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Structure–Activity Studies with Pheromone-binding Proteins of the Gypsy Moth, *Lymantria dispar*

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Abstract

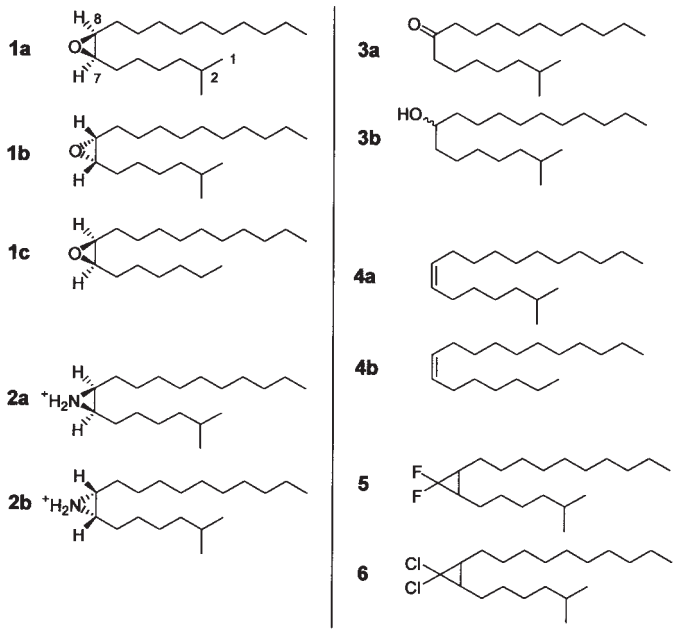
Pheromone olfaction in the gypsy moth, *Lymantria dispar*, involves accurate distinction of compounds with similar structure and polarity. The identified sex pheromone is (7*R*,8*S*)-2-methyl-7,8-epoxyoctadecane, **1a**, and a known antagonist is (7*Z*)-2-methyloctadec-7-ene, **4a**. The first step in pheromone olfaction is binding of odorants by small, soluble pheromone-binding proteins (PBPs), found in the pheromone-sensing hairs. We have studied the molecular determinants recognized by the two PBPs found in the gypsy moth, using three pheromone/PBP binding assays. Results indicate that (i) PBPs bind analogs of the pheromone with some discrimination; (ii) PBPs experience enhancement of binding when presented with **1a** or its enantiomer and **4a** simultaneously; and (iii) the binding enhancement is also seen at high ligand:PBP ratios. We found no evidence of allostery, so the synergistic binding effects and the concentration effect may only be explained by multimerization of PBPs with each other, which leads to more than one population of binding sites. We suggest that the enhanced ligand binding at high ligand:PBP ratios may serve to sequester excess ligand and thereby attenuate very strong signals.

Key words: binding assay, fluorescence, insect, odorant, olfaction, signal attenuation, synergy, transport

Introduction

In many species of moth, pheromones play a central role in reproduction. The female releases the pheromone, and the males follow the pheromone plumes upwind. The main sex pheromone component of the gypsy moth (*Lymantria dispar*) is (7*R*,8*S*)-2-methyl-7,8-epoxyoctadecane **1a** (Miller *et al.*, 1977). The antipode **1b** is believed to be a major component of the nun moth, *Lymantria monacha*, pheromone blend (Hansen, 1984). However, while **1b** strongly antagonizes the effect of **1a** in the gypsy moth, it has no effect on nun moth behavior (Grant *et al.*, 1996). The alkene **4a** is a strong antagonist in the gypsy moth (Gries *et al.*, 1996), but a synergist in the nun moth (Grant *et al.*, 1996). The nun moth also has the unbranched analogs of the epoxide **1c** and the alkene **4b** as synergistic components. Again, in the gypsy moth the unbranched compounds are antagonistic (Gries *et al.*, 1996). Thus, even though both species strongly respond to **1a**, the additional components of the nun moth blend are believed to be responsible for reproductive isolation between these two species. Because pheromones are gaining more widespread use as monitoring and control agents (Wyatt, 1997), it is of great interest to understand the molecular basis of the pheromone olfactory system (Plettner, 2002).

The pheromone olfactory system in male moths is housed in antennal sensory hairs (sensilla trichodea). Two or three olfactory neurons project their dendrites into the hollow space of one hair (Kaissling and Torson, 1980; Keil, 1982). The dendrites are bathed by sensillar lymph, an aqueous solution which contains pheromone-binding protein (PBP) (Vogt and Riddiford, 1981). The pheromone enters the sensilla through pores in the cuticle, and the abundant PBPs transport the hydrophobic pheromone through the aqueous lymph to the dendritic membrane (Vogt *et al.*, 1985). There, the odorants interact with G-protein-coupled transmembrane receptors, which initiate a nerve potential through several signal transduction steps (Krieger and Breer, 1999). The pheromone sensory hairs have two remarkable properties. (i) They exhibit a very high selectivity towards particular pheromone blend components or towards antagonists from related species (Mustaparta, 1997). (ii) They have a very broad range in sensitivity, from ~1.6×10^{-17} M/s (Kaissling and Priesner, 1970) to ~5×10^{-9} M/s (Kaissling, 1977) of



Scheme 1 Compounds used in this study. The carbon numbering referred to in the text begins at the end closer to the functional group, as shown for compound **1a**.

pheromone in the plume (see supplementary material). At this point it is not known how much each of the molecular components outlined above contributes to the selectivity and sensitivity of an olfactory hair.

The PBPs are not the only proteins that function in the transport of hydrophobic ligands through aqueous media. The general odorant binding proteins (GOBPs) (Maibeche-Coisne *et al.*, 1998; Vogt *et al.*, 1991), which are related in sequence to the PBPs (Vogt *et al.*, 1999) and the chemosensory specific proteins (CSPs), which are in a different protein family (Briand *et al.*, 2002) are either associated with chemosensory structures or have been shown to bind small ligands (Briand *et al.*, 2003; Mosbach *et al.*, 2003). It is not clear whether PBPs only act as pheromone transporters between the cuticle and the dendritic receptors, or whether PBPs are also scavengers, helping to clear the pheromone from the sensillar lymph.

