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Proteins that Smell: Pheromone Recognition and Signal Transduction†

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Abstract—Pheromone perception in Lepidoptera requires initial recognition and transport of the pheromone molecule by ligand-specific pheromone binding proteins (PBPs) in the moth antennae, followed by recognition of the ligand or PBP–ligand complex by a transmembrane G-protein-coupled odorant receptor protein. This signal is transduced by activation of a specific phospholipase C, intracellular release of inositol 1,4,5-trisphosphate (IP_3) and IP_3-gated opening of an ion channel. Individual pheromone-specific PBPs provide the initial ligand recognition event and encode ligand specificity. We have used photoaffinity labeling, cDNA library screening and cloning, protein expression, a novel binding assay and site-directed mutagenesis to define the ligand specificity of PBPs. Copyright © 1996 Elsevier Science Ltd

Introduction

How does the structure of a small volatile molecule become decoded as a 'smell' in the brain? The overall process of signal recognition and transduction by olfactory neurons[1–4] is crucial for survival of all organisms. In insects the olfactory sense is essential for location of a mate and food. When odorants enter the nasal mucosa of vertebrates or the antennae of insects, a complex set of equilibria and cascades of biochemical modifications occurs.[1] For the last 12 years at Stony Brook, we have focused our efforts on the structure of insect pheromone binding proteins, the specificity of ligand recognition and the involvement of PBPs in the overall signal transduction process. We began by synthesizing an electrophysiologically active,[5] tritium-labeled photoactivatable pheromone analogue[6] of the moth *Antheraea polyphemus* and then using this probe to selectively label PBPs of this moth.[7–9] Ten years elapsed from the first observation of specific photolabeling of a moth PBP to the identification of the ligand binding site of the recombinant protein.

The first contact between the signal and the sensory system occurs with the odorant binding proteins (OBPs),[10] two distinct families of small (ca. <20 kDa), soluble proteins that occur tissue-specifically at high concentrations in the nasal mucosa of vertebrates[11,12] and the antennal sensillum lymph of insects.[13,14] OBPs have roles in odorant transport, odorant degradation and clearance, and perhaps selective delivery to a membrane-associated receptor.[10,14,15] Odorant binding proteins are known in two insect orders, Lepidoptera[14] and Diptera,[16–18] and have more distant homologues in the Coleoptera.[19]

Key words: Ligand, binding, photoaffinity, cloning, olfaction.
†Dedicated to Professor Kenji Mori.

The PBPs of the Lepidoptera comprise one of three subfamilies[20–22] of the insect OBPs and it was proposed that PBPs should selectively recognize components of the female-produced sex pheromone blend. The two subfamilies of general OBPs (GOBPs) occur primarily in female antennae[21–23] and have been localized immunocytochemically in the sensilla basiconica.[24] PBPs are located primarily in the sensillum lymph of the pheromone-responsive sensilla trichodea[24] of the antennae of male moths and are presumed to mediate the delivery of hydrophobic sex pheromones to specific receptor proteins located in the dendritic membrane.[25] PBPs may also facilitate removal of pheromone metabolites after signal transduction occurs, as determined by photoaffinity labeling and enzyme kinetics experiments.[26,27]

Complete protein sequences are known for more than seven PBPs from six moth species (*Antheraea polyphemus, Antheraea pernyi, Heliothis virescens, Agrotis segetum, Lymantria dispar,* and *Manduca sexta*) and another five GOBPs (in two protein subfamilies) from three species have been described.[20–23,28–33] PBPs and GOBPs share several highly-conserved regions, including six conserved Cys residues that form intramolecular disulfide bridges.[27]

Expression of PBPs via ECPCR

The *A. polyphemus* male antennal PBP[13,34,35] became known as Apol-3 following the cDNA cloning and sequencing.[32] The recombinant protein was first expressed in a binding active, but potentially glycosylated form in baculovirus-infected insect cells,[36] but the disappointing yield could not provide sufficient protein for biophysical characterization. Thus, we employed expression-cassette PCR (ECPCR)[37] to develop an

Escherichia coli- based expression system for Apol-3.[27] The open reading frame (ORF) of the Apol-3 cDNA was copied and amplified into dsDNA equipped with (5′ to 3′) an *Eco* RI site, a ribosome binding site, a start codon, the ORF, a stop codon and a *Hind* III site as shown in Figure 1. The restriction-digested, purified cassette was ligated into a pBS-derived plasmid (pHN1+) equipped with a strong *tac* promoter upstream of an *Eco* RI site and a *rrn*BT_1T_2 transcription termination site downstream of the *Hind* III site. *Escherichia coli* XA90 cells were transformed and ampicillin-selected to give colonies containing the recombinant plasmid. Induction of growing cells with IPTG caused overproduction of a 14 kDa protein in both the soluble fraction and inclusion bodies.

The soluble recombinant PBP (rPBP) was readily purified by preparative isoelectric focusing (IEF) followed by gel filtration (GF) to give >95% homogeneous rPBP.[27] This material was indistinguishable from the moth antenna-derived PBP on native and SDS-PAGE, by Western blotting, and by specific photoaffinity labeling with [^{3}H](6*E*,11*Z*)-hexadecadienyl diazoacetate (*6E,11Z*-16:Dza). Approximately 5–10 mg/L of soluble recombinant protein could be obtained. An additional 100 mg/L of rPBP was in an insoluble, denatured form in the inclusion bodies; solubilization in 6 N guanidinium hydrochloride, reduction with DTT, refolding under aerobic conditions from a dilute cysteine–cystine solution, dialysis and chromatographic purification afforded refolded rPBP that possessed gel electrophoresis mobility, immunoreactivity and specificity of photolabeling essentially identical to the soluble rPBP. This refolded rPBP has proven to be very robust, having survived unchanged after storage in aqueous solution for over 3 years at 8 °C, as well as after 48 h in an NMR probe at temperatures up to 35 °C.

