Odorant Binding by a Pheromone Binding Protein: Active Site Mapping by Photoaffinity Labeling†

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ABSTRACT: The bacterially expressed recombinant pheromone binding protein (PBP) of *Antheraea polyphemus* was photoaffinity labeled with (6*E*,11*Z*)-[^{3}H]hexadecadienyl diazoacetate, a photoactivatable analog of the naturally occurring acetate pheromone. Radiolabeled peptides were separated from an endoproteinase Lys-C digestion by HPLC and characterized by Edman degradation. The label was exclusively found in the Asp^{39}–Lys^{58} fragment. Cleavage of this peptide (DDYVMTDRLAGCAINCLATK) with Arg-C gave a single radiolabeled peptide (DDYVMTDR), which was predicted to be α-helical. The adjoining LAGCAINCLATK fragment, which is highly conserved in PBP sequences, was predicted to be a hydrophobic β-strand and has been proposed to be important in recognition of the alkadienyl chain. Edman degradation confirmed the location of the covalently attached ligand at Thr^{44} of the smaller hydrophilic peptide. In addition, the synthesis of the newly identified pheromone component (4*E*,9*Z*)-tetradecadienyl acetate and its photoaffinity analog, (4*E*,9*Z*)-[^{3}H]tetradecadienyl diazoacetate, is also described. Mapping of PBP photoaffinity labeled by (4*E*,9*Z*)-[^{3}H]14:Dza revealed that the hydrophobic region Asp^{21}–Lys^{38} adjacent to the primary binding domain Asp^{39}–Lys^{58} contained a second modification site. The 14-carbon odorant molecule thus had two binding positions within the recognition site, while only a single binding position was available to the 16-carbon pheromone.

Pheromone binding proteins (PBPs)[1] form a subfamily (Vogt et al., 1991a; Krieger et al., 1993) of the insect odorant binding proteins (OBPs), in which ligand specificity is presumed to exist for the pheromone component(s) of the female-produced sex pheromone blend. These proteins, located in the sensillum lymph of the pheromone-responsive sensory hairs of the antennae of adult lepidopterans, mediate the delivery of hydrophobic sex pheromones to specific receptor proteins in the dendrite membrane (Prestwich, 1993a). PBPs may also facilitate removal of pheromone metabolites after signal transduction occurs (Vogt et al., 1985); indeed, equal affinities have been demonstrated for the pheromone acetate and its alcohol metabolite for the *Antheraea polyphemus* PBP (Prestwich, 1993b). To date, deduced protein sequences for five PBPs from four moth species and another five OBPs from three species have been described (Gyørgyi et al., 1988; Raming et al., 1989, 1990; Breer et al., 1990a; Krieger et al., 1991, 1993; Vogt et al., 1991b). PBPs and insect OBPs share highly conserved regions, including six conserved Cys residues. However, no direct evidence has been provided regarding the location of the pheromone or odorant binding site in any PBP or OBP.

The first high-yield expression and purification of a recombinant PBP (rPBP) was described recently (Prestwich, 1993b) for the Apo-3 PBP first characterized from the antennae of adult male *A. polyphemus* (Vogt & Riddiford, 1981). This 142-residue protein, which had been previously expressed in a potentially glycosylated form in baculovirus-infected insect cells (Krieger et al., 1992), was overexpressed in *Escherichia coli*, solubilized from inclusion bodies, and refolded into its pheromone-binding native form (Prestwich, 1993b). We now describe the first evidence for the location of the pheromone binding site in Apo-3 PBP using photoaffinity labeling by analogs of two pheromone components. For the 16-carbon analog, labeling occurs primarily within a hydrophilic domain adjacent to a highly conserved hydrophobic region recently suggested as a recognition site for the lipophilic hydrocarbon backbone of the pheromone (Krieger et al., 1993). The 14-carbon analog labels this position as a minor product, but exhibits a different primary site of covalent modification in a different peptide fragment. Thus, this shorter pheromone component was able to occupy two positions within the binding site.

EXPERIMENTAL PROCEDURES

Synthesis of Photoaffinity Analogs. (4*E*,9*Z*)-[9,10-^{3}H]-14:Dza was synthesized as shown in Scheme 1, with full experimental details provided in the supplementary material (Ng, 1990). This synthesis paralleled the earlier preparation of (6*E*,11*Z*)-[11,12-^{3}H]16:Dza (Ng, 1990; Prestwich et al., 1984). Both diazoacetate photolabels were obtained with specific activity ca. 40 Ci/mmol by selective reduction of alkynyl acetate precursors performed by Dr. D. G. Ahern (DuPont-New England Nuclear). Acetates were hydrolyzed and alcohols were converted to the diazoacetates as described (Prestwich, 1991a,b). Radiochemically pure materials were obtained by flash chromatography on a disposable column of

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[1] Abbreviations: PBP, pheromone binding protein; rPBP, recombinant PBP; HPLC, high-pressure liquid chromatography; (G)OBP, (general) odorant binding protein; (4*E*,9*Z*)-14:Dza, (*E*,*Z*)-4,9-tetradecadienyl diazoacetate; (6*E*,11*Z*)-16:Dza, (*E*,*Z*)-6,11-hexadecadienyl diazoacetate; TLC, thin-layer chromatography; FPLC, fast protein liquid chromatography; THP, tetrahydropyranyl; PPTS, pyridinium *p*-toluenesulfonate; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; Ac, acetate.