Gypsy moth PBPs exhibit 2- to 10-fold discrimination ability towards structurally related odorants, when only the dissociation constants are compared (Plettner *et al.*, 2000; Kowcun *et al.*, 2001). However, two ligands that bind to a protein with the same equilibrium constant may be exploiting very different molecular interactions, which may influence the conformational properties of the protein–ligand complex. In this paper we have studied a set of pheromone components and analogs, to address the molecular interactions that contribute to binding PBP–ligand complex. The functional role of molecular recognition by PBPs is not known. One possibility may be the exclusion of compounds that are irrelevant to pheromonal communication, while still providing flexibility to accommodate a variety of structurally related compounds. Understanding the structure–function relationships of the PBP–odorant interaction is an important step towards understanding the function of PBPs.

The gypsy moth has two different pheromone binding proteins, PBP1 and PBP2 (Vogt *et al.*, 1989; Merritt *et al.*, 1998). We have addressed the enantiomer selectivity of these two PBPs, and found that at pH 7.5 and low ionic strength PBP1 prefers **1b** while PBP2 prefers **1a** (Plettner *et al.*, 2000). Because pheromone olfaction has been studied extensively by electroantennography (EAG) in the gypsy moth, this species is a good model to elucidate the extent to which the PBPs contribute to the olfactory selectivity and sensitivity. In this study, we have performed three groups of experiments.

Experiment I

We addressed the question of PBP contribution to olfactory selectivity by examining binding of various analogs of epoxides **1a** and **1b** to PBPs, using three different pheromone/PBP binding assays. EAG data exist in the literature for all compounds examined (Schneider *et al.*, 1977; Hansen, 1984; Grant *et al.*, 1996; Dickens *et al.*, 1997). To assess the importance of the epoxide moiety we have studied analogs with NH_2^+ (**2a**, **2b**) and CX_2 (**5**, **6**) (Scheme 1) instead of the epoxide oxygen, or analogs lacking a three-membered ring between positions 7 and 8 (**3a**, **3b**, **4a**, **4b**). The protonated aziridines **2a** and **2b** have the opposite polarization to the epoxide O in **1a**, **1b** and **1c**: the aziridines have a full positive charge on the N, while the epoxides have a partial negative charge on the O. The alkenes **4a** and **4b** have a π-system instead of the epoxide and, consequently, a planar geometry around positions 7 and 8. To determine recognition of the 2-methyl group we have compared epoxides and alkenes with (**1a**, **1b**, **4a**) and without (**1c**, **4b**) this substituent. In an effort to understand the ligand binding interactions, we performed homology modeling based on the crystal structure of *Bombyx mori* PBP (Sandler *et al.*, 2000).

Experiment II

Because blend composition is so important in pheromone recognition, we have investigated the binding of binary combinations of **1a** or **1b** with **2–6** to PBPs.

Experiment III

To gain insights whether PBPs might act as scavengers, we measured and compared ligand binding at low ligand:PBP ratios to binding at high ligand:PBP ratios.

For experimental group I, our results indicate that the ligand binding selectivity of the PBPs studied here does not parallel the selectivity documented in EAG studies: ligands that bind strongly to PBPs do not necessarily evoke strong EAG responses. Importantly, differences in the dissociation

constant do not necessarily reflect a lack of ligand discrimination. Our binding and molecular modeling studies suggest that different molecular interactions are exploited by different ligands. For experimental group II we have observed binding synergy between certain combinations of ligands, and for experimental group III, we have observed significantly stronger binding at high ligand:PBP ratios than at low ratios. No cooperativity was observed in these binding studies, so we explain the observed ligand-mediated effects by the existence of multiple binding equilibria between the ligand, PBP monomers and PBP multimers. We propose that the effects observed herein explain the dual role of PBP as both odorant transporter and scavenger.

Materials and methods

Materials

PBPs were expressed in *Escherichia coli* (with the pHN1+ vector, supplied by L. Chen and G.L. Verdine, Harvard, via G.D.P.), reconstituted from inclusion bodies and purified as described elsewhere (Plettner *et al.*, 2000). The PBPs had the correct mass by MALDI-TOF MS and the expected pI. Purification of ditritiated (at C-5 and C-6) **1a** and **1b** (from G.D.P.) was as described (Plettner *et al.*, 2000); ditritiated (at C-7 and C-8) **4a** and **4b** were purified by chromatography on 5% $AgNO_3$ silica gel with heptane:toluene 3:1. Synthesis of the analogs **2**, **5** and **6** have been described in (Dickens *et al.*, 1997). A sample of (7*R*,8*S*)-epoxyoctadecane (**1c**) was obtained from Ms R. Gries (SFU).

We used three types of binding assays: (i) with radiolabeled pheromone: (a) with only radiolabeled ligand and (b) competition assays with radiolabeled ligand and non-radiolabeled analogs; (ii) with non-radiolabeled analogs by taking advantage of changes in the fluorescence of tryptophan 37 upon addition of ligand (Bette *et al.*, 2002); and (iii) by attaching a dansyl group to the PBP and monitoring changes in fluorescence upon addition of ligand.

Radioassays

In the radioassays, labeled pheromone was incubated at six different concentrations between 0.02 and 0.40 nM with 2 μM PBP in 10 mM Tris at pH 7.5. Protein-bound ligand was separated from unbound ligand by passage through a small column of Bio-Gel P-2 (Bio Rad, CA, 2 kDa exclusion limit). The protein was eluted from the mini-columns with assay buffer and the unbound ligand remained behind. The assay and its validation have been described in detail (Plettner *et al.*, 2000). Alkene (**4a**, **4b**) K_d values were determined with 80 μM CHAPS or 13 μM SDS (both 1% of the critical micelle concentration). Both detergents gave the same result.

Competition binding radioassays

Because the analogs **3–6** were not radiolabeled, we performed competition binding assays using radiolabeled **1a** or **1b** as the reporter ligand. Assays were performed as described previously (Plettner *et al.*, 2000), with the following modifications. PBP and non-radiolabeled analog were first equilibrated for 1 h, then radiolabeled ligand was added and the solution incubated for an additional hour. At that time, samples were passed through the P2 mini-columns to separate protein-bound from unbound ligand. Unless indicated otherwise, competitor concentration was 20 μM.