Photoaffinity labeling with [^{3}H]6*E*,11*Z*-16:Dza could be performed on rPBP in a variety of buffers between pH 6.0 and 8.5. Labeling of soluble rPBP and refolded PBP was equally competable by 6*E*,11*Z*-16:Ac, 6*E*,11*Z*-16:OH and 9*Z*-14:Ac, suggesting (albeit incorrectly; see below) that the rPBP could not distinguish the major pheromone component from a nonpheromone component nor a pheromone metabolite. This apparent lack of selectivity is an artifact of the irreversibility (i.e. nonequilibrium nature) of the photoaffinity labeling experiment. Using a new equilibrium binding assay,[38] we showed that the affinities for nonpheromone components can be over three orders of magnitude lower than for the cognate pheromone ligand.

Identification of pheromone binding site by photoaffinity labeling

Specific amino acids proximal to the reactive moiety of the photoaffinity analogue in the ligand binding site of a protein can become covalently modified when irradiation unmasks the reactive moiety. Peptide fragments of Apol-3 PBP bearing a radioactive lipid modification were sequenced and implicate key amino acids important to the recognition of the acetate functional group. Thus, Apol-3 rPBP was photoaffinity labeled with [^{3}H]6*E*,11*Z*-16:Dza and radiolabeled peptides were separated from an endoproteinase Lys-C digestion by HPLC and characterized by Edman degradation.[39] The label was exclusively found in the Asp^{39}-Lys^{58} fragment. Cleavage of this peptide with endoproteinase Arg-C gave a single radiolabeled peptide (DDYVMTDR), which was predicted to be α-helical; Edman degradation showed that the modification had occurred at Thr.[44]

Next, photoaffinity labeling of Apol-3 rPBP with

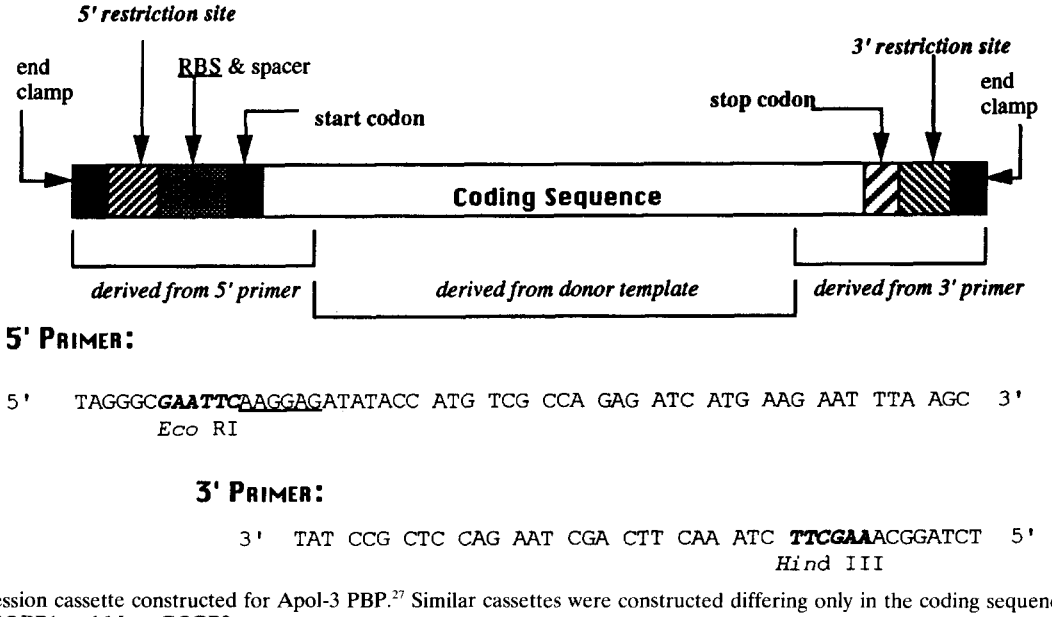

Figure 1. Expression cassette constructed for Apol-3 PBP.[27] Similar cassettes were constructed differing only in the coding sequences for Aper-1, Aper-2, Msex-GOBP1 and Msex-GOBP2.

