Molecular and Functional Characterization of an Odorant Binding Protein of the Asian Elephant, *Elephas maximus*: Implications for the Role of Lipocalins in Mammalian Olfaction†

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ABSTRACT: The sex pheromone present in the pre-ovulatory urine of female Asian elephants is the simple lipid (*Z*)-7-dodecen-1-yl acetate (Z7-12:Ac). Using radiolabeled probes, we have identified a pheromone binding protein that is abundant in the mucus of the trunk; this protein is homologous to a class of lipocalins known as odorant binding proteins (OBPs). To test five previously proposed roles for the OBP in chemosensory perception, we determined the equilibrium dissociation constant of the OBP−pheromone complex, as well as the association and dissociation rates. Using a mathematical model in conjunction with experimental data, we suggest that the binding and release of the pheromone by the OBP are too slow for the OBP to function in transporting the pheromone through the mucus that covers the olfactory sensory epithelium. Our data indicate that the elephant OBP only modestly increases the solubility of the pheromone in the mucus. Our results are most consistent with the notion that elephant OBP functions as a scavenger of the pheromone and possibly other ligands, including odorants. In light of these findings, and published results for other mammalian OBP−ligand complexes, a general model for the role of OBPs in mammalian olfaction is proposed. Moreover, the potential implications of these findings for interaction of *Z*7-12:Ac with insect antennal proteins are discussed.

The pre-ovulatory urine of female Asian elephants contains high concentrations (∼100 μM) of (*Z*)-7-dodecen-1-yl acetate (*Z*7-12:Ac)[1] (*1*). Male Asian elephants exhibit a wide range of behavioral and physiological responses in response to *Z*7-12:Ac, ranging from sniffing and checking with the tip of the trunk (check, place response) to pre-copulatory behaviors including flehmen, penile erections, and mounting attempts (*2*). Synthetic Z7-12:Ac alone is capable of eliciting the same range of behavioral responses as the pre-ovulatory female urine, although somewhat higher concentrations are needed. Therefore, *Z*7-12:Ac functions as a sex pheromone in the Asian elephant (*3*). The structure of the pheromone combined with the well-defined behavioral responses make the Asian elephant system an attractive model for studying perireceptor events in the perception of mammalian pheromones. Interestingly, *Z*7-12:Ac has also been identified as a component of pheromone blends in at least 35 insect species, and as an attractant in over 120 others of the 1700 listed in the Pherolist database (http://www-pherolist.slu.se/). Therefore, insect and mammalian systems that detect the same small molecule can be directly compared and contrasted.

In the elephant, the pheromone may be perceived by two separate chemosensory systems: the olfactory system and the vomeronasal system (VNO) (*4*). Both are well-developed: the surface area of the olfactory turbinates exceeds 800 cm^2, while the VNO is about 30 cm long and 1−2 cm in diameter. The surface of the sensory epithelium in both systems is covered by a protein-rich mucus layer; mucus is also present throughout the length of the trunk. Due to the lipophilic nature of the pheromone, we hypothesized that the mucus that covers the sensory epithelia contains proteins that would transport the lipophilic pheromone through the aqueous layer of mucus to the surface of the sensory cells.

The mucus associated with the olfactory system of many mammals has been shown to contain mammalian odorant binding proteins (OBPs) (*5*). Mammalian OBPs belong to the lipocalin protein family of small (15−21 kDa), water-soluble proteins. The three-dimensional structures of several lipocalins, including the bovine (*6*, *7*) and porcine (*8*) OBPs, have been solved. The dominant feature of the structure is a

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[1] Abbreviations: OBP, odorant binding protein; MUP, mouse urinary protein; GOBP, general odorant binding protein; PBP, pheromone binding protein; VNO, vomeronasal system; VOBA, volatile odorant binding assay; Z7-12:Ac, (*Z*)-7-dodecen-1-yl acetate; [^{3}H]Z7-12:Dza, (*Z*)-7-[^{3}H]-dodecen-1-yl diazoacetate.

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β-barrel, in which eight β-sheets surround a large hydrophobic binding pocket. Some OBPs exist as monomers, while others (notably the bovine OBP) form dimers under natural conditions. Mammalian OBPs have been shown to bind a diverse group of compounds, including aliphatic alcohols and aldehydes, aromatic compounds such as benzophenone, terpenoids, and heterocyclic compounds (*9*). Known dissociation constants of OBPs and their ligands are in the low micromolar range (*10*−*12*). The physiological function of OBPs remains unresolved, but five principal hypotheses have been set forth. First, the **carrier hypothesis** (*13*) postulates that OBPs transport lipophilic odorants through the aqueous layer of mucus to the olfactory sensory epithelium. Low solubility of many odorants in water, as well as moderate affinities of OBPs toward their ligands, is frequently cited in support of this commonly accepted hypothesis. Second, the **pheromone transport hypothesis** (*5*) argues that OBPs function as ligand-specific transporters for delivery of pheromones to receptor sites. Third, the **scavenger hypothesis** (*13*) contends that OBPs may function to remove odorants from the receptor after the transduction of the olfactory signal. Fourth, the **buffer hypothesis** (*13*) suggests that OBPs prevent saturation or overload of the receptors by binding excess ligand prior to receptor activation. Fifth, the **transducer hypothesis** (*13*) proposes that an OBP−ligand complex activates the olfactory receptor. Herein we present studies of the elephant OBP interactions with *Z*7-12:Ac, and we summarize biochemical and physicochemical evidence that establishes the most likely physiological role for the elephant OBP, with implications for the function of other mammalian OBPs.

In insects, roles analogous to those mentioned above have been proposed for insect pheromone binding proteins (PBPs) and related general odorant binding proteins (GOBPs), which are present in high concentrations (10 mM) (*29*, *30*) in the lymph that bathes the chemosensory neurons in the antennal sensilla. The insect PBPs and GOBPs are not related to the mammalian OBPs. The insect proteins, which are predominantly α-helical, have a fundamentally different three-dimensional architecture (*14*) as compared to the mammalian OBPs. The binding specificity of the insect proteins has been a subject of debate and discussion in the literature (*15*, *16*). Insect OBPs bind ligands with micromolar affinities, and appear to depend on pH, the presence of membranes (*17*), and possibly on the oxidation state of the cysteine residues present (*18*). Our experiments with *Z*7-12:Ac, combined with data from the literature on pheromone binding by insect PBPs and GOBPs, allowed us to extract kinetic data from binding experiments previously interpreted only statically. These data reveal intriguing analogies between the mammalian and insect proteins, possibly extending to their function.

