Discrimination of Pheromone Enantiomers by Two Pheromone Binding Proteins from the Gypsy Moth *Lymantria dispar*†

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ABSTRACT: The gypsy moth, *Lymantria dispar*, uses (7*R*, 8*S*)-*cis*-2-methyl-7, 8-epoxyoctadecane, (+)-disparlure, as a sex pheromone. The (−) enantiomer of the pheromone is a strong behavioral antagonist. Specialized sensory hairs, sensillae, on the antennae of male moths detect the pheromone. Once the pheromone enters a sensillum, the very abundant pheromone binding protein (PBP) transports the odorant to the sensory neuron. We have expressed the two PBPs found in gypsy moth antennae, PBP1 and PBP2, and we have studied the affinity of these recombinant PBPs for the enantiomers of disparlure. To study pheromone binding under equilibrium conditions, we developed and validated a binding assay. We have addressed the two major problems with hydrophobic ligands in aqueous solution: (1) concentration-dependent adsorption of the ligand on vial surfaces and (2) separation of the protein-bound ligand from the material remaining free in solution. We used this assay to demonstrate for the first time that pheromone binding to PBP is reversible and that the two PBPs from *L. dispar* differ in their enantiomer binding preference. PBP1 has a higher affinity for the (−) enantiomer, while PBP2 has a higher affinity for the (+) enantiomer. The PBP from the wild silk moth, *Antheraea polyphemus* (Apol-3) bound the disparlure enantiomers more weakly than either of the *L. dispar* PBPs, but Apol-3 was also able to discriminate the enantiomers. We have observed extensive aggregation of both *L. dispar* PBPs and an increase in pheromone binding at high (>2 μM) PBP concentrations. We present a model of disparlure binding to the two PBPs.

The gypsy moth, *Lymantria dispar*, is a widespread forest pest, which causes severe forest losses during outbreaks in Europe, Asia, and North America (*1*). Even though outbreaks occur approximately every 10 years, there is a need to closely monitor and control the gypsy moth. A key event in the moth's yearly cycle is the calling period in the spring, when the stationary females attract males from up to 1 km away, with a sex pheromone. The main component of this sex attractant in the gypsy moth is (7*R*, 8*S*)-*cis*-2-methyl-7,8-epoxyoctadecane, (+)-disparlure (*2*−*6*). Synthetic (+)-disparlure is able to attract males in the field, but the natural blend is more attractive, suggesting that there is/are minor synergistic component(s) (*6*). The antipode, (−)-disparlure, antagonizes the effect of (+)-disparlure and is slightly repellent by itself (*4*).

The female moths release the pheromone into the wind, causing it to disperse in nonhomogeneous plumes. The males detect the pheromone with their highly branched antennae and follow the plume upwind to the source. Pheromone structure and concentration are detected in specialized sensory hairs (sensilla trichodea) on the antennal side branches. Each sensillum houses dendrites from two or three distinct olfactory neurons, and each neuron is thought to respond to only one pheromone component (*7*). In the gypsy moth, different cells have been shown to respond to (+)-, and (−)-disparlure (*4*). In addition, different sensilla are known to have different concentration thresholds (*8*). This important feature enables moths to respond to 6 orders of magnitude in concentration, from 1 μM [3×10^6 molecules/sensillum in 1 s, (*9*)] to the behavioral threshold of 1 pM [1 molecule/sensillum on 25% of the 10 000 sensilla in 1 s (*10*)]. The molecular basis for different sensillar thresholds is not known.

The dendrites of pheromone olfactory neurons are bathed by sensillar lymph, which contains pheromone-degrading enzymes (*11*, *12*), as well as very high (millimolar) concentrations of pheromone binding proteins, PBPs (*13*). The PBPs are members of a homologous family of insect odorant binding proteins, OBPs (*14*, *15*). These binding proteins transport the hydrophobic pheromone components through the aqueous lymph. There is increasing evidence that PBPs and OBPs are not passive shuttles for hydrophobic compounds but may be involved in odor recognition and are essential for olfaction. Several studies have demonstrated moderate odorant selectivity of PBPs (*16*, *17*). For example, the PBP from *Antheraea polyphemus* binds (6*E*,11*Z*)-hexadeca-6,11-dienyl-1-acetate 33 times more strongly than (4*E*,9*Z*)- tetradeca-4,9-dienyl-1-acetate (*18*). Also, different PBPs from the same species differ in their pattern of pheromone affinities (*16*, *17*). A further example is seen in a recent study with honeybees, in which the antennal specific protein 1 (ASP1), a PBP found in workers and drones, specifically binds the aliphatic acid components of the queen

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mandibular pheromone. ASP2, a worker-specific OBP, has been shown to not bind any queen pheromone components (*19*). Finally, there is evidence which suggests that odorant binding proteins are essential for olfaction. One such observation was made in a study with a partially anosmic mutant of *Drosophila melanogaster*, which showed that even a water-soluble odorant such as ethanol requires its cognate odorant binding protein to elicit a behavioral response (*20*).

The gypsy moth has two major PBPs, PBP1 and PBP2, both of which have been cloned and sequenced (*15, 21*). Both PBPs have six cysteine residues that are highly conserved among insect PBPs and general odorant binding proteins (GOBPs) (*15, 19*). Similar to other insect PBPs, the gypsy moth proteins have molecular weights around 16 kDa and p*I* values around 5. Most PBPs have a highly variable stretch of amino acids between residues 39 and 58, which is thought to adopt a short β-sheet structure and may be involved in odor selectivity (*18*). There is one NMR solution structure of a PBP-like protein from *Tenebrio molitor* (*22*). This protein is mostly α-helical, consistent with circular dichroism data from PBPs (*23*) and related GOBPs (*24*). In the *T. molitor* protein, six α-helices form a hydrophobic cleft, which is rigidified by two disulfide bridges. Recently, the disulfide connectivity (*25*) and the crystallographic structure (*26*) of the PBP from *Bombyx mori* have been elucidated. The protein consists of six α-helices, four of which define the pheromone binding cleft. In contrast, the functionally related vertebrate OBPs consist of a β-barrel followed by a α-helix. They function as dimers, with putative odorant binding sites inside each barrel and possibly at the interface (*27, 28*). The honeybee ASP1 was shown to function as a dimer (*19*) and the PBP from *B. mori* forms a dimer in the solid state (*26*), but it is not clear whether all insect PBPs function as dimers.

A first step toward understanding pheromone selectivity and sensitivity of PBPs, and consequently their role in pheromone olfaction, is to study the affinity of various PBPs for different pheromone components. There are two problems with PBP/pheromone binding assays in aqueous solution. First, because the pheromone is so hydrophobic, it adsorbs on the vial surface (*29, 30*). Second, it has proven difficult to separate bound and free pheromone, because of the incompatibility of buffer and pheromone. Du and Prestwich (*18*) solved the first problem in their assay by coating plastic vials with 1-decanol. Separation of bound and free pheromone occurred on its own, by selective adsorption of the PBP−pheromone complex on the alcohol coating. With *L. dispar* PBPs this selective adsorption was not observed, so we devised and validated a gel-filtration method for the separation of bound and free pheromone.

