Protein Structure Encodes the Ligand Binding Specificity in Pheromone Binding Proteins†

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ABSTRACT: The ligand specificities and binding affinities of three recombinant pheromone binding proteins (PBPs) of two saturniid moths (genus *Antheraea*) were determined by using a novel binding assay in conjunction with two tritium-labeled constituents of the pheromone blend, [^{3}H]-6*E*,11*Z*-hexadecadienyl acetate and [^{3}H]-4*E*,9*Z*-tetradecadienyl acetate. The new binding assay, in which nonspecific adsorption to a plastic vessel is suppressed by presaturation of the surface with a 1-alkanol, allows measurement of dissociation constants (K_D) for lipophilic ligands for their carrier proteins. The three PBPs showed K_D values for [^{3}H]-6*E*,11*Z*-16:Ac and [^{3}H]-4*E*,9*Z*-14:Ac between 0.6 and 30 μM, as determined by Scatchard analysis. Importantly, two PBPs (Aper-1 and Aper-2) from one species showed opposite binding specificities for these two ligands. Aper-1, like Apol-3, showed 15-fold higher affinity for 6*E*,11*Z*-16:Ac than for 4*E*,9*Z*-14:Ac, while Aper-2 showed a 3.5-fold preference for binding the shorter chain compound. In addition, for the Apol-3 PBP, displacement of [^{3}H]-6*E*,11*Z*-16:Ac binding by other pheromone components or analogs showed a clear trend in relative binding affinity: 6*E*,11*Z*-16:Ac > 4*E*,9*Z*-14:Ac > 6*E*,11*Z*-16:Al ≈ 16:Ac > 6*E*,11*Z*-16:OH > 4*E*,9*Z*-14:OH. These data clearly demonstrate a >1000-fold range of binding affinities among these very similar structures and unambiguously demonstrate the specificity of the PBP−pheromone interaction. Moreover, this assay offers the potential for determining ligand specificities for odorant binding proteins and other proteins in the vertebrate lipocalin superfamily.

The pheromone binding proteins (PBPs) of the Lepidoptera provide a unique opportunity to explore the ligand specificity of proteins from vertebrates or invertebrates that bind to volatile, lipophilic odorants (Pelosi & Maida, 1990; Pelosi, 1994). These proteins, located in the sensillum lymph of the pheromone-responsive sensory hairs of the antennae of adult Lepidoptera (Vogt & Riddiford, 1981; Steinbrecht et al., 1992), mediate the delivery of hydrophobic sex pheromones to specific receptor proteins located in the dendritic membrane (Vogt, 1987; Prestwich, 1993b). Since a male moth responds to a conspecific female sex pheromone consisting of a blend of only a few lipids of defined structure, the PBPs may act as a primary filter in determining the overall specificity of the electrophysiological and behavioral responses (Prestwich & Du, 1995). PBPs may also facilitate the removal of pheromone metabolites after signal transduction occurs (Vogt et al., 1989). To date, deduced protein sequences for five PBPs from four moth species and another five general odorant binding proteins (GOBPs) from three species have been described (Gyørgi et al., 1988; Raming et al., 1989, 1990b; Breer et al., 1990; Krieger et al., 1991, 1993; Vogt et al., 1991a,b; Prestwich & Du, 1995). PBPs and GOBPs share several highly conserved regions, including six conserved Cys residues that form intramolecular disulfide bridges (Prestwich, 1993a).

Insect PBPs and GOBPs differ completely in primary, secondary, and tertiary structure from the 20-kDa odorant binding proteins (OBPs) that occur tissue specifically at high concentrations in the nasal mucosa of vertebrates (Pevsner et al., 1990; Pelosi, 1994). While the vertebrate OBPs primarily consist of 10 antiparallel β-strands and two short α-helices, the insect PBPs are over 45% α-helix. Although precise functions for vertebrate OBPs or insect PBPs and GOBPs in sensory transduction are not completely understood (Pelosi, 1994), biochemical data strongly suggest roles in odorant recognition, transport, degradation, and clearance (Prestwich et al., 1989) and perhaps also selective delivery to a membrane-associated receptor (Pelosi & Maida, 1990; Pelosi, 1994; Prestwich & Du, 1995).

We hypothesized that each insect pheromone component could have a unique high-affinity PBP, and the discovery of multiple PBPs in several moth species has provided support for this notion. Demonstration of specific PBP−ligand interactions required three components: an abundant source of purified PBPs of defined sequence, a supply of radiolabeled pheromone molecules, and a reliable assay for measuring binding affinity and specificity. The missing piece has been the availability of a quantitative and reproducible method for measuring the association of a long-chain alkenyl acetate to a protein in aqueous solution.

In this paper, we present a new approach to the problem of quantification of pheromone binding in aqueous solution. We also describe the expression and purification of two new recombinant PBPs (rPBPs), Aper-1 and Aper-2, originally cloned from *Antheraea pernyi*. These two rPBPs show 92% and 87% identity, respectively, with Apol-3 from *Antheraea polyphemus*, the third rPBP used in these measurements. The binding affinities of these three PBPs for two radiolabeled pheromone components were determined by Scatchard analysis using a novel reversed-phase binding assay. In addition, displacement experiments with the 16-carbon

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acetate, aldehyde, and alcohol and the 14-carbon acetate and alcohol were performed to show the ligand binding selectivity of the PBPs.