EXPERIMENTAL PROCEDURES

Synthesis of Probes. The tritiated pheromone (*Z*)-7-[^{3}H]-dodecen-1-yl acetate ([^{3}H]*Z*7-12:Ac) was synthesized at the National Tritium Labeling Facility, Lawrence Berkeley National Laboratory, Berkeley, CA, by reduction of 7-dodecyn-1-yl acetate with tritium gas, as described for similar compounds (*19*−*21*). The identity of the product was verified by radio-thin-layer chromatography, as well as by ^{1}H and ^{3}H NMR. The specific activity of the product was essentially equivalent to that of the T_2 gas used, that is, 57 Ci/mmol. The tritiated photoactivatable pheromone analogue (*Z*)-7-[^{3}H]dodecen-1-yl diazoacetate ([^{3}H]*Z*7-12:Dza) was synthesized from [^{3}H]*Z*7-12:Ac in two steps. First, the acetate was hydrolyzed to (*Z*)-7-[^{3}H]dodecen-1-yl alcohol by KOH in water/methanol. The alcohol was then converted to the diazoacetate as described (*19*).

Photoaffinity Labeling. In a typical photolabeling reaction, 0.5 μCi (0.88 nmol) of [^{3}H]*Z*7-12:Dza, dissolved in 0.5 μL of methanol, was added to 25 μL of male elephant trunk mucus or purified OBP. In competition experiments, a 1000-fold excess of *Z*7-12:Ac (Aldrich, >98% pure) was added prior to addition of the photoaffinity probe. After a 5 min incubation at room temperature, the mixture was irradiated at 254 nm for 1 min in a Rayonet UV reactor (4 lamps, 8 W each). After the photoreaction, the samples were analyzed by SDS−PAGE on a 15% polyacrylamide gel. Following Coomassie blue staining, the gel was impregnated with En^{3}Hance (NEN Life Sciences, Boston, MA), miniaturized with 50% PEG 8000 to increase the sensitivity of detection (*22*), and dried. The dried gel was exposed to a sheet of a BioMax MS film (Kodak, Rochester, NY) for 4−7 days at −80 °C, and the exposed film was developed.

Native PAGE/Electroblotting after Incubation with the Radiolabeled Pheromone. Electrophoresis/electroblotting of protein mixtures after incubation with the tritiated pheromone was carried out as described (*23*, *24*), with a few modifications. Typically, 1 mCi of [^{3}H]*Z*7-12:Ac was used. A methanolic solution of the radiolabeled pheromone (1 μL) was added to trunk mucus (25 μL), and the mixture was incubated at room temperature for 5−10 min prior to separation by PAGE under nondenaturing conditions on a 10% polyacrylamide gel. The proteins were electroblotted onto a PVDF membrane. The electrophoresis apparatus (Mini Protean II, BioRad Laboratories, Hercules, CA) was kept at 4 °C throughout the electrophoresis/electroblotting procedure. Radioactivity present on the blot was detected on film (BioMax MS, Kodak) with an exposure time of 1−4 days, which was sufficient to obtain a clear image using an intensifying screen (BioMax TranScreen HE, Kodak). After processing the film, the proteins present on the PVDF membrane were visualized by staining with Coomassie blue.

Western Detection. Rabbit antisera were raised against purified elephant OBP by Antibodies Inc. (Davis, CA). The Immunstar chemiluminiscent Western detection kit (BioRad) was used to visualize the elephant OBP in tissue and mucus samples. The anti-OBP antiserum diluted 2000-fold in the primary antibody dilution buffer was used, and the kit manufacturer's procedure was followed. Thus, tissue was rinsed with water, triturated in PAGE loading buffer, and centrifuged, and the supernatant was used for electrophoresis. Samples tested for presence of the OBP by Western analysis were the following: male and female trunk mucus, samples of male mucosal tissue collected in 25 cm intervals along the length of the trunk (the most proximal being about 25 cm from the olfactory sensory epithelium), tissue and mucus from female elephant olfactory sensory epithelium, and mucus from male and female VNO.

OBP cDNA Cloning. The N-terminal sequence of the elephant OBP (LEEPLLDEYCSEISGTWYTIYEA) was obtained by Edman sequencing of protein bands that exhibited pheromone binding in photolabeling and native PAGE/blotting experiments. An internal OBP sequence

(YTTNYEGKVDFSFIQQAKDFL) was obtained by Edman sequencing of peptides from *N*-chlorosuccinimide digestion.

Using Western blotting and antibodies raised against the elephant OBP, the OBP expression was localized to the proximal parts of the trunk. Total RNA was isolated from mucosal tissue of the upper parts of the trunk using the RNeasy kit (Qiagen) and the manufacturers' protocols. The RNA was reverse-transcribed using an oligo-dT primer and SuperScript II (Invitrogen) reverse transcriptase. A 249 bp fragment of elephant OBP cDNA was amplified using semi-nested PCR. The primers used in the first round of PCR were *ObpN* (ACNTGGTAYACNATHTAYGARGC, based on part of the N-terminal protein sequence) and $(dT)_{18}$. The primers used in the second round of PCR were *ObpN* and *ObpI* (AARTCYTTNGCYTGYTGDATRAA, based on part of the internal OBP protein sequence). The thermostable DNA polymerase was *Taq* Platinum (Invitrogen), used according to the manufacturers' protocols. In both rounds of PCR, the DNA was denatured at 94 °C for 2 min, and then subjected to 30 cycles of 55 °C (45 s), 72 °C (45 s), and 94 °C (45 s). The final extension was carried out at 72 °C for 5 min. An aliquot (10 nL) of the first PCR reaction was used as a template in the second round. The product was purified by 1.5% agarose gel electrophoresis and cloned into a pCR2.1 TOPO vector (Invitrogen) according to the manufacturers' instructions. The cloned fragment was used to design primers *3rac* (GAGGTGCTAAGTGATAACAGTC-CC), *5rac1* (CTAAGTGAGCGTCTCAGG-ACACGT), and *5rac2* (ACACGTGTCTGTATCAATGACTT). Primers *3rac* and $(dT)_{18}$ were used in PCR amplification of the 3′ end of the OBP cDNA. Primers *5rac1* and *5rac2* were used in PCR amplification of the 5′ cDNA end using the CapSelect method (*25*). PCR conditions were the same as above. *E. maximus* OBP has been assigned GenBank Accession Number BankIt490107, AF541941.

