A pheromone-binding protein from the cockroach *Leucophaea maderae*: cloning, expression and pheromone binding

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Odorant-binding proteins (OBPs) are thought to transport volatile compounds from air to their receptors through the sensillary lymph. In this protein family, the subgroup of pheromone-binding proteins (PBPs) is specifically tuned to the perception of the sexual pheromone. To date, the description of OBPs has been restricted to Endopterygota and Paraneoptera. Their expression in Orthopteroid has been hypothesized, but no evidence of OBP has been produced in this assemblage to date. In the present study, we describe the first OBP from a Dictyopteran insect that belongs to the cockroach *Leucophaea maderae*. The PBP of *L. maderae* (PBPLma) shares all the hallmarks of the OBP family and is expressed specifically in the female adult antennae, the sex that perceives the sexual pheromone. The affinity of the recombinant PBPLma produced

in the *Escherichia coli* periplasm for the pheromonal compounds has been tested by displacement of a fluorophore, 8-anilino-1-naphthalenesulphonic acid (ANS). Our results suggest that two chemically close compounds of the pheromonal blend (3-hydroxy-butan-2-one and butane-2,3-diol) are capable of displacing ANS, whereas two other pheromone components (E-2-octenoic acid and senecioic acid) and other alkyl volatile compounds are not capable of displacing ANS, indicating a certain filtering of binding, which can be correlated with the putative function.

Key words: Dictyoptera, fluorescent-binding assay, *Leucophaea maderae*, olfaction, pheromone-binding protein.

INTRODUCTION

Sexual behaviour and mate choice are based on different clues mainly mediated by the sexual pheromones. In *Leucophaea maderae*, as in all other Oxyhaloinae but in contrast with moths, the females perceive the sexual pheromone blend emitted by males [1]. The female must collect relevant information before mating; hence, olfactory and gustatory perceptions are used after the first step of these behaviours. The chemosensory receptors are expressed in small cuticular extensions on the dendrites of the specific neuron membrane called sensillae. Thousands of sensillae are spread on the antennae and palps, the main appendages involved in the chemical perception. The dendrites are bathed in a proteinaceous lymph in which a special protein family, the olfactory-binding proteins (OBPs), is found at a high concentration [2].

OBPs are acidic soluble proteins (14–17 kDa) with a six-conserved-cysteine residue pattern. They are involved in the chemoreception pathway, as they are supposed to transport odorants into and through the sensillary lymph to the chemosensory receptors and possibly act in odorant filtering [3,4]. It has been proposed that volatile molecules might trigger a response either by interacting with the receptor within an OBP–odorant complex and/or by docking on to a protein present on the dendritic membrane [5–7]. OBPs form a divergent family; the paralogy has been highlighted by the prediction of genes encoding putative OBPs through the genome analysis of *Droso-*

phila melanogaster (38 OBPs) [8,9] and *Anopheles gambiae* (at least 21 OBPs) [10,11].

A subgroup of OBPs, the pheromone-binding proteins (PBPs), is specifically tuned for the accommodation of sexual pheromones. They are present at high concentration in the lymph of individuals belonging to the sex perceiving the pheromone and attracted by the partner. The other OBP subgroups comprise the general odorant-binding proteins (GOBP1 and GOBP2) [3] and the antennal binding protein X [12,13], which are expressed in both sexes and are supposed to be involved in the perception of extraspecific semiochemicals. These subgroups have been identified by behavioural experiments and by gathering the sequences that share higher identity. Ligand-binding studies with photoaffinity- or radioactive-labelling experiments have been conducted with both subgroups, but only a few GOBPs have been demonstrated to bind odorants such as floral scents [14,15], whereas several PBPs have been demonstrated to bind pheromonal compounds [16–19]. Other binding experiments, based on endogenous fluorescence or displacement of a fluorophore, made it possible to determine the affinity constants accurately [20,21].