In this paper we describe an equilibrium binding assay with pure recombinant preparations of *L. dispar* PBP1 and PBP2 and radiolabeled (+), (−), and racemic disparlure. We present a model of pheromone−PBP binding interactions as well as a possible explanation for the existence of multiple PBPs with different pheromone binding preferences in the same species.

EXPERIMENTAL PROCEDURES

Materials. The pHN1+ plasmid was obtained from G. Verdine (Harvard) (*31*). Protein was assayed by the Bradford method with lysozyme as standard. Polyacrylamide gel electrophoresis was performed on 12−14% gels (49:1 bis, pH 8.8) with 4% stacking gels (pH 6.8). *A. polyphemus* PBP was from a previous study (*17*).

The radiolabeled disparlure used was synthesized in 1989 (*32*). Because ca. 50% of the material had radiodecomposed, it was purified by reaction with $NaBH_4$ followed by chromatography on silica gel with hexane/toluene 1:1. This process was repeated twice. The purified radiolabeled pheromone had the same retention factor as pure cold disparlure. It also showed the same chemical behavior in test reactions with HOAc, Al_2O_3, and aqueous 3% H_2SO_4, with $NaBH_4$, and with bis(trimethylsilyl)trifluoroacetamide as cold disparlure. Finally, the purified 10-year-old disparlure showed the same TLC and chemical behavior as racemic disparlure synthesized from fresh [7,8 $^3H_2$]-2-methyl-(*Z*)-7-octadecene by reaction with mCPBA.

Construction of Expression Vectors. For the construction of the PBP expression cassettes, RNA was extracted from fresh male *L. dispar* antennae by an acid guanidinium thiocyanate extraction protocol (*33*) and mRNA was obtained by use of the PolyATract system (Promega, Madison, WI). The mRNA was reverse-transcribed by AMV reverse transcriptase (Promega) and a C-terminal sequence-specific primer (5′ TCTAGGCAAAGCTTTTATGTATCAGCCAGGAGTTCTCCTAC 3′, PBP1; 5′ TCTAGGCAAAGCTTTTACTGTGATTCAGCTAAGAAGTCTGC 3′, PBP2). Expression cassettes were obtained by PCR with single-stranded antennal cDNA as template and a combination of sequence-specific C-terminal and N-terminal primers (5′ TAGGGCGAATTCAAGGAGATATACCATGTCGAAGCAAGTCATGAAACAAATGACC 3′, PBP1; 5′ TAGGGCGAATTCAAGGAGATATACCATGTCGAAGGATGTAATGCATCAGATGGCAC 3′, PBP2). For each reaction, 20−30 ng of template was mixed with 50−100 pmol of each primer. Reactions were run in 20 mM Tris, pH 8.4, with 50 mM KCl, 2.0 mM $MgCl_2$, 0.32 mM of each dNTP, and 0.4 μL of Taq polymerase (Gibco) in a total volume of 100 μL. Reactions were subjected to 40 cycles of 94 °C (2 min), 55 °C (2 min), and 72 °C (1 min), followed by 5 min at 72 °C and holding at 4 °C. The final PCR mixture was subjected to ethanol precipitation by addition of 50 μL of 7.5 M ammonium acetate and 450 μL of ethanol. The insert and vector were both digested with *Eco*RI and *Hin*dIII and purified by ethanol precipitation. Ligation was carried out with 40 pmol of each purified insert and vector, and T4 DNA ligase (Gibco) in a total volume of 10 μL. The reaction was subjected to 20 cycles of 4 °C (2 min) and 25 °C (2 min). Upon completion, 1 μL of this mixture was transformed into JM109 or XL1 Blue cells. Positive clones were identified by lysing a small sample from each colony in sterile water (78 μL) and performing PCR as described above. Plasmid from positive clones was then isolated and sequenced from both ends and internally.

Expression of PBPs. Clones containing the PBP cassette were transformed into *E. coli* (either XL1 Blue or JM 109) and grown in LB medium at 37 °C. Overnight cultures (10 mL) were used as inocula for 1 L cultures. When the optical density at 590 nm of the cultures reached 0.5−0.8, IPTG was added to give a final concentration of 1 mM, and the culture continued to grow at 20 °C for 4.5 h. Samples (1 mL) were taken before and after induction and analyzed by

SDS−PAGE and native PAGE in the presence of radiolabeled disparlure. Cells were harvested by centrifugation (3500 × g, 40 min, 4 °C) and kept at −80 °C until needed.

Native PAGE of PBP samples with radiolabeled disparlure was performed as described in refs *16* and *21*). Proteins were transferred to PVDF membranes, using Tris (25 mM) glycine (230 mM) buffer with 10% methanol. Transfer was effected at 100 V constant voltage for 1 h with cooling (buffer temperature at the end of the run was ca. 13 °C). The dried membranes were scanned on a Bioscan Scanner and also subjected to autoradiography.

Isolation of Active PBPs from Inclusion Bodies. The pellets from 2 L of culture were resuspended in 200 mL of lysis buffer (80 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, and 4% glycerol, pH 7.2, containing 2.4 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL chymostatin, 87 μg/mL PMSF, and 1 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma, St. Louis, MO)]. The suspension was passed through a French press twice (16,000 psi). RNase A (100 μg) was added to the crude lysate, and the lysate was incubated on ice for 1.5 h. Cell debris was removed by centrifugation (16,500 × g, 30 min, 4 °C). The pellet contained the crude inclusion bodies.

The inclusion bodies were solubilized and subjected to a denaturation/renaturation scheme with cysteine/cystine, as described previously (*31*). The renatured PBP was then purified either by preparative native PAGE or by gel filtration on Sephacryl S-100 HR. Details are described in the Supporting Information. Purified PBP was dialyzed against 20 mM ammonium acetate, pH 7.5 (3 × 2 L). The final dialysates were concentrated further (Centricon YM 10) to furnish solutions of 0.5−1 mg/mL. Final solutions were filter-sterilized and kept at −80 °C until needed.

Purification of PBP2 from the Soluble Fraction. Not all the PBP obtained during expression had precipitated as inclusion bodies. Material from the soluble fraction was purified by chromatography on Q-Sepharose, followed by gel filtration and finally by preparative electrophoresis, as described in the Supporting Information.

Thiol Titrations. I_2 reacts with thiols to give sulfenyl iodides, which can react further with a nucleophile. If the thiol is hindered, the nucleophile is water and the product is a sulfenic acid. If the thiol is not sterically hindered, the nucleophile can be another thiol group in the protein, and the product is a disulfide (*34*, *35*). Thus, the I_2:SH stoichiometry for very hindered thiols is 1:1 and for nonhindered ones 1:2. This method has been used to titrate very hindered thiols that do not react with Ellman's reagent (*36*).