MATERIALS AND METHODS

Chemicals. Synthesis of [11,12-$^3H_2$]-6*E*,11*Z*-16:Ac (Prestwich et al., 1984) and [9,10-$^3H_2$]-4*E*,9*Z*-14:Ac (Du et al., 1994), with final specific activities of ca. 40 Ci/mmol, was performed by C.-S. Ng (Stony Brook) using materials obtained by selective reduction of alkynyl acetate precursors performed by D. G. Ahern at Dupont New England Nuclear (Boston, MA). Radiochemically pure materials were obtained by flash chromatography on disposable columns of silica gel and silver nitrate-impregnated silica gel, with analysis by radio-TLC and by fluorography of En^{3}Hance-sprayed TLC plates on X-ray film. Radiolabeled acetates were stored at concentrations of 0.1−1.0 mCi/mL in 9:1 heptane−toluene solution at −20 °C. Dilutions of a stock solution of 2.5 μM tritiated pheromone in ethanol needed for performing the binding assays could be stored at −20 °C for up to 1 month. Unlabeled pheromones and analogs used in competition assays were repurified by silica gel chromatography from synthetic materials prepared at Stony Brook. Materials were homogeneous by TLC and at least 96% pure by GC. Working ethanolic solutions were prepared from stock 3 mM hexane solutions.

Recombinant Proteins. Apol-3 rPBP was expressed in *Escherichia coli* XA-90 cells transformed with pGDP-Apo3C (Prestwich, 1993a). Expression cassettes for Aper-1 and Aper-2 were prepared by PCR as previously described for Apol-3 (Prestwich, 1993a). Thus, custom 5′ primers consisted of an end clamp, an *Eco*RI site (underlined), a ribosome binding site (italic), a translation spacer, a start codon (bold), and 18 nucleotides of the coding region for each protein: for Aper-1, 5′-TAGGGCGAATTC*AAGGAG*ATATACC**ATG**TCGCCAGAGATCATAAAG-3′; for Aper-2, 5′-TAGGGCGAATTC*AAGGAG*ATATACC**ATG**TCACCAGAGGTCATGAAG-3′. These were paired with custom 3′ primers containing 21 nucleotides of the reading frame, a stop codon (bold), a *Hind*III site (underline), and an end clamp: for Aper-1, 5′-TCTAGGCAAAGCTT**TTA**AACTTCTGCTAAAACTTCGCC-3′; for Aper-2, 5′-TCTAGGCAAAGCTT**CTA**AACTTCAGCTAAGACCTCGCCTAT-3′. PCR was conducted as described previously (Prestwich, 1993a) using M13 plasmids (from H. Breer and J. Krieger, University of Hohenheim-Stuttgart) containing the cDNAs with the intact open reading frames for Aper-1 (Raming et al., 1990a) and Aper-2 (Krieger et al., 1991). ECPCR cassettes were gel-purified, enzymatically digested, and ligated into prepared pHN1+ vector (MacFerrin et al., 1993). *E. coli* XA-90 cells were transformed, and ampicillin selection provided colonies bearing the desired construct. The cDNA insert was sequenced to confirm the fidelity of the PCR amplification.

One-liter cultures of Aper-1, Aper-2, and Apol-3 transformants were each induced with IPTG, leading to overproduction of a 14-kDa protein in each case. The rPBPs were located in the soluble and inclusion body fractions, as described previously for Apol-3. Only the soluble rPBPs were employed in this study to eliminate possible differences in refolding among the three rPBPs. Thus, the crude cell-free lysate was subjected to preparative isoelectric focusing in a Rotofor, and the fractions between pH 4.5 and 5.0 were pooled, made 2 M with solid NaCl, and dialyzed against GF buffer [10 mM Tris-HCl (pH 7.2), 100 mM NaCl, and 1 mM EDTA]. Each rPBP was then purified by gel filtration using GF buffer and finally applied to an FPLC MonoQ column and eluted using a linear gradient of buffers A and B (buffer A, 10 mM Tris-HCl, pH 8.0; buffer B, buffer A plus 1 M NaCl). Purity was established on overloaded, Coomassie Blue-stained SDS−20% PAGE. Stock solutions of rPBP were prepared at 0.5−1 mg/mL in 20 mM Tris-HCl (pH 6.8) and stored at 4 °C.

Coating Procedure. A saturated solution of an *n*-alkyl alcohol in water−ethanol was prepared by adding water slowly to a 1% (w/v) solution of a 1-alkanol in absolute ethanol to the cloud point and then adding ethanol to clear the solution. 1-Octanol, 1-decanol, and 1-dodecanol were used in separate preliminary experiments. Twenty or thirty conical plastic microfuge tubes were filled with 1.5 mL of this solution, capped, and allowed to stand for 12 h at room temperature. The alkanol solution was emptied out, and the coated tubes were washed three times with deionized water and then air-dried.