To date, the description of OBPs has been restricted to Endopterygota and to Paraneoptera. In the present study, we report the first description of an OBP in an Orthopteroid insect, the cockroach *L. maderae*. This protein has been isolated and its expression in different tissues has been characterized. According to the dimorphism observed in the sex expression, this protein is supposed to be a PBP.

Abbreviations used: AMA, 1-aminoanthracene; ANS, 8-anilino-1-naphthalenesulphonic acid; OBP, odorant-binding protein; PBP, pheromone-binding protein; PBPLma, PBP of *Leucophaea maderae*; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

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The sexual pheromone blend of *L. maderae* is known and its components have been previously identified and behaviourally tested [1]. The blend is composed of four compounds: the 3-hydroxy-butan-2-one ($\text{CH}_3\text{CHOHCOCH}_3$) is the long-range attracting pheromone; the butane-2,3-diol ($\text{CH}_3\text{CHOHCHOHCH}_3$) seems to have no behavioural response and is supposed to be the precursor of the 3-hydroxy-butan-2-one; and the senecioic acid ($\text{CH}_3\text{C}(\text{CH}_3)=\text{CHCOOH}$) and the E-2-octenoic acid ($\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCOOH}$) are the pheromone compounds that stop the female at a close distance from the male. We have therefore determined, by fluorescence binding assays, the affinity of *L. maderae* PBP (PBPLma) for the compounds of the pheromonal blend.

EXPERIMENTAL

Material

Colonies of *L. maderae* were maintained at $25 \pm 2^\circ\text{C}$ and 70% relative humidity. The insects were fed with dry dog food and water was provided *ad libitum*. They were maintained under a 12 h light/dark cycle. Adults of both sexes were isolated from the colonies at the imaginal molt and reared individually in plastic boxes (9 cm $\times$ 6 cm $\times$ 2 cm) under the same conditions as the colonies.

RNA isolation, reverse transcriptase (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR

Total RNA from 500 adults or larvae (mixed from all instars), male or female tissues (antennae, labial and maxillary palps, cerci, tarsi, tergal gland, sternal gland and midgut) were extracted by the phenol/chloroform/3-methylbutan-1-ol method: tissues were crushed, 5 ml of lysis buffer [0.2 M EDTA, 0.1 M Tris/HCl (pH 9.5), 2% SDS, 5% ethanol, 1.4 mg/ml of K proteinase and 0.4 mg/ml of heparin] was immediately added, the samples were vortex-mixed and 5 ml of phenol [phenol/chloroform/3-methylbutan-1-ol in the ratio 25:24:1 (by vol.) saturated in 1 M Tris/HCl buffer (pH 7)] was added to the previous mixture. Samples were vortex-mixed and centrifuged at 10000 *g* for 10 min at 4°C . The upper layer was collected and the extraction was repeated three times: with 5 ml of phenol, with 50% phenol and 50% chloroform mixture [chloroform/3-methylbutan-1-ol in the ratio 24:1 (v/v)] and with 5 ml of chloroform. RNAs were precipitated in ethanol at -20°C overnight and harvested by centrifugation at 10000 *g* for 30 min at 4°C . Poly(A)-rich RNAs from adult female antennae and maxillary palps were purified by affinity with oligo(dT)-cellulose. The amount and the purity of each RNA sample were estimated using spectrophotometric measurements at 260 and 280 nm.

Poly(A)-rich RNAs from adult female antennae, labial and maxillary palps were used as templates for reverse transcription using Superscript II reverse transcriptase (Gibco Life Technologies, Eggenstein, Germany) and an oligo(dT)-anchor primer. The resulting cDNAs were used as templates in PCR with a degenerated primer (OBPcc) and an oligo(dT)-anchor primer.