[^{3}H]4*E*,9*Z*-14:Dza, the 14-carbon analogue of the minor pheromone component, followed by digestion with Lys-C and separation by HPLC showed three peaks of radioactivity in a 3:1:1 ratio. The major radioactive Lys-C fragment was assigned to Asp^{21}-Lys^{38}, with the modification located at residue Asp^{32}. The second peak was identified as the same Asp^{39}-Lys^{58} fragment labeled by the 16-carbon homologue; the last eluting peak was established as the C-terminal peptide, Leu^{125}-Val^{142}. The 14-carbon odorant molecule thus had two possible binding positions within the recognition site, while only a single binding position was available to the 16-carbon pheromone.[39]

The proposed binding of the two pheromone components in the pheromone binding site of Apol-3 PBP is diagrammed in Figure 2.[31,38,39] Anchoring of the ester carbonyl of the pheromone by the protonated guanidinium group of Arg^{46} occurs in conjunction with the hydrophobic hexadecadienyl chain interacting with a hydrophobic β-strand. While the carbene derived from the 16-carbon ligand ([^{3}H]6*E*,11*Z*-16:Dza) would covalently modify Thr^{44}, the carbene derived from the shorter fourteen-carbon ligand ([^{3}H]4*E*,9*Z*-14:Dza) would experience two competing binding interactions. Preference for the Arg^{46}–ester carbonyl interaction would lead to modification of Thr^{44}, while a preference for the hydrophobic recognition of the (*E*,*Z*)-diene and of the terminal butyl group would result in modification of Asp.[32] The 3:1 ratio of modification at Asp^{32} to Thr^{44} suggests a preference of ca. 0.7 kcal/mol for hydrophobic backbone binding. This region is the most hydrophobic domain in all PBPs and consists of a helix-sheet-helix motif. In Figure 3, the *RXXGCAXXC* motif is highly conserved in the primary sequences of the PBPs and the GOBPs.

Development of a pheromone-PBP binding assay

Early attempts to develop a binding assay to measure the specificity and affinity of pheromones for PBPs failed due to the low solubility of long-chain acetates in water and the adsorption of the hydrophobic ligand to the assay vessel.[9,40] Adsorption could not be prevented by surface pretreatment or protein additives.[9] One assay[41] used native Apol-3 PBP to solubilize [^{3}H]6*E*,11*Z*-16:Ac off the glass walls and into an aqueous buffer and estimated a K_D for 6*E*,11*Z*-16:Ac of ca. 10^{-8} M. A new 'reversed-phase' binding assay[38] was recently described and is shown diagrammatically in Figure 4. Using a plastic microfuge tube coated with