Optimization of the Binding Assay for rPBP. The assay development is described in detail for the binding of [^{3}H]-6*E*,11*Z*-16:Ac to Apol-3 rPBP. First, 200 μL of buffer (20 mM Tris-HCl, pH 6.8) was added to each coated assay tube, followed by 2 μL of a 2.5 μM ethanolic solution of the tritium-labeled pheromone. This gave a final concentration of 25 nM pheromone in aqueous solution. Radioactivity in solution was measured on an aliquot using an LKB Model 1218 liquid scintillation counter (LSC) with Scintiverse II (Fisher), with quench calibration based on external standards. Next, various concentrations of PBP were added and the solutions were incubated at either 4 or 25 °C. The time and temperature studies used 25 nM each of ligand and rPBP. Radioactivity remaining in solution after 15, 30, 45, 60, 90, and 120 min was determined by LSC of duplicate 50-μL aliquots. All subsequent assays were performed using 1-h incubations at 4 °C. The saturability of ligand binding was determined by adding various concentrations of tritiated pheromone to a 100 nM solution of rPBP.

In the absence of rPBP, solutions of 25 nM [^{3}H]-6*E*,11*Z*-16:Ac in decanol-coated tubes were prepared and held at 4 or 25 °C for 16 h. Duplicate aliquots were removed at 0.5, 1, 2, 4, 8, and 16 h and analyzed by LSC.

Determination of Dissociation Constants. The dissociation constants (K_D) were determined by Scatchard analysis. First, conditions were determined empirically for each rPBP to find an PBP concentration such that <10% of the ligand (1 μM [^{3}H]-6*E*,11*Z*-16:Ac or 1 μM [^{3}H]-4*E*,9*Z*-14:Ac) was bound. Then, with this concentration (e.g., 100 nM for Apol-3 with the 16-carbon pheromone), duplicate assay tubes were prepared with concentrations of [^{3}H]-6*E*,11*Z*-16:Ac or [^{3}H]-4*E*,9*Z*-14:Ac varying from 0.1 to 10 μM. Nonspecific binding was measured by adding a 300-fold excess of radioinert 6*E*,11*Z*-16:Ac or 4*E*,9*Z*-14:Ac to one of the duplicate tubes. The specifically bound, [B], and free, [F], concentrations were calculated and plotted as [B]/[F] vs [B]; the K_D value was determined from the slope of the plot. Each experiment was replicated three times and the data were averaged.

Determination of Ligand Specificity. In a competition assay, 25 nM [^{3}H]-6*E*,11*Z*-16:Ac was first incubated with

25 nM PBP for 1 h at 4 °C and then with various concentrations of competing ligands for an additional 30 min at 4 °C. Competitors were added in 2−5 μL of ethanol as stock solutions in decade concentrations. Ethanol concentrations up to 5% of the final volume did not affect the quality of data from the binding assay. The radioactivity was measured and equilibrium binding data were calculated. Each experiment was replicated three times, and the data reported are the means of three replicates.

Determination of Protein Exchange in Decanol-Coated Tubes. Three solutions of 25 nM photoaffinity-labeled rPBP, i.e., the covalent complex prepared by irradiation of [^{3}H]-6*E*,11*Z*-16:diazoacetate with Apol-3 rPBP (Du et al., 1994), were prepared. One was held at 4 °C for 16 h. The other two were treated with a 4-fold excess (100 nM) of unlabeled Apol-3 rPBP and held for 16 h at either 4 or 25 °C. Duplicate aliquots taken at 0.5, 1, 2, 4, 8, and 16 h for each experiment were analyzed by LSC.

RESULTS

The pheromone analogs [^{3}H]-6*E*,11*Z*-16:Ac and [^{3}H]-4*E*,9*Z*-14:Ac were prepared at a specific activity of 40 Ci/mmol by reduction of the appropriate enynyl acetate with tritium gas, using a quinoline-poisoned Pd/$BaSO_4$ catalyst in methanol as the solvent (Prestwich et al., 1984; Prestwich, 1987). The labeled dienyl acetates were purified by normal and argentation silica gel column chromatography to give the tritium-labeled pheromone components with >98% radiochemical homogeneity (Prestwich, 1991).

Two new rPBPs, Aper-1 and Aper-2, were expressed in bacterial cells transformed with an expression cassette produced by using PCR. All rPBPs were expressed by the introduction of a new start codon prior to the N-terminal residue. Thus, the rPBPs examined lack their signal peptides. The soluble rPBPs were purified by isoelectric focusing, gel filtration, and FPLC to obtain homogeneous proteins for the determination of binding affinities. As with Apol-3 rPBP, these two PBPs possessed three disulfide bonds since no free thiols were detected (Prestwich, 1993a). Aper-1 and Aper-2 were selected on the basis of the availability of the cDNA clones and the hypothesis that these different PBPs from the moth *A. pernyi* might encode specificity for two different pheromone components known to exist in that species. It is important to note that homogeneous PBPs must be employed for the binding assay described in the following to provide reproducible and accurate binding data.

Plastic microfuge tubes were coated with a saturated solution of one of three different 1-alkanols (octanol, decanol, and dodecanol) to block all nonspecific hydrophobic sites. Preliminary binding studies with Apol-3 rPBP used both refolded and soluble forms, but all subsequent assays employed only the soluble rPBP. The reproducibility of the data (±7.5% within and between replicated experiments) was superior with 1-decanol. 1-Dodecanol was nearly as good, but 1-octanol resulted in excessive (±15%) variability in replicate experiments.