Competition pH profiles

The pH profiles required 0.8 nM of tritium labeled **1a** or **1b** and 3 μM cold analog. Buffers with pH varying from 4.0 to 9.0 were of equivalent ionic strength (adjusted with KCl). For the buffer at pH 4.0, a 50 mM glycine–HCl solution was used. For buffers of pH 5.0, 5.5, and 6.0, 50 mM MES–Tris solutions were prepared. For buffers of pH 7.0, 8.0, and 9.0, 50 mM Tris–HCl solutions were prepared.

Fluorescence assays: tryptophan

Titrations were performed as described (Bette *et al.*, 2002) in 10 mM Tris, pH 7.5, presaturated with 0.8% ethanol, in a total volume of 1000 μl. The concentration of PBP1 was 1 μM, which gave a total of 1 nmol of PBP in the cuvette. Aliquots of ligand stock solutions in distilled ethanol (~300 μM) were added, such that titrations proceeded in increments of 0.15 nmol (=0.15 μM concentration increments), until 1–2 nmol had been added. After each addition, the fluorescence emission was monitored on a PTI (Quantum Master Model QM-1) fluorimeter. Excitation was at 295 nm and emission was monitored at 340 nm. Ligands **2a**, **2b**, **4a** and **4b** caused a decrease in fluorescence upon addition and ligand **1a** caused an increase in fluorescence.

Dansylation of PBP

PBP1 and PBP2 were coupled with dansyl chloride to produce the fluorescent protein. Six equivalents of DTT were added to 35 μM of PBP (which was in 10 mM ammonium acetate pH 7.5), under argon, and incubated for 1 h on ice. We have found that under these conditions only one disulfide bond is reduced (Kowcun *et al.*, 2001). The protein was then separated from unreacted DTT by filtration through P2 gel (Bio Rad) and elution with 30 mM potassium phosphate pH 7.7. The reduced protein was reacted with 18 equivalents of dansyl chloride and incubated for 20 min on ice. At the pH of the reaction mixture (~7.6), the lysine residues are protonated, but the newly formed thiols are largely deprotonated and are the strongest nucleophile on the protein. Unreacted dansyl chloride was removed by filtering twice through the gel column with 100 mM potassium phosphate, pH 5.0. MALDI-TOF MS and thiol titration revealed that only one of the two thiol groups coupled with a dansyl moiety. A number of experiments indicate that a cysteine thiol and not an amino group has been dansylated (see Appendix). Structural details of the dansyl modified PBP will be published elsewhere.

Dansyl-PBP titrations

Titrations were performed in 10 mM Tris, pH 7.5, in a total volume of 1000 µl. Two series of titrations were done: (i) with 3 µM dansylated PBP and **1a**, **1c**, **2a**, **2b**, **4a** and **4b** and (ii) with 1 µM dansylated PBP and **1a**, **1c**, **4a** and **4b**. Stock solutions of the ligands were prepared in distilled ethanol and were in the range of 300 µM. Aliquots were added to the cuvette such that titrations proceeded in 0.1 µM increments. Excitation was at 340 nm and emission was at 506 nm. Fluorescence intensity increased significantly upon addition of ligand. An ethanol control titration was performed where 1 µl aliquots of ethanol were added to a 3 µM dansylated PBP solution. The presence of ethanol had a minimal effect on the change in fluorescence intensity (about a 10% increase of the total fluorescence change after 7 µl ethanol was added).

Homology modeling

Models of *L. dispar* PBP1 and PBP2 were obtained using the X-ray structure of the *Bombyx mori* PBP (Sandler *et al.*, 2000) as a template. The 143 amino acid sequence of PBP1 and the 145 amino acid sequence of PBP2 were submitted to the fully automated SWISS-MODEL server using the 'Iterative magic fit' algorithm available within the Swiss-PDB Viewer interface (http://www.expasy.ch/spdbv/) (Guex and Peitsch, 1997). Comparative protein modeling was used to generate a protein model since both proteins exhibit significant sequence identity with *B. mori* PBP (61% and 48% with PBP1 and PBP2, respectively).

Models of the complexes of pheromones **1a** and **1b**, as well as aziridine analogs **2a** and **2b**, with PBP1 and PBP2 were constructed as follows. The ligands were built using Insight/Discover software (Accelerys, Inc.) implemented on a Silicon Graphics O2 platform. Ligands were constructed assuming all-*trans* conformations for the alkyl chains and were subjected to brief energy minimizations to obtain reasonable structures before docking. The aziridine analogs were constructed in protonated form, consistent with the biochemical data. Energy minimizations were performed using the CVFF force field (Dauber-Osguthorpe *et al.*, 1988). Ligands were docked by manual positioning within the binding site, at a similar location to the position of bombykol within the binding site of *B. mori* PBP (Sandler *et al.*, 2000). These complexes were then optimized by energy minimizations (200 steps, using the conjugate gradient algorithm) before examination of the intermolecular interactions (hydrogen bonds, hydrophobic interactions and cation–π interactions).

Results

I a. Importance of the functional group at position 7 and 8

The alkene **4a** has a π-system in place of the epoxide. Consequently, the geometry around positions 7 and 8 is planar, which is different from the geometry in an epoxide (Figure 1). Also, the electron density in an alkene is extended from C-7 to C-8, above and below the plane of the carbon bond framework. In the epoxide, electron density is located in the oxygen lone pairs, which are more diffuse (i.e. point further away from the atom) and are located ~55° relative to the plane of the epoxide. A similarity between the epoxides **1a**/**1b** and alkene **4a** is the rigidity around the C7–8 bond. Because of the subtle geometric and electronic differences, as well as the biological function of **4a**, we were interested in the PBP binding of this alkene.