Protein Purification for Binding Studies and Antibody Preparation. Proteins of male elephant trunk mucus were separated by preparative nondenaturing electrophoresis of male trunk mucus on a 12.5% polyacrylamide gel using the Protean II setup (BioRad). The gel was then electroeluted using the Whole Gel Eluter (BioRad) following the recommended procedure. The eluted fractions were desalted and transferred into PBS buffer by ultrafiltration on Centriprep YM-10 (Millipore). Protein concentration was determined spectrophotometrically using the modified Edelhoch method (*26*), as well as by Bradford assay. The UV spectrophotometer employed was a diode array based Hewlett-Packard model HP8453, connected to a PC equipped with the UV−Visible Chemstation software v. A.02.05.

Volatile Odorant Binding Assay (VOBA). The assay was carried out as described (*10*), with a few modifications. Mucus or OBP purified by preparative native polyacrylamide gel electrophoresis (50 μL) was incubated overnight at 37 °C in a closed chamber containing a drop of *Z*7-12:Ac (Aldrich, >98% pure). Aliquots (25 μL) were injected under a layer of heptane (100 μL), and an aqueous solution of Proteinase K (Invitrogen, 10 μL of 1 mg/mL) was added. The mixture was incubated overnight at 25 °C. The amount of pheromone in the heptane extract was determined by quantitative gas chromatography on a Hewlett-Packard 5890 Series II gas chromatograph equipped with an HP-5 column (30 m × 0.32 mm × 0.25 μm film). The system was precalibrated with triplicate serial dilutions of *Z*7-12:Ac in heptane, in the concentration range from 373 nM to 382 μM. The load/response curve was linear throughout this range, with $R^2 = 0.9992$.

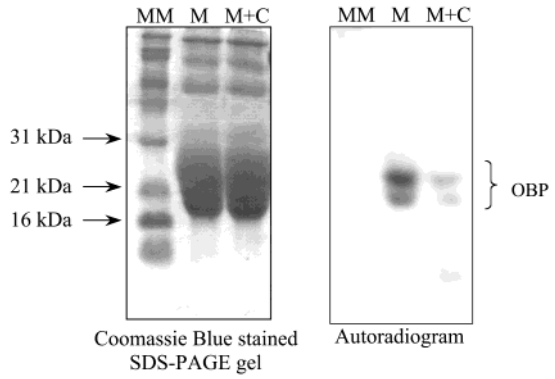

FIGURE 1: Photoaffinity labeling of male trunk mucus. M: male trunk mucus photolabeled with [^{3}H]*Z*7-12:Dza in the absence of a competitor; M+C: male trunk mucus photolabeled with [^{3}H]*Z*7-12:Dza in the presence of the nonradioactive pheromone (*Z*7-12:Ac) as a competitor. The arrows indicate areas of strongest photolabeling, corresponding to proteins homologous to known OBPs.

Gel Filtration-Based Binding Assay. The assay was based on published methods (*27*, *28*), with several modifications. The column used for separation of protein-bound and free ligand was HiTrap Desalting 5 mL (Pharmacia). To determine the K_d of the OBP/*Z*7-12:Ac complex, a solution of 0.1 μCi of [^{3}H]*Z*7-12:Ac (1.8 pmol) in methanol (0.8 μL) was added to OBP dissolved in PBS (1 mL, 40 nM to 40 μM in OBP). After incubation at 37 °C for 10 min, the samples were applied onto the gel filtration column. The protein fraction was eluted with 2.2 mL of PBS. Free ligand was eluted with 20 mL of PBS. Aliquots of the eluted fractions were mixed with liquid scintillation cocktail (Scintisafe Econo 1, Fisher Scientific), and the radioactivity was counted. To determine the off-rate, 1 μCi of [^{3}H]*Z*7-12:Ac (18.2 pmol) in 1 μL of methanol was added to 200 μL of male elephant trunk mucus diluted 10× in PBS, and the system was equilibrated at 37 °C for 10 min, during which the initial equilibrium was reached. Then, 9.8 mL of prewarmed PBS (37 °C) was added, disturbing the initial equilibrium. The onset of a new equilibrium via dissociation of the OBP/Z7-12:Ac complex was monitored: a 1 mL aliquot was removed every 15 min, the free and bound pheromone were separated by chromatography as described above, and the radioactivity present in the high and low molecular mass fractions was determined by liquid scintillation counting.

RESULTS

Homologues of Known OBPs Bind the Pheromone in the Elephant Trunk Mucus. To visualize pheromone binding by the proteins in the trunk mucus, two experiments were carried out. First, photoaffinity labeling of the complete protein mixture with the radiolabeled pheromone analogue [^{3}H]*Z*7-12:Dza (Figure 1) was performed. Second, native PAGE/electroblotting was performed with mucus that had been preincubated with the radiolabeled pheromone [^{3}H]*Z*7-12:Ac (Figure 2).

A broad protein band of molecular mass ca. 20 kDa was photoaffinity labeled by the pheromone analogue. Addition

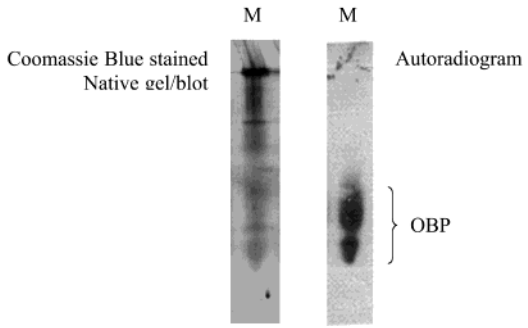

FIGURE 2: Native gel/blot of male trunk mucus. M: male trunk mucus + [^{3}H]*Z*7-12:Ac. Arrows indicate areas of strongest binding of the radiolabeled pheromone. N-Terminal sequences obtained from the corresponding protein bands are identical to the N-terminal sequence of the elephant OBP.