LAP Lygus, the OBP from *Lygus lineolaris* GB-AF091118 [7], and TSPLma, an OBP-like protein from *L. maderae* GB-AY116617 [38], were used to design the primer OBPcc (GCNTGYGCNGGNCARTGYT). The products were amplified by 30 cycles of PCR. Each cycle includes 1 min at 94°C , 1 min at annealing temperature (58°C) and 1 min at 72°C , and then 10 min at 72°C for the last-cycle extension step. The reaction was performed in 20 μl containing 50 pmol for each primer, 0.3 mM deoxynucleotides and 0.1 unit of *Taq* polymerase

(Life Technologies, Basel, Switzerland) in the appropriate enzyme buffer. Separation of PCR products was performed by 1% agarose-gel electrophoresis, and DNA purification was performed on an anion-exchange column after solubilization of the gel-containing band (CONCERTTM, Rapid Gel Extraction System; Gibco BRL, Cergy-Pontoise, France). Fragments were subcloned in a T/A cloning plasmid (pGEM-T Easy; Promega, Charbonnières, France) and sequenced on both strands by MWG sequencing services (MWG-Biotech, Ebersberg, Germany).

Based on the sequence of the fragment obtained by RT-PCR, sets of specific primers were synthesized and used for RACE. The first-strand cDNA was 3'-tailed using terminal deoxynucleotidyl transferase and dCTP. This matrix was used for the second-strand cDNA synthesis, using 1 μM poly(G) adapter-primer, 50 μM dNTPs in 1 PCR cycle with 0.1 unit of *Taq* polymerase (Life Technologies). A 30-cycle-nested PCR was performed using the poly(G) adapter-anchor primer in combination with specific primers (PBP5'1, GACGCAATCGGTGTAGTTCT; PBP5'2, TCGTTGAAACCGTACAGTCG). A 30-cycle nested PCR was performed using the anchor primer in combination with specific primers. Each of the 30 cycles included 1 min at 94°C , 1 min at annealing temperature and 1 min at 72°C . The amplified product was purified, subcloned and sequenced on both strands as described above for RT-PCR (Figure 1).

Sequence analysis

Database searches were performed using Gapped BLAST [22]. Multiple sequence alignments were performed using CLUSTALW [23] with standard parameters and a Blossum matrix. The hydrophobicity profile was determined by the method of Kyte and Doolittle [24]; the putative signal peptide prediction was done using SignalP [25]. The secondary structure was predicted using Jpred [26] and the tertiary structure using 3D-ppsm [27].

Northern-blot analysis and *in situ* hybridization

As described in the RNA isolation subsection, total RNAs were separated on a formaldehyde-agarose (0.8%) gel and transferred on to a Hybond-N⁺ nylon membrane (Amersham). Samples were hybridized with a random-primed α -³²P-labelled 361-bp cDNA probe amplified with OBPcc and dT-anchor primer in a hybridization buffer [6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 0.5% SDS and 100 $\mu\text{g}/\text{ml}$ of salmon sperm DNA] at 42°C overnight, and then washed in 0.2 $\times$ SSC/0.1% SDS at 42°C and autoradiographed.

The same α -³²P-labelled random-primed cDNA fragment as the one mentioned above was used for *in situ* hybridization analysis. Cryosections were deposited on poly(L-lysine)-coated slides. After three washes in PBS (130 mM NaCl/7 mM Na_2HPO_4 /3 mM NaH_2PO_4 ; pH 7), the sections were incubated in 0.2 M HCl for 10 min, acetylated using acetic anhydride and then dehydrated in increasing concentrations of ethanol (50, 75, 95 and 100%). Desiccated sections were incubated for 1 h at 38°C in a prehybridization buffer [4 $\times$ SSC/1 $\times$ Denhardt's (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA)]. The probe was dissolved in a hybridization buffer [50% (v/v) formamide, 10% (w/v) dextran sulphate, 1 $\times$ Denhardt, 4 $\times$ SSC and 0.5 mg/ml of salmon sperm DNA] and deposited on each section for 16 h at 38°C . After six washes (20 min each) in 4 $\times$ SSC and one wash (45 min) in 2 $\times$ SSC, slides were desiccated, coated with Ilford K5 emulsion and exposed for approx. 6 days at 4°C before developing.