Table 1 Binding of alkenes to gypsy moth PBPs

Pheromone binding protein	Radiolabeled ligand	Non-labeled ligand	K_d (µM)
L. dispar PBP1	**4a**	none	4.7 ± 1.6[a]
	4b	none	8.7 ± 0.2[a]
L. dispar PBP2	**4a**	none	4.3 ± 1.2[a]
	4b	none	7.1 ± 2.6[a]
L. dispar PBP1	**1b**	**4a**	1.5
	1b	none	2.8

[a]Means ± SE of three replicates.

Radioassays revealed that both PBPs bind alkene **4a** in the 4–5 µM range (Table 1), 2× more weakly than their respective preferred epoxide enantiomer (Plettner *et al.*, 2000). Binding of **1a** and **4a** was also examined by endogenous tryptophan fluorescence titrations. Titration of PBP1 with **1a** led to an increase in tryptophan fluorescence intensity. However, titration with **4a** led to a decrease in fluorescence intensity. In both cases, PBP1 reached saturation after about 1 equivalent (1 µM) of the corresponding ligand had been added to solution. In all cases the number of binding sites per polypeptide was one and, as expected for a protein with one binding site, no significant cooperativity (Hill coefficients ~ 1) was detected. Surprisingly, binding of both ligands under high ligand:PBP conditions was 9–10× stronger (Table 2) than observed with the radioassay (Table 1). The same observation was made with both dansylated PBPs and **4a** (Table 2).

To test whether the neutral or protonated forms of aziridines **2a** and **2b** bind to PBP, we determined the pH dependence of the competition between radiolabeled **1a** or **1b** and cold **2a** or **2b**. The pH profile of labeled **1a** with cold **2a** and PBP2 reveals strongest competition in the neutral to basic pH ranges (Figure 2A). A similar result was obtained with radiolabeled **1b** and cold **2b** (Figure 2B). The pK_a of aziridinium ion is ~8.3 (O'Rourke *et al.*, 1956) and the strongest binding of **2a** or **2b** is at pH < 8. These results suggest that PBP preferentially binds **2a** and **2b** in their protonated forms, which is surprising. Electronically, protonated **2a** and **2b** are opposite to epoxides **1a** and **1b**

Table 2 Binding of alkenes to gypsy moth PBPs determined by fluorescent assays

Assay	PBP	PBP conc. (μM)	Ligand	K_d (μM)[a]	No. of binding sites[a]	Hill coefficient[a]
Dansyl PBP[a]	PBP1	1	**1a**	0.2	1.2	1.4
		3		0.1	1.0	1.1
		1	**1c**	0.2	1.1	1.1
		3		0.1	1.0	0.8
		3	**2a**	0.5 ± 0.2	1.4 ± 0.2	1.1 ± 0.1
		3	**2b**	0.8 ± 0.5	1.40 ± 0.03	1.06 ± 0.01
		3	**4a**	0.5 ± 0.2	1.2 ± 0.1	1.2 ± 0.5
		3	**4b**	0.17 ± 0.01	1.14 ± 0.04	1.1 ± 0.5
	PBP2	3	**2a**	0.7 ± 0.3	1.3 ± 0.2	1.0 ± 0.1
		3	**2b**	0.7 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
		3	**4a**	0.5 ± 0.3	1.3 ± 0.1	1.0 ± 0.2
		3	**4b**	0.2 ± 0.1	1.04 ± 0.04	1.1 ± 0.3
Tryptophan	PBP1	1	**1a**	0.3	1.2	0.4
		1	**2a**	0.2	0.9	0.6
		1	**2b**	0.4	1.4	1.6
		1	**4a**	0.5 [b]	[b]	[b]
		1	**4b**	0.3	1.2	1.3

[a]Where errors are given, values represent means ± SE of three independent assays.
[b]Data very scattered because change in fluorescence intensity was small.

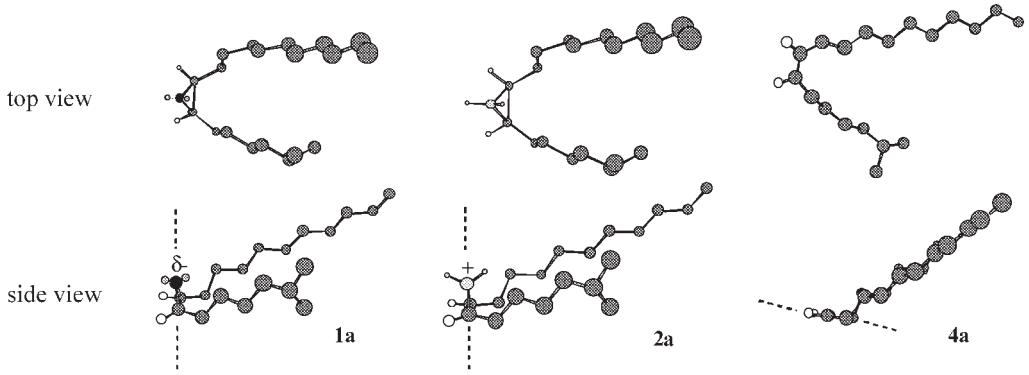


Figure 1 Energy-minimized conformers of the epoxide **1a**, protonated aziridine **2a** and alkene **4a**. In the side view, the plane of the three-membered ring (**1a** and **2a**) or of the alkene (**4a**) has been emphasized by the dotted line. Partial and full charges on the heteroatom have been indicated for **1a** and **2a**, respectively.

(Figure 1): the O carries a partial negative charge (δ–) and the N of protonated **2a** and **2b** carries a full positive charge.

To examine the strength of aziridine binding we performed competitive radioassays, in which a fixed amount of **1a** was displaced with increasing amounts of non-labeled **2a** or **2b**. The aziridines displaced **1a** from PBP2 with a saturation around 0.1 μM. Fluorimetric titrations of 1 μM dansylated PBP1 or PBP2 with increasing concentrations of **2a** or **2b** revealed similar affinities, one binding site and no cooperativity (Figure 3, Table 2). Very similar K_d values were obtained by titration of 1 μM PBP1 with either **2a** or **2b** and by monitoring the decrease in endogenous tryptophan fluorescence with increasing ligand concentrations, suggesting that PBP dansylation does not affect the binding properties significantly. The advantage of monitoring dansyl fluorescence over tryptophan fluorescence is that the dansyl group is a stronger fluorophore and the change in fluorescence on ligand binding is larger (Figure 3). The dissociation constants obtained show that **2a** bound to PBP1 twice as strongly as **2b** (Table 2). Thus, some enantiomer discrimination is taking place even though the protonated aziridines are of opposite polarity to the epoxides. PBP2 bound **2a** and **2b** equally, consistent with previous observations that PBP2 is less discriminating than PBP1 (Plettner *et al.*, 2000).