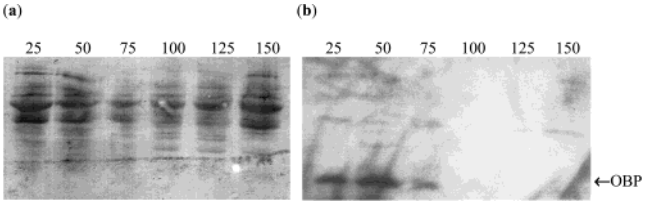

FIGURE 3: OBP expression in the trunk mucosal tissue. (a) Coomassie blue stained blot; (b) Western detection with anti-OBP antiserum. Numbers indicate the approximate distance (cm) of samples from the olfactory sensory epithelium.

of the pheromone to the reaction mixture prior to irradiation reduced the intensity of the labeling, thereby demonstrating the specificity of the binding (Figure 1). Edman sequencing of different parts of the photolabeled protein band uniformly revealed the main amino acid sequence as LEEPLLDEYC-SEISGTWYTIYEASENIE. In addition, several lower intensity sequences that were N-terminally truncated by 3−10 amino acids were obtained in this heterogeneous protein band. The localization on the gel of individual truncated sequences varied between samples. The extent of truncation did not directly correlate with the molecular mass as observed by SDS−PAGE, suggesting an additional source of the observed heterogeneity.

Native PAGE/electroblotting of male elephant trunk mucus after incubation with the radiolabeled pheromone revealed strong binding of the pheromone by a very broad protein band (Figure 2). Edman sequencing of proteins from different parts of the labeled band revealed N-terminal sequences identical to those obtained from the photoaffinity labeling experiment. No significant differences between the sexes were observed in trunk mucus protein composition or pheromone binding (data not shown). Western blot analysis revealed the presence of the elephant OBP in the proximal parts of the trunk (Figure 3).

Using semi-nested PCR, a 249 bp OBP cDNA fragment was amplified. The cDNA termini were amplified using 3′ and 5′ RACE. The resulting cDNA encoded a 162 amino acid protein (Figure 4) of deduced molecular mass 18 535 Da. Database search (Blastp, nr database) revealed similarity to proteins of the lipocalin family. Alignment of the elephant OBP sequence with the nine most similar lipocalins is shown in Figure 4. The deduced protein sequence was closest to the mouse (35% identity, 58% similarity) and rat (34% identity, 56% similarity) probasins, lipocalins of unknown function produced in the prostate. The next most similar proteins are the porcine OBP (34% identity, 56% similarity) and rat OBP (33% identity, 54% similarity). Bovine and equine allergen lipocalins, bovine OBP, and hamster aphrodisin exhibit a slightly lower similarity (29−31% identity, 45−50% similarity). The localization of the protein expression by Western blotting to the proximity of the olfactory epithelium led us to classify the elephant protein as a homologue of other mammalian OBPs. The deduced amino acid sequence contains one putative *N*-glycosylation site at Asn-61. *N*-Glycosylation has been observed in other lipocalins (*29*, *30*). It is therefore likely that both proteolytic processing and glycosylation contribute to the observed molecular mass heterogeneity of the elephant OBP.

The Elephant OBP Is Present in the Mucus of the Trunk and Possibly Also the VNO. Apart from the trunk mucus, we were also able to detect binding of the pheromone by proteins present in the male and female VNO mucus. Native PAGE/electroblotting after incubation with the radiolabeled pheromone revealed binding mainly by an as yet unidentified protein in the 60 kDa range; however, a significant portion of the pheromone was bound by proteins of low abundance in the lipocalin range (Figure 5). Immunodetection performed on the same blot, using antisera raised against the elephant OBP, demonstrated strong immunoreactivity in parts of the blot coinciding with the pheromone binding lipocalins. Therefore, a protein identical, or at least very similar, to the trunk mucus OBP is also present in the VNO mucus. When Western detection was performed on a rinse of the olfactory sensory tissue, as well as on the tissue homogenate, no OBP immunoreactivity could be detected (Figure 6). However, since only a limited amount of tissue was available for the experiment, we cannot exclude the possibility that mucus covering some parts of the olfactory sensory epithelium does contain the OBP.

Elephant OBP Binds the Pheromone with Low Micromolar Affinity. Binding of the pheromone by the elephant OBP was quantitatively investigated by a gel filtration based assay (*27*). Serial dilutions of the OBP were equilibrated with the radiolabeled pheromone, and the free pheromone was separated from the protein fraction by gel filtration on a HiTrap Desalting 5 mL column (Pharmacia). The results are shown in Figure 7. The dissociation constant of the OBP/pheromone interaction was inferred by nonlinear regression analysis, using the Prism software package, to be 0.8 ± 0.1 μM. The affinity was found to vary little within the pH range of 5.0−9.0. The stoichiometry of binding was determined by VOBA to be one *Z*7-12:Ac per OBP molecule.

Pheromone Aqueous Solubility Is in the Micromolar Range. Pheromone solubility was measured using a VOBA. PBS buffer (a protein-free solution) was exposed to air saturated with *Z*7-12:Ac vapors overnight. The equilibrium pheromone concentration in the buffer was determined using quantitative gas chromatography as described under Experimental Procedures. The assay was carried out in triplicate, at pH values between 5.0 and 9.0. Under the conditions of the assay, the pheromone concentrations in the buffer reached 10−15 μM, depending in part on the pH of the buffer (*31*).