The quantity of [^{3}H]-6*E*,11*Z*-16:Ac in solution versus that adsorbed to the surface of the tube was monitored for 16 h (data not shown). At 4 °C in 1-decanol-coated tubes, 5% was adsorbed on the surface at the end of the 1-h incubation period. This percentage rose to 7.5% at 4 h, 9% at 8 h, and 13% at 16 h. At 25 °C, this exchange process was somewhat more rapid and may account for the higher variability in our preliminary room temperature binding assays. Thus, during the course of the experiment, no substantial exchange occurred between the labeled and unlabeled ligands and the alkanol surface coating.

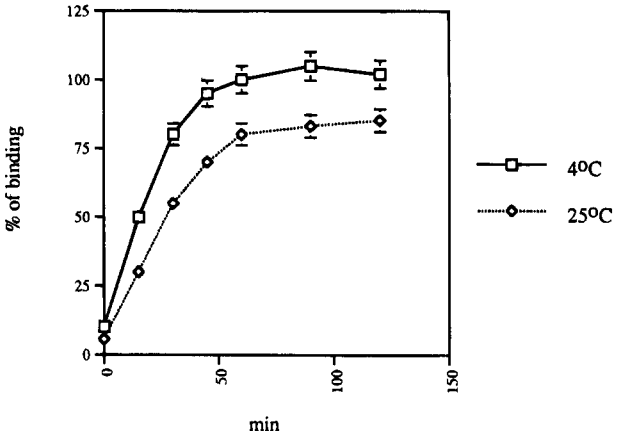

FIGURE 1: Temperature and incubation time dependence of binding of 25 nM [^{3}H]-6*E*,11*Z*:16:Ac to 25 nM Apol-3 rPBP. Data shown represent the averages of three independent determinations.

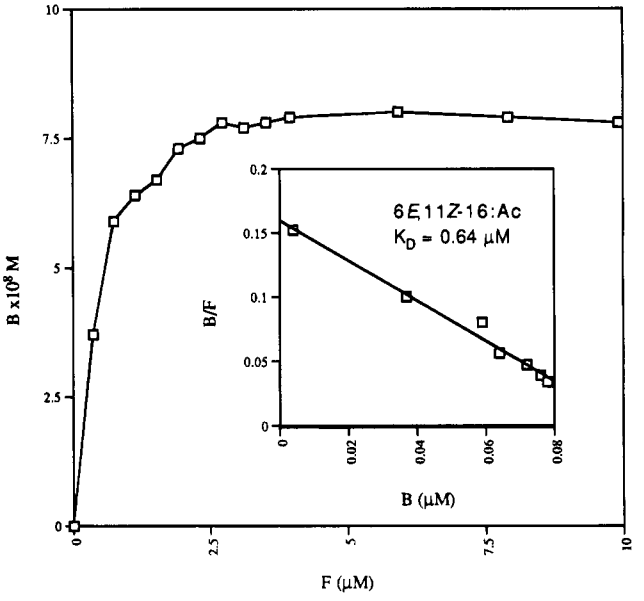

FIGURE 2: Binding of 0.1−10 μM [^{3}H]-6*E*,11*Z*:16Ac to 100 nM Apol-3 PBP. Specific binding ([B]) is plotted as a function of free ligand remaining ([F]). Scatchard analysis of the data is shown in the inset. Data shown represent the averages of three independent determinations.

The time course and saturability of binding were investigated using a variety of concentrations of pheromone incubated with purified Apol-3 rPBP. Figure 1 shows the time and temperature dependence for 25 nM Apol-3 rPBP with 25 nM [^{3}H]-6*E*,11*Z*-16:Ac in 20 mM Tris-HCl (pH 6.8). The percentage of specific binding at 4 °C appeared somewhat higher than that at 25 °C, but both temperatures showed similar time dependencies. After 15 min, binding reached the half-maximal value, and binding reached a maximum value after 45 min (Figure 1). With 100 nM Apol-3 rPBP, the binding of increasing concentrations of [^{3}H]-6*E*,11*Z*-16:Ac was saturable (Figure 2).

Scatchard analysis for Apo-3 rPBP revealed a single binding site with a calculated K_D of 0.64 μM for 6*E*,11*Z*-16:Ac (Figure 2, inset) and a K_D of 21.0 μM for 4*E*,19*Z*-

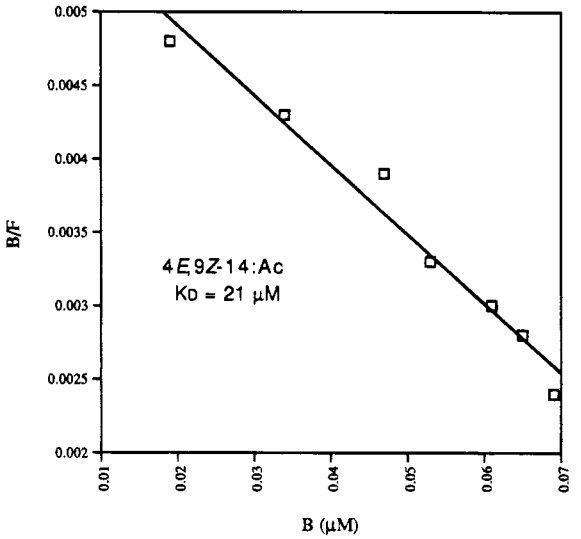

FIGURE 3: Scatchard plot for binding of [^{3}H]-4*E*,9*Z*-14:Ac to Apol-3 rPBP. The K_D value is 21 μM for 4*E*,9*Z*-14:Ac.