I b. Importance of the 2-methyl substituent

Three different assays were performed using **4a** and **4b**, PBP1 and PBP2. First, radioassays using labeled **4a** and **4b**

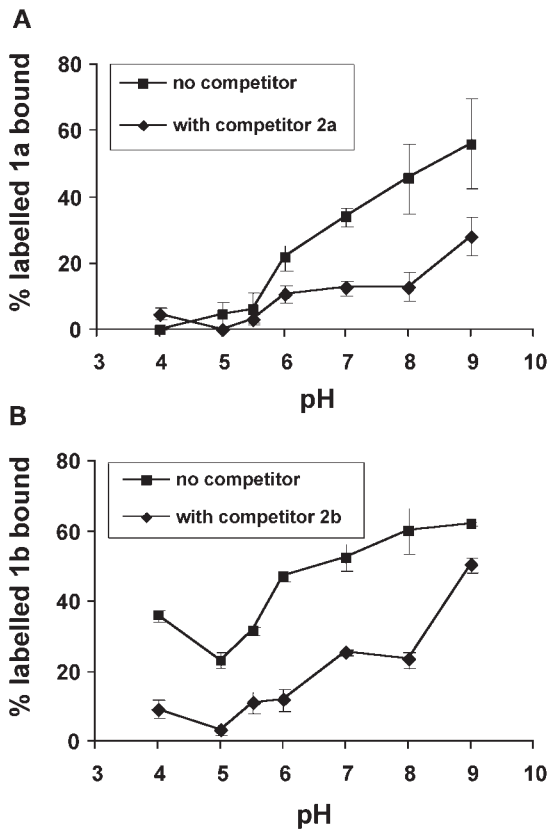


Figure 2 Effect of pH on pheromone binding to PBP2 in the presence of the aziridine competitor at constant ionic strength. **(A)** Shown are the pH profiles of radiolabeled pheromone **1a** alone (squares) and **1a** incubated with cold aziridine **2a** (diamonds). **(B)** Shown are the pH profiles of radiolabeled pheromone **1b** alone (squares) and **1b** incubated with cold aziridine **2b** (diamonds). Each point is the average of three replicates and the bars denote the standard error.

revealed dissociation constants in the 6 μM range (Table 1). At this low ligand concentration relative to PBP, **4a** appeared to bind more strongly than **4b** to both PBP1 and PBP2. Second, fluorimetric titrations using 3 μM dansylated PBP1 and PBP2 with these ligands at saturating concentrations yielded K_d values 10-fold smaller than in the radioassay (Table 2). In addition, at this high ligand:PBP concentration ratio, **4b** had a higher affinity for both PBP1 and PBP2 than **4a**. Again, the same binding selectivity was obtained with endogenous tryptophan fluorescence titrations (Table 2). Thus, at low alkene concentration relative to PBP we observed the opposite ligand binding preference than at high alkene:PBP concentration ratio.

The dissociation constants obtained using the radioassay were significantly different from values obtained by fluorescence measurements. The radioassays with **4a** and **4b** were performed using detergents, either SDS or CHAPS, at 1% critical micelle concentration (CMC). To determine if these detergents were interfering with binding we performed experiments with 1 μM dansylated PBP1 in the presence of either SDS or CHAPS (data not shown). No major difference to detergent-free preparations was observed, showing that the detergents used in the radioassays were not responsible for the much weaker binding observed. The large difference between the K_d obtained with **4a** and **4b** in the radioassay and in fluorescent assays can only be explained by the difference in ligand concentration. In radioassays the ligand concentration is in the low nanomolar range with a large (μM) excess of protein. In fluorescent assays the ligand is present in stoichiometric quantities relative to the protein. We hypothesize that ligand binding to PBP influences the multimerization of protein and this, in turn, perturbs binding of further ligand.

II. Competition between pairs of ligands

In the assay with PBP1, radiolabeled **1b** and non-labeled analogs **3–6**, we detected a 2- to 3-fold increase in **1b** binding in the presence of analogs (Figure 4A). In the competition binding assay using PBP2 and **1a**, only **4a** and **5** gave a slight but significant enhancement of **1a** binding (Figure 4B). Similar results were obtained in two other assays with different lots of PBP. Compound **4a** gave the strongest enhancement with both PBPs. Since this is of potential behavioural significance, we investigated binding of alkenes to the PBPs further. First, the affinity of PBP for radiolabeled **1b** was nearly twice as large in the presence of cold **4a** as in the absence, again suggesting an enhancement of binding (Table 1). Second, the pH profiles of radiolabeled **1a** (Figure 5A) and **1b** (Figure 5B) competing with cold **4a** show that the enhancement occurs only above pH 6.5 for both PBPs.

III. Effect of PBP concentration on ligand binding

Previous experiments explored the binding of pheromone (nM) at increasing concentrations of excess PBP (μM). Surprisingly, a 3-fold increase in pheromone binding between 2 to 3 μM was observed (Plettner *et al.*, 2000). PBPs equilibrate between monomeric, dimeric and higher-order multimeric forms. This is apparent in native electrophoresis gels where dimers, trimers and tetramers can easily be detected (Plettner *et al.*, 2000). Dimeric forms have also been observed in gel filtration experiments (Maida *et al.*, 1993). The effect of these equilibria should be more noticeable at higher PBP concentrations, and to test this, fluorimetric titrations were performed using either 1 or 3 μM dansylated PBP1 and **1a**, **1c** (Table 2), **4a** and **4b** (data not shown) as titrants. Again, all experiments revealed one binding site per polypeptide and no cooperativity. K_d values for 1 μM PBP1 were ~2-fold higher than with 3 μM protein, suggesting slightly stronger binding at higher PBP concentrations. As presented above, the strongest binding enhancements were seen in the fluorimetric assays, where the ligand:PBP ratio was high.