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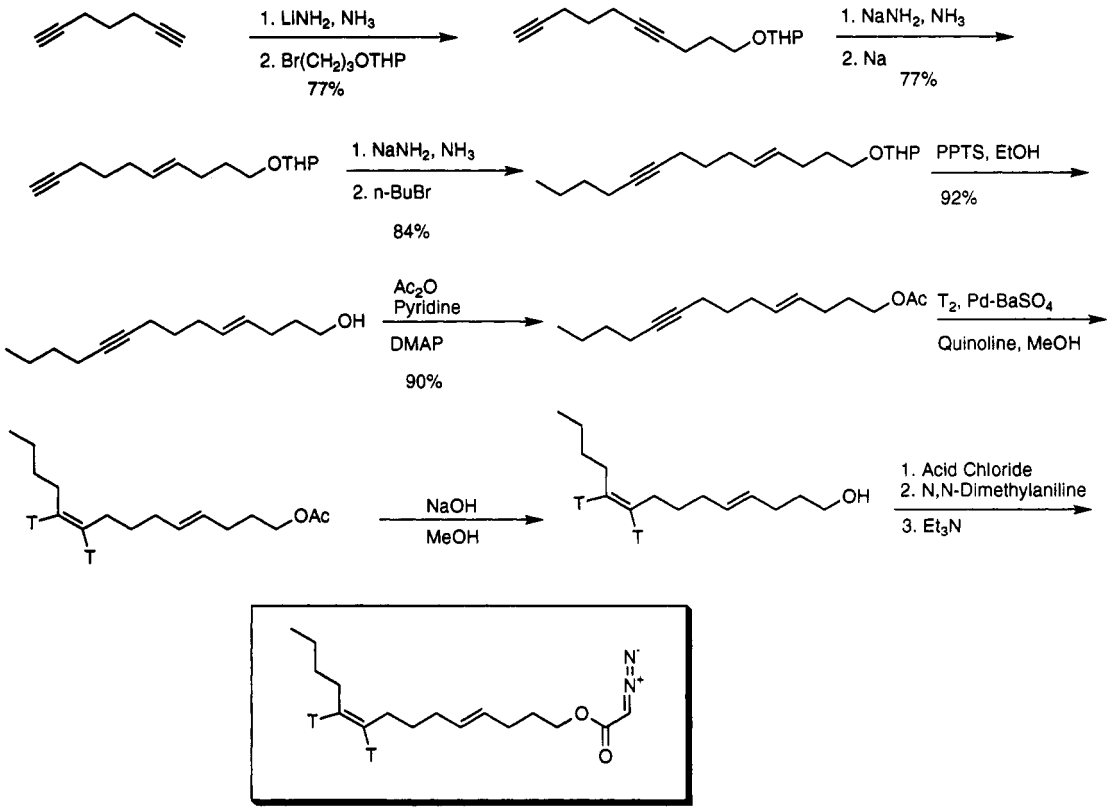
Scheme 1: Synthetic Route to (4*E*, 9*Z*)-[^{3}H]Tetradecadienyl Diazoacetate[a]



[a]Details are available as supplementary material.

silica gel with analysis by radio-TLC by fluorography of EnHance-sprayed TLC plates on X-ray film. Radiolabeled diazoacetates were stored at concentrations of 0.1–1.0 mCi/mL in 1:1 toluene–heptane solution at –20 °C (Prestwich, 1987, 1991a).

Photoaffinity Labeling of PBP. Recombinant PBP (rPBP) was expressed in XA-90 *E. coli* transformed by plasmid pGDP-Apo-3c. The PBP from inclusion bodies was solubilized, refolded, and purified by preparative isoelectric focusing and gel filtration as described previously (Prestwich, 1993b).

Photolabeling was performed as described previously (Prestwich et al., 1984; Prestwich, 1987, 1993b; Vogt et al., 1988). Briefly, PBP samples were incubated for 30 min at 4 °C in quartz tubes with 300 nM photolabel. This solution was prepared by addition of 2 μL of an ethanolic stock solution of (6*E*,11*Z*)-[11,12-^{3}H]hexadecadienyl diazoacetate [(6*E*,11*Z*)-[^{3}H]16:Dza] (specific activity, 40 Ci/mmol) or (4*E*,9*Z*)-[9,10-^{3}H]tetradecadienyl diazoacetate [(4*E*,9*Z*)-[^{3}H]14:Dza] to a solution of 300 nM PBP in 20 mM Tris-HCl buffer, pH 6.8, at 4 °C. The PBP-photolabel solutions were irradiated in a Rayonet reactor (6 lamps, 8 W each, 254 nm) for 40 s. The labeled rPBP was purified by ion-exchange chromatography on an FPLC Mono-Q column (Pharmacia).

Proteolytic Digestions. The purified, photolabeled PBP was denatured in 8 M urea and 0.4 M NH_4HCO_3, reduced with excess dithiothreitol, and then alkylated with iodoacetamide. Reduced and carboxamidomethylated PBP was recovered by ethanol precipitation. The photolabeled, carboxamidomethylated PBP was incubated with 0.1 molar equiv of endoproteinase Lys-C (Wako) in 20 mM Tris-HCl and 1 mM EDTA, pH 9.0, for 12 h at 30 °C. The enzyme was inactivated by adding 3 vol of 0.1% TFA. The digested peptides were lyophilized and then separated using reverse-phase HPLC. Arg-C (Boehringer Mannheim) digestion of peptides was performed using a 1:50 ratio of enzyme to substrate by weight in 90 mM Tris-HCl, 8.5 mM $CaCl_2$, 5 mM DTT, and 0.5 mM EDTA, pH 7.6, for 18 h at 37 °C. The reaction was quenched by freezing and lyophilization.

HPLC Purification and Radioassay. The digested peptides were separated by reverse-phase HPLC on a 4.6 mm × 22.5 cm Aquapore RP-300 C_{18} column (Applied Biosystems, Inc. (ABI)). A linear gradient of 0.1% TFA in water to 0.1% TFA in 80% CH_3CN was employed with a flow rate of 0.8 mL/min. The eluate was monitored for absorbance at 220, 254, and 280 nm. Aliquots from collected fractions were analyzed for total radioactivity by liquid scintillation counting (see below).

