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Bacterial expression and photoaffinity labeling of a pheromone binding protein

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Abstract

The first high-level production of a binding-active odorant binding protein is described. The expression cassette polymerase chain reaction was used to generate a DNA fragment encoding the pheromone binding protein (PBP) of the male moth *Antheraea polyphemus*. Transformation of *Escherichia coli* cells with a vector containing this construct generated clones which, when induced with isopropyl β-D-thiogalactopyranoside, produced the 14-kDa PBP in both the soluble fraction and in inclusion bodies. Purification of the soluble recombinant PBP by preparative isoelectric focusing and gel filtration gave >95% homogeneous protein, which was immunoreactive with an anti-PBP antiserum and exhibited specific, pheromone-displaceable covalent modification by the photoaffinity label [^{3}H]6*E*,11*Z*-hexadecadienyl diazoacetate. Recombinant PBP was indistinguishable from the insect-derived PBP, as determined by both native and denaturing gel electrophoresis, immunoreactivity, and photoaffinity labeling properties. Moreover, the insoluble inclusion body protein could be solubilized, refolded, and purified by the same procedures to give a recombinant PBP indistinguishable from the soluble PBP. Proton NMR spectra of the soluble and refolded protein provide further evidence that they possess the same folded structure.

Keywords: *Antheraea polyphemus*; expression cassette PCR; Lepidoptera; olfactory signal transduction; pheromone binding protein; pheromone perception; photoaffinity labeling; protein refolding

Insects offer an ideal model system for studying the molecular details of olfaction. Mate-seeking behavior in moths (Insecta, Lepidoptera) is under strong selection pressure, and each moth species responds with exquisite sensitivity to a limited number of fatty acid-derived pheromones. Perception of the female sex pheromone by male moths is mediated by proteins located in the sensory hairs of the antennae (Vogt, 1987; Vogt et al., 1990b; Prestwich, 1992). These include soluble pheromone binding proteins (Vogt & Riddiford, 1981), pheromone degrading enzymes (Vogt et al., 1985), putative membrane-associated pheromone receptors (Vogt et al., 1988), and several membrane-associated transductory proteins (Breer et al., 1990a) involved with G-protein-mediated activation of a phospholipase C (Boekhoff et al., 1990) and a putative inositol trisphosphate-gated ion channel (Breer et al., 1990b). Among these proteins, the PBPs are the most abundant, and are most likely to be involved in solubilization and transport of the lipophilic pheromone in the sensillum lymph. The availability of milligram quantities of recombinant PBPs for structural studies would enable investigators to gain new chemical insight into how proteins can discriminate among odorants on the basis of chain length, functional group, and alkene geometry. In addition, the functional role of these so-called transport proteins in transduction of an olfaction signal could also be addressed.

Recently, PBPs have been cloned and sequenced from male antennae of the moths *Antheraea polyphemus* (Raming et al., 1989), *Antheraea pernyi* (Raming et al., 1990), *Manduca sexta* (Gyørgi et al., 1988), and *Heliothis virescens* (H. Gänßle, J. Krieger, K. Raming, & H.

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Abbreviations: Apo-3, *Antheraea polyphemus* cDNA clone or expressed protein; Apr-1, *Antheraea pernyi* cDNA clone or expressed protein; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CTAB, cetyl trimethylammonium bromide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ECPCR, expression cassette PCR; GdnHCl, guanidinium hydrochloride; GF, gel filtration buffer, 10 mM Tris HCl, 100 mM NaCl, 1 mM EDTA, pH 7.2; GOBP, general odorant binding protein; IEF, isoelectric focusing; IPTG, isopropyl β-D-thiogalactopyranoside; LBA, 10 g tryptone, 5 g yeast extract, 10 g NaCl, 100 mg ampicillin/L; OBP, odorant binding protein; PBP, pheromone binding protein; PCR, polymerase chain reaction; rPBP, recombinant PBP; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBE, 89 mM Tris-borate, 2 mM EDTA, pH 8.0; TE, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Breer, unpubl.), and it is clear that they form a new family of genes encoding lipid transporting proteins with substantial allelic microheterogeneity even in a single species (Raming et al., 1991). In addition, a new class of female antennal-specific proteins, the general odorant binding proteins, also belong to this family as demonstrated by cDNA sequencing (Breer et al., 1990c) and N-terminal protein sequencing (Vogt et al., 1990a). Although this family of proteins has no significant sequence similarity to the odorant binding proteins from vertebrates (Pevsner et al., 1988) or the homologous insecticyanin from caterpillars (Riley et al., 1984), it is conceivable that these families of proteins are structurally analogous, representing evolutionary convergence to a common domain structure. In order to test this hypothesis and to further explore the binding kinetics and physiological role of the PBPs, access to recombinant PBP was required. The baculovirus-mediated expression of an *A. pernyi* cDNA (Apr-1) in Sf9 cells (Krieger et al., 1992) produced modest levels of protein which, although biochemically active as a PBP, appeared to be modified by glycosylation relative to the native protein. In order to scale up protein expression and to obtain substantial quantities of isotopically labeled protein for 3D NMR analysis, a bacterial expression system was required. This paper describes the use of expression cassette polymerase chain reaction (MacFerrin et al., 1990) to engineer a bacterial overproducer (Schreiber & Verdine, 1991) of the *A. polyphemus* Apo-3 PBP, and documents that both the soluble and refolded recombinant PBP are indistinguishable from the native PBP isolated from moth antennae.

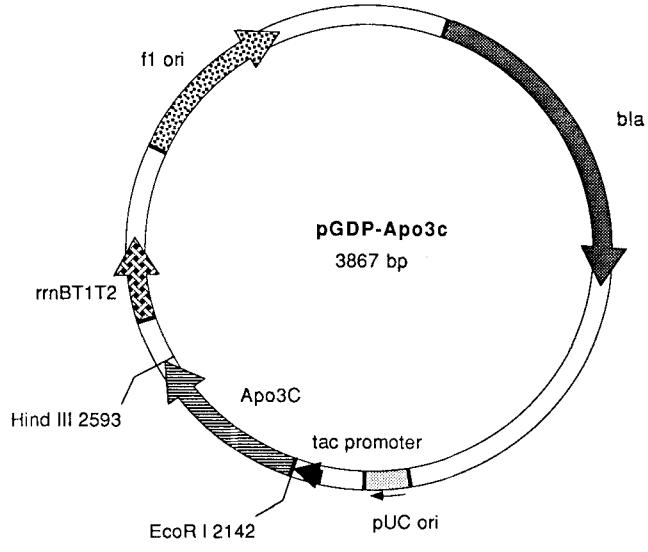

Fig. 1. Construct for expression of *A. polyphemus* PBP Apo-3 using pHN1+ vector and expression cassette shown in Figure 2.