Elephant OBP Is Present in the Trunk Mucus in High Micromolar Concentrations. The concentration of the OBP in trunk mucus (three different batches collected by inducing a forceful exhalation of the animal, and originating predominantly from proximal parts of the trunk) was estimated from

FIGURE 4: ClustalW alignment of the amino acid sequence of the elephant OBP with other lipocalins. The light gray box indicates >50% similarity; the dark gray box indicates >50% identity. A '+' sign indicates the predicted *N*-glycosylation site. Prime characters (′) indicate termini of truncated proteins.

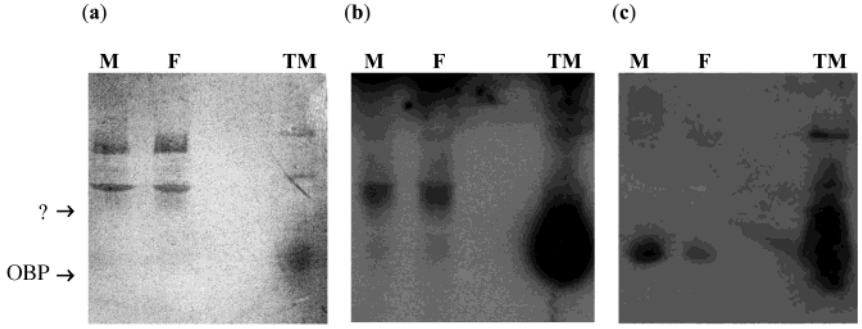

FIGURE 5: Native gel/blot of male and female VNO mucus. (a) Coomassie blue stained blot; (b) autoradiogram; (c) Western detection with anti-OBP antiserum. M: male VNO mucus + [^{3}H]Z7-12:Ac; F: female VNO mucus + [^{3}H]Z7-12:Ac; TM: male trunk mucus. OBP indicates the OBP molecular weight range; a question mark (?) indicates an unidentified protein exhibiting binding of the pheromone.

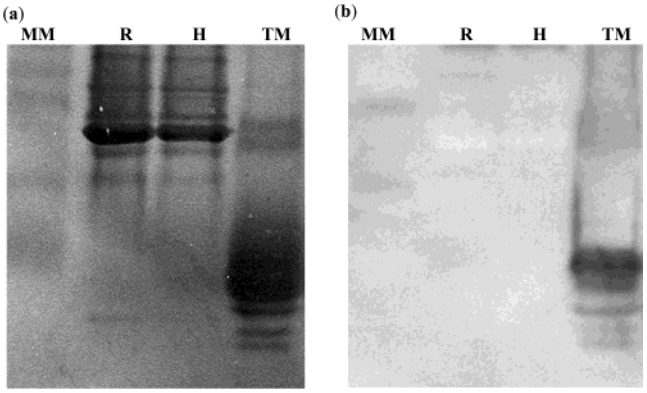

FIGURE 6: Western detection of the OBP in the tissue of the elephant olfactory sensory epithelium. (a) Coomassie blue stained blot of olfactory sensory tissue; (b) Western detection with anti-OBP antiserum. MM: molecular weight markers; R: rinse of the tissue; H: tissue homogenate; TM: trunk mucus

Coomassie-stained SDS−PAGE gels to be ca. 1 μg/μL (50 μM). To ascertain the proportion of the OBP present capable of binding the pheromone, a VOBA experiment on whole trunk mucus was carried out. The concentration of *Z*7-12:Ac dissolved in trunk mucus under the conditions of VOBA was 62 ± 12 μM. Assuming that the concentration of the free (unbound) pheromone in the mucus is similar to that in PBS (10−15 μM), and that no other proteins in the mucus contribute significantly to pheromone binding, the concentration of OBP capable of binding the pheromone in the trunk mucus is ca. 50 μM. This value indicates that essentially all OBP present in the mucus is functional.

The Half-Life of the Pheromone−OBP Complex Is Close to 30 min. The decay of the OBP−pheromone complex was observed by the gel filtration method described in detail under Experimental Procedures. The decay exhibited first-order (exponential) kinetics. The first-order off-rate constant of the complex determined in this way is $k_{off} = (3.75 \pm 0.84) \times 10^{-4}$ s^{-1}. This corresponds to a half-life of 30.8 ± 5.6 min (Figure 8).

Formation of the Pheromone−OBP Complex Occurs in Tens of Seconds. From the values of the dissociation constant (K_d) and the off-rate constant (k_{off}), the association rate constant (k_{on}, a second-order rate constant) was calculated:

$$k_{on} = \frac{k_{off}}{K_d} = 469\ \mathrm{dm^3\,mol^{-1}\,s^{-1}}$$

To calculate the time required for the ligand to become bound by the OBP, the formation of the complex PL from the free

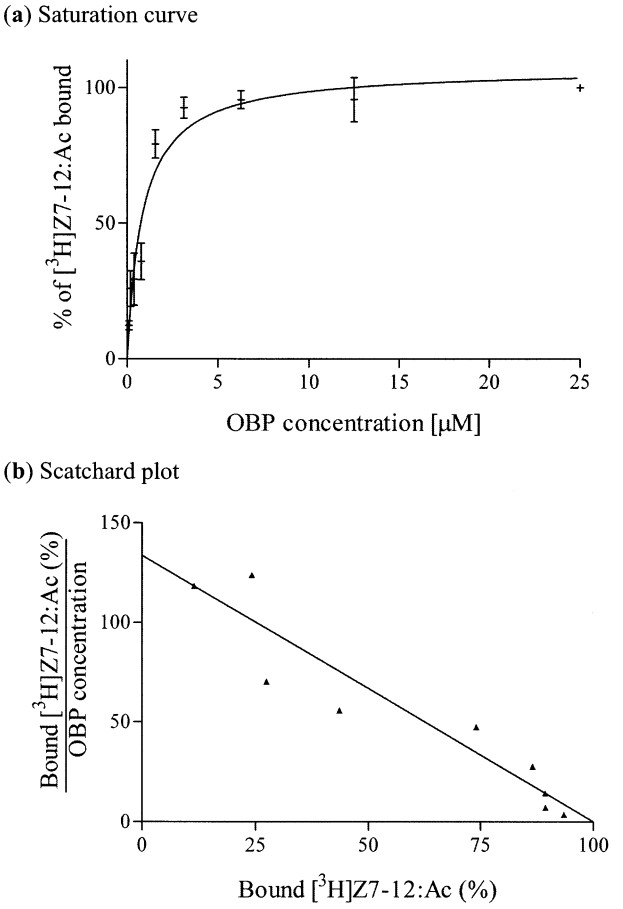

FIGURE 7: Determination of the OBP/pheromone dissociation constant. (a) Nonlinear regression analysis of the equilibrium binding data was used to determine the K_d of the OBP/pheromone interaction. (b) Scatchard plot of the equilibrium binding data.