Protein Sequence Analysis. The radiolabeled peptides were sequenced by Edman degradation using an ABI Model 475A pulsed liquid phase sequencer. Thus, HPLC fractions containing the modified peptides were neutralized with 4.7 μL of 30% ammonium hydroxide per 0.2 mL of sample. The solution was placed in Immobilon-CD Ultrafree devices (Millipore Corp.) and centrifuged in a Sorvall RC-3 centrifuge (800 rpm, 15 min). The Ultrafree device was dried (Savant SpeedVac), and the peptide-containing Immobilon-CD membrane was trimmed and placed in the ABI blot cartridge for sequencing. The PTH-amino acid from each sequencing cycle was eluted from the blotted membrane with *n*-butyl chloride and then lyophilized. The samples were redissolved in DMSO, and radioactivity was measured using an LKB Model 1218 liquid scintillation counter with Scintiverse II (Fisher).

RESULTS

Two photoaffinity analogs were synthesized in radiochemically homogeneous form following a common synthetic scheme (Ng, 1990). The preparation of (6*E*,11*Z*)-[^{3}H]16:Dza followed the procedure of Prestwich et al. (1984). An analogous preparation of lower homolog, (4*E*,9*Z*)-[^{3}H]14:Dza, was accomplished as shown in Scheme 1. Thus, the lithium

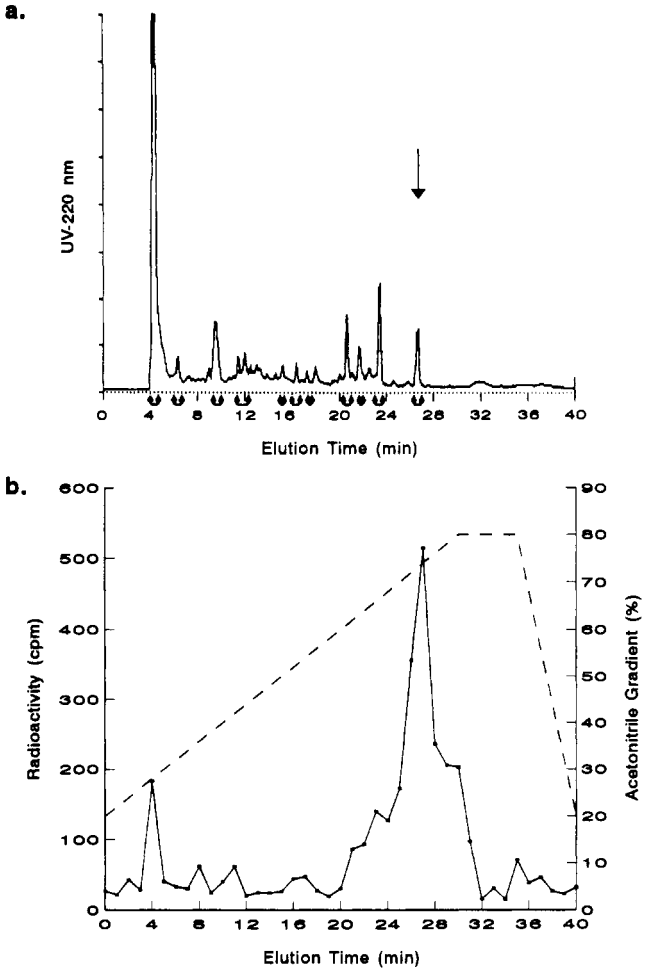


FIGURE 1: HPLC separation of Lys-C-digested peptides from Apo-3 rPBP photolabeled with the ligand (6*E*,11*Z*)-[^{3}H]16:Dza. Panel a, purification of peptides on a reverse-phase HPLC Aquapore RP-300 C_{18} column (see text for details); panel b, radioactivity (cpm) in aliquots of HPLC fractions. The major peak at 27 min (arrow) contains the purified radiolabeled peptide that was sequenced.

acetylide of 1,6-heptadiyne was prepared in liquid ammonia and alkylated in 77% yield with the tetrahydropyranyl ether of 3-bromopropanol. Sodium reduction of the monosodium acetylide salt, followed by alkylation with bromobutane, gave the enyne ether in 58% yield (two steps). Deprotection followed by acetylation gave the (4*E*)-tetradec-9-yn-1-yl acetate; semihydrogenation with quinoline-poisoned Pd/$BaSO_4$ in methanol gave the unlabeled (4*E*,9*Z*)-14:Ac in 97% yield. The radiosynthesis was accomplished by tritium gas reduction of the enyne acetate in methanol with quinoline-poisoned Pd/$BaSO_4$. The tritium-labeled dienyl acetate was purified by argentation silica gel column chromatography to give the desired (4*E*,9*Z*)-[^{3}H]14:Ac. The labeled dienyl acetate was hydrolyzed with methanolic potassium carbonate and then converted to the diazoacetate with the acid chloride of glyoxylic acid tosylhydrazone in methylene chloride solution using *N*,*N*-dimethylaniline for ester formation followed by triethylamine to complete the diazotization.

The purified rPBP was photoaffinity labeled under conditions selected to optimize covalent modification of the protein without causing nonspecific attachment of the photolabel to sites other than the true pheromone binding site. The photoaffinity-labeled rPBP was purified by MonoQ FPLC and then reduced and carboxamidomethylated prior to subsequent proteolytic digestions. Since the six cysteines in the Apo-3 PBP sequence are involved in three disulfide linkages (Krieger et al., 1993; Prestwich, 1993b), reduction and carboxamidomethylation reactions were necessary to complete the enzymatic digestions. Reduced and carboxamidomethylated rPBP was recovered by ethanol precipitation from solutions containing detergents, denaturants, and organic solvents.

The (6*E*,11*Z*)-[^{3}H]16:Dza-labeled rPBP was digested with endoproteinase Lys-C under conditions that gave complete cleavage. Separation of fragments by reverse- phase HPLC gave a single major peak of radioactivity (Figure 1). As anticipated from the additional hydrophobicity imparted by the covalently attached radioligand, the modified radioactive peptides had longer retention times than the unmodified fragments. This fraction was sequenced by Edman degradation from Immobilon-CD bound peptide, and the sequence of this fragment established its identity as the Lys-C fragment Asp^{39}–Lys^{58} (Figure 3a).