Results

Two oligonucleotides based on the N-terminal and C-terminal amino acid sequences encoded by the cDNA for *A. polyphemus* antennal PBP Apo-3 (Raming et al., 1989) were used for ECPCR (Fig. 1) (MacFerrin et al., 1990). The N-terminal primer, a 55-mer, had an *Eco* RI site, an AAGGAG ribosome binding site, an ATG start codon, and codons for Ser^1 to Ser^9. The C-terminal 41-mer, encoded Ile^{135} to Val^{142}, the ATC stop codon, and a *Hind* III site. In addition to the 55-mer N-terminal primer shown here, a 60-mer primer using Met^5 as the start signal and codons for Lys^6 to Lys^{14} was used to construct an alternative cassette.

Thirty cycles of PCR amplification of the Apo-3 cDNA insert contained in a double-stranded M13(mp18) plasmid (Raming et al., 1989) using the 55-mer and 41-mer led to a ca. 550-bp PCR product. After purification by extraction and precipitation, and digestion with *Eco* RI and *Hind* III, the gel-purified cassette was ligated into pHN1+ prepared by digestion with *Eco* RI and *Hind* III. Competent XA-90 *Escherichia coli* cells were transformed and two positive ampicillin-resistant colonies were obtained that showed the correct DNA restriction fragments. This plasmid construct, pGDP-Apo3c (Fig. 1), differs from plasmid pGDP-Apo3d, which employed a 60-mer N-terminal primer lacking codons for Ser^1 to Ile^4 along with the same 41-mer C-terminal primer. The 5′ linker, coding region, and 3′ linker regions of pGDP-Apo3c were sequenced using a *tac* primer, the 55-mer PCR primer, the 41-mer PCR primer, and a primer downstream from the *Hind* III site (Fig. 2).

```
     Eco RI  RBS          MET Ser Pro Glu Ile Met Lys Asn Leu Ser
     GAATTCAAGGAGATATACC ATG TCG CCA GAG ATC ATG AAG AAT TTA AGC

10   Asn Asn Phe Gly Lys Ala Met Asp Gln Cys Lys Asp Glu Leu Ser
     AAT AAT TTT GGT AAA GCT ATG GAT CAG TGT AAG GAC GAG CTT AGT

25   Leu Pro Asp Ser Val Val Ala Asp Leu Tyr Asn Phe Trp Lys Asp
     CTC CCT GAT TCC GTT GTC GCC GAT TTG TAC AAC TTT TGG AAG GAT

40   Asp Tyr Val Met Thr Asp Arg Leu Ala Gly Cys Ala Ile Asn Cys
     GAC TAT GTG ATG ACC GAT AGG TTA GCA GGA TGT GCC ATA AAC TGT

55   Leu Ala Acc Lys Leu Asp Val Val Asp Pro Asp Gly Asn Leu His
     TTG GCC ACC AAG CTA GAT GTA GTC GAT CCT GAT GGA AAT CTC CAC

70   His Gly Asn Ala Lys Asp Phe Ala Met Lys His Gly Ala Asp Glu
     CAT GGA AAC GCA AAG GAC TTC GCG ATG AAG CAT GGA GCT GAT GAA

85   Thr Met Ala Gln Gln Leu Val Asp Ile Ile His Gly Cys Glu Lys
     ACC ATG GCG CAG CAA CTG GTG GAT ATT ATA CAC GGA TGT GAA AAG

100  Ser Ala Pro Pro Asn Asp Asp Lys Cys Met Lys Thr Ile Asp Val
     TCT GCT CCA CCT AAT GAC GAT AAA TGT ATC AAG ACT ATA GAT GTT

115  Ala Met Cys Phe Lys Lys Glu Ile His Lys Leu Asn Trp Val Pro
     GCG ATG TAT TTC AAA AAA GAG ATC CAT AAG CTG AAC TGG GTT CCT

130  Asn Met Asp Leu Val Ile Gly Glu Val Leu Ala Glu Val END
     AAC ATG GAC CTT GTA ATA GGC GAG GTC TTA GCT GAA GTT TAG

     HindIII
     AAGCTT
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Fig. 2. Nucleotide and derived amino acid sequence of the expression cassette for Apo-3 showing 5′ and 3′ restriction sites (underlined) used for insertion into pHN1+. The bold, italicized Met^6 was the start codon in the pGDP-Apo3d cassette.

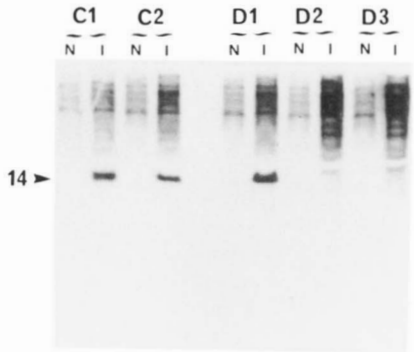

Fig. 3. Induction of Apo-3 rPBP synthesis by IPTG. N, not induced; I, induced with IPTG; C1, C2, colonies expressing pGDP-Apo3c; D1–D3, colonies expressing pGDP-Apo3d, the cassette encoding an N-terminally truncated Apo-3 protein.

Protein expression was induced in the pGDP-Apo3c (or pGDP-Apo3d) containing cells with IPTG, and total cellular protein was analyzed by SDS-PAGE (Fig. 3). A 14-kDa protein was observed in both the soluble fractions and cell pellets (data not shown), and this protein co-eluted with the native *A. polyphemus* PBP (Fig. 4). Similarly, a 14-kDa protein was also present in induced cultures of pGDP-Apo3d-containing cells (Fig. 3).

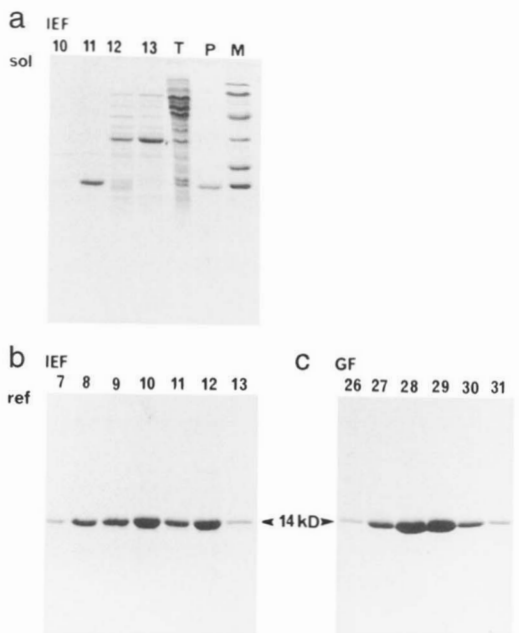

Fig. 4. Purification of soluble and refolded Apo-3 rPBP by Rotofor (IEF) and by gel filtration (GF). **a:** SDS-PAGE of IEF fractions for soluble rPBP, from pH 4.51 (fraction 10) to pH 5.94 (fraction 13), with focusing of rPBP at pH 4.80 (fraction 11). Lane T indicates total soluble protein before IEF separation; lane P is authentic antennal PBP; lane MW contains the molecular mass markers (97, 66, 45, 33, 21.5, and 14.4 kDa). **b** (IEF): SDS-PAGE showing IEF of refolded rPBP from pH 4.23 (7) to pH 4.85 (13); only fractions from pH 4.5 to 4.85 were combined. **c** (GF): SDS-PAGE for homogeneous refolded, IEF fractions of rPBP eluted from Sephacryl S-100.