14:Ac (Figure 3). The data shown were obtained by calculating specific binding, i.e., total binding minus nonspecific binding, in the presence of 300-fold excess pheromone. Bound ligand and free ligand were physically separated during this new assay protocol. The free ligand remained in solution, while ligand bound to the rPBP was adsorbed on the surface of the decanol-coated tube. Indeed, the entire binding assay was effectively conducted on surface-adsorbed protein. For this reason, it was crucial that the protein employed was homogeneous to avoid interference by other binding sites with different affinities. Moreover, only soluble rPBPs, rather than refolded rPBPs, were employed. Immunological detection of free rPBP in solution (25 nM) indicated that less than 2 nM was detectable in solution.

The rates of exchange of bound protein and unbound protein on the surface of the decanol-coated tubes were examined using [^{3}H]-6*E*,11*Z*-16:diazoacetate photoaffinity-labeled Apol-3 rPBP as the reporter. Interestingly, 25 nM photolabeled PBP showed ca. 8% protein in solution at 1 h, with an extremely shallow rise to ca. 10% over 16 h at 4 °C (data not shown). The addition of 100 nM unlabeled PBP caused a temperature-dependent rise of ^{3}H-labeled PBP in solution over a 4-h period to 16% at 4 °C to 21% at 25 °C. Thus, less than 10% of the total PBP was free in solution, and the protein component of the assay experienced more rapid exchange with the surface coating than did the ligand.

The ligand specificity of the Apol-3 rPBP binding site was determined by competitive displacement of [^{3}H]-6*E*,11*Z*-16:Ac with a variety of pheromone analogs (Figure 4). The three pheromone components present in *A. polyphemus* and *A. pernyi* blends are 6*E*,11*Z*-16:Al, 6*E*,11*Z*-16:Ac, and 4*E*,9*Z*-14:Ac. Figure 4 clearly shows that these components differ in IC_{50} values for displacement over 1 or 2 orders of magnitude, with affinities for Apol-3 rPBP in the order 6*E*,11*Z*-16:Ac > 4*E*,9*Z*-14:Ac > 6*E*,11*Z*-16:Al. Moreover, the action of the sensillar esterase (Vogt et al., 1985) rapidly degrades the acetate to 6*E*,11*Z*-16:OH. This metabolite showed a 1000-fold lower affinity for the binding site under these assay conditions. The saturated analog 16:Ac (data not shown) bound with an affinity intermediate to the aldehyde and the alcohol metabolite. These data confirm

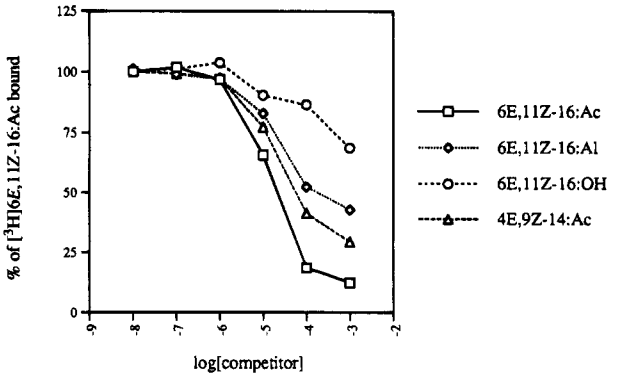

FIGURE 4: Competitive displacement of binding of 25 nM [^{3}H]-6*E*,11*Z*:16Ac to 25 nM rPBP by 6*E*,11*Z*:16Ac (□), 4*E*,9*Z*-14:Ac (△), 6*E*,11*Z*:16:Al (◇), and 6*E*,11*Z*-16:OH (○).

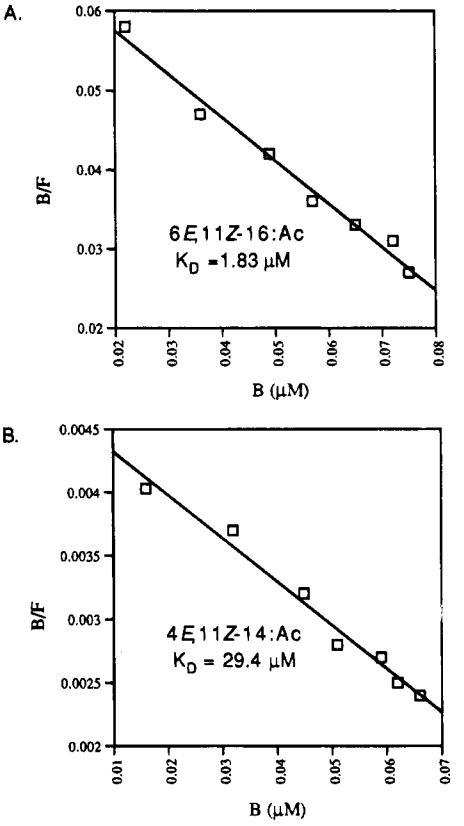

FIGURE 5: Scatchard analysis for binding of [^{3}H]-6*E*,11*Z*-16:Ac (A) and [^{3}H]-4*E*,9*Z*-14:Ac (B) to Aper-1 rPBP.

that functional group, ligand length, and double bonds all contribute to the specificity of the ligand–protein interaction.