The Lys-C fragment DDYVNTDRLAGCAINCLATK, containing the binding site of the photoaffinity ligand, was cleaved by endoproteinase Arg-C, and the two fragments were separated by reverse-phase HPLC (Figure 2). A single labeled peptide was purified, and its shorter HPLC retention time suggested that the labeling occurred in the more hydrophilic

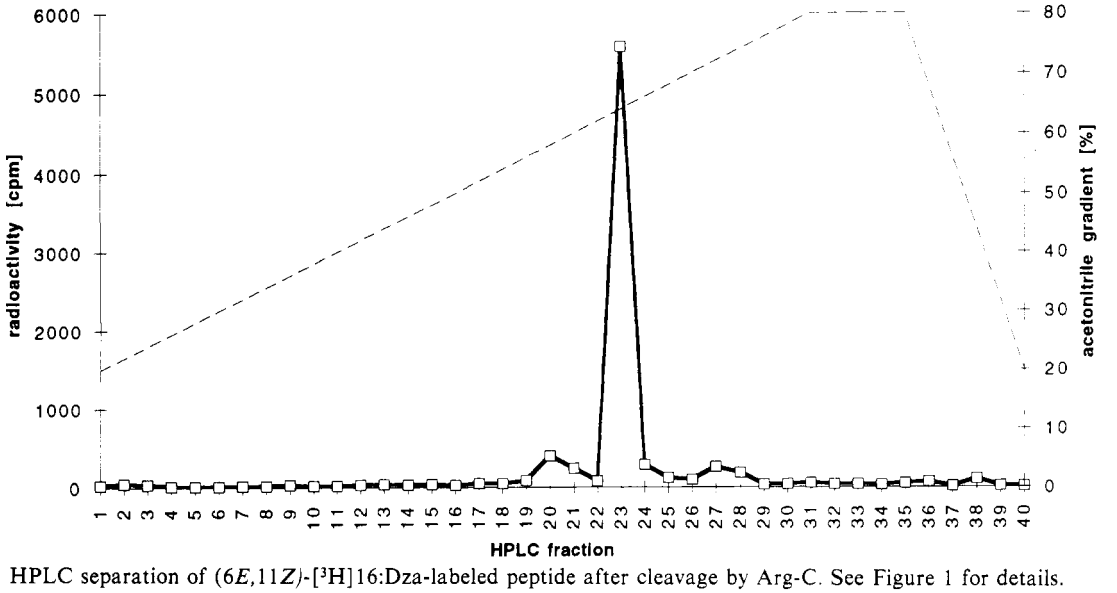


FIGURE 2: HPLC separation of (6*E*,11*Z*)-[^{3}H]16:Dza-labeled peptide after cleavage by Arg-C. See Figure 1 for details.

a. Edman degradation

Cycle No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AA	D	D	Y	V	M	T	D	R	L	A	G	-	A	I	N	-	L	-	-	-
Apo-3	D	D	Y	V	M	T	D	R	L	A	G	C	A	I	N	C	L	A	T	K

b. Radiosequencing

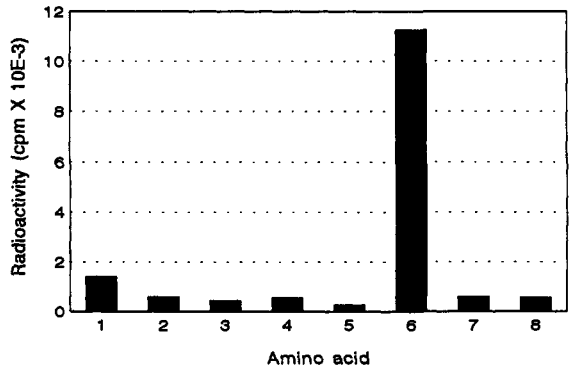


FIGURE 3: Amino acid sequence (Edman degradation) and radioassay of eluted PTH-amino acids for two peptides from (6*E*,11*Z*)-[^{3}H]-16:Dza-labeled rPBP. Panel a, sequence of the radiolabeled Lys-C fragment; panel b, radioactivity of each cycle from Edman degradation sequencing of the labeled Arg-C fragment.

fragment, i.e., DDYVMTDR. This was confirmed by Edman degradation; the products collected from the sequencer at each cycle were redissolved in 1-chlorobutane, and the radioactivity was measured using a liquid scintillation counter. The sixth cycle showed 20 times higher radioactivity than all the other fractions (Figure 3b). From the sequence, this residue corresponded to Thr^{44}. Thus, Thr^{44} has been identified as the unique, covalently modified residue in (6*E*,11*Z*)-16:Dza-labeled PBP.

A similar photolabeling protocol gave rPBP labeled with (4*E*,9*Z*)-[^{3}H]14:Dza. This substrate was digested by Lys-C and separated by HPLC under the same conditions (Figure 4). Three peaks of radioactivity were obtained. Edman degradation of each fragment was performed. The major radioactive Lys-C fragment was assigned to Asp^{21}–Lys^{38}; the second peak corresponded to the same Asp^{39}–Lys^{58} fragment labeled by the 16-carbon homolog; and the peak at 35 min was established as the C-terminal peptide, Leu^{125}–Val^{142}. For the major peak, the radioactivity of each sequence cycle was measured, and the maximum labeling was detected in the 12th cycle, corresponding to modification of the residue Asp^{32} (Figure 5).

DISCUSSION

An understanding of the molecular recognition processes in soluble odorant binding proteins (OBPs) is a necessary prelude for comprehending the sequential events leading to the activation of the odorant receptor-coupled, G-protein-mediated signal transduction cascade in olfaction (Boekhoff et al., 1993; Breer et al., 1990b; Raming et al., 1993; Prestwich, 1993a). OBPs are small and water-soluble, and they are uniquely present in olfactory tissues of vertebrates and insects (Pelosi & Maida, 1990; Pevsner et al., 1988; Pevsner & Snyder, 1990). In contrast to the OBPs of vertebrates that recognize a large number of odorants of diverse structure, insect OBPs possess odorant specificities for chemical structures and terminal functionalities. The insect general odorant binding proteins (GOBPs) (Vogt et al., 1991a,b; Breer et al., 1990a), which are expressed in both male and female antennae, showed over 95% conserved amino acid sequence among lepidopterous species. In contrast, the male-specific antennal PBPs showed highly conserved overall structures with 35–65% sequence identities, but retained significant interspecific differences. It has been believed that this macromolecular diversity may correspond to the diversity of small molecules in the structures of lepidopteran pheromones. Overall, pheromone perception may provide a valuable model system to study how specific unsaturated straight-chain fatty acid derivatives could be distinguished by proteins.