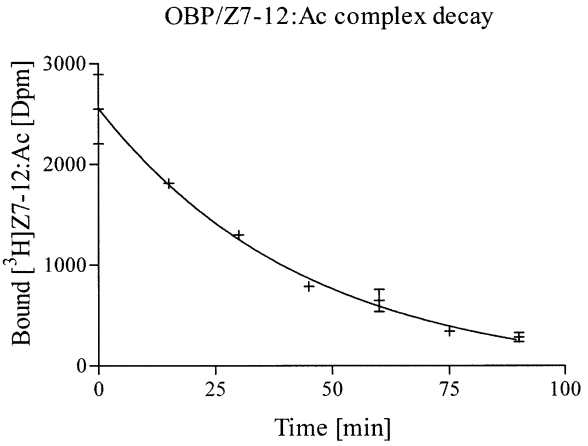

FIGURE 8: Dissociation of the radiolabeled pheromone from the OBP. The gel filtration assay was used as described in the text.

protein P and the free ligand L was described by the following kinetic equation:

$$\frac{d[PL]}{dt} = k_{on}[P][L] - k_{off}[PL] = k_{on}(p - [PL])(l - [PL]) - k_{off}[PL]$$

where p and l are the total protein and ligand concentrations, respectively. The solution of this kinetic equation for the concentration of the protein−ligand complex can be found in the following form:

$$[PL] = \frac{2k_{on}lp[e^{D^{1/2}t} - 1]}{[k_{off} + k_{on}(l + p)][e^{D^{1/2}t} - 1] + \sqrt{D}[e^{D^{1/2}t} + 1]}$$

where $D = -4k_{on}{}^2lp + [k_{off} + k_{on}(l + p)]^2$.

The corresponding expression for the time required for the complex concentration to reach the value [PL] is

$$t = \frac{1}{\sqrt{D}} \ln\left\{\frac{2k_{on}lp - [k_{off} + k_{on}(l + p) - \sqrt{D}][PL]}{2k_{on}lp - [k_{off} + k_{on}(l + p) + \sqrt{D}][PL]}\right\}$$

This expression can be used to calculate the time required for 50% of the ligand to become bound by the OBP. When values of k_{on}, k_{off}, and p obtained above are used, the time needed for 50% of the pheromone to become bound by the OBP is between 30 s (when the pheromone concentration is very small) and 43 s (when the concentration of the pheromone is equal to that of the OBP).

Transport of the Pheromone through Mucus Is Faster than Binding by the OBP. To investigate the significance of the experimentally determined OBP/pheromone association rate, the rate of transport of the pheromone (alone and bound to the OBP) by diffusion through a layer of mucus covering an epithelium was estimated using the standard model, the model of diffusion into a semi-infinite slab (*32*). In this model, the concentration c_z of the diffusing compound at distance z from the surface of the mucus, at time t can be calculated:

$$c_z = c_0 \times erfc\frac{z}{\sqrt{4Dt}}$$

where D is the diffusion coefficient, c_0 is the concentration of the diffusing compound at the surface of the mucus, and *erfc* is the complementary error function. Conversely, the time t at which the concentration of the diffusing compound at distance z from the surface reaches the value c_z can be calculated using the following equation:

$$t = \frac{z^2}{4Dinverfc^2\left(\frac{c_z}{c_0}\right)}$$

When typical literature values of the required parameters are used [thickness of the layer of mucus = 10 μm (*33*), diffusion coefficient for pheromone-like compounds = 1×10^{-9} m^2 s^{-1} (*34*)], the calculated time necessary for the ligand concentration at the surface of the epithelium to reach half of that at the outer surface of the mucus is 0.1 s. Thus, if the pheromone, and possibly other OBP ligands as well, diffuses through an OBP-rich mucus to reach the sensory cell, significant concentrations at the surface of the olfactory sensory cell will be reached before binding by the OBP occurs. Should the pheromone diffuse through the mucus bound to an OBP (diffusion coefficients of proteins similar in size and molecular weight to mammalian OBPs are close to 0.07×10^{-9} m^2 s^{-1}) (*34*), the process would take approximately 1.4 s.

DISCUSSION

Typical early responses of male Asian elephants to the female pheromone include the check, place, and flehmen

responses. Behavioral data suggest the involvement of the vomeronasal system in detection and recognition of the pheromone; however, a role played by the main olfactory system, especially in the early responses, cannot be excluded.

The mucosal tissue of the upper parts of the trunk, in the proximity of the olfactory sensory epithelium, produces copious protein-rich mucus. The proximity of the mucus-secreting tissues to the olfactory epithelium indicates a possible role of the trunk mucus in olfactory perireceptor events. During early pheromone responses (place, check), the mucus-laden end of the trunk comes in direct contact with the pheromone-containing urine. During a later pheromone response, the flehmen response, the end of the trunk is placed inside the mouth, close to the openings of the vomeronasal ducts. This suggests a possible role of the trunk mucus in vomeronasal perireceptor function. Finally, the presence of the mucus along the whole length of the trunk is consistent with the mucus functioning directly in the trunk, rather than in the chemosensory organs.

