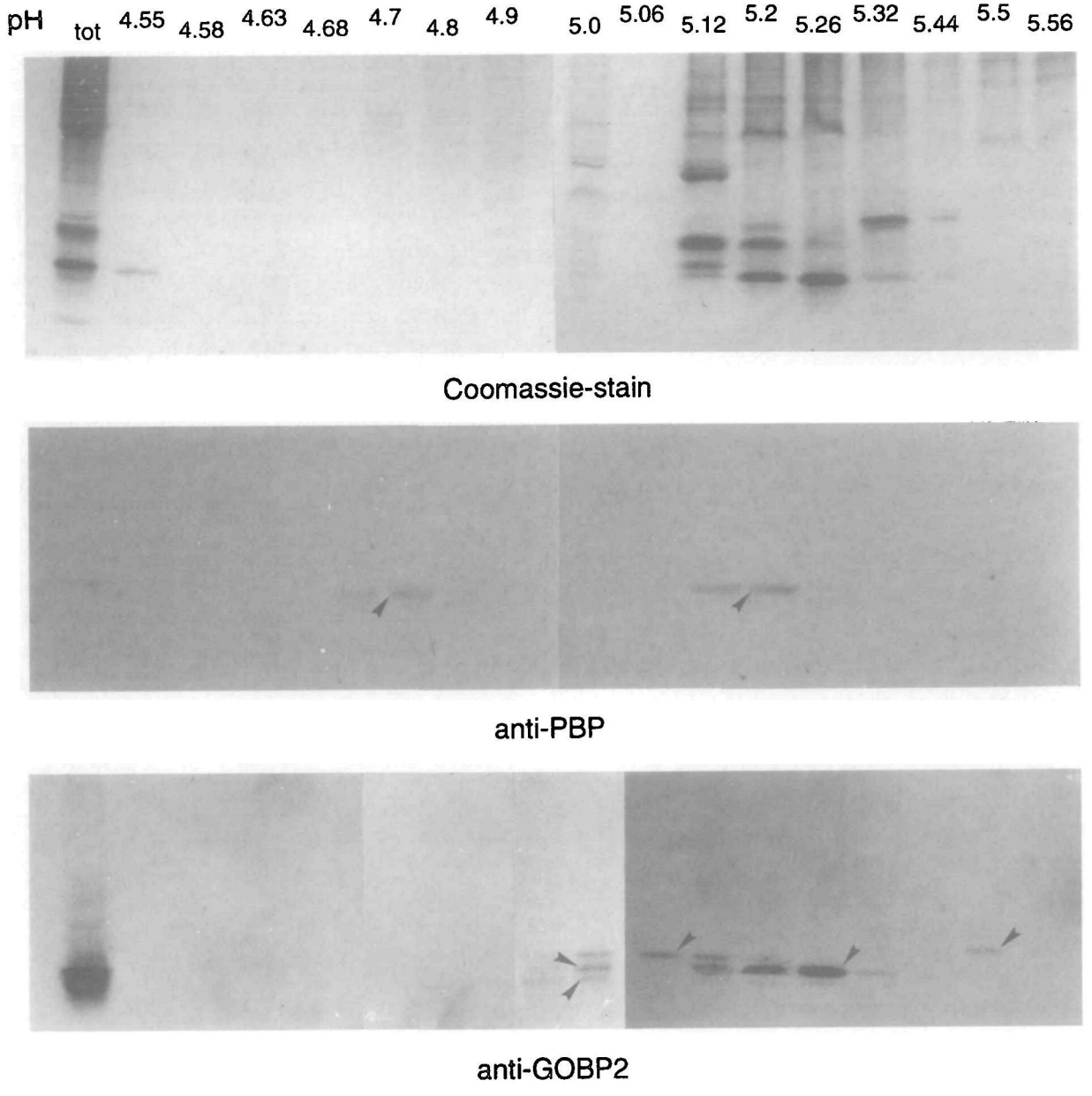

Figure 4 Top: CHIEF fractions from female *B. mori* antennae analysed in 15% native PAGE. Protein patterns were visualized by Coomassie stain. Middle: immunoblotting with the anti-PBP antiserum (dilution 1:1000). Bottom: immunoblotting with the anti-GOBP2 antiserum (dilution 1:1000). tot represents the total antennal homogenate. Arrows indicate the different PBPs and GOBPs at the pH where they show the maximum level of cross-reactivity.