The Apo-3 PBP consists of 142 amino acid residues and has an isoelectric point, p*I*, of 4.80. Interestingly, pheromone binding was only observed between pH 6.0 and 9.0, well above

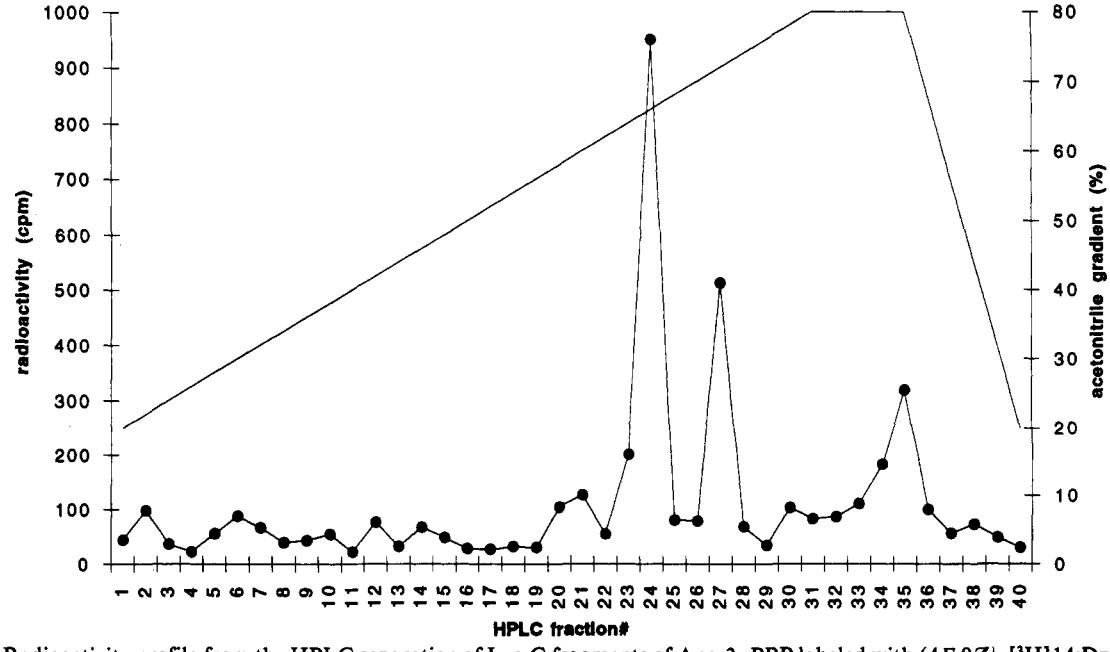


FIGURE 4: Radioactivity profile from the HPLC separation of Lys-C fragments of Apo-3 rPBP labeled with (4*E*,9*Z*)-[^{3}H]14:Dza. See Figure 1 for details.

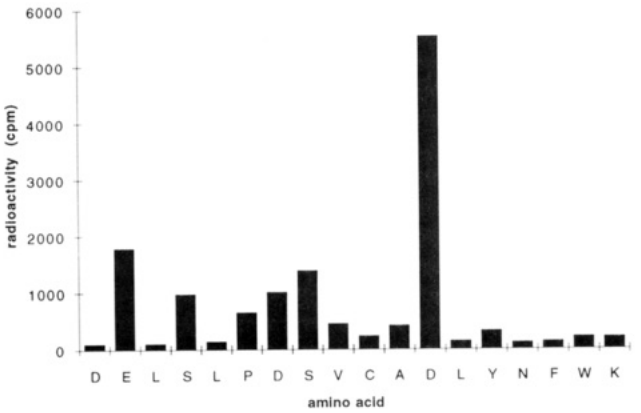


FIGURE 5: Amino acid sequence (Edman degradation) and PTH amino acid radioassay for the major (24 min) Lys-C fragment of (4*E*,9*Z*)-[^{3}H]14:Dza-labeled rPBP.

the p*I* value for the apoprotein (Prestwich, 1993b). The hydrophobicity profile of Apo-3 PBP showed a typical pattern of hydrophobic and hydrophilic domains for OBPs (Raming et al., 1989; Figure 6). The predicted secondary structure, when compared with PBPs of other species (Krieger et al., 1993), provided key information for the tentative assignment of particular regions as participants in the active odorant recognition site.

Mapping of the binding site by microsequencing analysis of the major radiolabeled peak from Lys-C digestion of photoaffinity-labeled rPBP revealed that Asp^{39}–Lys^{58} contained the covalent attachment site. This region appeared to be the major hydrophobic domain in all insect antennal binding proteins, and appeared to comprise a helix–sheet–helix motif (Krieger et al., 1993). On the basis of a comparison of the primary sequences of three classes of OBPs (Figure 7), the hydrophobic domains containing amino acids 39–58 are highly conserved in GOBPs, but show certain variations in PBPs. This is consistent with the notion that PBPs associate principally with species-specific sex pheromones, i.e., that each species would have a slightly different PBP for each different pheromone structure. In contrast, the GOBPs of the same class are virtually identical, suggesting that they may interact with the general odorants, e.g., plant volatiles, of common importance among moth species. It was proposed that the hydrophobic domain contained within the amino acid fragment Asp^{39}–Lys^{58} could comprise the binding pocket for "general" odorants as well as the binding pocket for specific pheromones (Krieger et al., 1993).