More than 90% of the total recombinant PBP produced by the cells was present in the inclusion bodies of the transformed and induced cells. Induction of PBP production at 30 °C for 10 h did not substantially improve the yield of soluble PBP. Two methods were examined for refolding this insoluble PBP after dissolving the protein in 6 N guanidinium hydrochloride: a standard cysteine–cystine redox couple technique (De Bernardez-Clark & Georgiou, 1991; Seeley & Young, 1991) and a novel DMSO-mediated oxidation method developed by Tam et al. (1991). The former method provided higher yields of properly refolded protein. The rPBP samples were examined by photoaffinity labeling and then purified by preparative IEF followed by gel filtration.

Recombinant PBP was purified by preparative IEF in the pH 4.0–6.0 range, a technique previously employed for purification of a recombinant *A. pernyi* PBP from baculovirus-infected Sf9-cells (Krieger et al., 1992). This accomplished a several hundred-fold purification in a single rapid process, as illustrated by the stained SDS-PAGE gels in Figure 4. Preparative IEF was performed with both soluble and refolded rPBP. The latter experiment served to precipitate and separate oligomeric or incorrectly folded rPBP from the correctly folded and biologically active rPBP. Both soluble and refolded rPBP focused between pH 4.5 and 4.8. Removal of ampholytes by ultrafiltration in the presence of 1 M NaCl was followed by passage of the retentate through a Sepharose S-100 gel filtration column to give >95% homogeneous PBP (Fig. 4). Soluble and refolded rPBP showed identical elution times from Sepharose S-100.

To authenticate the biological activity and authenticity of the refolded rPBP, three tests were performed: immunoblotting, native gel electrophoresis, and photoaffinity labeling. The first recognizes various epitopes of the intact native PBP, the second can demonstrate whether a single globular protein or a family of refolded rPBPs is present, and the third provides a diagnostic test for correct conformation of the pheromone binding site. Thus, a Western blot of SDS-PAGE separated proteins from the bacterial expression (crude soluble protein and IEF-purified PBP) and, as a control, authentic male antennal *A. polyphemus* PBP showed clear immunoreactivity of the rPBP with the antibody to the *Lymantria dispar* PBP2 (data not shown). Native PAGE, followed by Western blot analysis, also revealed that the refolded rPBP had as its major component a protein with migration and immunochemical properties identical to those of the native PBP (Fig. 5). A higher mass, immunoreactive protein in lanes 1 and 2 appeared to be a refolding artifact and was readily removed by gel filtration.

Next, the native moth PBP (purified from moth sensory hairs by preparative native PAGE), soluble rPBP, and refolded rPBP were photoaffinity labeled with [^{3}H]6*E*, 11*Z*-hexadecadienyl diazoacetate (Prestwich et al., 1984; Vogt et al., 1988) in the presence or absence of excess

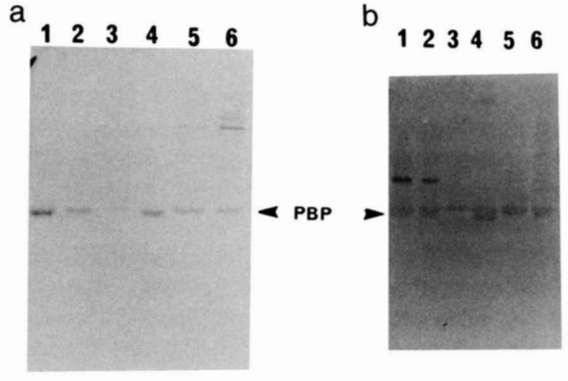

Fig. 5. Western blot analysis of Apo-3 PBP samples separated by native PAGE. **a:** Coomassie blue-stained gel. **b:** AP-detected immunoreactive bands. Lane 1, refolded and IEF purified rPBP; lane 2, refolded by CysSH–CysSSCys redox couple; lane 3, refolded by DMSO method; lane 4, *A. polyphemus* antennal PBP; lanes 5, 6, soluble rPBP purified by IEF (two independent preparations).

competitors (Fig. 6). The native moth PBP and the IEF-purified soluble and refolded rPBPs were each covalently modified by the photoaffinity analogue in the absence of competitors, suggesting that the binding site was intact. The weak competition for the refolded rPBP was attributed to the higher concentration of protein and the oligomeric rPBP still present in this sample prior to purification by gel filtration. In the crude bacterial proteins, only the PBP was labeled, showing selectivity, but labeling was difficult to detect by fluorography due to the low concentration of labeled PBP in the total protein loaded on a gel.

Using the pheromone itself, 6*E*,11*Z*-hexadecadienyl acetate, and the corresponding alcohol, 6*E*,11*Z*-16:OH, as competing ligands at increasing concentrations from 12 to 400 μM (corresponding to a 40–1,280-fold excess relative to [^{3}H]6*E*,11*Z*-16:Dza), it appeared that the pheromone acetate and alcohol were equivalent as competitors

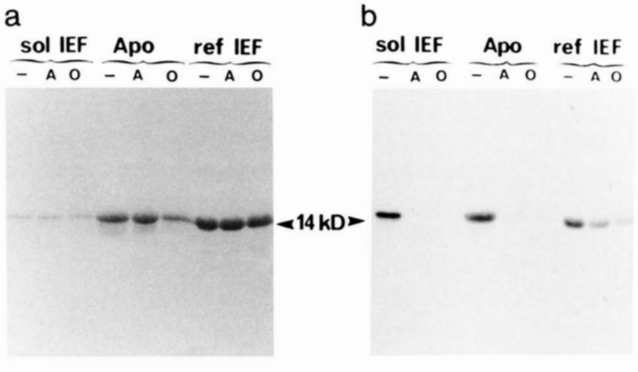

Fig. 6. Photoaffinity labeling of Apo-3 PBP at 300 nM [^{3}H]6*E*,11*Z*-16:Dza. **a:** Coomassie blue-stained gel. **b:** Fluorogram. –, no competitor; A, 200-fold excess 6*E*,11*Z*-16:Ac; O, 200-fold excess 6*E*,11*Z*-16:OH; sol IEF, soluble rPBP after Rotofor; ref IEF, refolded rPBP after Rotofor; Apo, moth-derived PBP.