Protein (200 μL of ≥50 μM), 200 μL of 0.25 M phosphate buffer, pH 8.4, and 400 μL of 0.6 M NaI were mixed in a quartz cuvette. To this mixture, 0.25 mM I_2 in 0.2M NaI was added in 1 μL aliquots with thorough mixing after each addition. The absorbance at 355 nm (λ_{max} for I_3^-) was recorded after every addition. The reference mixture contained 200 μL of water instead of protein. Once equivalence had been reached, absorbance at 355 nm increased with every addition.

Other Protein Characterization (IEF and MALDI-TOF MS). IEF was performed on 6.5% acrylamide with 0.35% bisacrylamide, 0.5% BioLite (pH 3−10 ampholyte solution), and 1.9% Pharmalyte (pH 4−6.5 ampholyte solution), run with 20 mM NaOH in the upper buffer chamber and 20 mM H_3PO_4 in the lower chamber. The following pI standards (Sigma) were used: trypsin inhibitor (4.55), β-lactoglobulin A (5.13), carbonic anhydrase B (5.85, 6.57), and myoglobin (6.76, 7.16).

Samples for MALDI-MS were prepared by applying 0.5 μL of the protein stock solution (ca. 10 pmol/μL) to a gold plate. After the protein had dried in the sample well, 0.5 μL of matrix solution [sinnapinic acid (10 mg/mL) in acetonitrile, 3% TFA, and deionized water (3:1:6)] was added to the well and left to dry. MALDI mass spectra were obtained on a reflectron MALDI/TOF mass spectrometer (model: Perseptive Voyager-DE STR, PE Applied Biosystems Co., Framingham, MA) equipped with a time lag focusing ion source and a pulsed linear detector. A nitrogen laser (337 nm, 3 ns pulse width) was used to desorb the ions from the source. Spectra were obtained in linear mode with delayed extraction at an acceleration voltage of 25 kV. The instrument was operated at less than 1×10^{-7} Torr. Ion detection and signal amplification was achieved with a hybrid conversion microchannel plate-discrete dynode electron multiplier assembly. Mass spectra obtained were the average of 256 individual spectra, each obtained from one laser pulse.

Equilibrium Binding Assays, General. Assays were performed in 1.5 mL Eppendorf tubes that had been coated with 1-tetradecanol, as described before (*17*). The total volume was always 300 μL per replicate, and the buffer used most frequently was 20 mM Tris, pH 7.5. The other buffer tested in this study was receptor lymph ringer, which consisted of 22 mM sucrose, 172 mM KCl, 9 mM KH_2PO_4, 11 mM K_2HPO_4, 3 mM $MgCl_2$, 1 mM $CaCl_2$ (modified after ref *7*). After equilibration of PBP and radiolabeled pheromone (see below), each vial was sampled once (50 μL) for total radioactivity in solution and twice (100 μL) for radioactivity bound to PBP. Each 100 μL aliquot was passed through a small (30 mg, 50 μL void volume) column of Bio-Gel P-2 (Bio-Rad, Hercules, CA, 2 kDa exclusion limit) in a 200 μL pipet tip with a glass wool plug. Each tip was flushed once with an additional 100 μL of clean buffer. The entire filtrate was collected in a scintillation vial. Determination of the protein concentration in filtrates obtained in this manner indicated that all the protein eluted from the minicolumns. Scintillation samples were mixed with 8 mL of Scinti Safe Econo 1 (Fisher) and counted for 2 min.

Equilibration of Pheromone and PBP (Association Reaction). In this assay the equilibrium between pheromone (D) and PBP, PBP + D ⇄ PBP·D, was approached from the side of the reactants. To determine the equilibrium constant for dissociation of PBP·D, K_d = [PBP][D]/[PBP·D], PBP concentration was kept constant (1−3 μM) and the pheromone concentration was varied (6−8 concentrations between 0.02 and 0.40 nM). Each concentration was tested in three replicates with and without PBP. Coated vials were preincubated with buffer for 20 min on ice. Ethanolic pheromone stock solutions (300 × for each concentration to be tested) were prepared from hexane/toluene (1:1) storage stocks. For the most concentrated solution in the series, the appropriate amount of hexane/toluene stock was dispensed into a glass vial, the hexane/toluene was evaporated under nitrogen, and the residue was brought up in ethanol. The other pheromone stock solutions were prepared by dilution of the most concentrated sample. To each buffer aliquot, 1 μL of the appropriate ethanolic pheromone stock was added, and the

mixture was incubated on ice for at least 30 min. Next, the appropriate volume of concentrated PBP stock solution (typically 40−80 μM) was added to the treatments and all samples were incubated on ice for at least 1 h before sampling.

Equilibration of Pheromone−PBP Complex (Dissociation Reaction). To approach the dissociation equilibrium from the pheromone−PBP complex (PBP·D), the complex was first isolated and then reequilibrated in fresh buffer. Concentrated PBP stock in 20 mM ammonium acetate, pH 7.5 (200−250 μL of 40−80 μM), was incubated on ice with pheromone (80−160 nM, final concentration in the incubation mixture) for at least 1 h. The incubation mixture was then filtered through two columns of Bio-Gel P2 (120 mg each, 200 μL void volume) in tandem. The two-column system was rinsed with 2 × 200 μL of buffer, the entire filtrate was combined and the volume was determined. The protein concentration of the filtrate was determined by the Bradford assay and was found to be the same as the value calculated from the original protein concentration and the dilution factor, consistent with complete elution of protein from the column. A control with the same volume of buffer and the same pheromone concentration was always prepared in parallel and was used to verify that all the free pheromone was retained on the two columns.

For determinations of K_d, freshly prepared PBP·D complex was diluted into 20 mM Tris, pH 7.5, to give different final concentrations of D (0.04−1.28 nM). Each series of samples was topped up with untreated PBP to give a constant final concentration of PBP (1−1.5 μM). Controls for each incubation were prepared by diluting an equivalent volume of control filtrate in Tris buffer. The samples were incubated for 2 h at room temperature, followed by 1 h on ice before sampling.

RESULTS

Expression, Purification, and Characterization of PBP. Detailed results from expression and purification are given in the Supporting Information. The major difficulty encountered during expression and purification of the *L. dispar* PBPs was aggregation of the protein when concentrations of 2 mg/mL were exceeded (Figure 1). Analysis of concentrated renatured PBP2 by native PAGE revealed monomeric, dimeric, and higher multimeric forms of PBP2 with three visualization methods: (1) Coomassie staining, (2) autoradiography of a sample that was electrophoresed with radiolabeled pheromone, and (3) Western blotting with a PBP2-specific antibody (Figure 1). Furthermore, N-terminal sequencing of the various bands revealed that they all had the intact PBP2 sequence. PBP1 behaved in a similar manner.