OBPs and PBPs may play active roles in the initial biochemical recognition step leading to the perireceptor events of odorant perception (Prestwich, 1993a). Support for this notion comes from the known microheterogeneity of DNA encoding such proteins in moths (Krieger et al., 1991) and the association of specific OBP subtypes with distinct classes of olfactory receptor neurons (Vogt et al., 1991a). Recently, multiple forms of vertebrate OBPs were characterized from rats by cDNA cloning (Dear et al., 1992) and by analysis of olfactory proteins from the Old World porcupine (Felicioli et al., 1993), further supporting a discrimination function for odorant processing in the vertebrate nasal mucosa. Ultrastructural (Keil & Steiner, 1991; Steinbrecht, 1993), immunocytochemical (Maida et al., 1993), developmental (Vogt et al., 1989), and electrophysiological studies (Van der Berg &

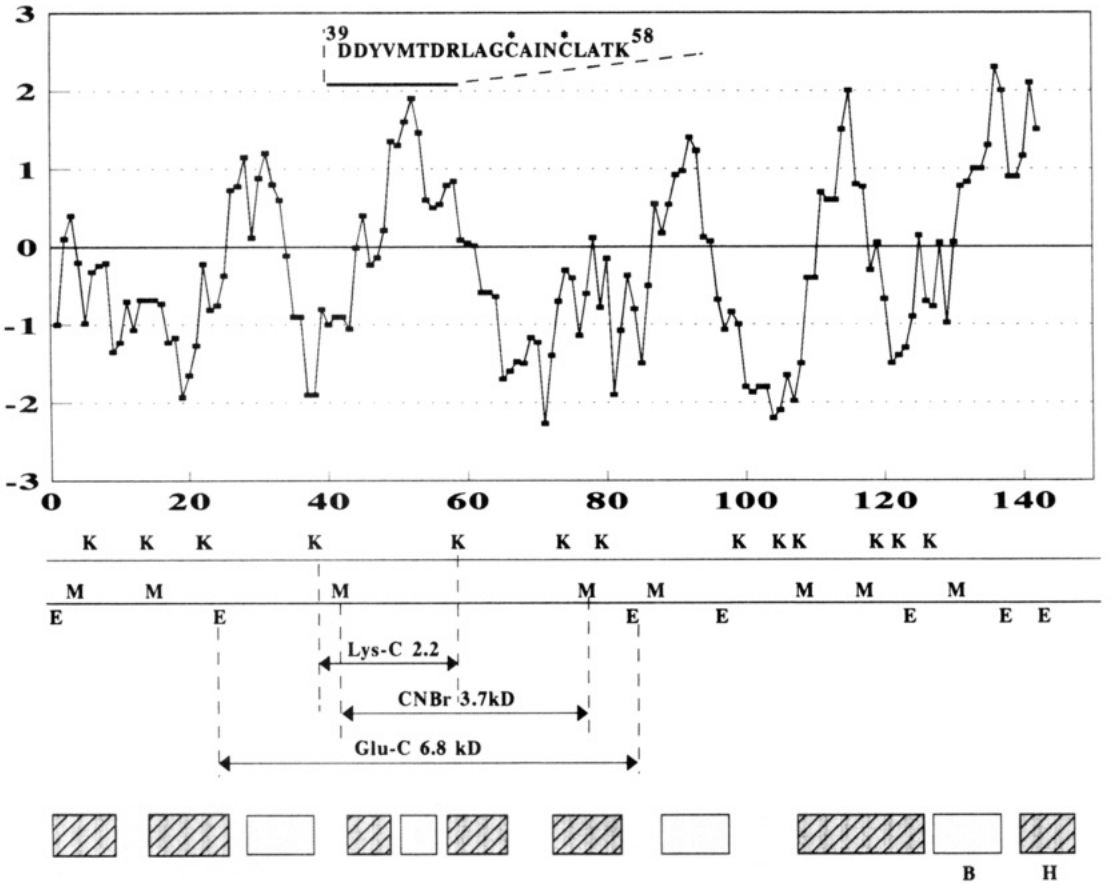


FIGURE 6: Peptide map for Apo-3 PBP. Hydrophobicity was predicted by the Kyte–Doolittle algorithm; positive values indicate hydrophobicity, and negative values indicate hydrophilicity. Secondary structure was predicted by the Chou–Fasman method. H corresponds to an α-helical structure and B indicates β-strand regions. The locations of Lys (K), Glu (E), and Met (M) are shown, and the location and amino acid sequence of the radioactive peptide Asp^{39}–Lys^{58} obtained from the (6*E*,11*Z*)-[^{3}H]16:Dza-labeled rPBP are indicated.

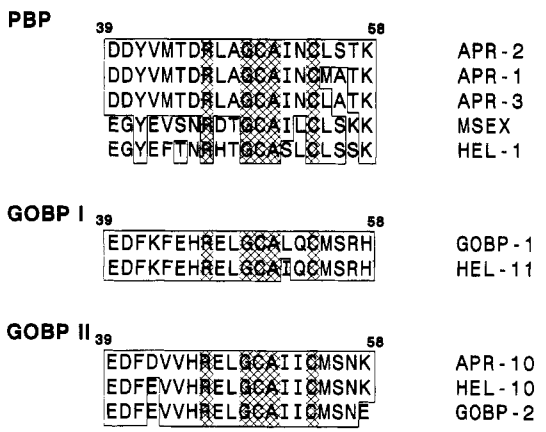


FIGURE 7: Sequence analysis of the hydrophobic domains (amino acids 39–58) of the three classes of insect odorant binding proteins in various species: PBP, pheromone binding proteins; GOBP, general odorant binding proteins. Boxed residues indicate identical amino acids within a single class. Amino acid identities among the three classes are indicated by shaded columns. Domains: APR-1 (Raming et al., 1990) and APR-2 from *A. perneyi* (Krieger et al., 1991); Apo-3 from *A. polyphemus* (Raming et al., 1989); MSEX from *Manduca sexta* (Gyørgyi et al., 1988); HEL-1, HEL-10, and HEL-11 from *Heliothis virescens* (Krieger et al., 1993); GOBP-1 and GOBP-2 from *M. sexta* (Vogt et al., 1991b); APR-10 from *A. perneyi* females (Breer et al., 1990).