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ccaacacgaatgCGGTCTCTGCAACTAACCTGTTTGCTAGCAGTAGCTACAGCCACTCTG 60
      M R S L Q L T C L L A V A T A T L
gcgactctaccagagctacaaggacgctatggggccgctggttaagagagtgcattggc 120
      A D S T Q S Y K D A M G P L V R E C M G
agtgtctctgccactgaagacgacttcaaaacggttttgaacaggaaccctctggaatca 180
      S V S A T E D D F K T V L N R N P L E S
aggacagctcagtgcttagcctgCGCCCTGGACAAGTGGGCCTATCTCACCAGAA 240
      R T A Q C L L A C A L D K V G L I S P E
ggcgccatctatacaggagatgacctgatgcctgtcatgaaccgactgtacgggttcaac 300
      G A I Y T G D D L M P V M N R L Y G F N
gacttcaagacagtcagtgaggcgaagccgtgaacgactgcgccaatcaagtgaacgg 360
      D F K T V M K A K A V N D C A N Q V N G
gcgtatccggacaggtgtgatcttcaagaacttcaccgattgcgtccgcaactcctac 420
      A Y P D R C D L I K N F T D C V R N S Y
tgatactaggggaagcatccagttgtagccgatacagtaactactgtatgtttgcactgca 480
      *
cttgctttgtatggattcatacacttaataaaatcactgtaatccgaaaaaaaaaaaaa 540

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Figure 1 Nucleotide cDNA sequence and predicted amino acid sequence of PBPLma

The signal peptide and the polyadenylation signal are underlined. The stop codon is indicated with an asterisk. The predicted α -helices are shaded grey.

Expression of PBPLma in pET22b(+)

PBPLma was amplified by PCR using ECO/STQ (AAA-GAATTCCTCTACCCAGAGCTACAAG) and HIN/Stop (AAAAAGCTTTCAGTAGGAGTTGCGGAC) (restriction sites are underlined and the stop codon is in boldface). The amplified fragment was digested by *EcoRI* and *HindIII* before being cloned between the same restriction sites in pET22b(+) (Novagen, Madison, WI, U.S.A.). pET22b(+)/PBPLma was sequenced using the T7 promoter to check the sequence of the amplified fragment and the correct frame reading of the construct. The recombinant protein PBPLma was produced in *Escherichia coli* BL21(DE3) at 37 °C in Luria–Bertani medium (2 litres) with ampicillin (50 μ g/ml). The expression was induced with 0.3 mM isopropyl β -D-thiogalactoside when the A_{600} reached 1.8. Induced cultures were grown at 18 °C to optimize the yield of soluble protein; cells were harvested by centrifugation after 20 h when $A_{600} > 4$.

Purification and characterization of the recombinant PBPLma

All purification steps were performed at 4 °C. The periplasmic proteins were released by osmotic shock as described in the pET System Manual (available from <http://www.novagen.com>). The periplasmic fraction was dialysed overnight against a 10 mM Tris/10 mM NaCl buffer (pH 8) and purified by anion exchange on a ResourceQ column (Amersham Pharmacia Biotech) pre-equilibrated in the same buffer. The elution was performed in two steps, with a first wash using 50 mM NaCl, followed by a linear gradient of 50–250 mM NaCl in 10 mM Tris (pH 8). PBP was eluted with approx. 100 mM NaCl. The fractions (1.5 ml) containing PBPLma were pooled and concentrated by a 3 kDa Microsep (Filtron, Northborough, MA, U.S.A.). The concentrated proteins were loaded on to a preparative Superdex 200 gel-filtration column (Amersham Pharmacia Biotech) pre-equilibrated with a 10 mM Tris/150 mM NaCl buffer (pH 8). The elution was performed at 2.5 ml/min. PBPLma-containing fractions were pooled and concentrated.