The expressed PBPs had molecular masses within ≤20 amu from the expected mass (Table 1), and they exhibited p*I* values very close to the value expected from the sequence. They all had the correct N-terminal sequence, and the inserts in the expression vectors chosen for expression exhibited no mutations. Thiol titrations with I_2, which reacts even with very sterically hindered thiols that do not react with Ellman's reagent, revealed that renatured PBP1 and PBP2 had a low percentage (between 4% and 10%, depending on the stoichiometry; see Experimental Procedures) of free thiol groups (Table 1). Considering that each PBP has six thiols, this means that 0.7−1.7% of the PBP contained free SH groups. PBP2 obtained from the soluble fraction and *A. polyphemus* PBP did not contain any detectable free thiol groups.

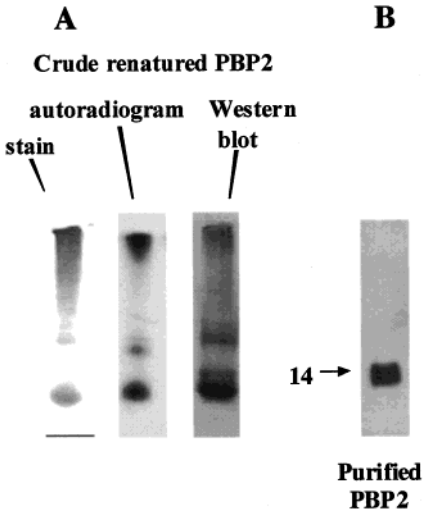

FIGURE 1: (A) Native PAGE of crude renatured PBP2, run with radiolabeled pheromone. Monomeric, dimeric and multimeric bands were cut out from a Coomassie-stained blot and sequenced. (B) SDS−PAGE of gel-purified monomeric PBP2 with silver staining. The aggregated forms of PBP stain more strongly with silver nitrate than the monomeric form. This staining method was therefore useful for visualizing traces of multimeric PBP in purification samples.

Validation of the Binding Assay. Adsorption of disparlure on the plastic vials used for the assay depended on four factors: (a) the coating of the vial, (b) the type of buffer, (c) the target concentration of pheromone (= the total amount of pheromone added), and (d) the hydrophobicity of the test compound. Comparison of coated and noncoated vials indicated that coating decreased adsorption of disparlure by 10%, once the system had stabilized. Comparison of different coating materials revealed that tetradecanol was optimal for the particular pheromone used in this study (Supporting Information). Comparison of receptor lymph ringer, a concentrated phosphate buffer (see Experimental Procedures), to a dilute Tris buffer revealed that adsorption was less severe in Tris buffer (60% on average) than in ringer (70% on average). The most important factor in the adsorption of a given compound was the total amount of pheromone added to the aqueous phase. Adsorption in Tris buffer ranged from 30% for the lowest target concentration (0.01 nM) to 80% for the highest (1.3 nM) (Supporting Information). Finally, the hydrophobicity of the solute had a marked effect on adsorption. Disparlure alkene [(7*Z*)-2-methyloctadec-7-ene] adsorbed 75% at 0.16 nM, a concentration at which disparlure typically adsorbed 40%. Disparlure was the most hydrophobic compound we were able to assay in Tris buffer without the addition of detergent.

A time course of disparlure adsorption on the vial revealed that the aqueous mixture stabilized within 30 min of addition of the ethanolic stock solution. Further time course studies revealed that the system remained stable for up to 20 h (data not shown).

The sampling scheme for the assay took into consideration the adsorption phenomena discussed above. Because of the concentration-dependent adsorption of the pheromone, the nonadsorbed pheromone was sampled for every replicate (Figure 2 A). Once the total nonadsorbed pheromone had

Table 1: Characterization of Recombinant PBPs

PBP	preparation	molecular mass: expected [a]	molecular mass: observed (by MALDI-MS)	PI: expected [a]	PI: observed	% free thiols by I_2 titration
PBP1	renatured	16 145	16 145	4.99	4.8 ± 0.1 [b]	4−8
PBP2	renatured	16 146	16 124	5.28	5.2 ± 0.1 [b]	5−10
PBP2	soluble	16 146	16 153	5.28	5.0	0
A. poly. PBP	renatured	15 955	15 934	4.56	4.7	0

[a] Calculated from the amino acid sequence with McVector (Oxford Molecular Group, Oxford UK). [b] Average ± SE from three calibrated IEF runs.

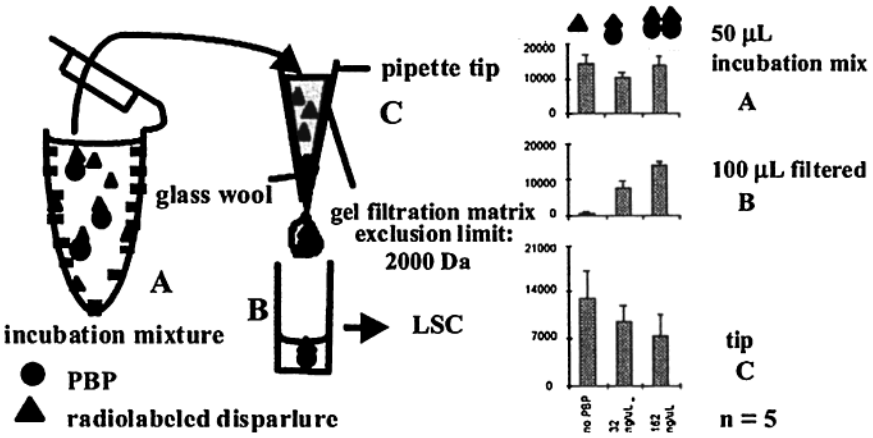

FIGURE 2: Equilibrium binding assay for binding proteins and water-insoluble pheromones and analogues. (A) First, PBP (●) is equilibrated with the radiolabeled pheromone (▲). (B) Bound and free pheromone is separated by gel filtration, and the bound pheromone is quantitated by liquid scintillation counting (LSC). (C) Free pheromone remains behind in the filter. Right panel: results from an experiment with 0, 32, and 162 μg/mL (0, 2, and 10 μM) PBP and constant radiolabeled pheromone (2 nM). The graphs show the total activity (disintegrations per minute, dpm) of 50 μL of nonfiltered sample(top), 100 μL of filtered incubation mixture (middle) and the material remaining in the filter (bottom). Bars represent the mean ± S. E. of 5 replicates.

been sampled, the concentration of bound pheromone was determined by gel filtration (Figure 2B,C). Gel filtration proved to be a reliable method of separating the bound from free pheromone. As shown in the right panel of Figure 2, the total concentration of nonadsorbed pheromone was similar in treatments with 0, 32, and 162 ng/μL (0, 2, and 10 μM) PBP and 2 nM racemic disparlure in solution. The pheromone recovered in the filtrate (B) increased with increasing concentration of PBP, while the pheromone left behind in the filter (C) decreased with increasing PBP concentration. The activity disintegrations per minute (dpm), of the material recovered in the filtrate was very close to the activity calculated from the K_d of racemic disparlure (Table 2), the concentration of total PBP and of disparlure, suggesting that dissociation of PBP·D during the filtration step is negligible. This observation is consistent with a 12 h half-life we have measured for PBP·D (these kinetic studies will be presented in another paper).