Ziegelberger, 1991) have established the cellular locations of PBPs and have confirmed their importance in mediating pheromone detection by moths. What remains unknown is the extent to which PBPs are involved in odorant discrimination and in odorant recognition at the putative dendritic membrane receptor protein.

Photoaffinity labeling allows identification of specific amino acids in the ligand binding site of a protein. The covalently modified amino acids are presumed to be proximal to the reactive moiety of the photoaffinity analog when it is properly situated in the binding site. PBPs from several species have been characterized using photoaffinity analogs of pheromones (Prestwich, 1991b). In the *A. polyphemus* PBP described herein, we have taken advantage of the ligand specificity of the diazoacetate analogs of two pheromone components to achieve covalent attachment of the photoaffinity analogs to the pheromone binding site of pheromone. Subsequently, we could obtain and sequence covalently modified peptide fragments of the PBP, which permitted the identification of the amino acids involved in the recognition of the acetate functional group for these two analogs.

The initial proteolytic digestion of the (6*E*,11*Z*)-[^{3}H]16:Dza-labeled rPBP produced a single radiolabeled Lys-C fragment, ^{39}DDYVMTDRLAGCAINCLATK^{58}. Further digestion of this fragment with endoproteinase Arg-C, which cleaves the peptide on the carboxyl side of the arginine, gave two fragments. The hydrophilic subfragment DDYVMTDR was found to contain the radiolabeled residue. This peptide was predicted to be an α-helix and possessed a number of nucleophilic residues that could be involved in interaction of the acetyl group of the pheromone molecule with the protein. Moreover, the hydrophobic subfragment LAGCAINCLATK was predicted to consist of a highly conserved β-sheet region (Figures 6 and 7), and it appeared likely that such a region would participate in forming the hydrophobic binding pocket necessary for recognition of the lipophilic, alkene-rigidified backbones of the pheromone molecules (Krieger et al., 1993). The site of covalent modification was then determined by microsequencing of the labeled peptide DDYVMTDR with concomitant measurement of the radioactivity of each fraction collected from the Edman reaction chamber. While the other cycles showed background levels, the sixth cycle showed a clear peak of radioactivity (11 000 cpm) corresponding to the threonine in position 44 (Figure 4). Thus, Thr^{44} was confirmed as the active site residue that was modified by the carbene produced during photolysis. Although a three-dimensional structure from either X-ray or NMR experiments is not yet available, some important conclusions may be reached on the basis of comparisons of the primary structures of the PBP family (Figure 7) and on the basis of observations of other lipid binding proteins of known structure (Zhang et al., 1992; Petrou et al., 1993). In particular, the unique, conserved Arg^{46} that is proximal to the modified Thr residue is well-positioned to be involved in recognition of the ester carbonyl group of pheromone acetates.

A second acetate pheromone component, (4*E*,9*Z*)-14:Ac, has been identified in the pheromone blend of *Antheraea perneyi*, and corresponding responsive sensillar receptor neurons have been found in both *A. polyphemus* and *A. perneyi* (Bestmann et al., 1987). The photoaffinity analog for this component, (4*E*,9*Z*)-[^{3}H]14:Dza, was synthesized (Ng, 1990) in order to probe the existence of a second member of the *A. polyphemus* PBP family in the sensillum lymph. The 14-carbon pheromone analog covalently modified Apo-3 rPBP during photoaffinity labeling, although the efficiency of covalent modification was slightly lower than for the 16-carbon pheromone analog. As observed for the labeling of rPBP by (6*E*,11*Z*)-[^{3}H]16:Dza, increasing levels of competitive displacement were observed with 10–300-fold molar excesses of both (6*E*,11*Z*)-16:Ac and (4*E*,9*Z*)-14:Ac. This data supported the notion that both pheromone components (and their photolabile analogs) were specifically recognized at the same ligand binding site.

Proteolysis of the (4*E*,9*Z*)-[^{3}H]14:Dza-modified PBP was performed by following the procedures developed for the longer pheromone analog. In this case, endoproteinase Lys-C digestion resulted in the appearance of three radioactive peptide fragments (Figure 4). Each of the three was sequenced by Edman degradation, revealing three separate regions of covalent modification of the PBP by this ligand. The peptide bearing the highest degree of labeling corresponded to Asp^{21}–Lys^{38}, that is, just "upstream" from the region labeled by the 16-carbon analog. Microsequencing and radioassay of the eluted amino acid derivatives confirmed that Asp^{32} had been covalently modified. The two minor sites included the Asp^{39}–Lys^{58} region and the C-terminal region, Leu^{125}–Val^{142}.

On the basis of these data, we propose a model to rationalize the binding of the two pheromone components in the pheromone binding site of Apo-3 PBP (Figure 8). We postulate that the ester carbonyl of the acetate functionality of the pheromone forms a hydrogen bond with the protonated guanidinium group of Arg^{46}. This "anchors" the hydrophilic head of the pheromone in the hydrophilic helical region of the PBP and allows the hydrophobic hexadecadienyl chain to properly associate with the hydrophobic sheet in the binding site. Photolytic production of the reactive carbene occurs several angstroms away from the Arg^{46}–carbonyl interaction; thus the 16-carbon ligand (6*E*,11*Z*)-[^{3}H]16:Dza generates a carbene in the binding site that could covalently modify Thr^{44} in two ways: (i) by direct insertion into the O–H bond of Thr^{44} or (ii) by nucleophilic trapping of a rearranged ketene by the same hydroxyl group of the Thr^{44} side chain. The 14-carbon ligand [(4*E*,9*Z*)-[^{3}H]14:Dza] is approximately 2.6 Å shorter in its extended conformation. As a result of the deletion of two methylene units between the (diazo)acetate and the first double bond, this ligand may have two competing