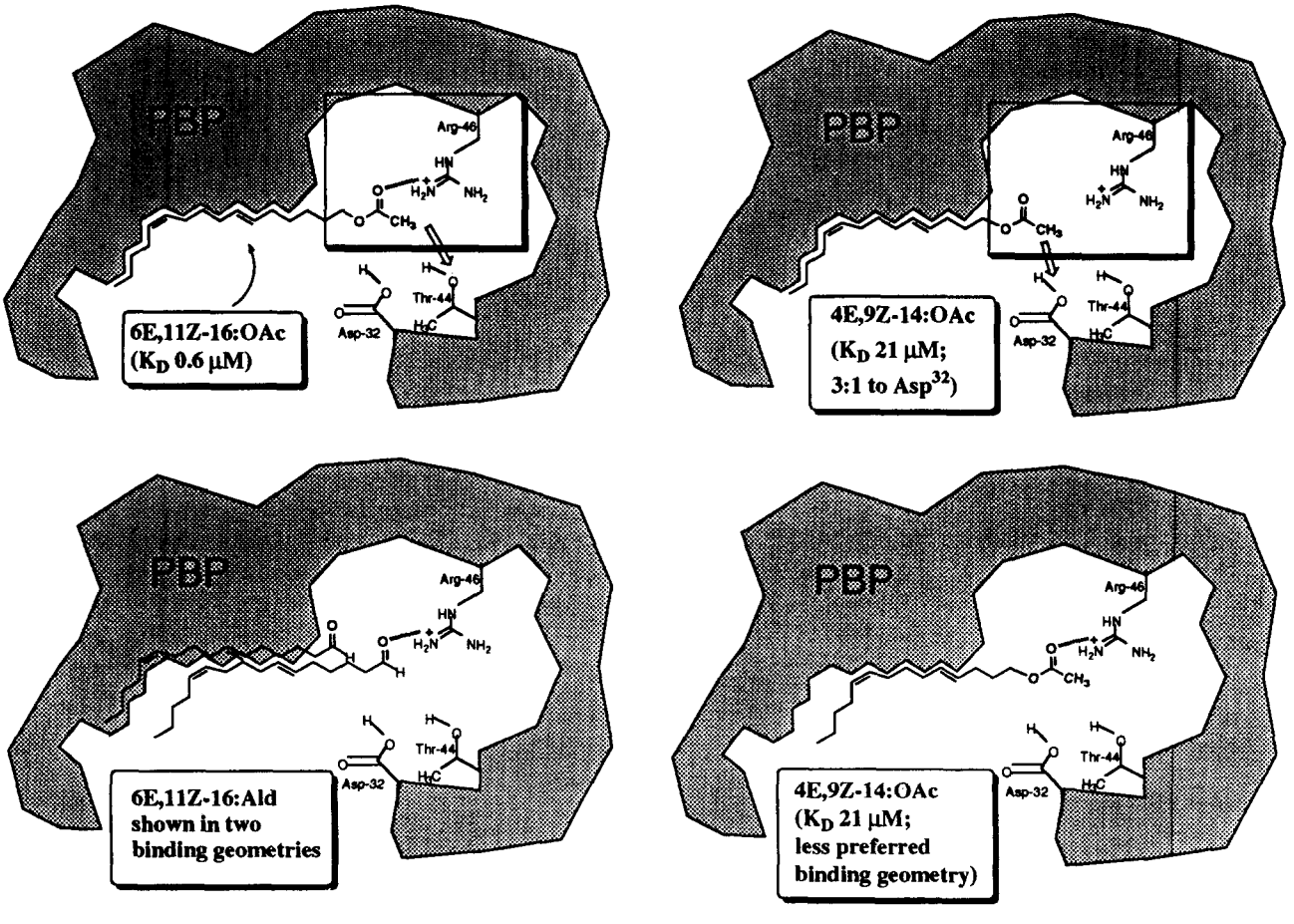

Figure 2. Apol-3 appears optimized for 6*E*,11*Z*-16:Ac. Top: The preferred orientation of 6*E*,11*Z*-16:Ac in Apol-3 PBP leads to modification of Thr^{44} (open arrow) when the acetate is replaced a diazoacetate and photolyzed; similarly, Asp^{32} is preferrentially modified by the 4*E*,9*Z*-14:Ac analogue. Bottom right: The less preferred binding geometry for the 4*E*,9*Z*-14:Ac maintains the carbonyl–Arg^{46} interaction; similarly the 6*E*,11*Z*-16:Ald (bottom left) may bind to Apol-3 PBP in an analogous location.

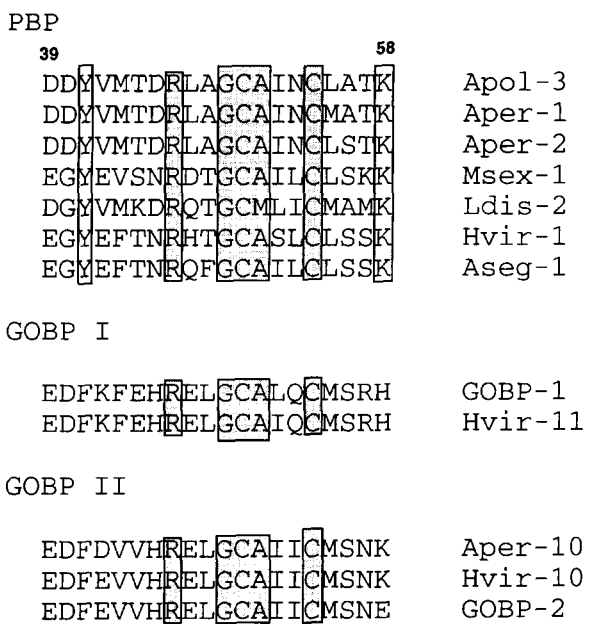

Figure 3. Comparision of the Apol-3 binding site sequence (top) with five other PBPs and five GOBPs. Open boxed residues are conserved within PBP subfamily; shaded boxes denote residues conserved among all subfamilies. Key: Aper-1, *A. pernyi*,[33] Aper-2,[29] Aper-10,[23] Msex-1; *M. sexta*,[28] Hvir-1, Hvir-10, Hvir-11, *H. virescens*,[20] GOBP-1, GOBP-2, *M. sexta*,[22] Ldis-2, *L. dispar* PBP-2,[31] Aseg-1, *A. segetum*.[31]

1-dodecanol, up to 100 nM [^{3}H]6*E*,11*Z*-16:Ac was >95% dissolved in aqueous buffer. When Apol-3 rPBP was added, the tritiated pheromone was removed from the solution, since the PBP–pheromone complex then adsorbed to the amphiphilic surface coating.

The optimal parameters for the binding assay are summarized below. Using these conditions, Scatchard analysis of the binding of [^{3}H]6*E*,11*Z*-16:Ac to Apol-3 showed a single saturable site with $K_D = 0.6$ μM; the K_D was 21 μM for [^{3}H]4*E*,9*Z*-14:Ac (see Figure 2). Displacement of [^{3}H]6*E*,11*Z*-16:Ac binding by other pheromone components (6*E*,11*Z*-16:Ald and 4*E*,9*Z*-14:Ac), a non-pheromone component (16:Ac) and pheromone catabolites (6*E*,11*Z*-16:OH and 4*E*,9*Z*-14:OH) showed a clear trend in relative binding affinity: 6*E*,11*Z*-16:Ac >4*E*,9*Z*-14:Ac >6*E*,11*Z*-16:Ald≈16:Ac >6*E*,11*Z*-16:OH >4*E*,9*Z*-14:OH. These data clearly demonstrate a >1000-fold range of binding affinities among these very similar structures and unambiguously demonstrate the specificity of the PBP–pheromone interaction. As suggested in Figure 2, the lower affinities of the analogues reflect a misalignment of critical alkene bonds, hydrophobic chains or carbonyl groups in the Apol-3 active site.

Optimized PBP-pheromone binding assay

- Coat microfuge tuges with saturated solution of 1-decanol in ethanol–water.
- Use 25 nM PBP and 25 nM [^{3}H]-labeled pheromone for competitive displacement.
- Equilibrium of bound and free ligand achieved in 1 h at 4 °C.
- Essentially no exchange of [^{3}H]ligand with surface coating.
- Over 95% of PBP adsorbs to surface coating while ligands stay in solution.
- Very slow exchange of adsorbed and free PBP.
- Add competitors in ethanolic solution.