To account for variability in the pipetting and filtration procedures, each replicate was sampled twice for bound pheromone, and the values for the two samplings were averaged. Variability between the two samplings averaged 27%, and reproducibility of the entire incubation scheme gave standard errors of 17% on average.

Determination of K_d. Both the association and dissociation assays (see Experimental Procedures) gave linear plots of bound (PBP·D) vs total disparlure. Attempts to plot the same data in Scatchard format yielded no linear relationship (Supporting Information). Because of the concentration-dependent pheromone adsorption, it was not possible to vary the pheromone concentration over a sufficiently broad range to obtain meaningful Scatchard plots. Binding constants were therefore obtained from plots of bound disparlure [PBP·D] vs total disparlure $[D]_{total}$:

$$K_d = [PBP][D]/[PBP\cdot D]$$
$$[PBP]_{total} = [PBP\cdot D] + [PBP] \quad (1)$$
$$[D]_{total} = [PBP\cdot D] + [D]$$

where the items measured in the assay are: [PBP·D], $[PBP]_{total}$, and $[D]_{total}$. Because the total amount of PBP used was typically in large excess (10^3-10^5 fold) over the total amount of pheromone added to the system, $[PBP]_{total} \gg [PBP\cdot D]$, and therefore, $[PBP] \approx [PBP]_{total}$.

Substituting into eq 1:

$$K_d = [PBP]_{total}([D]_{total} - [PBP\cdot D])/[PBP\cdot D] \quad (2)$$

Solving for [PBP·D]:

$$[PBP\cdot D] = [PBP]_{total}[D]_{total}/(K_d + [PBP]_{total}) \quad (3)$$

Thus, a plot of [PBP·D] vs $[D]_{total}$ is linear with a slope of $[PBP]_{total}/(K_d + [PBP]_{total})$, as long as the assumption that the total pheromone binding protein far exceeds the bound pheromone holds. In both association and dissociation binding assays, $[PBP]_{total}$ was typically between 1 and 3 μM,

Table 2: Binding of Disparlure to Different Pheromone Binding Proteins

pheromone binding protein	preparation	disparlure	type of assay	pH	K_d (μM)[a]	average K_d[b] (μM) (pH 7.5)
L. dis. PBP1	renatured	(+)	association	7.5	6.9 ± 0.2	7.1 ± 0.4
		(+)	dissociation	7.5	7.3 ± 0.2	
		(−)	association	7.5	1.8 ± 0.2	2.2 ± 0.4
		(−)	dissociation	7.5	2.6 ± 0.3	
		racemic[c]	association	7.5	7.8 ± 0.2	
L. dis. PBP2	renatured	(+)	association	7.5	2.6 ± 0.1	1.8 ± 0.4 A
		(+)	association	7.5	1.0 ± 0.1	
		(+)	dissociation	7.5	2.0 ± 0.1	
		(+)	association	6.8[d]	1.5 ± 0.1	
		(−)	association	7.5	3.6 ± 0.1	3.2 ± 0.5 B
		(−)	association	7.5	3.7 ± 0.2	
	soluble	(−)	association	7.5	3.4 ± 0.2	
		(−)	dissociation	7.5	1.9 ± 0.1	
L. dis. PBP2	renatured	racemic[e]	association	7.5	4.7 ± 0.2[f]	4.9 ± 0.1 C
		racemic[g]	association	7.5	4.8 ± 0.2	
		racemic[c]	association	7.5	5.1 ± 0.2	
A. polyphemus PBP	renatured	(+)	association	7.5	21.5 ± 0.3	

[a] Calculated as described under Experimental Procedures. Unless indicated, the error reflects a 10% error in the protein concentration, which ranged from 1 to 3 μM. All assays at pH 7.5 were done in 20 mM Tris. [b] Average of all K_d values obtained above pH 6.0 for each form of disparlure. Errors are the range ($n = 2$) or the SE ($n \geq 3$). Averages followed by different letters differ significantly (t-test, $p < 0.05$). [c] Freshly synthesized. [d] Receptor Lymph Ringer (see Experimental Procedures). [e] Synthesized in 1989 and purified the same way as the (+)- and (−)-disparlure. [f] Average ± SE of three independent determinations with different protein lots. [g] Mixture of 43% (+)- and 57% (−)-disparlure.

while the concentration of pheromone was 3−5 orders of magnitude smaller.

Comparison of Equilibrium Constants. The constants obtained with the association and dissociation assays are nearly the same (Table 2), suggesting that pheromone binding by PBPs is reversible. The mode of preparation (recovery from the soluble fraction or renaturation from inclusion bodies) had no effect on the pheromone binding, as seen for the values obtained with an association assay for renatured and soluble PBP2 with (−) disparlure. The age of the disparlure (freshly synthesized or purified 12-year-old material) made no difference to pheromone binding, as seen by the values obtained for PBP2 and racemic disparlure. The (+)- and (−)-disparlure were synthesized by a different route than the racemic material (*32*), and therefore impurities expected in a 1:1 mixture of the two enantiomers and the racemic material were different. The observation that all three racemic preparations gave the same binding constants indicated that none of them contained impurities that interfered with binding. Furthermore, the tritiated pheromones were chromatographically pure and reacted as expected for disparlure in test reactions (see Experimental Procedures). Finally, binding of (+)-disparlure to PBP2 did not differ significantly in 20 mM Tris buffer, pH 7.5, and in receptor lymph ringer, pH 6.8, suggesting that the slight drop in pH and the increase in ionic strength did not alter binding extensively. Thus, the Tris buffer was a valid substitution for receptor lymph ringer in the equilibrium binding assay. The effect of pH variations over a wide range (4−11) is presented elsewhere.

Enantiomer Discrimination. When affinities of the PBPs for the two disparlure enantiomers were compared, PBP1 had a higher affinity toward the (−) enantiomer while PBP2 preferred the (+) enantiomer (Table 2). The racemic pheromone gave a higher apparent K_d than either enantiomer for both PBPs. Binding of racemic disparlure to PBP, where both enantiomers compete for the same binding site, can be described by

$$\text{PBP}\cdot\text{D}(-) \underset{K_d(-)}{\overset{D(-)}{\rightleftharpoons}} \text{PBP} \underset{K_d(+)}{\overset{D(+)}{\rightleftharpoons}} \text{PBP}\cdot\text{D}(+) \qquad (4)$$

where $[\text{D}(+)] + [\text{D}(-)] = [\text{D}]$, $[\text{PBP}\cdot\text{D}(+)] + [\text{PBP}\cdot\text{D}(-)] = [\text{PBP}\cdot\text{D}]$, and

$$K_d(\text{racemic}) = [\text{PBP}][\text{D}]/[\text{PBP}\cdot\text{D}] \qquad (5)$$

$$= [\text{PBP}]([\text{D}(+)] + [\text{D}(-)])/([\text{PBP}\cdot\text{D}(+)] + [\text{PBP}\cdot\text{D}(-)])$$

where $[\text{PBP}]_{total} = [\text{PBP}] + [\text{PBP}\cdot\text{D}(+)] + [\text{PBP}\cdot\text{D}(-)]$