Mass analysis was obtained by the MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) method with Voyager-DE RP (PerSeptive Biosystems, Wiesbaden-

Nordenstadt, Germany). A sample (0.7 μ l containing 15 pmol) was mixed with an equal volume of sinapinic acid matrix solution and spotted on the target, then dried at room temperature (20 °C) for 10 min. The molecular mass standard was apomyoglobin (molecular mass of 16.9516 kDa).

CD spectra were measured on a Jasco J-810 spectrophotometer in a 10 mM Na/Na₂PO₄ buffer (pH 7.5) at 20 °C between 260 and 180 nm (20 nm/min). The cell length was 1 mm and the protein concentration 0.1 mg/ml. Prediction of deconvolution and secondary structure was done using CDNN.

Fluorescence assays

The four sexual pheromonal compounds were purchased from Interchim (Montluçon, France). 8-Anilino-1-naphthalene-sulphonic acid (ANS) and 1-aminoanthracene (AMA) were purchased from Sigma (St. Louis, MO, U.S.A.). Fluorescence emission spectra and binding assays using ANS were performed on a CARY Eclipse Varian™ fluorescence spectrophotometer at 25 °C in front-face configuration with a 1 cm light-path fluorimeter quartz cuvette with an excitation slit of 5 nm and an emission slit of 10 nm. The effects of ANS displacement by ethanol and methanol have been tested by adding increasing amounts of alcohol to the ANS–PBPLma complex solution. Ethanol was found to be more competitive than methanol. Assays were made by dissolving ANS and water-non-miscible competitors in methanol at a fixed 0.8% final concentration in 1 ml of a 10 mM Tris buffer (pH 8) at 25 °C (excitation at 380 nm, emission between 400 and 620 nm).

All values were obtained from three independent measurements. The data for the ANS–PBPLma complex formation were obtained by titration of 1 μ M protein with increasing concentrations of ANS. Competition assays were made by monitoring the fluorescence signal variation of an ANS (4 μ M)–PBPLma (1 μ M) complex on addition of increasing concentrations of competitors. Before data analysis, fluorescence at the maximum emission (465 nm) was corrected for the extent of ANS fluorescence decrease due to the methanol displacement effect.

The affinity of the pheromonal compounds and other chemicals for PBPLma was estimated by plotting the decrease in ANS

fluorescence intensity at the maximum emission, calculated as $(I - I_{\min}) / (I_0 - I_{\min})$, against the competitor concentration, where I is the fluorescence intensity after the addition of aliquot of the competitor, $I_{\min}$ the fluorescence intensity at the saturation concentration of the competitor and I_0 the maximum fluorescence intensity of the ANS-PBPLma complex at 465 nm. The IC_{50} values were estimated from the direct plot by non-linear regression with the equation corresponding to a single binding site using Prism 3.02 (GraphPAD Software for Science, San Diego, CA, U.S.A.). The K_d values were calculated according to $K_d = IC_{50} / (1 + [ANS] / K_{ANS})$ where $[ANS]$ is the free concentration of ANS and K_{ANS} is the dissociation constant for ANS-PBPLma.

RESULTS

Identification and purification

A PCR approach coupled with 3'- and 5'-RACE was developed to isolate the full-length cDNA encoding the PBPLma. Electrophoretic analysis showed an amplified cDNA band of 362 bp. Specific primers deduced from the corresponding sequence were used for the amplification of the 5'-ends. The PBPLma complete cDNA sequence with an open reading frame of 414 nucleotides was obtained. The deduced protein is composed of 137 amino acids with a calculated molecular mass of 13.12 kDa and a pI of 4.82 (Figure 1; PBPLma GenBank® accession no. AY116618). The hydrophobicity profile determined by the method of Kyte and Doolittle [24] revealed that the first 19 amino acids are hydrophobic and probably correspond to a signal peptide with a putative cleavage site between residues 18 and 19 (TLA-DS).