Proteins encode ligand specificity

The critical test for our new binding assay was to find a PBP that would show higher affinity for the shorter ligand. A potential system was available for *A. pernyi*, since two cloned cDNAs from the same species were available that encoded PBPs with important sequence divergences. Figure 5 shows a comparison of the three amino acid sequences for Apol-3, Aper-1 and Aper-2. Aper-1 is most similar to Apol-3 with 92% identity for 142 residues. Eight of the 12 changes (M5I, N10Q, V30I, L55M, D75E, T85S, L133V, I135L) are highly conservative with respect to hydrophobicity, size and charge. Aper-1 was expected to show selectivity for the 16-carbon pheromone component. In contrast, Aper-2 showed somewhat lower (87%) similarity to Apol-3, with the differences localized in patches in the primary structure (underlined and double-underlined in Figure 5). Our hope was that Aper-2 would thus have a different ligand selectivity than Aper-1. Interestingly, the binding region as identified in Apol-3 was essentially completely conserved in Aper-1 and Aper-2, unlike the divergence seen in binding regions of the noctuid, sphingid and lymantriid PBPs (see Figure 3) that recognize pheromone components with different functional groups, chain lengths or alkene regio- and stereochemistry.

Aper-1 and Aper-2 PBPs were obtained as described for Apol-3 PBP.[27] Thus, the ORFs of the appropriate cDNAs (from J. Krieger and H. Breer) encoding Aper-1 and Aper-2 [29,33] were copied into dsDNA equipped with (5′ to 3′) an *Eco* RI site, a ribosome binding site, a start codon, the ORF, a stop codon, and a *Hind* III site. The restriction-digested, purified cassette was ligated into pHN1+, transformed into *E. coli* XA90 cells and ampicillin-resistant colonies were picked. Induction of protein expression gave a 14 kDa protein in both the soluble and insoluble fractions for each PBP. For the binding assays, only soluble rPBPs were employed to avoid any variation due to the refolding of different rPBPs. Thus, the soluble rPBPs Aper-1 and Aper-2 were purified by preparative IEF, GF and Mono-Q FPLC (as with Apol-3) to give >95% homogeneous rPBP.[27] Highly-purified PBPs are crucial for obtaining reproducible results in the new binding assay.[38]

As seen in Figure 6, both Aper-1 and Apol-3 show 20- to 30-fold higher binding affinity for 6*E*,11*Z*-16:Ac relative to 4*E*,9*Z*-14:Ac. In contrast, Aper-2 shows a 3.5-fold binding preference for the shorter 4*E*,9*Z*-14:Ac over the longer 6*E*,11*Z*-16:Ac component, demonstrating for the first time that two PBPs in the same moth bind two pheromone components with different