Because PBP is present in a large excess over D

$$[\text{PBP}]_{total} \approx [\text{PBP}] \qquad (6)$$

and

$$^1/_2[\text{D}] = [\text{D}(+)] + [\text{PBP}\cdot\text{D}(+)] = [\text{D}(-)] + [\text{PBP}\cdot\text{D}(-)] \qquad (7)$$

where K_d(racemic) can be estimated from the K_d values for the individual enantiomers by substituting eqs 6 and 7 into eq 5:

$$K_d(\text{racemic}) = \frac{\dfrac{[\text{PBP}]_{total}\,K_d(+)}{(K_d(+) + [\text{PBP}]_{total})} + \dfrac{[\text{PBP}]_{total}\,K_d(-)}{(K_d(-) + [\text{PBP}]_{total})}}{2 - \dfrac{K_d(+)}{(K_d(+) + [\text{PBP}]_{total})} - \dfrac{K_d(-)}{(K_d(-) + [\text{PBP}]_{total})}} \qquad (8)$$

When $[\text{PBP}]_{total} \gg K_d(+)$ and $K_d(-)$, the racemic K_d is approximately the numerical average of the K_d values for the individual enantiomers. From eq 8 and $[\text{PBP}]_{total} = 1$

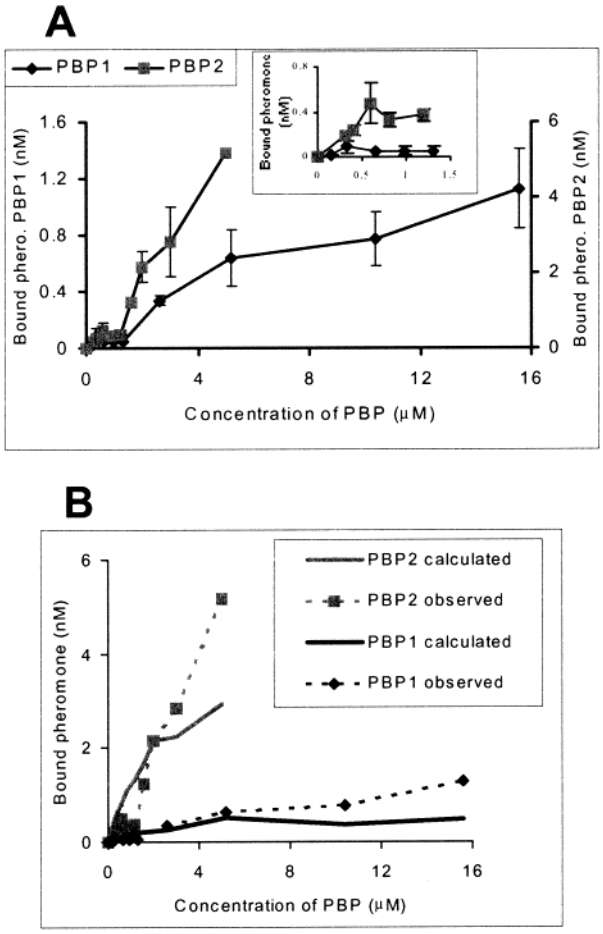

FIGURE 3: (A) Effect of increasing PBP1 (◆) or PBP2 (■) concentration on binding of (+)-disparlure (total added 9 nM). Each point is the average of three replicates. Error bars denote the standard error. Inset: low PBP concentration region of the plot. (B) Same data as in panel A compared to the concentration of bound pheromone calculated from the K_d (Table 2) and the measured concentration of pheromone in solution. The concentration of nonadsorbed pheromone was 2.0 ± 0.1 nM for the PBP1 assay and 3.5 ± 0.1 nM for the PBP2 assay. $[PBP{\cdot}D] = [PBP]_{total}[D]_{total}/K_d(1 + [PBP]_{total}/K_d)$.

μM, the estimated K_d(racemic) value for PBP2 is 2.3 μM and for PBP1 is 3.5 μM. The values observed are significantly higher than these expected values. An explanation for this behavior will require knowledge of the PBP structure with the pheromone enantiomers bound.

Given that the assay was carried out at PBP concentrations of 1−3 μM, but the PBP concentration in the antenna is 10 mM, we investigated the effect of increasing the PBP concentration beyond that used in the assay while keeping a low pheromone concentration (nanomolar). The binding profile obtained for (+), and (−)-disparlure was biphasic, with a low concentration region up to approximately 2 μM, followed by a dramatic increase in the concentration of bound pheromone above 2 μM (Figure 3 A). In all experiments of this type, the bound pheromone concentration was close to or less than the expected value from the K_d up to PBP concentrations of 2 μM, but was significantly higher than expected from the K_d above the 2 μM threshold (Figure 3B and Supporting Information). Despite this apparent increase in pheromone binding, PBP2 bound (+)-disparlure with higher affinity than PBP1, throughout the range of concentrations tested.

We have also compared the species selectivity. The PBP from *A. polyphemus* (Apol-3) had a significantly lower affinity for (+)-disparlure than either of the two PBPs from *L. dispar* (Table 2). Despite its lower affinity for disparlure, *A. polyphemus* PBP was also able to discriminate enantiomers. In parallel tests at a single concentration of pheromone, *A. polyphemus* PBP bound 15% ± 5% of the total (+)-disparlure added and 30% ± 5% of the total (−)-disparlure ($n = 4$).

DISCUSSION

Binding Assay. In this paper we present an equilibrium binding assay, which allowed us to study the reversible binding of a very hydrophobic pheromone in aqueous solution. We have addressed the major problem of such assays: adsorption of the hydrophobic ligand on the vial surface. Many biologically important processes involve protein-mediated transport of hydrophobic compounds through aqueous solutions. This includes not only transport of odorants (*29*, *37*) but also of hydrocarbons, wax esters (*38*, *39*), steroids, and other lipids (*40*). Transport of a ligand requires two properties of the binding protein−ligand interaction. First, binding must be reversible, and second, the ligand must bind more tightly at the source than at the target. Because the binding equilibrium can be approached from either side in our assay, the procedure can be used to test for binding reversibility. Also, our assay allows variation of the pH and buffer composition. Finally, the assay is sufficiently robust to detect subtle variations in binding selectivity. This flexibility and sensitivity is not available with the widely used native PAGE assay. Similarly, the solution assay previously published did not permit testing of reversibility and relied on the selective adsorption of the PBP−pheromone complex (*18*). This phenomenon was not observed in this study and may have been a property of the particular PBP preparation used at that time. Our assay allowed us to study the enantiomer selectivity of the two *L. dispar* PBPs.