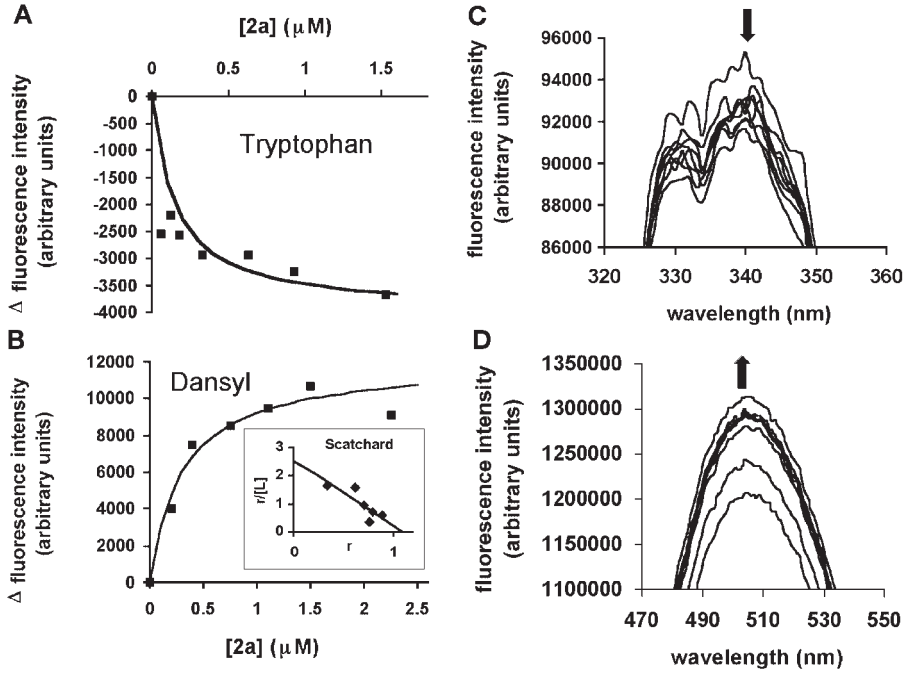


Figure 3 **(A)** Fluorimetric titration with PBP1 and aziridine **2a**, monitoring endogenous tryptophan fluorescence. **(B)** Fluorimetric titration with dansylated PBP1 and **2a**. Inset: data replotted, where [L] is the ligand concentration and r is the proportion of bound ligand. **(C)** Endogenous tryptophan fluorescence curves for titrations with PBP1 and **2a**. Emission was monitored at 340nm. **(D)** Fluorescence curves for titrations with dansylated PBP1 and **2a**. Emission was monitored at 506 nm.

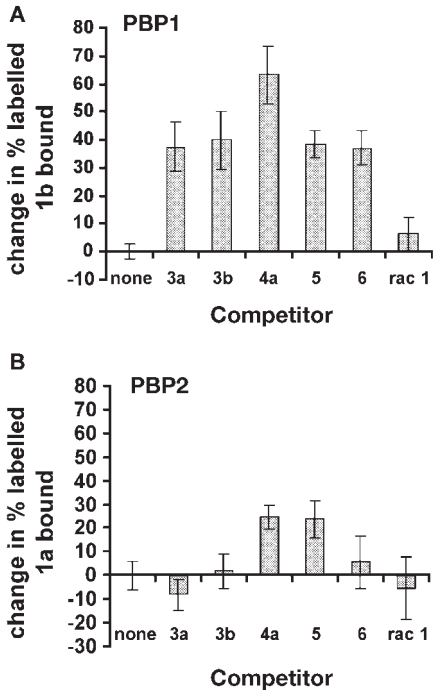


Figure 4 Effect of pheromone binding to PBP1 and PBP2 in the presence of hydrophobic ligands. **(A)** Radiolabeled **1b** binding to PBP1 in the presence of non-labeled analogs **3–6**. **(B)** Radiolabeled **1a** binding to PBP2 in the presence of non-labeled **3–6**. Racemic disparlure is denoted as 'rac 1'. Each column is the average of three replicates and the bar denotes the standard error.

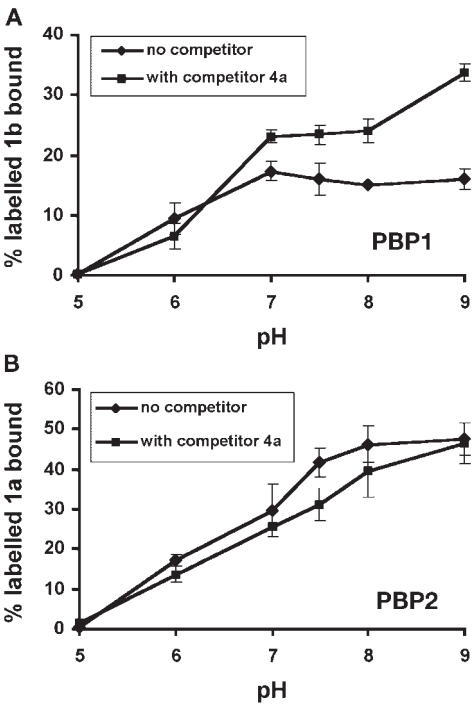


Figure 5 Effect of pH on pheromone binding to PBP1 and PBP2 in the presence of **4a** at constant ionic strength. **(A)** PBP1 pH profiles of **1b** alone (diamonds) and **1b** incubated with **4a** (squares). **(B)** PBP2 pH profiles of **1a** alone (diamonds) and **1a** incubated with **4a** (squares). Each point is the average of 3 replicates and the bar denotes standard error.