for displacement of the diazoacetate photoanalogue from the binding site (Figs. 7, 8). Interestingly, the monounsaturated homologue 9*Z*-tetradecenyl acetate, which is related to the 4*E*,9*Z*-tetradecadienyl acetate component of the pheromone for this species, also showed competitive displacement of the photolabel at a concentration equivalent to that of the 16-carbon pheromone component. The fluorograms (Fig. 7) and densitometric scans (Fig. 8) demonstrate that the refolded rPBP and soluble rPBP had essentially indistinguishable binding affinities for the photolabel and competitors. The high affinity of the alcohol, produced in vivo by the action of a sensillar esterase on the pheromone acetate, suggests that it too may be transported by the PBP (Vogt et al., 1985, 1988). A similar result was obtained with the Sf9-cell produced recombinant Apr-1 PBP (Krieger et al., 1992). In a separate set of experiments (data not shown), it was determined that the affinity of refolded, purified rPBP for [^{3}H]6*E*,11*Z*-16:Dza was essentially unchanged over the range of pH 6.0–8.5. However, at pH 5.5 and lower, photoaffinity labeling efficiency was reduced dramatically, suggesting either a change in conformation or an aggregation effect that diminished the ability of the binding site to recognize pheromone.

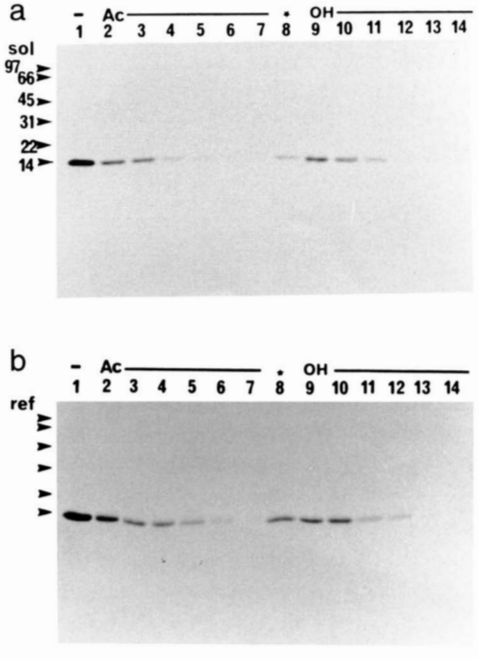

Fig. 7. a: Fluorogram of photoaffinity labeling of IEF-purified soluble rPBP, showing competitive displacement of [^{3}H]6*E*,11*Z*-16:Dza by acetate (Ac, lanes 2–7) and alcohol (OH, lanes 9–14) derivatives of pheromone (see Fig. 6) using 40-, 80-, 160-, 320-, 640-, and 1,280-fold excess during incubation period prior to photolysis. Lane 1 shows the photolabeling in the absence of any competing ligand; lane 8 (starred) shows competition by a 160-fold excess of *Z*9-tetradecenyl acetate. **b:** Photoaffinity labeling of IEF/GF purified refolded rPBP under identical conditions. Equal quantities of PBP were present in each lane (protein-stained gels not shown).

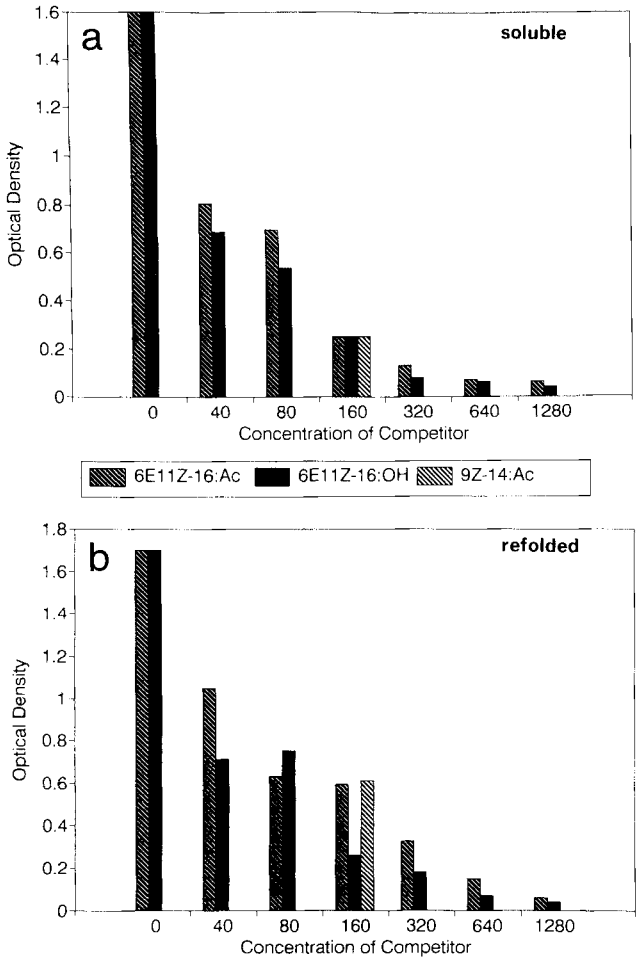

Fig. 8. Densitometric scan of Figure 7 fluorograms of photolabeled rPBP. **a:** Soluble rPBP. **b:** Refolded rPBP.

Experiments designed to detect free thiols (i.e., cysteines not involved in disulfide bonds) unambiguously showed the complete absence of free thiols (less than 0.02 free thiol per cysteine present in the refolded, gel filtered rPBP). This is the first confirmation of the anticipated role of the six conserved Cys residues in forming disulfides that constrain the tertiary structure. It does not, however, indicate which pairs of Cys residues are involved in each disulfide linkage.

Preliminary 1D proton NMR studies of the rPBP samples were conducted to assess their suitability for complete structural assignment using 2D, 3D, and 4D NMR methodologies. A spectrum was first obtained at pH 5.3, which was selected to be near the PBP isoelectric point and low enough to minimize exchange of amide NH protons. Broadened lines suggested aggregation or a conformational change at this pH. This was consistent with the failure of the rPBP to undergo photoaffinity labeling at pH values below 5.6. Observation of the 1D NMR at pH 6.0, 6.5, and 7.0 showed good dispersion in the amide region and a wealth of partially resolved C-alpha protons. The 500-MHz proton spectra of the refolded, purified rPBP at pH 7.0 and of soluble, purified rPBP at pH 7.0 were nearly indistinguishable (Fig. 9).