single bands of apparent molecular weight of 15–16 kDa (Figure 3, bottom). In females, anti-GOBP2 immunoreactive bands were found at pH 5.0 (two bands), 5.06 (one band), 5.26 (one band) and 5.5 (one band) (Figure 4, bottom). As with PBPs, different GOBPs were also present in very different amounts. The Coomassie staining of proteins from female antennae (Figure 4, top) showed only the anti-GOBP2 immunorective protein in the fraction of pH 5.26, while the other anti-GOBP2 immunoreactive proteins were not clearly visible. This suggests that these proteins are probably expressed at lower levels than the 'classic', dominant OBPs. The number of cross-reacting proteins at different values of pH in males and females is summarized in Table 1.

Immunolocalization of PBP and GOBP2 in olfactory sensilla

Immunolabelling experiments with the anti-PBP and the anti-GOBP2 antisera yielded specific labelling patterns among olfactory sensilla in the antennae of *B. mori*. Each

Table 1 Identified odorant-binding proteins in antennal homogenates of *B. mori* after CHIEF fractionation and native PAGE

pH	Number of cross-reacting proteins in native PAGE			
	Males		Females	
	Anti-PBP	Anti-GOBP2	Anti-PBP	Anti-GOBP2
4.30				
4.40				
4.50				
4.55				
4.60	1			
4.70			1	
4.75	1			
4.80	1			
4.90		1		
5.00	1			2
5.05		1		1[a]
5.15				
5.20			1	
5.25		1		1[a]
5.30				
5.40				
5.50				1

[a]In females at pH 5.06 and 5.26 respectively.

sensillum was either specifically labelled by one of the two antisera or not labelled at all. Double-labelling by both antisera did not occur. Long sensilla trichodea, which are the most numerous sensilla on the antenna, exhibited strong, uniform labelling in both sexes. In males they were labelled by anti-PBP, whereas in females they were labelled by anti-GOBP2 (Figure 5). Labelling of medium-sized sensilla trichodea and sensilla basiconica was more heterogeneous. Most of these sensilla were labelled by anti-GOBP2, some were labelled by anti-PBP or remained unlabelled. The anti-PBP labelling was more frequent among medium-sized sensilla trichodea, whereas the proportion of unlabelled sensilla was higher among sensilla basiconica than among medium-sized sensilla trichodea (Figure 5). Olfactory sensilla coeloconica, which are structurally different from sensilla trichodea and sensilla basiconica, were never labelled by the two antisera. In addition to the complex distribution of different OBP types among olfactory sensilla, we were able to distinguish at least two classes of labelling densities with each of the antisera. Specific immunolabelling of sensilla basiconica and medium-sized sensilla trichodea by the anti-GOBP2