Secondary structure of PBPLma

The predicted secondary structures of the mature protein include six α -helices (45%) and random coils (55%), in agreement with the fact that PBPLma shares significant sequence similarity with OBPs from different orders and with Lepidopteran PBPs. The secondary structure content has been determined by CD measurement. The CD spectra show a typical shape with two minima at approx. 210 and 225 nm, indicating that PBPLma is well folded and has a majority of α -helices (results not shown). The α -helices composition obtained by the computer program CDNN, approx. 40%, is slightly lower than the Jpred prediction of 45%.

RNA expression profile in antennae and subcuticular histological localization

A 361-bp cDNA probe amplified with OBPcc and dT-anchor primer was used for Northern-blot analysis. This probe was used to check the tissue specificity in adults as well as in larvae in the different appendages (see above). The transcript was specifically detected in the adult female antennae (Figure 2). No transcript was detected either in larvae, in other tested female tissues or in males. The PBPLma transcript was not detected in the tergal and sternal glands (which compose the pheromonal gland in males) of the adult and larvae of both sexes (results not shown).

In situ hybridization analysis was undertaken with antennae and maxillary palp sections of adults of both sexes. The hybridization revealed a labelled area located in the subcuticular area of the female antennae (Figure 3A) corresponding to the epidermic-cells layer. No labelling was observed in maxillary palps (Figure 3C) or the male antennae section (Figure 3B), used as negative control.

Figure 2 Northern-blot analysis of female adult, male adult and larvae with total RNAs (7 µg/lane)

RNAs were extracted from different tissues, as indicated: midgut, tarsi, cerci, antennae and maxillary palp, which was divided in proximal part (prox) corresponding to the four basis articles and distal part (dist) corresponding to the last article for the adult tissues. For the larvae, the whole labium and labial palp were collected. rRNAs are indicated to verify the equal amount of RNA loaded in each lane.

E. coli periplasmic expression of PBPLma and purification

A special feature of the pET22b(+) vector is the expression of PBPLma as a fusion protein with *pelB*, the signal peptide targeting the protein to the periplasm of the bacteria. The *pelB* signal is cut off during translocation to the periplasm where the oxidative environment is favourable to the formation of disulphide bonds. The recombinant PBPLma was composed of 129 amino acids, the first 11 residues coming from the multi-cloning site of the pET22b(+) vector, with a calculated molecular mass of 14.15 kDa and an acidic pI of 4.68.

Purification of the recombinant protein yielded 9 mg/l of pure protein/culture. The PBPLma molecular masses obtained by gel filtration under native conditions and by MS were 13.3 and 14.14 kDa respectively. As the calculated PBPLma molecular mass is 14.15 kDa, we can conclude that PBPLma is monomeric. SDS/PAGE analysis of the whole cell protein showed a significant increase in the intensity of two bands at approx. 15 and 17 kDa on induction (results not shown). The 17 kDa band is not found in the periplasmic fraction but is present in the cytoplasmic fraction, suggesting that it corresponds to the PBPLma with its *pelB* signal peptide. The 15 kDa band present in the periplasmic fraction corresponds to the PBPLma without its *pelB* signal peptide and confirms good translocation of the protein.

Pheromone binding with fluorescence spectroscopy

ANS fluorescence (excitation at 380 nm, emission between 400 and 620 nm) is influenced by its environment: in an aqueous environment, ANS emits a low level of fluorescence at 465 nm, but in a hydrophobic environment, the fluorescence emission is

Figure 3 *In situ* hybridization with a PBPLma α - 32 P-labelled random-primed cDNA probe in adult tissue

PBPLma expression analysis in female antennae (A), in female or male maxillary palp longitudinal and transversal sections (C1 and C2) and negative control hybridized in male antennae (B).

enhanced. This feature proved to be useful in displacement experiments with OBPs and related proteins that bind their ligand in a hydrophobic pocket [28]. The ANS–PBPLma complex formation, using increasing concentrations of ANS, can be described by a hyperbolic curve as expected for a one-site binding model (Figure 4A) with a calculated K_d of $2.14 \mu\text{M}$.