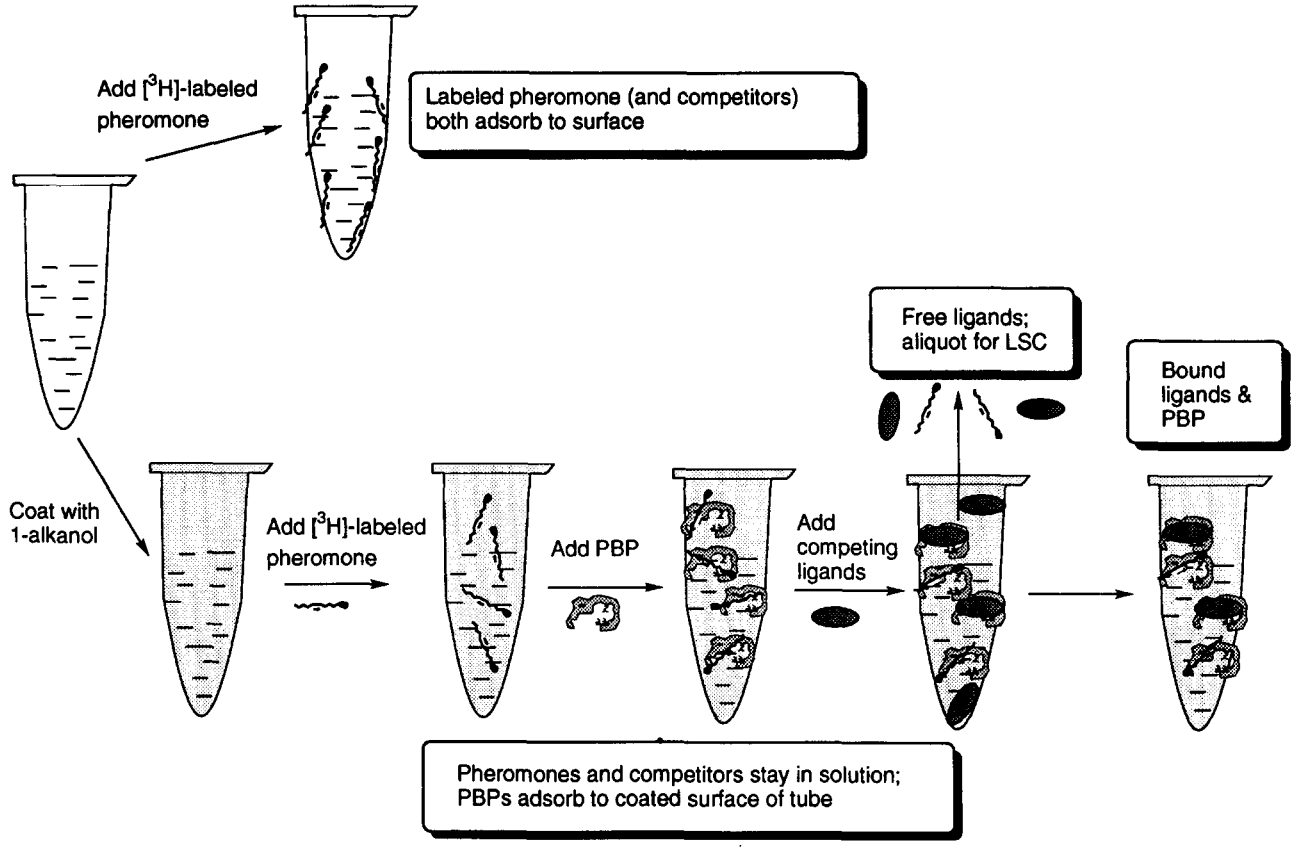

Figure 4. Schematic depiction of the novel pheromone binding assay. To avoid adsorption of the tritiated pheromone, the surface was saturated with a nonspecific binder capable of creating a hydrophilic-like coating. 1-Decanol gave better data than 1-octanol or 1-dodecanol. Labeled pheromone stays dissolved and addition of 1 molar equiv of PBP results in the formation of 1:1 complexes that adsorb to the surface. Addition of a competing ligand displaces some of the tritium-labeled pheromone into solution and after 1 h an aliquot of the solution is counted to directly measure free tritiated ligand.

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         10        20        30        40        50        60        70        80
         |         |         |         |         |         |         |         |
SPEIMKNLSNNFGKAMDQCKDELSLPDSVVADLYNFWKDDYVMTDRLAGCAINCLATKLDVVDPDGNLHHGNAKDFAMKH
---*I---*Q-*C-------Q--NI----I----------------------M*----*----------E-----
---V#---CM-Y#-------Q--N#----I----------------------#S----I----------E-----