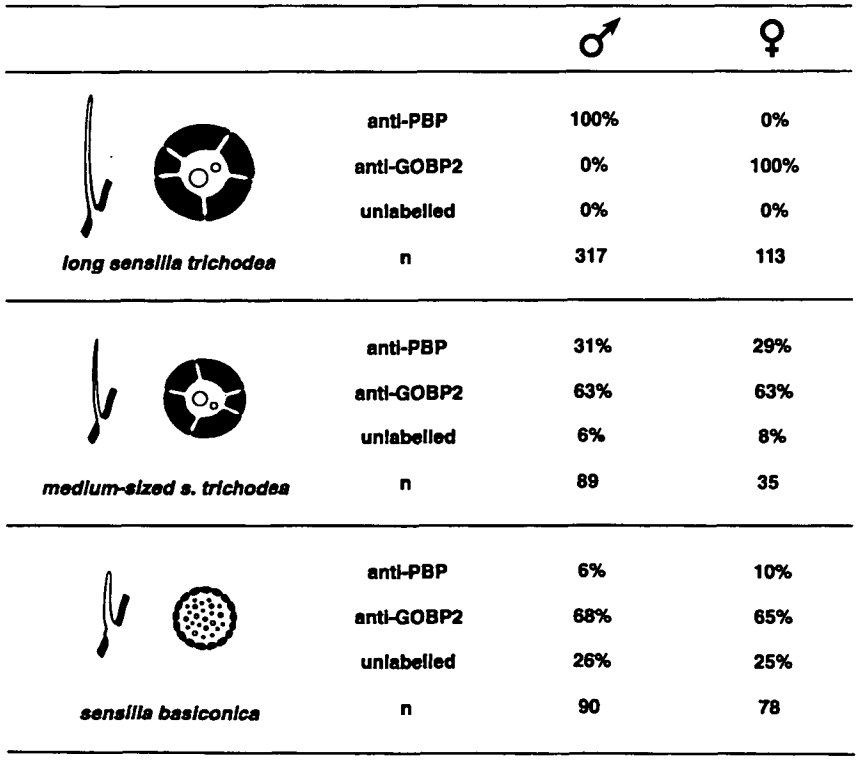

Figure 5 Distribution of anti-PBP and anti-GOBP2 labelling among subtypes of olfactory sensilla in male and female *B. mori*. Schematic drawings of sensory hairs of different sensillum subtypes (longitudinal sections on the left, cross-section on the right side). Subtypes were distinguished according to the length of the sensory hair, the thickness of the hair cuticle, the pore density, the number of the receptor cells and the morphology of the dendrites (i.e. branched or unbranched), according to Steinbrecht (1970, 1973).

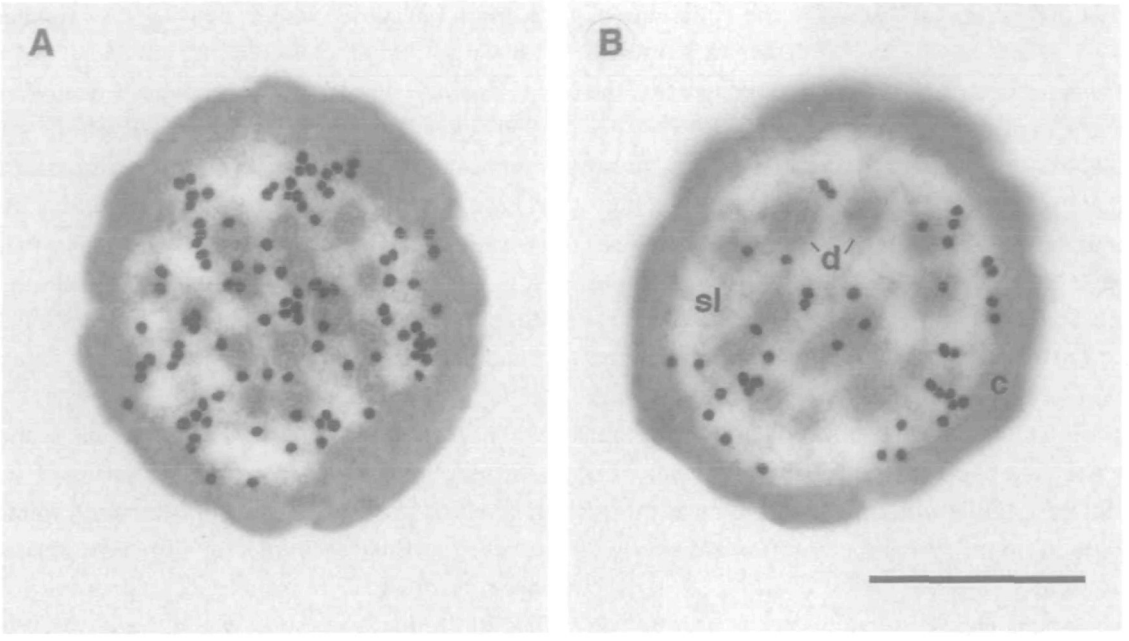

Figure 6 Sensory hairs of olfactory sensilla basiconica in cross-section, labelled in the same experiment with the anti-GOBP2 antiserum (dilution 1:9000). In the sensillum on the left side **(A)** the sensillum lymph (sl) is clearly labelled more intensely than in the sensillum on the right side **(B)**. c = cuticle; d = dendrite. Scale bar = 0.5 μm.