PBPLma affinity for the four components of the pheromonal blend and for other ligands has been tested by ANS displacement. Addition of the pheromone components, 3-hydroxy-butan-2-one or butane-2,3-diol, produced a significant quenching of the fluorescence, with IC_{50} of $10.9 \mu\text{M}$ ($K_d = 3.8 \mu\text{M}$) and $7.1 \mu\text{M}$ ($K_d = 2.5 \mu\text{M}$) respectively (Figure 4B). Two other components of the pheromonal blend, the senecioid acid and the E-2-octenoic acid, do not produce a quenching of the fluorescence emission. An alcohol (12-bromo-dodecane-1-ol) and long-chain fatty acids [lauric acid (C_{12}), palmitic acid (C_{18})] were also tested and do not produce any quenching of the fluorescence emission.

DISCUSSION

We describe for the first time a PBP in the Dictyoptera. In contrast with the Lepidoptera, PBPLma is produced by females to accommodate the pheromones emitted by the male. The description of an OBP in cockroach extends the hypothesis, proposed by Vogt et al. [7], to the Orthopteroidea that the insect OBPs have an ancestral form, common for the Endopterygota and the Paraneoptera, and it suggests that OBPs may be

widespread throughout these three divisions, unless they were secondarily lost.

The first PBPs described in *Antheraea polyphemus* [19] and later on in other Lepidoptera [20,21] and in Coleoptera [29] were detected specifically in the antennae of the males and had the capacity to bind the major component of the pheromone. More recently, some PBPs have been detected in both sexes and shown also to bind pheromone compounds [30,31]. The possible capacity for females to have a feedback control of their pheromone release has been proposed by Vogt et al. [31] to account for this evidence.

The presence of six α -helices has been described in the three-dimensional structure of a PBP from *Bombyx mori* [32] and confirms secondary structure predictions based on many PBP sequences. The presence of these six conserved helices suggests that all PBPs, despite low sequence identity, might share the same fold as the *B. mori* PBP. Six cysteine residues conserved in sequence alignments form three disulphide bridges C1–C3, C2–C5 and C4–C6, which enforce the organization of the α -helices [32]. In the *B. mori* PBP structure, helices $\alpha 1$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ form a 'binding pocket' and helix $\alpha 3$ closes one end of this pocket; the bombykol pheromone is bound in this cavity by numerous hydrophobic interactions [32]. A pH switch changing the conformation from a 'closed' form binding the odorant at pH 6.5 to an 'open' form unable to bind odorants at pH 4.5 has been proposed [32,33]. Horst et al. [34] described the formation of a seventh α -helix at the C-terminal end of *B. mori* PBP at

Figure 4 Fluorescence studies with PBPLma

(A) Titration of PBPLma with ANS. Relative fluorescence intensity is plotted as a function of ANS concentration. Conditions were as follows: 1 μ M PBPLma in a 10 mM Tris buffer (pH 8) at 25 °C. ANS fluorescence was monitored at the emission maximum at 465 nm with excitation at 380 nm. Data represent the means for three independent measurements. Error bars indicate S. D. The curve corresponds to the theoretical binding curve for $n = 1$ and $K_d = 2.14 \mu$ M. Inset: fluorescence emission spectra of ANS in the presence of PBPLma [1 μ M in a 10 mM Tris buffer (pH 8) at 25 °C]. The titration curves of PBPLma were obtained with increasing amounts of ANS (0–13 μ M). Excitation wavelength was 380 nm and the emission was recorded between 400 and 620 nm with a maximum at 465 nm. (B) Competition assays by pheromone compounds with the ANS–PBPLma complex. The decrease in ANS fluorescence intensity at the emission maximum (465 nm) with increasing amounts of competitors is reported. PBPLma (1 μ M) was pre-equilibrated with ANS (4 μ M). Data represent the means for three independent measurements.

acidic pH (4.5), filling the binding site of the PBP and mimicking the ligand position. Interestingly, this seventh helix lacks in PBPLma sequence (Figure 1).