         90        100       110       120       130       140
         |         |         |         |         |         |
GADETMAQQLVDIIHGCEKSAPPNDDKCMKTIDVAMCFKKEIHKLNWVPNMDLVIGEVLAEV          Apol-3
---AS--**-----------*-------*--*-*--------------D--V-L-------          Aper-1
---DG--HE-----------S-------I--M-I--------------#--#-V-------          Aper-2
```

Figure 5. Amino acid sequences of Aper-1 and Aper-2 are compared with that of Apol-3. Dashes indicate identical residues in all three proteins. Asterisks (*) indicate identical residues for Apol-3 with Aper-1 only; number signs (#) indicate identical residues for Apol-3 and Aper-2 only. Underlined and double-underlined regions are surface regions with high divergence of primary amino acid sequence among the three proteins; the double-underlined region was employed for preparation of Aper-1 and Aper-2 specific antisera. The highly conserved region (dotted underline) containing the photoaffinity labeled Thr^{44} and the unique Arg^{46} for orientation of the ligand carbonyl and the hydrophobic binding surface for the alkadienyl chain appears not to encode ligand specificity.

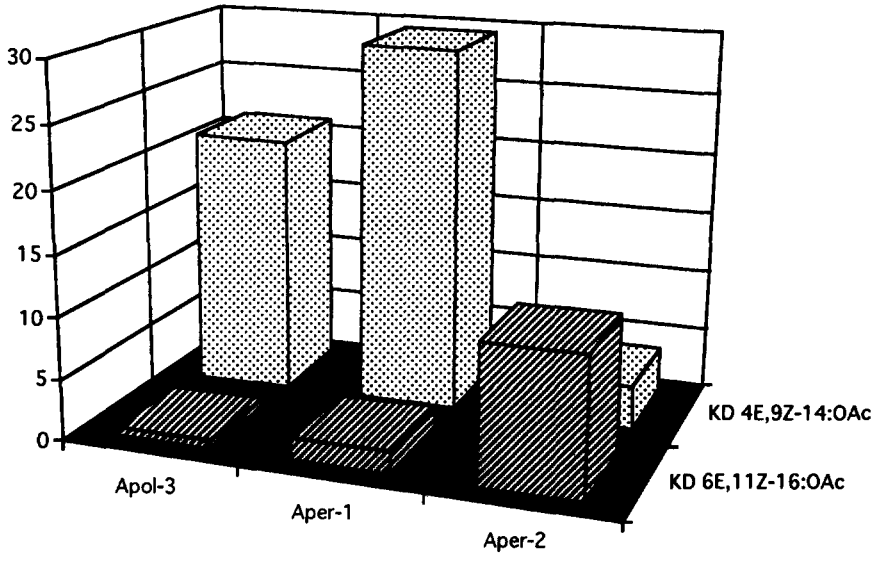

Figure 6. Dissociation constants (K_D) for two pheromone components with three purified, soluble recombinant PBPs. Note that Apol-3 and Aper-1 selectively bind 6*E*,11*Z*-16:Ac, while Aper-2 shows a selectivity for 4*E*,9*Z*-14:Ac.

and complementary affinities. Interestingly, Aper-2 shows 85% identity with Aper-1 and 87% identity with Apol-3. The majority of the 21 differences between Aper-1 and Aper-2 are conservative, but the least conservative changes occur in two distinct regions, underlined and double-underlined in Figure 5: residues 9–13 (SQNFC in Aper-1, CMNYG in Aper-2) and residues 84–89 (ASMAQQ in Aper-1, DGMAHE in Aper-2). We have hypothesized that the latter pair of polypeptides are crucial in ligand differentiation and the predicted surface location of these sequences suggested a strategy (see Section 7, below) for localizing these two PBPs in moth antennae.

Finally, we have recently used ECPCR to produce bacterial expression systems (L. Feng and G. Du, unpublished results) for the *M. sexta* GOBP 1 and GOBP 2 from cloned cDNAs provided by R. G. Vogt.[22] Although the natural ligands for these proteins are unknown, the high conservation of protein sequence throughout the Lepidoptera and the localization to female sensilla basiconica suggest one or more ligands of plant origin that female moths must perceive to signal suitable sites for oviposition. Several tritiated ligands will be used in the new binding assay and a wide variety of potential ligands will be used as competitors to identify potential natural odorants for the GOPBs.

Structural Studies with PBPs

In contrast to the β-barrel form of the vertebrate OBPs,[10] the PBPs are predominantly helical proteins. Deconvolution of the circular dichroism (CD) for Apol-3 allows prediction of 49% α-helix and 8% β-sheet.[30] Chou–Fasman and Garnier algorithms predict 47–48% helix content. The CD spectra appear to be invariant with PBP concentration from 1 mM to 0.01 mM, suggesting that no aggregation occurs. CD studies in progress on the titration of PBP with pheromone to give the 1:1 PBP:pheromone complexes will be informative, particularly if conformational changes occur when the ligand binds.

A high-resolution NMR structure is also accessible. Refolded Apol-3 rPBP has proven to be very robust, surviving essentially unchanged after prolonged storage at 8 °C, and several days at 35 °C. Apol-3 has been expressed from minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source to give uniformly labeled [^{15}N]-rPBP (G. Prestwich, G. Du and L. Feng, unpublished results). Refolded, purified [^{15}N]-PBP showed ca. 90 of the expected nitrogens in a single quantum coherence experiment (M. Rosen, S. Mohanty and G. D. Prestwich, unpublished results).

How are PBPs distributed in the antenna?

PBPs most likely play an active role in the initial biochemical recognition step leading to the perireceptor events of odorant perception.[25,42] Evidence for this proposal includes: the microheterogeneity of DNA encoding such proteins in moths,[29] the association of specific OBP subtypes with distinct classes of olfactory receptor neurons,[21] the immunohistochemical localization of GOBPs and PBPs in separate sensilla,[24] the segregation of OBP types in *Drosophila* olfactory hairs,[16,17] and the different binding affinities of Apol-3 and Aper PBPs for a variety of pheromone structures.[30] Ultrastructural,[43,44] developmental[45] and electrophysiological studies[46] have established the cellular locations[47] of PBPs and have confirmed their importance in mediating pheromone detection.[48]

To address the issue of where ligand-specific PBPs are located in antennae, we identified *A. pernyi* as a