Pheromone Binding Specificity. Even though the basis for the binding assay used here differed from that used by Du and Prestwrich (*18*), the order of magnitude of the K_d values (μM) obtained was the same. In both assays, however, the PBP concentration was 3−4 orders of magnitude lower than in the antenna. We found that the affinity of *L. dispar* PBPs for both enantiomers of disparlure dramatically increased at PBP concentrations above 2 μM. Despite the increase in affinity, the data in Figure 4 show that PBP2 had a higher affinity for (+)-disparlure than PBP1, which suggests that the ligand preference did not change with increasing PBP concentration. From native PAGE experiments with PBP and pheromone it is clear that both monomeric and aggregated PBP bind the pheromone. Thus, it is possible that at higher PBP concentrations additional binding sites become available at the interfaces of aggregated PBP monomers. This possibility is consistent with the observation that several PBPs form active dimers (*19*, *26*, *41*). Alternatively, aggregation may bring about a conformational change, which permits stronger ligand/PBP interactions. A third alternative is that at high PBP concentration an increased amount of pheromone is desorbed from the vial surface.

Disparlure bound with higher affinity to the *L. dispar* PBPs than to the PBP of *A. polyphemus*, Apol-3. The structural

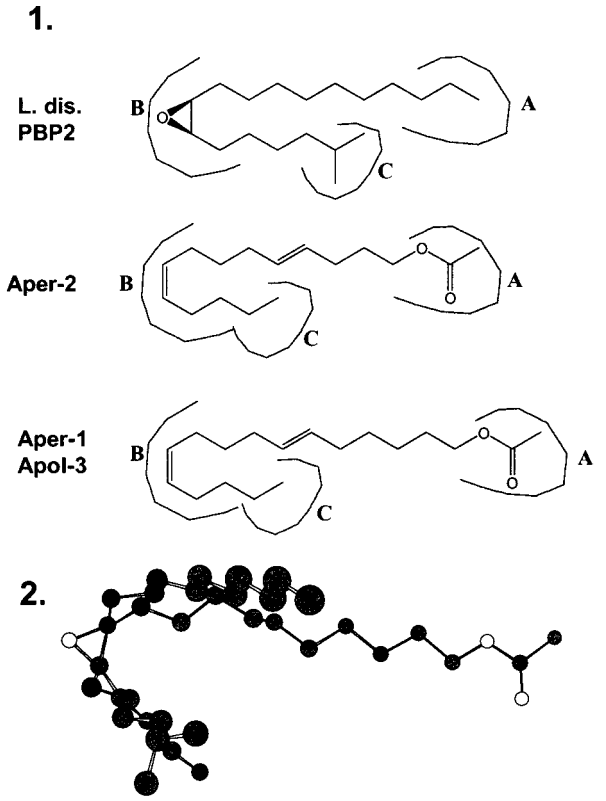

FIGURE 4: (1) Model of pheromone binding to *L. dispar* PBP. The long arm of disparlure would bind in pocket A, which in Apol-3, Aper-1, and Aper-2 binds the acetate group (*17*). Pocket B would recognize the bend in the structure of these pheromones, and pocket C would accommodate the shorter arm. Chain length would be detected by different placement of the three pockets relative to each other. Chirality of disparlure would be detected by the shape of pocket B. (2) Superposition of (6*E*,11*Z*)-hexadeca-6,11-dienyl-1-acetate and (+)-disparlure (view from the top with the epoxide in the plane of the paper). Structures were energy-minimized and superimposed with Chem-3D (CambridgeSoft Co., Cambridge, MA). The long arm of the acetate projects significantly further away from the rigid bend than the long arm of disparlure, suggesting that pocket A will be further away from B and C in Apol-3 than in the *L. dispar* PBPs. Apol-3 binds the acetate much more tightly than (+)-disparlure, possibly because the pockets are not suitably aligned to accommodate disparlure.

differences between the major pheromone components of *L. dispar* and *A. polyphemus* are subtle. Both pheromones have a rigid bend in the chain and both have a longer and a shorter arm, but they differ in their functional group type and placement (Figure 4). Different PBPs are clearly able to distinguish such structural nuances. Previous studies have shown similar intra- and interspecific binding selectivity of PBPs. For example, in *Antheraea pernyi*, one PBP (Aper-1) preferentially bound (6*E*,11*Z*)-hexadeca-6,11-dienyl-1-acetate over the 14-carbon homologue and the other PBP (Aper-2) had the opposite selectivity. Apol-3 bound the 16-carbon acetate preferentially over the 14-carbon homologue (*18*). In *Mamestra brassicae* the major male-specific PBP, Mbra-1′, appeared to bind the pheromone more strongly than Mbra-1, which is found in males and females. The other major PBP, Mbra-2, did not bind the pheromone at all (*16*). In another study, the PBP from *Bombyx mori* only bound bombykol and failed to bind structurally unrelated pheromone components from scarab beetles (*23*). Finally, in this study, *L. dispar* PBP1 had a lower affinity for racemic disparlure than PBP2, consistent with the findings from a previous study (*21*).

The most important finding in this study was that *L. dispar* PBP1 and PBP2 differed in their affinity for the enantiomers of disparlure. PBP2 preferentially bound (+)-disparlure, while PBP1 preferentially bound (−)-disparlure. To our knowledge, this is the first demonstration of enantiomer discrimination by a PBP. The ability to discriminate enantiomers was not unique to the *L. dispar* PBPs. Apol-3 slightly preferred (−)-disparlure. A previous study with two species of scarab beetle, which use opposite enantiomers of japonilure as pheromone, failed to detect enantioselectivity in the PBPs from these two species (*42*). Similarly, a previous study with *L. dispar* PBP1 and PBP2 failed to reveal the enantiomer preferences of the two PBPs. One possible explanation for these results is that the native PAGE binding assay may not have been sensitive enough to detect subtle differences in affinity.

Sequence comparison of the three PBPs featured in this study reveals low sequence homology between them (55% PBP1/2, 61% PBP1/Apol-3, 55% PBP2/Apol-3). The region between the first two conserved cysteine residues exhibits considerable sequence variability between distantly related PBPs (*14*), but not between closely related ones that bind structurally related ligands (*29*). This region reacts with a photolabile pheromone analogue in Apol-3 (Figure 5) (*17*) and is therefore thought to interact with the acetyl group of the pheromone. This region is flanked by sets of highly conserved cysteine residues, as well as two highly conserved pairs of positively and negatively charged amino acids that may be involved in salt bridges (Figure 5). It is possible that the highly conserved features provide a template for a pocket that is involved in ligand recognition. Given that this particular region is highly conserved in Aper-1, Aper-2, and Apol-3, all of which bind homologous acetate pheromones, this pocket is not likely responsible for recognition of chain length by itself. The relative positioning of pockets that recognize individual features, such as the acetate group and the *Z* double bond, is probably responsible for chain length recognition (Figure 4). The observation that Apol-3 weakly binds disparlure, which is similar in overall shape to (6*E*,-11*Z*)-hexadeca-6,11-dienyl-1-acetate, suggests that perhaps the pocket that interacts with the acetate group accommodates the long arm of disparlure. The pocket that recognizes the bend possibly accommodates the *cis*-epoxide moiety, and perhaps a third pocket detects the smaller arm in these pheromones. If this is the case, then chirality should be recognized mainly by the second pocket (B, Figure 4).