The ligand specificities of the two *A. pernyi* proteins were determined for the binding of both pheromone components to Aper-1 rPBP and Aper-2 rPBP. Figure 5 shows the Scatchard analyses for Aper-1 with 6*E*,11*Z*-16:Ac and 4*E*,9*Z*-14:Ac. Figure 6 illustrates the same analyses for Aper-2. These data show quite clearly that Aper-1 has a 15-fold higher affinity for the 16-carbon pheromone, while Aper-2 has a 3.5-fold higher affinity for the 14-carbon pheromone. The dissociation constants for all rPBPs are summarized in Table 1.

DISCUSSION

Early attempts to measure the binding affinity for a pheromone in aqueous solution were frustrated by the low

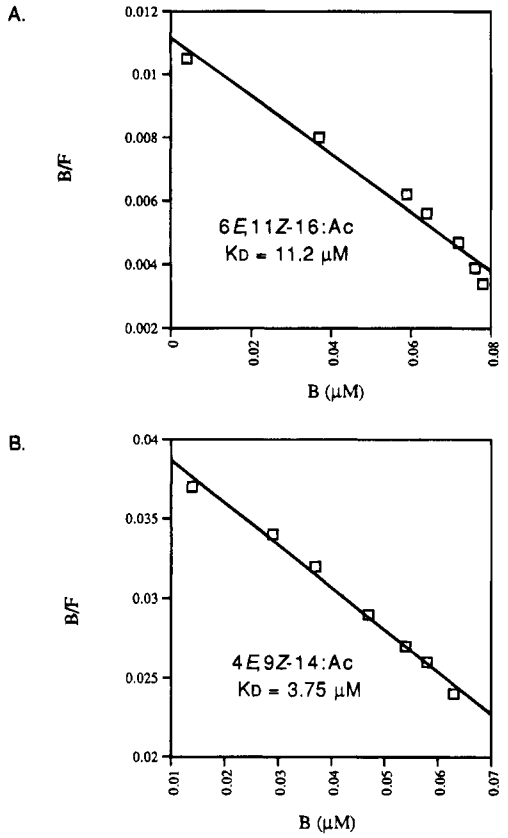

FIGURE 6: Scatchard analysis for binding of [^{3}H]-6*E*,11*Z*-16:Ac (A) and [^{3}H]-4*E*,9*Z*-14:Ac (B) to Aper-2 rPBP.

Table 1: Dissociation Constants (μM) for Binding of Pheromone Acetates to Apol-3, Aper-1, and Aper-2 Recombinant PBPs

	pheromone binding protein		
pheromone	Apol-3	Aper-1	Aper-2
6*E*,11*Z*:16:Ac	0.64	1.83	11.2
4*E*,9*Z*:14:Ac	21.0	29.4	3.75

solubility of long-chain acetates in water (estimated with [^{3}H]-11*Z*-16:Ac to be <10 nM) and by the tendency of the hydrophobic ligand to adsorb to the glass or plastic walls of the assay vessel (Prestwich, 1987). The addition of albumins did not solve this problem, nor did the use of poly(ethylene glycol) coating or silanization procedures. Native Apol-3 PBP was used to solubilize [^{3}H]-6*E*,11*Z*-16:Ac into an aqueous buffer from its adhesion to the glass vessel (De Kramer & Hemberger, 1987). Affinities and selectivities were determined with such an assay, and the K_D for 6*E*,11*Z*-16:Ac was estimated at 10^{-8}, but this assay might not accurately reflect true *in vivo* protein−ligand affinities.

A preliminary experiment showed that, by coating a plastic microfuge tube with 1-dodecanol, the hydrophobicity of the plastic was reversed. Thus, in a solution of ca. 50 nM [^{3}H]-6*E*,11*Z*-16:Ac in aqueous buffer, virtually all (>95%) of the pheromone was still dissolved. We rationalized this as follows: the alkyl chain of the 1-alkanol would adsorb to the vessel walls, as did the hydrophobic pheromones, while the hydroxy terminus would be displayed into the aqueous phase. Basically, this takes the liability of nonspecific binding to the surface and converts it into an asset. Interestingly, when rPBP was added, the tritiated pheromone was removed from the solution. This observation provided the basis for an extremely facile separation of bound and free ligands, since the PBP−pheromone complex adsorbed to the amphiphilic surface coating while the free ligand (and competitors) remained in solution. The exchange of [^{3}H]-pheromone (and, by inference, the exchange of the competing ligands) with the surface coating was negligible. In contrast, the bound and free proteins can undergo slow but measurable exchange during the course of the assay. Displacement of bound tritiated ligand by a competitor therefore should report the true binding affinity of the ligand for the PBP, although surface effects on PBP−ligand affinities cannot be ruled out in any binding assay methodology.