suitable model system. Electrophysiological data were available to define which sensilla trichodea responded to 6*E*,11*Z*-16:Ac and which to 4*E*,9*Z*-14:Ac; moreover, Aper-1 and Aper-2 proteins had been implicated in differential binding of these two ligands. Thus, we identified regions in these two proteins with maximal sequence difference between the two PBPs: ADASMAQQLV (Aper-1) and ADDGMAHELV (Aper-2) were surface-exposed 10 mers with no more than two consecutive identical amino acids between them (see Figure 5). Synthetic peptides were prepared with an N-terminal Cys residue, the peptides were coupled through the Cys residue to keyhole limpet hemocyanin using a heterobifunctional linker and polyclonal antisera were obtained from rabbits. Western blots with purified IgG fractions and crude antisera showed essentially no cross-reactivity with recombinant Apol-3 (sequence ADETMAQQLV in the same region) for either antiserum. Aper-1 and Aper-2 rPBPs showed low cross-reactivity with the heterospecific antiserum (G. Du and L. Feng, unpublished results). Antisera purified by subtractive peptide affinity chromatography will be employed for immunocytochemical studies. The most likely outcomes are: (1) both Aper-1 and Aper-2 are in all sensilla, regardless of electrophysiological response profile; or (2) Aper-1 and Aper-2 segregate partially or completely to sensilla responding to a particular component. This result will move us one step closer to connecting the arrival, transport and recognition stage of pheromone perception to the perireceptor events in signal transduction.

A model for pheromonal signal transduction

Understanding molecular recognition in PBP–pheromone complexes is the first step in deciphering the sequential events leading to the activation of the pheromone receptor-coupled, G-protein-mediated signal transduction cascade in olfaction.[25,31,42,49–52] While the OBPs of vertebrates recognize a large number of odorants of diverse structure,[10] the PBPs and GOBPs possess odorant specificities for a limited set of chemical structures and terminal functionalities. The GOBPs,[21–23] which are expressed primarily in female antennae, are over 95% conserved in the Lepidoptera; PBPs have highly-conserved overall protein folds with 32–92% sequence identities.

Figure 7 illustrates a model[30,31] in which pheromones are displayed to receptors; a similar process was envisaged for vertebrate OBPs.[10,14] The key points of this model are:

PBPs encode ligand specificity. The 30-fold difference in binding affinities for two components corresponds to over 2 kcal difference in free energies of

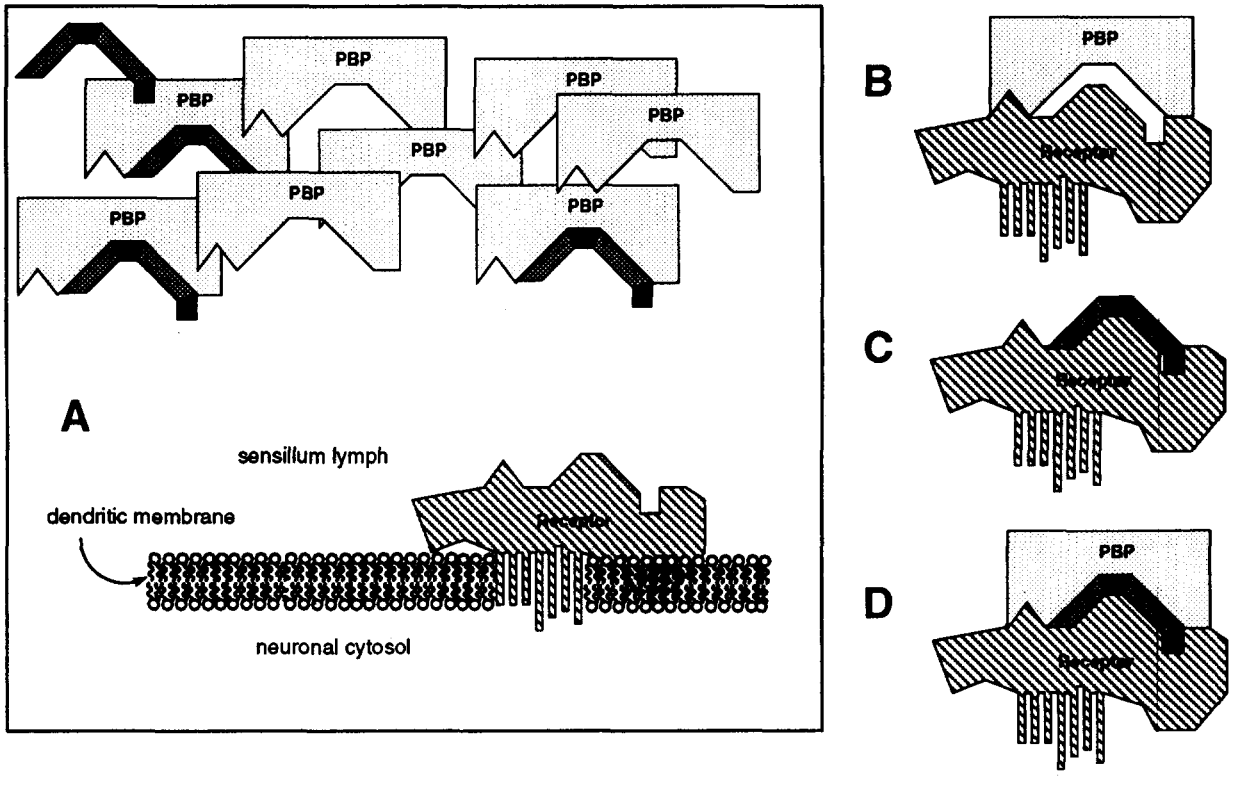

Figure 7. Model of the pheromone(P)–PBP–receptor (R) interaction. Panel A: An excess of pheromone binding protein is always present relative to incoming pheromone molecules; with a K_D value of ca. 1 μM, essentially all pheromone is bound to PBP. The putative dendritic receptor is membrane-associated via a hypothesized seven-transmembrane domain coupled to a heterotrimeric G-protein for signal transduction. Panel B: PBP–R complex causes no signal transduction. Panel C: P–R complex will be transient since all P is present as PBP-P. Panel D: PBP–P–R complex proposed as a productive signal transducer; PBP presents a conformationally restricted P to R, while R recognizes parts of both P and PBP.

interaction between the two protein–ligand complexes. This corresponds to a hydrogen bond or to one or two pi–pi or hydrophobic interactions; conformational distortion for steric reasons would be still another source of this 2 kcal difference. The existence of at least two PBPs in a single moth species that interact specifically with two different pheromone components suggests a pre-selection or filtering role for PBPs in odor discrimination.

PBPs transport for *all* hydrophobic molecules entering the sensillum lymph via the pores and pore tubules. The ratio of protein to ligand is sufficiently high[13] to ensure that even low affinity (mM) ligands are present in bound form or else do not even enter the lumen. PBPs also deliver pheromones and nonpheromonal molecules to sensillar enzymes for inactivation,[26,53,54] although the apparent presence of sensory esterase-lacking insects is still problematic.[55]

PBPs also bind pheromone metabolites and may assist in their removal from the sensillum lymph. The rapid and continuous turnover of PBP in male *L. dispar* antennae[45] suggests such an active role. The subcellular localizations of PBPs and their presence in supporting cells is consistent with this hypothesis as well.

PBPs may present the pheromone ligand to the G-protein-coupled receptor located in the membrane of the pheromone-specific dendrite. As shown in Figure 7, PBPs could bind specific pheromones in such a conformation as to present a composite pheromone–PBP surface to the receptor protein. Interactions from *both* the bound ligand and ligated protein would be required for receptor activation; neither the PBP nor the pheromone alone is sufficient to activate the receptor. We have observed that for both *A. segetum* (C. Lôfstedt and G. D. Prestwich, unpublished results) and *A. polyphemus*,[8] putative dendritic membrane receptors were specifically labeled in the presence of PBPs. The possibility that each PBP contains both a pheromone-selective binding site which, when properly occupied, correctly orients and exposes an 'anticodon' loop capable of conveying confirmatory or synergistic information to accomplish selective receptor activation, is fully consistent with our observations thus far. The surface epitopes described for the Aper-1 and Aper-2 immunocytochemical project above are candidates for such a region.

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