PBPLma shares a percentage of sequence identity in the range 14–25% with several Lepidopteran PBPs. PBPLma possesses the hallmarks of the OBP family; however, its low molecular mass (13.12 kDa), its acidic pI (4.82) and its six-cysteine pattern were conserved in alignment. *In situ* hybridization localized the transcript of PBPLma in an area just below the cuticle, and Northern-blot analysis of PBPLma localized the mRNA specifically in the antennae of an adult female (Figures 2 and 3).

In *L. maderae*, at least two pheromonal compounds, the senecioic acid and the E-2-octenoic acid, have a dual function in the behaviour of this cockroach [1]. In females, they act as a sexual pheromone by stopping the female at a close distance from the male, whereas in males they are involved in the dominance relationships. We, therefore, decided to test the binding of these pheromones with PBPLma. Binding of pheromones to PBPs has been tested previously by fluorescence

studies [20,21]. Depending on the case, intrinsic fluorescence or fluorescent ligand displacement was used. In the case of PBPLma, intrinsic fluorescence has been unsuccessful since there is no tryptophan residue in the sequence. Furthermore, AMA previously used with different Lepidopteran PBPs [21] and with the mammalian OBPs' functional homologues [35,36] did not bind to PBPLma. We subsequently tried ANS, previously used with *B. mori* PBP and a locust chemosensory protein [33,37]. ANS excitation at 380 nm in the presence of PBPLma gave a strong fluorescence peak at 465 nm, suggesting the formation of an ANS–PBPLma complex. Moreover, this suggests that ANS fits well into the hydrophobic cavity (with $K_d = 2.14 \mu$ M; Figure 4A). The formation of a fluorophore–PBP complex seems to be very sensitive to interactions in the cavity, as demonstrated by Campanacci et al. [21] by the formation of a complex between AMA and wild-type *Mamestra brassicae* PBP and not with a 6-amino-acid residues mutated form (Mbra1-M6).

To investigate the affinity of PBPLma for the sexual pheromone blend compounds, competitive binding assays were performed in which ANS was to be displaced. Addition of both 3-hydroxy-butan-2-one or butane-2,3-diol produced a significant decrease in the fluorescence (Figure 4B). These two compounds are chemically very close, as they differ only by one functional group, an hydroxide or a ketone. Although a unique binding site has been reported in PBPs, multiple binding in the cavity cannot be excluded due to the small size of the ligands. The K_d values should therefore be considered as overall apparent values. The *M. brassicae* PBP1 and the *A. polyphemus* PBP1 [21] have been described to bind several odorants with no obvious correlation between the presence, the position and the number of double bonds, between different functional groups of the first carbon and even between different chain lengths.

PBPLma is not capable of discriminating between 3-hydroxy-butan-2-one and butane-2,3-diol, but it discriminates the chemical functional groups or the chain lengths, as the two pheromonal acids did not displace the ANS. Other ligands such as 12-bromododecane-1-ol, lauric acid (C_{12}) and palmitic acid (C_{18}) were also tested and did not displace the bound ANS. These observations suggest that PBPLma might be capable of filtering ligands and is more specific than other PBPs mentioned above. Strikingly, PBPLma binds the 3-hydroxy-butan-2-one and butane-2,3-diol that are water-soluble molecules, in contrast with most PBPs described previously, which bind, almost exclusively, very hydrophobic compounds. This observation extends the molecular repertoire of PBPs to small hydrophilic molecules and might be based on different molecular binding mechanisms as compared with Lepidoptera PBPs.

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Received 4 December 2002/9 January 2003; accepted 15 January 2003

Published as BJ Immediate Publication 15 January 2003, DOI 10.1042/BJ20021877