Discussion

OBPs have been characterized in vertebrates using molecular cloning and immunohistochemical methods and in insects using molecular cloning and protein photoaffinity labeling approaches. Two fundamental questions regarding OBPs remain unsolved. First, why are they present? It is generally assumed that these OBPs in vertebrates and the PBPs and GOBPs in insects facilitate solubilization of hydrophobic molecules within an aqueous environment and also mediate the transport of the odorants to a membrane-bound receptor. However, it is conceivable that binding a pheromone alters the conformation of the PBP, and that this "activated" PBP is actually responsible for receptor activation. That is, PBPs may undergo a gain of function change analogous to that which occurs for the immunosuppressant-immunophilin complexes (e.g., FK506-FKBP) (Schreiber, 1991). Second, how do OBPs and PBPs selectively bind specific odorants? Clearly, very

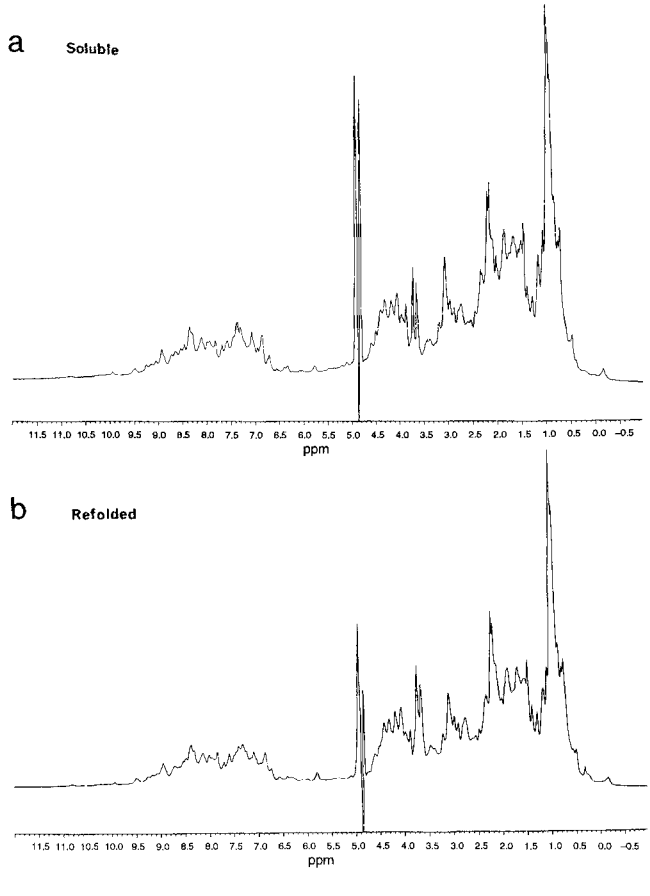

Fig. 9. Proton NMR spectra (500 MHz) of soluble (**a**) and refolded (**b**) rPBP (1 mM) in 50 mM sodium phosphate, 10% D_2O, pH 7.0, 30 °C.

subtle structural differences in PBPs are needed for recognition of different chain lengths, different alkene geometry or regiochemistry, or different C-1 functionality within a very homogeneous group of odorant structures (such as the lepidopteran pheromones). To address these questions, access to substantial quantities of protein for structural studies was required, and an expression system needed to be developed that could conveniently provide the natural OBP as well as isotopically labeled proteins and potential mutant proteins.

ECPCR is a convenient technique that allows rapid construction of an expression cassette for the production of a protein by chemists and obviates the need for post-expression proteolytic processing (MacFerrin et al., 1990; Schreiber & Verdine, 1991). This technique has been successfully used to produce proteins in 20–200-mg quantities for the study of specific protein–ligand interactions using protein crystallography and multidimensional protein NMR. Thus, we exploited this method to produce two separate plasmids, one of which carried the full-length amino acid sequence of a PBP, Apo-3 PBP, originally cloned from a cDNA library prepared to mRNA from the antennae of the wild silkmoth, *A. polyphemus* (Raming et al., 1989). Transformation of *E. coli* XA-90 cells and induction of protein synthesis with IPTG led to high-level production of the desired rPBP in both the soluble and inclusion body fractions of the cells. Soluble protein could be purified readily in two steps by preparative IEF followed by gel filtration; insoluble protein could be solubilized, refolded, and purified by the same two steps. The analysis of the purified, soluble rPBP, as well as the refolded rPBP, indicates that they have the molecular size, immunoreactivity, and isoelectric point identical to that of native *A. polyphemus* PBP (Klein, 1987). Most importantly from a physiological point of view, both soluble and refolded rPBP have identical binding affinities for pheromone as determined by photoaffinity labeling experiments.

The native structure of PBP represents a global energy minimum and a preferred folding geometry: insoluble rPBP from inclusion bodies can be solubilized and refolded to give rPBP with all the biological and physical properties appropriate to the native PBP and the soluble rPBP. The lack of free cysteine thiols strongly supports the notion that the six conserved Cys residues in the PBP family are involved in three disulfide linkages that constrain the tertiary structure and give rise to the pheromone binding site. Indeed, preliminary studies of the soluble and the refolded protein by 1D proton NMR experiments at pH 7.0 (Fig. 9) suggest that the overall structures are identical. The high-field proton NMR spectra taken at 500 or 600 MHz at pH 5.3 in deuterated sodium acetate (50 mM) showed a drastic change in structure relative to that observed in sodium phosphate (50 mM) at pH 6.0, 6.5, 7.0, and unbuffered at pH 8.0. Photoaffinity labeling of proteins over a pH range of pH 5.5–8.5 confirmed that below pH 6.0, the protein is unable to bind pheromone effectively.

Sequential assignments for the amino acids and determination of the secondary structure and tertiary structure by NMR (Wüthrich, 1990) are in progress using ^{15}N-labeled PBP generated by this expression method (G.D. Prestwich, unpubl.). In addition, rPBP will be valuable in developing binding assays (de Kramer & Hemberger, 1987) and functional electrophysiological assays involving perfusion of intact sensilla (Kaissling et al., 1991; van den Berg & Ziegelberger, 1991). Through a combination of structural and physiological studies, a better understanding of the molecular recognition of pheromones and the molecular transduction should soon be realized.

Materials and methods

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized in the trityl-off mode on an Applied Biosystems Model 381A DNA synthesizer, cleaved from the resin in concentrated NH_4OH (65 °C, 12 h), and purified by electrophoresis (300 V, 6 h) on a 20% acrylamide–40% urea gel in TBE. The bands were excised and DNA was isolated by adsorption to C_{18} Sep-pak and elution with 30% acetonitrile–70% 0.05 M Et_3NHCO_3.