We have attempted to locate the region in the primary structure that may be involved in recognition of chirality by identifying residues that are common to PBP1 and Apol-3 but differ in PBP2. Both Apol-3 and PBP1 preferentially bound (−)-disparlure and were the most closely related pair among the three pairs of PBPs in this study. The first striking feature is a cluster between residues 65 and 79 (Figure 5). Another interesting observation is that threonine 44, which is thought to be part of pocket A (Figure 4), on the basis of previous photolabeling results (*17*), is common to PBP1 and Apol-3 but differs in PBP2 (Figure 5). This suggests that the relative alignment of pockets on the protein also plays a role in the recognition of chirality. The crystal structure of the *B. mori* PBP reveals a heart-shaped pheromone binding

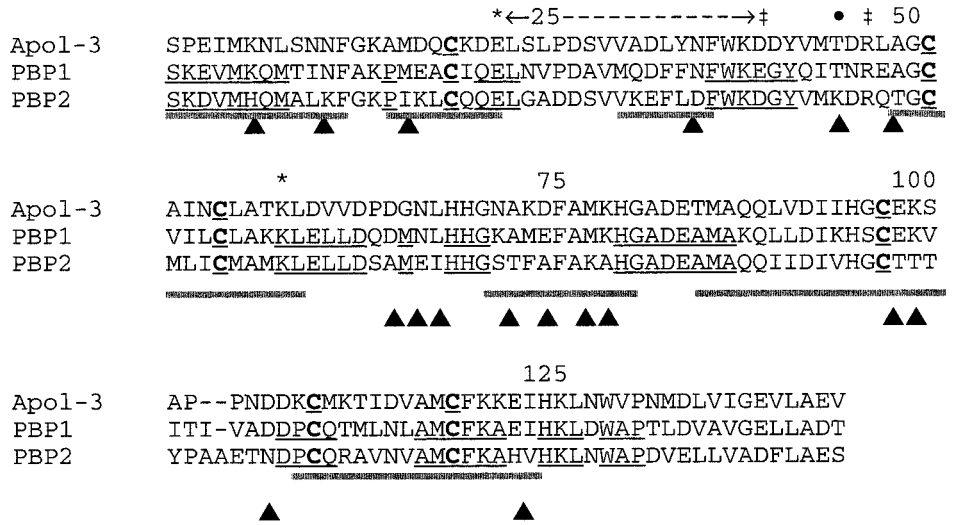

FIGURE 5: Aligned sequences of *L. dispar* PBP1 and PBP2 and *A. polyphemus* PBP (Apol-3) without signal sequences. The six conserved C residues are shown in boldface type; stretches of residues conserved between PBP1 and PBP2 are underlined. Two salt bridges that are highly conserved among PBPs are indicated with ‡ and *. (●) Residue of Apol-3 photolabeled with (6*E*,11*Z*)-[^{3}H] hexadecadienyl diazoacetate, a mimic of the major pheromone component, and Broken arrow indicates residues labeled by (4*E*,9*Z*)-[^{3}H]tetradecadienyl diazoacetate (*18*). The gray bars indicate α-helical regions in *B. mori* PBP (*25*). Residues that are common to PBP1 and Apol-3 but differ in PBP2 are marked ▲ (see Discussion).

site with three pockets: one accommodates the long arm, the other, the short arm, and the third pocket accommodates the bend. Two highly conserved phenylalanine residues (12 and 118) stack above and below the π system of the pheromone. All these structural elements ensure that the pheromone binds in a unique orientation (*26*). If the *L. dispar* PBPs have a binding pocket of similar shape and also orient the long and the short arm of the pheromone uniquely, then recognition of chirality should take place mainly in the pocket that accommodates the bend.

The pheromone olfactory system in the gypsy moth is able to recognize five molecular features of disparlure: (1) the chain length and in particular the relative size of the long and the short arms; (2) the cis configuration of the epoxide (the trans isomers give much weaker or no responses) (*43*); (3) the position of the methyl group relative to the epoxide (*44*); (4) the chirality (*4*); and (5) the nature of the three-membered ring (*45*). In this study we have clearly demonstrated that the two *L. dispar* PBPs are able to discriminate chirality. We are currently studying PBP-mediated recognition of other molecular features.

Why Multiple PBPs? Most insects studied so far have more than one PBP, regardless of whether the pheromone consists of a single major component or of a multicomponent blend (*19, 21, 29, 37, 46*). Furthermore, the PBPs within a species are often divergent in sequence. There are two possible reasons for having multiple divergent PBPs in one species. First, different PBPs may be required to distinguish major and minor pheromone components or pheromones and behavioral antagonists. Different PBPs may act as a slightly selective filter, binding compounds that are behaviorally relevant and preventing completely unrelated odorants from interfering. Second, PBPs with subtly different binding selectivities may modulate the detection threshold of the sensillum they are expressed in. The latter function may be crucial in determining the range of pheromone concentrations an insect is able to respond to.

The detection threshold of a sensillum is expected to be dependent on both the odorant transport efficiency of the PBP and the specificity of the receptor neurons. In the gypsy moth, each sensillum is thought to house 2−3 neurons of different specificity. One type of neuron is thought to be specific for (+)-disparlure, while the other responds to (−)-disparlure and the alkene (*4*). If each PBP is expressed in a different subset of sensilla, independently of the type of receptor, then a mosaic of sensilla with different thresholds will result. For example, a sensillum with a (+)-disparlure-responsive neuron and PBP2 would have a lower detection threshold for (+)-disparlure than a sensillum with PBP1. On the other hand, the sensillum with PBP2 would saturate at a lower concentration of (+)-disparlure than the sensillum with PBP1. Under this model, having two populations of (+)-disparlure-specific sensilla with different detection and saturation thresholds should enable a moth to detect a significantly wider range of (+)-disparlure concentrations than a single population of sensilla. To verify this hypothesis, three major questions need to be addressed. First, does the number of PBPs correlate with the detection range of a given species? Second, is only one PBP expressed per sensillum? Third, is PBP expression independent of the type of neuron housed in a sensillum?

In conclusion, we have developed and validated a robust binding assay for the study of the interaction between hydrophobic ligands and their cognate proteins in aqueous solution. Using this assay, we have demonstrated for the first time that pheromone binding to PBP is reversible and that the two PBPs from *L. dispar* differ in a subtle but statistically discernible manner in their preference for the enantiomers of disparlure. We have proposed sites on the sequence of the PBPs that may be responsible for the recognition of chirality.

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SUPPORTING INFORMATION AVAILABLE

Text, table, and figures with details of expression, purification, and characertization of PBP. This material is available free of charge via the Internet at http://pubs.acs.org.

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