antiserum resulted mainly in high labelling densities, but some sensilla displayed lower densities (Figure 6). In the case of labelling with the anti-PBP antiserum, almost all sensilla showed high labelling densities. Only a few medium-sized sensilla trichodea exhibited lower labelling densities. Differences in labelling densities were more obvious after freeze-substitution with osmium tetroxide. There was no morphological difference between sensilla of different labelling densities within a subtype, and labelling of sensillum-lymph appeared to be homogeneous regardless of labelling density.

Discussion

Fractionation of proteins according to their isoelectric point prior to separation by native PAGE permitted an identification of odorant-binding proteins at a higher resolution than by native PAGE and/or SDS–PAGE alone. Since all OBPs have a similar molecular weight, immunoblots of unfractionated antennal homogenates showed only one or two immunoreactive proteins. In addition, CHIEF fractionation of a large number of homogenized antennae (i.e. up to 2000) permitted the identification of proteins present in minute amounts. The total number of OBPs could be even higher, since some low-molecular-weight proteins with low pI did not cross-react with our two antisera, indicating that they might belong to the GOBP1 class.

At the beginning of this investigation, one PBP and two GOBPs were described for *B. mori* (Vogt *et al.*, 1991a). The PBP of *B. mori* males was biochemically characterized by Maida *et al.* (1993) as a protein of 16 kDa and pI 4.9. Recently, Krieger *et al.* (1996) cloned a PBP gene of *B. mori* antennae. The encoded PBP has a molecular weight of 17.3 kDa and a pI of 4.7, as estimated from the deduced amino acid sequence. Nevertheless, it was suggested by these authors that this PBP could be the same as that already characterized by Maida *et al.* (1993), because the differences in their chemical properties might be due to the different approaches used in their identification.

In the present study we have identified at least four PBPs in males which differ in their isoelectric points. It appears very likely that the two PBPs described above represent two different PBPs, since we detected two major anti-PBP cross-reacting proteins which eluted at similar pH (4.8 and 5.0 respectively) to the two described PBPs (4.7 and 4.9 respectively). In females, only two PBPs were detected. One of them eluted at a significantly higher pH than the four PBPs of the male, suggesting the exclusive expression of this protein in females. We also found several different anti-GOBP2 cross-reacting proteins in both sexes. But in contrast to PBPs, GOBP2s are more heterogeneous in females (five different proteins) than in males (three dif-

ferent). The results presented here give the first hint of heterogeneity of OBPs in *B. mori*. Further experiments should affirm this heterogeneity by microsequencing of the immunoreactive proteins.

Heterogeneity of OBPs was also found in other moth species (Maida *et al.*, 1995 and in preparation; Nagnan-Le Meillour *et al.*, 1996) and in *Drosophila* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). It appears to be a common feature not only in insects but also in vertebrates. The presence of several OBPs within one species was demonstrated by fractionation of proteins according to their pI using chromatofocusing techniques (Felicioli *et al.*, 1993; Pes and Pelosi, 1995). The heterogeneity of OBPs is consistent with the idea that different OBPs with different binding affinities might participate in odour discrimination (for a review see Prestwich *et al.*, 1995).