In the N-terminal primer (5′ TAGGGCGAATTC *AAGGAG*ATATACC ATG TCG CCA GAG ATC ATG AAG AAT TTA AGC 3′), the *Eco* RI site is underlined and the Shine-Dalgarno consensus ribosome binding site sequence is italicized. In the C-terminal anticoding primer (3′ TAT CCG CTC CAG AAT CGA CTT CAA ATC TTCGAAACGGATCT 5′), the *Hind* III site is underlined. With the exception of the ATG start codon, all three-base codons, including the ATC stop codon, are identical to those in the target cDNA template. A second N-terminal primer (5′ TAGGGCGAATTCTTAACC *AGGGAG*CTGATT ATG AAA AAT TTA AGC AAT AAT TTT GGT AAA 3′)—utilizing an existing internal Met^5 as the start codon, an AAA codon for Lys^6, and modified translational spacers—was employed to prepare a second expression cassette. Translational spacers were based on optimal sequences (MacFerrin et al., 1993) used most recently by Liu et al. (1990).

PCR amplification, cassette purification, digestion, ligation, and transformation

The 55-mer N-terminal PCR primer and 41-mer C-terminal anticoding primer (10 μL of 10 pmol/mL) were combined with 80 ng template DNA (M13mp18 plasmid containing the Apo-3 cDNA insert), 5 μL each of 2.5 mM dNTPs (N = C, G, A, T), 0.5 μL of *Taq* I polymerase (5 U/μL), 5 μL of 10× Taq I buffer, 10 μL of 1% Triton X-100, and 10 μL of 10× gelatin, and 38 μL of water and amplified by 30 cycles of PCR (Innes & Gelfand, 1990)

(94 °C, 5 min; cycle: 94 °C, 2 min; 42 °C, 2 min; 70 °C, 3 min) under standard conditions. The ca. 550-bp PCR product was isolated and purified by phenol extraction and precipitation of the DNA from a 3 M sodium acetate, pH 6.0, solution using ethanol (Sambrook et al., 1989). After digestion with *Eco* RI and *Hind* III (2 h, 37 °C) and purification on a 1% agarose gel, the sticky-ended cassette DNA was isolated by adsorption to silica (Gene-Clean, Bio101) (Vogelstein & Gillespie, 1979). An overexpresser construct in the plasmid pHN1+ (provided by L. Chen & G.L. Verdine, Harvard University; H.M. Nash & G.L Verdine, unpubl.) was similarly digested and the linearized, sticky-ended plasmid was isolated using Gene-Clean. This vector is a modified pBS+ (Stratagene) in which the insert is under the control of a strong *tac* promoter sequence upstream of the *Eco* RI site and contains the *rrnB*T_1T_2 transcription termination sequence downstream of the *Hind* III site. The cassette was ligated with the plasmid vector using T4 ligase (16 °C, 12 h), and competent XA-90 *E. coli* cells were transformed by 90 s heat shock at 42 °C followed by growing for 1.5 h at 37 °C in LB medium. LB agar plates containing ampicillin were inoculated and incubated overnight at 37 °C.

Cell culture, induction, and protein purification

Two ampicillin-resistant colonies were obtained. Each colony was grown in 3 mL of LB-ampicillin (LBA) medium for 12 h at 37 °C and replated to obtain single clones. Cells grown to early log phase (A_{600} = 0.4–0.6) were induced with 1 mM IPTG, and cells from both induced and uninduced cultures were isolated by centrifugation. Aliquots were analyzed for protein production and for the presence of the desired Apo-3 cassette insert. Thus, cells were lysed by sonication or by lysozyme–freeze–thaw methods (Sambrook et al., 1989), and soluble and insoluble proteins were analyzed by SDS-PAGE (see below). Plasmid DNA was isolated by the CTAB (cetyl trimethylammonium bromide) miniprep technique and digested as above with *Eco* RI and *Hind* III. Inserts were visualized in 1% agarose gels containing ethidium bromide.

Large scale fermentations were conducted in 1-L flasks in LBA medium using starter cultures from overnight 10-mL incubations. After induction and protein synthesis (4–6 h, 37 °C), cells were harvested in 1-L bottles at 3,500 × *g*, lysed in a 80 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 4% glycerol, pH 7.2, buffer containing 0.5 mM PMSF using a French press (14,000 psi, 4 °C, 3–4 passes), and soluble proteins were isolated by ultracentrifugation (30,000 × *g*) followed by ultrafiltration with 20 mM Tris, pH 7.2, buffer.

The soluble proteins were purified by preparative IEF using a Rotofor (Bio-Rad) apparatus (Krieger et al., 1992). Thus, 55 mL of desalted soluble protein containing 2% Pharmalyte 4-6 (or Biolyte 4/6) ampholytes was focused into 20 chambers at 12 W constant power, 300–1,000 V, 4 °C, during 6 h. The fractions were harvested by suction and analyzed in three ways: pH, immunoreactivity, and SDS-PAGE. The pH 3.8–5.0 range was expected to contain Apo-3 protein, whose predicted pI value is 4.43 (Raming et al., 1989) and measured pI is 4.7 (Klein, 1987). Dot blots on PVDF were performed for each fraction, for crude protein, and with known amounts of native purified Apo-3 protein. Immunochemical detection was achieved with an antiserum (rabbit) to *L. dispar* PBP2 (Vogt et al., 1989), which was known to cross-react with the *A. polyphemus* PBP, and with goat anti-rabbit antiserum coupled to alkaline phosphatase. SDS-PAGE confirmed the presence of PBP in core fractions of the immunoreactive-pI range. Pooled PBP-containing fractions were diluted with 1 M NaCl and ultrafiltered (YM10) with three washes of 10 mM Tris, 1 mM EDTA, and 100 mM NaCl, pH 7.2 (GF buffer).

The final purification step for both soluble and refolded rPBP was gel filtration on a 3 × 100-cm column of Sephacryl S-100 high resolution preequilibrated with GF buffer and run at 30 mL/h with collection of 10-mL fractions. Fractions containing homogeneous rPBP as judged by SDS-PAGE were concentrated by ultrafiltration. Purified rPBP can be stored in 10–50-mg/mL solutions in water or in buffers of pH 5.0–8.0 at 4 °C with no detectable change in binding activity or homogeneity during 1 month.