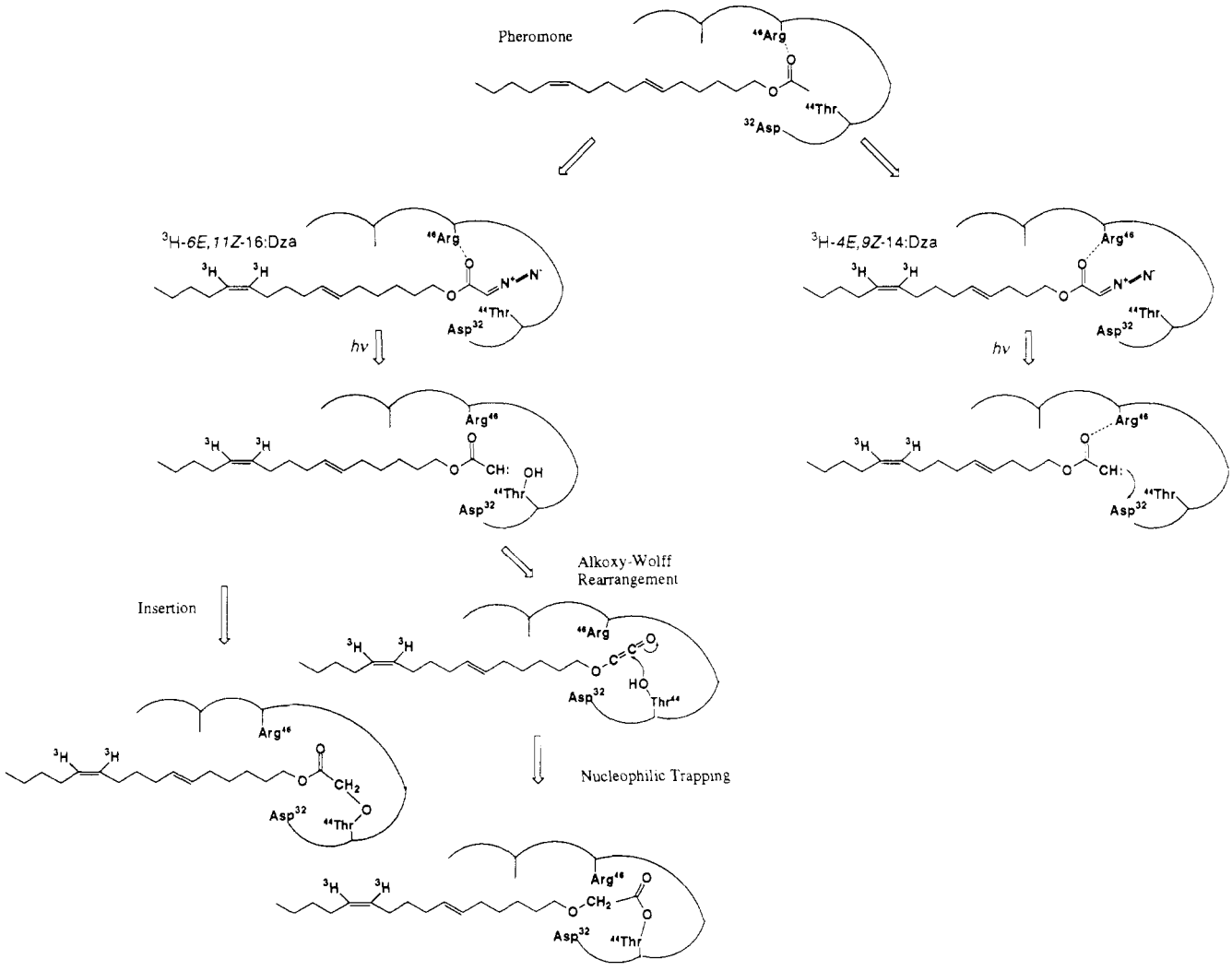


FIGURE 8: Proposed mechanism for photoaffinity labeling by (6*E*,11*Z*)-[^{3}H]16:Dza and (4*E*,9*Z*)-[^{3}H]14:Dza.

binding interactions that can determine its position in the binding site. On one hand, it may possess the Arg^{46}–ester carbonyl interaction to anchor the acetate head group in the binding site; this mode does not optimally satisfy the recognition of the hydrocarbon chain. In the photolabeling experiment, this would result in modification of Thr^{44}. An alternative binding mode, which optimizes the recognition of the *E*,*Z*-diene and the positioning of the terminal butyl group, would locate the reactive carbene derived from photolysis of the diazoacetate in a different position. In this alternative binding mode, a different amino acid, Asp^{32}, would be modified. Apparently, both Asp^{32} and Thr^{44} are located in the pheromone binding pocket, perhaps within 2.6 Å of one another and in close proximity to the carbene groups generated upon diazoacetate photolysis. Since the PBP serves to bind and solubilize the extremely hydrophobic odor molecules and facilitate transport of pheromones into and through the aqueous environment around olfactory receptor dendrites, the hydrophobic domains of PBPs are believed to account for the specific recognition of the pheromone backbone. The seven putative helical domains in PBP (Krieger et al., 1993) may fold to form a central hydrophobic pocket for pheromone binding. Moreover, it is possible that receptor activation requires recognition of a pheromone-PBP complex for signal transduction to occur. Evidence has been reported for microheterogeneity in PBPs from moths with multiple pheromone components (Krieger et al., 1991; Prestwich, 1991c, 1993a; Vogt et al., 1989; Vogt & Prestwich, 1987). Thus, we propose that a second PBP with a higher binding specificity for the 14-carbon pheromone should be present in the *A. polyphemus* moth antenna and should be important for blend recognition.

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SUPPLEMENTARY MATERIAL AVAILABLE

Synthesis protocol and spectroscopic data relevant to the synthesis of the pheromone analog (4*E*,9*Z*)-[^{3}H]tetradecadienyl diazoacetate (11 pages). Ordering information is given on any current masthead page.

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