Using photoaffinity labeling, as well as native electrophoresis of proteins preincubated with radiolabeled pheromone, we detected a broad band of 18−21 kDa proteins present in the trunk mucus that bound the pheromone. The N-terminal sequences obtained from different regions of this band were essentially identical, differing only in the N-terminal starting point. These sequences were similar to known mammalian OBPs and other proteins of the lipocalin protein family. The elephant OBP cDNA was cloned from a male trunk mucosal RNA using PCR techniques. The cDNA sequence encodes a 18.5 kDa protein, consistent with the OBP molecular mass observed. This molecular mass is also consistent with the size of the OBP detected in the mucosal tissue (rather than in the mucus) by Western blotting. The presence of a sharp OBP band in the mucosal tissue indicates that posttranslational modification is the most probable source of the observed heterogeneity of the OBP present in the trunk mucus. The presence of proteins smaller than predicted in the elephant trunk mucus could be attributed to truncation of the OBP N-terminus, revealed by the N-terminal sequencing. Proteins larger than the predicted molecular mass are likely to be modified by glycosylation, as observed in other members of the lipocalin protein family (*23*). Some heterogeneity at the cDNA level has also been observed (data not shown), which could be attributed either to OBP expression from multiple alleles or to PCR artifacts. The degree of similarity to other lipocalins was moderate, considering the diversity of the lipocalin protein family, as well as the large evolutionary distance (100 MY) between elephants (clade Afrotheria) and most other mammals, including all common laboratory mammalian model animals (clade Laurasiatheria) (*28*).

The experimental investigation of the equilibrium binding and the binding kinetics of *Z*7-12:Ac to the *E. maximus* OBP as determined by a gel filtration-based assay demonstrated that the pheromone bound to the OBP with moderate (micromolar) affinity, well within the range of values published for other mammalian OBP−ligand interactions (*9*−*12*, *22*). The modified gel filtration assay also determined the rates of formation and dissociation of the OBP−pheromone complex. The value of the dissociation rate constant k_{off} was determined to be 3.75×10^{-4} s^{-1}, corresponding to a half-life of 31 min for the OBP−*Z*7-12:Ac complex. This result is consistent with our observation that the OBP−pheromone complex can endure native electrophoresis. Some kinetic data have already been published for other mammalian OBPs and their ligands (*21*). Unfortunately, the published data contain inconsistencies between the values of k_{on}, k_{off}, and K_d, and are therefore of limited value. However, the dissociation rates of other mammalian OBPs and their ligands are likely to be slow (similar to those observed herein), as exemplified by the fact that several complexes of lipocalins similar to the elephant OBP and their ligands have been purified and crystallized for X-ray studies with ligands still present (*33*, *34*). We were unable to directly measure the association rate, since the time required for the gel filtration experiment was comparable to, or larger than, the time of complex formation. Therefore, the association rate (on-rate) constant of the elephant OBP−pheromone complex, k_{on}, was calculated from the values of K_d and k_{off}. The association time was then calculated from the on-rate constant and the concentration of the OBP in the trunk mucus, as estimated from VOBA experiments. The time required for association of 50% of the pheromone with the OBP in the trunk mucus was calculated to be between 30 and 43 s, depending on the pheromone concentration. Of the binding characteristics that were measured, the value of the off-rate constant is likely to be the most accurate, since the half-life of the complex does not depend on the protein concentration, which would need to be experimentally determined. In the natural environment of the viscous trunk mucus, both the on-rate and the off-rate would be expected to be slower than in PBS buffer. However, the value of K_d would be minimally affected. Only small differences in binding properties of different parts of the broad OBP band were observed in our experiments (native PAGE/electroblotting, photoaffinity labeling, VOBA, dissociation rate assay), even though the existence of small populations of the OBP with different binding properties cannot be excluded.

The binding data on the elephant OBP/pheromone system, combined with published information on OBP structure and ligand binding properties, can be employed to test the five hypotheses proposed for the physiological function of OBPs. According to our measurements and calculations, the binding of the pheromone occurs rather slowly (in about 30 s). This contrasts with the much shorter time (0.1 s) necessary for the pheromone to diffuse through the layer of mucus covering the olfactory epithelium, as calculated using published values of diffusion coefficients for similar compounds, as well as olfactory mucus thickness observed in other mammals. We have also found that the water solubility of *Z*7-12:Ac allows the pheromone to reach micromolar concentrations (10−15 μM) without assistance from a binding protein. Since the OBP concentration in the trunk mucus is also in the micromolar range (50 μM), binding by an OBP can only cause a moderate increase in solubility of the pheromone in the mucus. Even if the mucus that covers the olfactory epithelium contained concentrations of the OBP as high as in the trunk mucus, the slow on-rate, high pheromone diffusion speed, relatively high pheromone solubility, and slow dissociation rate would argue against the OBP functioning as a carrier through the olfactory or trunk mucus. Combined with the fact that at least some of the mucus covering the olfactory epithelium does not contain the OBP,

our data appear to falsify the carrier hypothesis, which posits that OBPs are needed to transport lipophilic ligands through the aqueous mucus to the olfactory sensory epithelium.

Our data cannot exclude the possibility that OBPs increase the rate of transport of ligands across the air/mucus interface. However, the solubility experiments reported herein show that the concentration of the pheromone in PBS reaches values that are 2 orders of magnitude higher than the saturated concentration of the pheromone in the air, implying that at room temperature the free energy of the pheromone in the aqueous environment is considerably lower than that of the pheromone in the gas phase; i.e., the pheromone 'prefers' water to air. Therefore, in the absence of a proven complex mechanism of entry of the pheromone (and other odorants) into the aqueous environment, we conclude that the rate-limiting step of the transfer is diffusion of the pheromone from the surface of the liquid phase. Our experiments also do not exclude the possibility of a specialized mechanism of releasing ligands in the vicinity of the receptor. However, the lack of changes in known OBP structures upon ligand binding (*7*, *9*) makes it improbable that a protein in the receptor cell membrane can distinguish between a liganded and free OBP molecule. Thus, a hypothetical ligand-releasing protein would have to bind with a significant affinity, open, and release all present OBP molecules, of which only a small fraction would contain a ligand. Such a system would require energy input and be very inefficient. In the insect PBPs, pH has been shown to influence binding affinities toward pheromones (*17*); however, our experiments have not demonstrated a significant effect of pH on Z7-12:Ac binding by the OBP. We therefore conclude that our results on the elephant OBP properties, in conjunction with published data, contradict the carrier hypothesis.