Protein refolding

Insoluble protein from cell lysates from a 1-L culture was washed with 0.2% Triton X-100 in 50 mM Tris buffer (pH 6.8) and then dissolved in 5 mL of 6 N guanidinium hydrochloride. Two refolding protocols were attempted. The redox method (De Bernardez-Clark & Georgiou, 1991; Seeley & Young, 1991) was performed as follows. To 2.5 mL of solubilized inclusion body protein was added 2.5 mL of 10 mM DTT in 200 mM Tris-HCl (pH 8.0). After reduction of the solubilized protein at room temperature for 30–60 min, 1 mL of 100 mM cystine (dissolved in 0.5 N NaOH) was added to give ca. 14 mM cyst(e)ine. After incubation for 10 min to oxidize remaining DTT, this mixture was added in portions to 10 volumes of 5 mM cysteine in 100 mM Tris-HCl, pH 8.0. The final mixture, containing 0.5–2.0 mg/mL protein, and ca. 5–6 mM cyst(e)ine was shaken in air on an orbital shaker for 24 h at room temperature. The precipitated, aggregated protein was removed by centrifugation (6,000 × *g*, 30 min), and the supernatant was dialyzed overnight against 200 mM Tris pH 8.5. The sample was concentrated and further desalted by ultrafiltration (YM10). The final concentration for ca. 50 mL was 1.5 mg/mL. This protocol was later repeated on a 4-L scale fermentation by a fourfold scaleup to obtain ca. 6 mL at 40 mg/mL.

In the second method, DMSO was employed as the stabilizer and oxidant during protein refolding (Tam et al.,

1991). Thus, addition of 2.5 mL of solubilized inclusion body protein above was done in 100-μL aliquots to 30 mL of 25% DMSO in 75% 100 mM MES (pH 5.6). Although a white precipitate was formed, the mixture was shaken gently for 24 h at room temperature, insoluble material was removed by centrifugation, and the supernatant was dialyzed against 50 mM Tris, pH 6.8, and then concentrated by ultrafiltration. The final concentration for ca. 10 mL solution was 0.7 mg/mL.

Refolded protein was examined for the integrity of its pheromone-binding activity by photoaffinity labeling (see below) and for preservation of antigenic regions by Western blot immunodetection. In addition, refolded protein was subjected to preparative IEF to monitor extent of correct refolding and to purify the correctly folded isoform. Gel filtration as a final step allowed removal of oligomeric forms produced during reformation of disulfide bonds.

Photoaffinity labeling

Samples containing PBP were photoaffinity labeled with [^{3}H]6*E*,11*Z*-hexadecadienyl diazoacetate (Prestwich et al., 1984) as previously described (Prestwich, 1987) using 150–600 nM photolabel in the absence or presence of increasing concentrations of either the unlabeled acetate (6*E*,11*Z*-16:Ac) or alcohol (6*E*,11*Z*-16:OH) as competing ligands. The tritium-labeled diazoacetate was stored at −20 °C in either hexane or heptane containing 2–10% toluene to retard radiolytic decomposition. Unlabeled competitors were prepared as 0.1, 1.0, and 10 mM solutions in hexane at stored at −20 °C. In a typical experiment, 1.5 μL of 17 μM photolabel and either 0 (−) or 5 μL (+) of 1 mM competitor was added to the bottom of 12 × 100-mm quartz tubes, followed by 20 μL of hexane to rinse in the ligands. Solvents were removed with a gentle stream of N_2, and then 3 μL of ethanol was added. The diluted, chilled protein solution was added (80 μL), the tube was vigorously vortex-mixed, and samples were incubated for 1 h at 4 °C. Samples were irradiated 20 s at 254 nm with a germicidal lamp, and aliquots were treated with 2× sample buffer, boiled, and separated by SDS-PAGE.

Photoaffinity labeling was conducted over a pH range of 5.5–8.5 to evaluate the stability of the binding-active conformation. Thus, 30 mM MES was prepared at pH 5.5, 6.0, and 6.5, and 30 mM Tris-HCl buffers were prepared at pH 7.0, 7.5, 7.0, 8.0, and 8.5. An aliquot of pheromone from a 50 mM sodium deuterioacetate NMR solution was diluted with two volumes of the indicated buffer, and incubation, labeling, electrophoresis, and fluorography were performed as usual.

Electrophoresis and fluorography

DNA was separated on 0.8% or 1.5% agarose gels in TBE containing 10 mg/L ethidium bromide in the buffer and in the gel with visualization of the oligonucleotides by UV. Proteins were separated by SDS-PAGE using modified Laemmli conditions on a 20% acrylamide gel with a 200:1 ratio of acrylamide to bisacrylamide, using a minigel apparatus and 25–30 mA per 1.5 mm gel or 15–18 mA per 0.75 mm gel. Western blots were performed on unstained, unfixed gels using PVDF (Immobilon-P) membranes, 10 mM CAPS, pH 11.0, at 50 V, 30 min, followed by blocking (casein) and antibody-mediated visualization (goat anti-rabbit IgG coupled to alkaline peroxidase) was employed for the dot blots. Fluorography was performed by impregnation of the gels with 15% 2,5-diphenyloxazole (PPO) in glacial acetic acid and exposure of the rinsed and dried gel to XAR-5 film for 2–5 days at −70 °C (Skinner & Griswold, 1983). Increased sensitivity and shorter exposure times were possible when gels were miniaturized with PEG 1,500 prior to drying and fluorography (Mohamed et al., 1989).

DNA sequencing

Plasmid DNA was isolated by the CTAB method and double-stranded DNA was sequenced by the dideoxy termination method (Sanger et al., 1977) using the Sequenase kit (U.S. Biochemicals). Primers employed included the PCR primers used in construct production and vector-specific complementary upstream and downstream oligonucleotides.

Thiol determinations

Free cysteines were measured by adding 100 μL of 10 mM DTNB in 100 mM sodium phosphate (pH 8.0) to rPBP in 2 mL buffer containing 5 mM EDTA. Stock solutions of 10 mM reduced glutathione were employed as standards, and A_{412} values were recorded.

NMR spectroscopy

One-dimensional NMR spectra were obtained on IEF/GF purified proteins (1–3 mM) in 50 mM sodium phosphate buffer, pH 6.0, 6.5, or 7.0, containing 10% D_2O for lock, using either a Bruker AM-500 spectrometer (Harvard) or a Bruker AMX-600-MHz instrument (Stony Brook).

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References

Boekhoff, I., Raming, K., & Breer, H. (1990). Pheromone-induced stimulation of inositol trisphosphate formation in insect antennae is mediated by G-proteins. *J. Comp. Physiol. B 160*, 99–105.

Breer, H., Boekhoff, I., Strotmann, J., Raming, K., & Tareilus, E. (1990a). Molecular elements of olfactory signal transduction in insect antennae. In *Chemosensory Information Processing* (Schild, D., Ed.), pp. 77–86. Springer-Verlag, Berlin.

Breer, H., Boekhoff, I., & Tareilus, E. (1990b). Rapid kinetics of second messenger formation in olfactory transduction. *Nature 345*, 65–67.

Breer, H., Krieger, J., & Raming, K. (1990c). A novel class of binding proteins in the antennae of the silk moth *Antheraea pernyi*. *Insect Biochem. 20*, 735–740.