The assay was optimized with respect to the choice of alcohol used for the coating procedure, the incubation time to reach complete occupancy of binding sites, the temperature for maximal binding, and the concentrations of ligand and protein needed for determining dissociation constants and competitive IC_{50} values. 1-Decanol seemed to give the most reproducible data, that is, the spread of the replicate values was ±8% within and between assays. The selection of a 1-h incubation at 4 °C and protein and tritiated ligand concentrations of 25 nM proved to be the most advantageous for determining ligand specificity. This assay may be more generally applicable to the study of poorly water-soluble ligands with their cognate binding proteins.

The first rPBP studied with this new assay was Apol-3 rPBP, produced by bacterial expression and purified to homogeneity in milligram quantities (Prestwich, 1993a). Apol-3 rPBP had biochemical characteristics identical to those of native Apol-3 PBP isolated from *A. polyphemus* male antennae (Prestwich, 1993a). This PBP was previously employed to map the binding site for 6*E*,11*Z*-16:Ac by photoaffinity labeling with a photolabile analog, [^{3}H]-6*E*,11*Z*-16:Dza, followed by sequencing of covalently modified peptide fragments (Du et al., 1994). This work demonstrated a single site of covalent modification at Thr^{44}, close to a highly conserved Arg^{46}. Moreover, a second pheromone component, 4*E*,9*Z*-14:Ac, had been identified in the pheromone blend of *A. polyphemus*, and a corresponding responsive sensillar receptor neuron has been found (Kaissling, 1986; Bestmann et al., 1987). The photoaffinity analog for this component, [^{3}H]-4*E*,9*Z*-14:Dza, was synthesized (Ng, 1991) in order to determine its binding site in Apol-3 and other *Antheraea* PBPs. This 14-carbon pheromone analog covalently modified Apol-3 PBP during photoaffinity labeling, although the efficiency of covalent modification was slightly lower. Importantly, three covalently modified peptides were isolated by HPLC. The major site of modification was Asp^{32}, with Thr^{44} modification accounting for 25% of the labeling and an undetermined residue in the C-terminal Leu^{125}−Val^{142} accounting for about 15% of the labeling. The 14-carbon pheromone component thus had two possible binding positions within the recognition site, while only a single binding position was available to the 16-carbon pheromone (Du et al., 1994).

The proposed binding site of the two pheromone components in the pheromone binding site of Apol-3 rPBP is shown diagrammatically in Figure 7 (Prestwich & Du, 1995). The two competing binding interactions experienced by the 14-carbon pheromone component appeared to differ by approximately 0.7 kcal/mol, with the hydrophobic chain interaction dominating. This region of the protein primary sequence is the most hydrophobic domain in all PBPs and is predicted to have a helix−sheet−helix motif. The

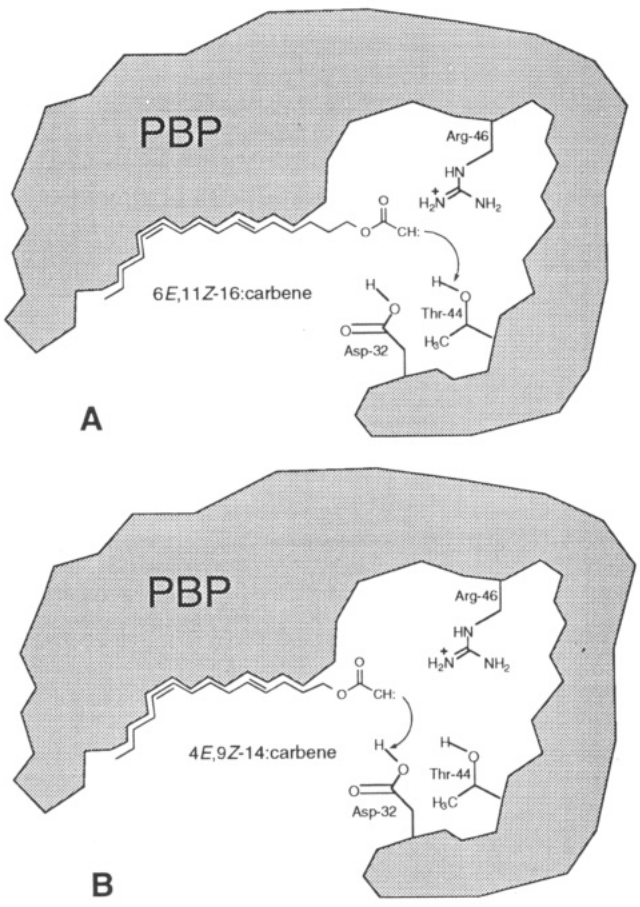

FIGURE 7: Binding of 6*E*,11*Z*-16:Ac and 4*E*,9*Z*-14:Ac to the Apol-3 rPBP binding site. Panel A: Binding interactions of 6*E*,11*Z*-16:carbene (derived from photolysis of the diazoacetate) with PBP mediated by Arg^{46} electrostatic interaction with ester carbonyl and by hydrophobic interaction with the alkadienyl hydrocarbon chain. Panel B: Preferred binding mode for 4*E*,9*Z*-14:Ac with PBP; hydrophobic interactions with the *E*,*Z*-tetradecadienyl chain position the carbene to interact with Asp^{32} [redrawn from Prestwich and Du (1995)].