Slow ligand binding and release are most consistent with the scavenger hypothesis, in which OBPs function to remove ligands from the odorant receptor after the transduction of the olfactory signal. According to our calculations, the pheromone (and possibly other ligands as well) can diffuse through the layer of mucus covering the olfactory epithelium before becoming bound by the OBP. Therefore, it seems plausible that the pheromone (or other OBP ligand) diffuses through the mucus and interacts with receptors prior to complexation with the OBP. Only pheromone molecules present in the mucus for an extended period (tens of seconds) will become bound by the OBP; due to the long dissociation rates, these molecules in effect become sequestered from further olfactory receptor interactions. This model is also corroborated by the observation that small hydrophobic molecules are structurally sequestered in the hydrophobic interior of the OBP (*7*, *9*). Sequestration by an OBP could be an important mechanism of signal termination, preventing reactivation of sensory cells by odorant molecules released from the receptor, or activation by molecules that have been trapped in the nasal passages. Our results indicate that the main location of the OBP is distinct from the olfactory sensory epithelium. Similar findings have been reported in other mammalian OBPs as well (*22*). Therefore, the function of the mammalian OBPs as scavengers may, in fact, not be primarily localized to the olfactory sensory epithelium, but to the nonsensory epithelia of the chemosensory organs. In the elephant, this could include the tip of the trunk that is in direct contact with the pheromone-containing urine during a check or a place response, prior to a flehmen response. The elephant OBP would, by binding the pheromone that remained on the tip of the trunk as well as in the VNO, terminate the pheromone spike and prepare both the tip of the trunk and the VNO for another flehmen. This is consistent with the typical interval of 1−5 s between the tip of the trunk touching the urine and being placed in the mouth, as well as with the typical interval between flehmen responses (15−60 s).

The calculations herein show that binding by the OBP occurs slowly even in the presence of high concentrations of the pheromone. Therefore, even high concentrations of OBPs will probably not prevent olfactory receptor overload, as proposed by the buffer hypothesis. The slow on-rates, as well as the closed binding cavity of OBP-like lipocalins, render the transducer hypothesis unlikely. Finally, our data provide partial support for the pheromone carrier hypothesis, since the mechanism of delivery of the pheromone to the VNO during a flehmen response (on the tip of the trunk) differs from that to the olfactory epithelium (directly from air). After all, the elephant OBP was identified in the trunk mucus on the basis of its binding to the pheromone. It is possible that the pheromone binds to the OBP at the tip of the trunk during early pheromonal responses (place, check), and the pheromone−OBP complex is introduced into the VNO during the flehmen response. The slow pheromone binding by the OBP could be considered consistent with the slow buildup of pheromone-induced behavior (typically between 30 s and several minutes from a first check response to a first flehmen response). However, the slow release of the pheromone by the OBP in the absence of a known receptor cell-mediated release mechanism remains inconsistent with the requirement of this hypothesis that the pheromone be transported and released prior to olfactory receptor activation. An interesting result in this context is the presence of another protein distinct from the OBP in the VNO mucus that is capable of binding the pheromone.

Since *Z*7-12:Ac is not only the elephant sex pheromone, but also a component of many insect sex pheromone blends, some of our experimental results can be applied to insect olfaction. Dissociation constants of interactions of several insect pheromones and other compounds with PBPs have been determined. The K_d values are similar to those published for mammalian OBPs/ligands (∼1 μM) (*31*, *32*). Published estimates of the on-rate constants range between 170 and 170 000 $dm^3\ mol^{-1}\ s^{-1}$; estimates of the off-rate constants are within the range 10^{-2}−$10^{-4}\ s^{-1}$ (*33*). However, published native PAGE/electroblotting experiments after incubation with the radiolabeled pheromone (*17*), analogous to those carried out by us on the elephant proteins, indicate that the dissociation rate of the PBP/pheromone complex is similar or even slower than that of the elephant OBP/pheromone, implying that the association rate constants are also similar to the mammalian system. Since the concentrations of insect PBPs and GOBPs in the sensillar lymph are considerably higher than concentrations of mammalian OBPs in the mucus, the rates of ligand binding by the insect proteins are going to be much faster than in mammalian species. Our calculations for physiologically relevant pheromone concentrations (0−50 nM) indicate that 50% of the pheromone will be bound by the insect PBPs within 0.2 s. The physiological

rates of off-loading are uncertain, as various off-loading mechanisms have been proposed. Due to the minuscule size of the sensilla, rates of transport of the pheromone by diffusion are likely to be much higher than in mammalian systems. According to the semi-infinite slab model applied to a sensillum of a 2 μm diameter, the concentration of the pheromone (by diffusion alone) at the dendrite will reach 50% of that on the outer surface of the lymph in 0.016 s. For ligands bound to a small protein (PBP or GOBP), the transport by diffusion will take about 0.13 s. Importantly, in both cases, the transport by diffusion is faster than binding by PBPs or GOBPs. If mixing of the lymph due to passive movements of the sensillum occurs, transport of the pheromone will become even faster, while rates of binding will stay unaffected. Our solubility measurements indicate that aqueous Z7-12:Ac concentrations, without assistance from a protein, can reach values 6−9 orders of magnitude higher than necessary for activation of insect pheromone sensing cells, casting doubts on pheromone solubilization as the PBP function. Our results are more consistent with an earlier model of insect perireceptor events in which the PBPs act as scavengers (*29*, *34*), than with later models that assume binding of the pheromone by the PBPs occurs essentially instantaneously (*33*).

In conclusion, the results herein demonstrate that the *E. maximus* OBP is the major protein of the elephant trunk mucus that binds the elephant pheromone. Our equilibrium and kinetic studies suggest that the pheromone binding and release by the OBP are too slow for the OBP to transport the pheromone through the aqueous mucus and release it at the olfactory receptor cells. Slow association and dissociation rates may be a general characteristic of mammalian OBP/ligand interactions. This would have major implications for the role of mammalian OBPs in the perireceptor processes of olfactory perception. Comparison of our experimental results on the elephant OBP with published data for insect PBPs reveals similarities in kinetics of binding of *Z*7-12:Ac, with possible implications for our understanding of the perireceptor events of pheromone detection in insects.

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