Discussion

I. Binding interactions

In both competition assays and in direct binding assays with dansylated PBPs we found that both PBPs bind to both **2a** and **2b**. Our homology models based on the *B.mori* PBP crystal structure suggest that a hydrogen bond from the side chain hydroxyl group of threonine 9 to the epoxide moiety of **1b** may contribute to binding of the compound to PBP1. This effect was not detected for **1a**, because the epoxide points away from the threonine hydroxyl group. Also, this effect was not observed for PBP2 with either **1a** or **1b** because PBP2 has an alanine at position 9. By analogy, it was expected that **2a** should bind with no hydrogen bond, while **2b** should hydrogen bond at basic pH. However, we found that both aziridines bind in protonated form, which precludes hydrogen bond donation from the PBP to the ligand. The protonated aziridines have the opposite polarity of the epoxides **1a** and **1b**, and the ability of the PBPs to accommodate such a dramatic change in the functional group that is recognized is remarkable. Docking of protonated **2a** and **2b** into the homology models suggests that the aziridines are held in place by one or more π-cation interactions in the PBP binding site (Figure 6). These interactions are thought to be nearly the same strength as salt bridges in solvents of intermediate polarity and approximately twice as strong in water (Gallivan and Dougherty, 2000). The aziridines could intercalate between the highly conserved phenylalanines 12 and 119 in the middle of the binding pocket and could interact with the conserved tryptophan 37 and phenylalanine 36 at the base of the binding pocket. The same interactions were predicted for PBP2, consistent with the binding data which shows no significant difference between PBP1 and PBP2 in the affinity for the aziridines. Interestingly, **2a** and **2b** were active in electroantennogram (EAG) studies, but much less than **1a** or **1b** (Dickens *et al.*, 1997). Thus, even though the PBPs bind the aziridines with approximately the same strength as the pheromone **1a**, the aziridines elicit very weak EAG responses and no behavioural responses, possibly because they do not activate a transmembrane receptor.

All pH profiles of PBP–ligand binding we have examined exhibit a sharp increase in binding between pH 6.0 and 6.5. (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Oldham *et al.*, 2000; Kowcun *et al.*, 2001). Previous studies suggest that a large conformational change occurs in that range (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Horst *et al.*, 2001) and that the monomer/dimer equilibrium is shifted towards the dimer at pH > 6.0 (Leal, 2000). Furthermore, recent studies (Maida *et al.*, 2000; Deyu and Leal, 2002) suggest that PBPs populate more than one stable conformer at pH > 6. This is apparent in native electrophoresis, where the monomeric forms of many insect PBPs and OBPs run as two closely spaced bands. Ligand binding could cause a change in the conformer population in two ways: (i) by an induced-fit mechanism or (ii) by preferentially binding to one of the existing conformers and shifting the conformational equilibrium towards one form by mass-action. The changes in fluorescence intensity of tryptophan [this study and (Bette *et al.*, 2002)] or of the appended dansyl group we

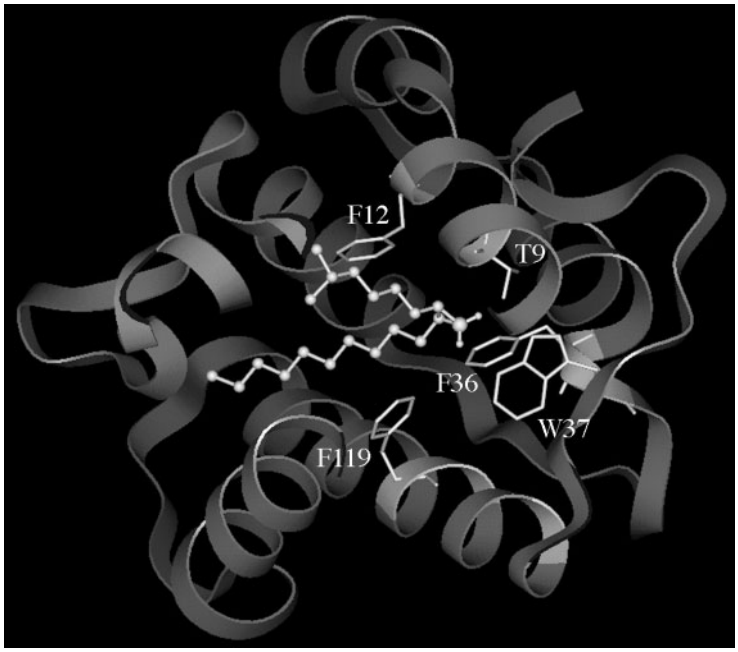


Figure 6 Homology model of PBP1 with aziridine **2b** docked into the binding pocket. The protein backbone is shown as a ribbon. For clarity, a portion of the protein has been cut away to reveal the ligand in the binding pocket.

observed in our titrations probably reflect a change in conformation of the PBP upon ligand binding.

The studies with **1c**, **4a**, and **4b** were conducted to observe steric effects within the binding sites of both PBP1 and PBP2. PBPs bound **4b** slightly more than **4a** in the fluorescence assays, suggesting that the 2-methyl group makes a small contribution to PBP binding, although it is probably too small to account for the difference in the behavioural effect or the EAG (Gries *et al.*, 1996). Similarly, the K_d values of **1c** and **1a** with PBP1 were not significantly different, suggesting that PBP1, which so far has been the more selective of the two PBPs, does not recognize the presence or absence of the methyl group. Interestingly, the gypsy moth shows virtually no behavioural response to **1c** and **4b** and EAG responses are very weak (Gries *et al.*, 1996). Thus, the transmembrane receptors must be tuned very precisely to the presence or absence of the 2-methyl group.