sequence RXXGCAXXC is conserved in the primary sequences of all PBPs and GOBPs sequenced to date, including unpublished sequences for PBPs from *Lymantria dispar* and *Agrotis segetum* (G. Du and S. LaForest, personal communication). We reasoned that Apol-3 had evolved specificity for the 16-carbon pheromone; however, two confirmatory experiments were required. First, a direct demonstration that Apol-3 had different binding affinities for the two native pheromone molecules was needed. Second, evidence was needed for the existence of two proteins from the same moth species with different binding specificities for the two pheromone components. To this end, we expressed Aper-1 and Aper-2, two male-specific PBPs from *A. pernyi*, a species that employs different proportions of the same three pheromone components as *A. polyphemus* in its attractive blend.

The comparison of the amino acid sequences of Aper-1 and Aper-2 to that of Apol-3 is illustrated in Figure 8. Aper-1 is most similar to Apol-3, with 92% identity for 142 residues. Eight of the twelve changes (M5I, N10Q, V30I, L55M, D75E, T85S, L133V, and I135L) are highly conservative with respect to hydrophobicity, size, and charge. Importantly, both Aper-1 and Apol-3 show 15−28-fold higher affinity for 6*E*,11*Z*-16:Ac relative to 4*E*,9*Z*-14:Ac (Table 1). This suggests that these two proteins are "tuned" to the 16-carbon pheromone in these sister species.

In contrast, Aper-2 shows a 3.5-fold preference for the 14-carbon acetate over the 16-carbon component. *This is the first unambiguous demonstration that two PBPs in the same species have different affinities for two distinct pheromone components. Indeed, this is the first demonstration of odorant specificity encoded into any odorant binding protein.* Interestingly, Aper-1 shows 85% identity with the homospecific protein Aper-2 and 92% identity with the heterospecific protein Apol-3; Aper-2 shows 87% identity with Apol-3. The majority of the 21 differences between Aper-1 and Aper-2 are highly conservative, but the least conservative changes occur in two distinct regions (Figure 8): residues 9−13 (SQNFC in Aper-1 and CMNYG in Aper-2) and residues 84−89 (ASMAQQ in Aper-1 and DGMAHE in Aper-2). Three-dimensional structural studies of the pheromone−PBP complexes will likely implicate these hypervariable patches in the differentiation of chain length specificity.

Olfaction, the ability to sense and to discriminate among a large number of airborne molecules, plays an important role in the feeding and mating behaviors of most terrestrial animals (Pelosi & Maida, 1990). Earlier biochemical approaches led to the characterization of proteins capable of binding odorants, namely, pyrazine odorants, in vertebrates and pheromones in moths. OBPs in general, and PBPs specifically, may play active roles in the initial biochemical recognition step leading to the perireceptor events of odorant perception (Vogt, 1987). Support for this notion comes from

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

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

FIGURE 8: Comparison of amino acid sequences of Aper-1 and Aper-2 with that of Apol-3. Dashes (−) indicate identical residues in all three proteins. Asterisks (*) indicate identical residues for Apol-3 with Aper-1 only; number signs (#) indicate identical residues for Apol-3 and Aper-2 only.

the known microheterogeneity of DNA encoding such proteins in moths and the association of specific OBP subtypes with distinct classes of olfactory receptor neurons (Laue et al., 1994). Recently, multiple forms of vertebrate OBPs were characterized from the old-world porcupine, further supporting a discrimination function for odorant processing in the vertebrate nasal mucosa (Felicioli et al., 1993). Ultrastructural (Steinbrecht, 1992; Steinbrecht et al., 1992), developmental (Vogt et al., 1989), and electrophysiological studies (van den Berg & Ziegelberger, 1991) have established the cellular locations of PBPs and confirmed their importance in mediating pheromone detection by moths.

In conclusion, a novel binding assay has been presented for accurate and reproducible determination of binding affinities for essentially water-insoluble lipids to their specific binding proteins in aqueous solution. This assay has been used to demonstrate unambiguous evidence for ligand selectivity of recombinant pheromone binding proteins by displacement of tritium-labeled pheromone components by pheromone analogs and and by direct measurement of the dissociation constants for the pheromone components themselves. Moreover, this assay has been established that ligand specificity is indeed encoded in the structures of the proteins, that is, that unique PBPs have evolved to bind unique ligands. The corollary of this discovery is that the microdiversity of PBP structures could correspond to the number of pheromone components perceived. Such a result could not be obtained previously, since insect-derived preparations of PBPs contain often inseparable mixtures of PBP isoforms with different binding affinities. Finally, our data offer strong support for the notion that PBPs play an *active* role in pheromone perception at the molecular level, not merely acting as passive, nonselective carrier molecules. This active role may involve serving as selective filters for molecules relevant to a given sensillum or participating in protein-mediated ligand delivery to the putative G-protein-coupled receptors in the dendritic membrane.

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