De Bernardez-Clark, E. & Georgiou, G. (1991). Inclusion bodies and recovery of proteins from the aggregated state. In *Protein Refolding* (Georgiou, G. & De Bernardez-Clark, E., Eds.), pp. 1–20. American Chemical Society, Washington, D.C.

de Kramer, J.J. & Hemberger, J. (1987). The neurobiology of pheromone reception. In *Pheromone Biochemistry* (Prestwich, G.D. & Blomquist, G.J., Eds.), pp. 433–472. Academic Press, Orlando, Florida.

Gyørgi, T.K., Roby-Shemkovich, A.J., & Lerner, M.R. (1988). Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: A tissue-specific developmentally regulated protein. *Proc. Natl. Acad. Sci. USA 85*, 9851–9855.

Innes, M.A. & Gelfand, D.H. (1990). *PCR Protocols*. Academic Press, Orlando, Florida.

Kaissling, K.-E., Keil, T.A., & Williams, J.L.D. (1991). Pheromone stimulation in perfused sensory hairs of the moth *Antheraea polyphemus*. *J. Insect Physiol. 37*, 71–78.

Klein, U. (1987). Sensillum lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturnidae). *Insect Biochem. 17*, 1193–1204.

Krieger, J., Raming, K., Prestwich, G.D., Frith, D., Stabel, S., & Breer, H. (1992). Expression of a pheromone-binding protein in insect cells using a baculovirus vector. *Eur. J. Biochem. 203*, 161–166.

Liu, J., Albers, M.W., Chen, C.M., Schreiber, S.L., & Walsh, C.T. (1990). Cloning, expression, and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteins by site-directed mutagenesis. *Proc. Natl. Acad. Sci USA 87*, 2304–2308.

MacFerrin, K.D., Chen, L., Terranova, M., Schreiber, S.L., & Verdine, G.L. (1993). Protein overproduction using the expression-cassette polymerase chain reaction. *Methods Enzymol. 217*, 79–102.

MacFerrin, K.D., Terranova, M.P., Schreiber, S.L., & Verdine, G.L. (1990). Overproduction and dissection of proteins by the expression-cassette polymerase chain reaction. *Proc. Natl. Acad. Sci. USA 87*, 1937–1941.

Mohamed, M.A., Lerro, K.A., & Prestwich, G.D. (1989). Polyacrylamide gel miniaturization improves protein visualization and autoradiographic detection. *Anal. Biochem. 177*, 287–290.

Pevsner, J., Reed, R.R., Feinstein, P.G., & Snyder, S.H. (1988). Molecular cloning of odorant-binding protein: Member of a ligand carrier family. *Science 241*, 336–339.

Prestwich, G.D. (1987). Chemical studies of pheromone reception and catabolism. In *Pheromone Biochemistry* (Prestwich, G.D. & Blomquist, G.J., Eds.), pp. 473–521. Academic Press, Orlando, Florida.

Prestwich, G.D. (1992). Chemical studies of pheromone receptors in insects. *Arch. Insect Biochem. Physiol.*, in press.

Prestwich, G.D., Golec, F.A., & Andersen, N.H. (1984). Synthesis of a highly tritiated photoaffinity labeled pheromone analog for the moth *Antheraea polyphemus*. *J. Labelled Compd. Radiopharm. 21*, 593–601.

Raming, K., Krieger, J., & Breer, H. (1989). Molecular cloning of an insect pheromone-binding protein. *FEBS Lett. 256*, 215–218.

Raming, K., Krieger, J., & Breer, H. (1990). Primary structure of a pheromone-binding protein from *Antheraea pernyi*: Homologies with other ligand-carrying proteins. *J. Comp. Physiol. B 160*, 503–509.

Raming, K., Krieger, J., & Breer, H. (1991). Cloning of genomic and complementary DNA encoding insect pheromone binding proteins: Evidence for microdiversity. *Biochim. Biophys. Acta 1088*, 277–284.

Riley, C.T., Barbeau, B.K., Keim, P.S., Kezdy, F.J., Heinrikson, R.L., & Law, J.H. (1984). The covalent protein structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta* L. *J. Biol. Chem. 259*, 13159–13165.

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74*, 5463–5467.

Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science 251*, 283–288.

Schreiber, S.L. & Verdine, G.L. (1991). Protein overproduction for organic chemists. *Tetrahedron 47*, 2543–2550.

Seeley, R.J. & Young, M.D. (1991). Large-scale refolding of secretory leukocyte protease inhibitor. In *Protein Refolding* (Georgiou, G. & De Bernardez-Clark, E., Eds.), pp. 206–216. American Chemical Society, Washington, D.C.

Skinner, M.K. & Griswold, M.D. (1983). Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. *Biochem. J. 209*, 281–289.

Tam, J.P., Wu, C.-R., Liu, W., & Zhang, J.-W. (1991). Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and application. *J. Am. Chem. Soc. 113*, 6657–6662.

van den Berg, M.J. & Ziegelberger, G. (1991). On the function of the pheromone binding protein in the olfactory hairs of *Antheraea polyphemus*. *J. Insect Physiol. 37*, 79–85.

Vogelstein, B. & Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci USA 76*, 615–619.

Vogt, R.G. (1987). The molecular basis of pheromone reception: Its influence on behavior. In *Pheromone Biochemistry* (Prestwich, G.D. & Blomquist, G.J., Eds.), pp. 385–431. Academic Press, Orlando, Florida.

Vogt, R.G., Koehne, A.C., Dubnau, J.T., & Prestwich, G.D. (1989). Expression of pheromone binding proteins during development in the gypsy moth *Lymantria dispar*. *J. Neurosci. 9*, 3332–3345.

Vogt, R.G., Prestwich, G.D., & Lerner, M.R. (1990a). Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol. 22*, 74–84.

Vogt, R.G., Prestwich, G.D., & Riddiford, L.M. (1988). Sex pheromone receptor proteins: Visualization using a radiolabeled photoaffinity analog. *J. Biol. Chem. 263*, 3952–3959.

Vogt, R.G. & Riddiford, L.M. (1981). Pheromone binding and inactivation by moth antennae. *Nature 293*, 161–163.

Vogt, R.G., Riddiford, L.M., & Prestwich, G.D. (1985). The kinetic properties of a pheromone degrading enzyme: The sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci. USA 82*, 8827–8831.

Vogt, R.G., Rybczynski, R., & Lerner, M.R. (1990b). In *Chemosensory Information Processing* (Schild, D., Ed.), pp. 33–76. Springer-Verlag, Berlin.

Wüthrich, K. (1990). Protein structure determination in solution by NMR spectroscopy. *J. Biol. Chem. 265*, 22059–22062.