The immunocytochemical results presented here provide further evidence for this idea. The presence of a certain OBP is clearly not associated with the morphological sensillum type (i.e long sensillum trichodeum, medium-sized sensillum trichodeum and sensillum basiconicum), but rather with the specificity of the receptor cell. The best support for this hypothesis comes from the predominant long sensilla trichodea present in male and female antennae. In males, these sensilla contain receptor cells which respond to the two pheromone components bombykol and bombykal, and always express PBP, whereas in females they possess receptor cells responding to the plant odours benzoic acid and linalool (Priesner, 1979; Heinbockel and Kaissling, 1990), while always expressing GOBP2. In this case, the same morphological sensillum type expresses different classes of OBPs, each being associated with receptor cells of highly different specificity classes. The medium-sized sensilla trichodea and sensilla basiconica do not show a uniform expression of a single OBP class, exhibiting mostly GOBP2-, sometimes PBP- or no cross-reactivity with our antisera. This non-uniform expression pattern of OBPs corresponds to the non-uniform electrophysiological specificity of the receptor cells. In the related silkmoth *Antheraea*, these sensilla respond to a variety of non-pheromonal odours with overlapping specificity (Schneider *et al.*, 1964; Kafka, 1987). For a direct correlation of OBP expression and specificity of receptor cells in these sensilla, further characterization of electrophysiological specificity and OBP content in identified sensilla must be performed. Unlabelled sensilla, found in both sexes, are morphologically indistinguishable from labelled ones. They might contain an OBP which does not cross-react with our antisera—perhaps a GOBP1 (see above).

Sensilla coeloconica, which have been recently identified as olfactory sensilla (B. Pophof, submitted for publication), showed no cross-reactivity with our antisera. These sensilla belong to another sensillum 'Bauplan' than sensilla trichodea and sensilla basiconica mainly possessing different stimulus conducting structures (Keil and Steinbrecht, 1984). It would be of particular interest to elucidate whether sensilla coeloconica express odorant-binding proteins at all.

The biochemical data presented here show that our antisera cross-react with multiple forms of an OBP class. Therefore, it is not possible to determine whether immunolabelled sensilla encounter one or more forms of an OBP class, but we can exclude co-expression of PBPs and GOBP2s. Most sensilla which were specifically labelled showed strong labelling densities, but some of the medium-sized sensilla trichodea and sensilla basiconica displayed significantly lower labelling densities. Even without exact quantitation, the specific labelling densities could be put into three categories: 'strong', 'intermediate' and 'zero'. The reason for this intermediate labelling might be a reduced expression level of respective OBPs, or a reduced cross-reactivity of all or of some of the OBPs encountered. In *Drosophila*, the related OBPs OS-E and OS-F (McKenna *et al.*, 1994) are always co-expressed in olfactory sensilla trichodea of the funiculus (Hekmat-Scafe *et al.*, 1997). Thus, it is likely that at least OBPs of a given class are co-expressed in olfactory sensilla of moths, too. In this case a stepwise difference in labelling density could be attributed to a different composition of OBP forms, which have different levels of affinity to our antisera. The presence of multiple forms of OBPs in different combinations would offer the possibility of selective transport of different odorants towards the receptor molecules, which are presumably situated in the dendritic membrane.

A prerequesite for such selective transport would be that OBPs possess a differential affinity for odorants. Only a few studies have provided data which suggest that a certain binding specificity is encoded in OBPs. The PBP of *A. polyphemus* binds the hexadecadienyl acetate (6E, 11Z-16:Ac) pheromone much more strongly than the corresponding alcohol (De Kramer and Hemberger, 1987; Du and Prestwich, 1995). Moreover, in the related *A. pernyi*, the two recombinant PBPs showed different binding preferences for the two pheromone components of this

species (Du and Prestwich, 1995). Additional evidence for a certain binding specificity encoded in OBPs comes from a comparative immunolabelling study in species from different moth families (Steinbrecht, 1996). The results revealed differences in the cross-reactivity of the anti-PBP antiserum with the PBPs in pheromone-sensitive sensilla trichodea of males of different moth species. Similar cross-reactivity is correlated more with the similarity of the pheromone components used by these species than with their taxonomical relatedness, indicating a certain specificity of the different PBPs for pheromone structure.

Taken together, these data, the OBP heterogeneity and the specific binding of odorants by OBPs argue for the idea that OBPs could contribute as a first step to odour discrimination, selecting odorants to a certain extent prior to their transport towards the presumed membrane-bound receptor molecules, where they or a complex of OBP and odorant are specifically recognized.

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