II. Synergistic binding effects as explained by multiple equilibria

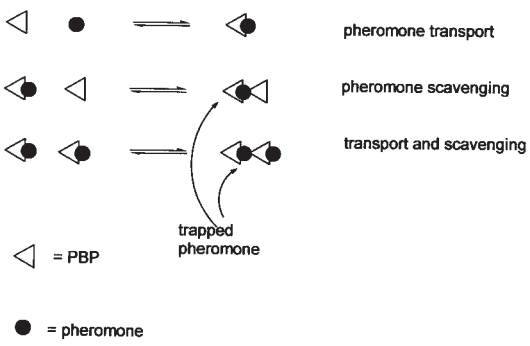
Incubation of PBP1 and **1b** or PBP2 and **1a** with hydrophobic analogs yielded very surprising results. Some competing ligands act as positive effectors, enhancing the binding of **1b** or **1a**. The enhanced binding was more pronounced for PBP1 than for PBP2. The two PBPs showed a different selectivity pattern: while PBP1 showed a significant binding enhancement with **3a**, **3b**, **4a**, **5** and **6**, PBP2 showed an enhancement only with **4a** and **5**. Scatchard analysis of PBP binding with various individual ligands reveal one binding site/PBP and no significant cooperativity. The only way to explain the binding enhancement is that binding of hydrophobic ligands perturbs the multiple equilibria between PBPs and PBP–ligand complexes. For a ligand-mediated binding enhancement to occur, the affinity of one PBP–ligand complex for another complex has to be higher with multiple ligands than with one ligand alone. The converse must be true when an inhibition of binding occurs, as was seen previously for a 1:1 mixture of **1a**:**1b** (racemic **1**) (Plettner *et al.*, 2000).

There is evidence to support the idea of PBPs forming reversible multimers in a ligand-dependent manner. The X-ray structure of *B.mori* PBP reveals a bundle of α-helices with one binding site in the middle (Sandler *et al.*, 2000). There is no obvious second binding site on the monomeric PBP unit, although new binding sites could form at protein/protein interfaces in the oligomers. The possibility of dynamic oligomerization is supported by the direct observation of dimers and higher order multimers of PBPs. First, the *B.mori* PBP crystallized as a head-to-tail dimer (Sandler *et al.*, 2000). Second, dimers have been observed by gel filtration (Leal, 2000; Maida *et al.*, 1993) and native polyacrylamide gel electrophoresis (Plettner *et al.*, 2000). Third, at constant pheromone and increasing PBP concentration a sharp, 3-fold increase in pheromone binding can be seen between 2 and 3 μM of PBP (Plettner *et al.*, 2000). In a gel filtration assay, significantly more pheromone binding occurred at high PBP concentration than in dilute preparations (Maida *et al.*, 1993). If PBP oligomerization had no effect on pheromone binding, then the sharp increase would not be observed. The 2-fold increase in binding strength seen when binding of 3 μM dansylated PBP is compared to binding of 1 μM PBP to ligand is also consistent with this concentration dependence. Finally, observations from another group also support the existence of ligand-mediated effects in olfactory systems. In *Helicoverpa zea*, a significant increase in the frequency of action potentials of pheromone-responsive neurons was observed when the preparations were exposed simultaneously to (11*Z*)-hexadecenal and (11*Z*)-hexadecen-1-yl acetate or to (11*Z*)-hexadecenal and linalool (Ochieng *et al.*, 2002).

Binding enhancement of **1a** or **1b** was only seen with more hydrophobic ligands such as **4a** and at pH values above the pH transition, parallel to individual ligand binding (Kowcun *et al.*, 2001; Oldham *et al.*, 2000) and to dimerization (Leal, 2000). Interestingly, PBP1 exhibited this effect much more strongly than PBP2. As discussed above, insect odorant-binding proteins and PBPs have more than one stable conformer at pH > 6. Different ligands may bind preferentially to one of these conformers and these complexes, in turn, may form different multimers with each other. With the ligands studied here, it may be possible to preferentially stabilize one of these conformers and to obtain structural information. The functional significance of these multiple ligand effects may be to provide a very early integration of blend information.

III. Binding at high pheromone concentrations

The dissociation constant values obtained using the different assays depended on the relative protein to ligand concentration. Radioassays performed at low ligand:PBP ratios resulted in much weaker binding than fluorescent titrations performed at high ligand:PBP ratios. The binding enhancement seen at high pheromone concentrations is probably due to a phenomenon similar to the dual-ligand effect discussed above. Namely, at high pheromone concentration, multimers of PBP–ligand complexes form. If the multimers form in a head-to-tail fashion as seen in the crystal structure (Sandler *et al.*, 2000), then pheromone will be trapped in the multimeric PBP and can leave the PBP only if the multimer dissociates first (Scheme 2). The functional significance of this effect may be to sequester excess pheromone at high stimulus doses, thereby preventing an overloading of the receptors. Through this mechanism, the PBP may provide an automatic stimulus attenuation: at low doses the protein acts as a carrier and at high doses it acts as a carrier for a portion of the material and sequesters the excess. The PBP is thought to occur in the lymph in ~1–10 mM concentration (Vogt *et al.*, 1989) and the pheromone concentration would depend upon the dose in



Scheme 2 Possible role of PBP multimerization in pheromone scavenging.

the airstream. The lowest limit of pheromone in the airstream, that elicits behavior, is 1.6×10^{-17} M/s and the high limit, at which adaptation occurs, is ~2.8×10^{-5} M/s. The concentration of pheromone within the sensillum is not known, because very little is known about transport efficiency on cuticular structures and about pheromone accumulation rates within sensory hairs. Assuming a 0.1% transfer efficiency from airstream to sensory hair, the range of pheromone concentrations in the sensillum is 16 pM–28 M, and the ligand:PBP ratios would vary from 1.6×10^{-8} to 2.8×10^{-2} (Appendix). The range of ligand:PBP ratios covered in our experiments was 10^{-5}–1. Thus, we have covered a meaningful portion of the potential biological range of ligand:PBP ratios. The multimerization mechanism proposed here may explain the remarkable concentration range to which moths respond.

To conclude, we have described three important findings with respect to PBPs in the gypsy moth. (i) The PBPs bind a variety of ligands that are structurally related to the pheromone. The PBPs show subtle differences in binding constants, which are not sufficient to explain the strong preferences detected for the same set of ligands in electroantennogram studies. However, the ligands studied require very different molecular interactions, in order to bind. These different binding modes may give rise to different protein conformations for the PBP-ligand complexes. (ii) We have observed binding synergy between different ligands when these are presented simultaneously. This effect may represent a very early interpretation of odor blends. (iii) By far the strongest differences in ligand binding are seen at different ratios of ligand:PBP. This dependence of the binding affinity on the ligand:PBP ratio may explain why PBP may act both as odorant transporter and as scavenger.

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Appendix: supplementary material

Supplementary material can be found at http://www.chemse.oupjournals.org.

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