Revisiting the Specificity of *Mamestra brassicae* and *Antheraea polyphemus* Pheromone-binding Proteins with a Fluorescence Binding Assay*

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In the context of molecular recognition, the perception of pheromones by male moths is a system that shows both high specificity and significant affinity, resulting in mating within the same species and with the response being elicited by relatively low concentrations of pheromone. In a generally recognized scheme, the hydrophobic pheromone molecules enter the antennal sensilla (sensory hairs) through pores in the cuticle and traverse the aqueous sensillum lymph transported by low molecular mass (15–17 kDa) pheromone-binding proteins (PBPs), finally reaching receptors located in the dendritic membranes of the olfactory neurons, where they are recognized either free or in association with the PBP (1–5).

PBPs are present in the sensillum lymph at millimolar concentrations and were originally identified by their ability to bind radiolabeled compounds of the pheromone blend (1) and by their N-terminal sequence (6). The primary structures of PBPs from various moth species, established from molecular cloning (7–14), have indicated diversity among PBPs and their classification into a large family of insect pheromone/odorant-binding proteins, which also includes two classes of general odorant-binding proteins 1 and 2 (10, 11, 15), antennal-binding proteins Xs (11, 14, 16), and antennal-binding proteins (14). Insect PBPs and OBPs are α-helical proteins (17), thus completely different from mammalian OBPs which belong to the lipocalin superfamily and have a β-barrel fold (18–20).

Although molecular cloning has rapidly increased the information available on moth PBPs primary structures, cloned genes have not greatly increased our knowledge of their binding affinities and specificities. Heterologously expressed PBPs from *Antheraea polyphemus* (ApollBPB1), *Antheraea pernyi* (AperBPB1 and -2), and *Lymnaea dispers* (LdisBPB1) have already been used to develop quantitative binding assays with photoactivable derivatives of the pheromone or radioactive pheromones (8, 21–24). However, synthesis of photoactivatable or radioactive pheromones is difficult and limits the candidate compounds tested, usually to the main components of pheromone blends identified in behavioral studies.

In the present study, we have examined by fluorescence the binding affinity of 1-aminoanthracene (AMA) to recombinant PBPs of *A. polyphemus* (ApollBPB1), *Mamestra brassicae* (MbraBPB1), *Bombyx mori* (BmorBPB), and a mutant of MbraBPB1 (Mbra1-M6) mutated at residues in the internal cavity to mimic that of BmorBPB, using the fluorescence probe 1-aminoanthracene (AMA). AMA binds with high affinity to ApollBPB1 and ApollBPB1, however, no binding was observed with either BmorBPB or Mbra1-M6. The latter result indicates that relatively limited modifications to the PBP cavity actually interfere with AMA binding, suggesting that AMA binds in the internal cavity. Several pheromones are able to displace AMA from the MbraBPB1- and ApollBPB1-binding sites, without, however, any evidence of specificity for their physiologically relevant pheromones. Moreover, some fatty acids are also able to compete with AMA binding. These findings bring into doubt the currently held belief that all PBPs are specifically tuned to distinct pheromonal compounds.

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The abbreviations used are: PBP, pheromone-binding protein; OBP, odorant-binding protein; Mbra, *Mamestra brassicae*; Apoll, *Antheraea polyphemus*; Aper, *Antheraea pernyi*; PCR, polymerase chain reaction; AMA, 1-amino-anthracene; C16-OH, cetyl alcohol; Z9-C16-COOH, palmitoleic acid; Z9-C16-COOH, palmitoleic acid; E6,Z11-C16-Ac, (E6,Z11)-11-hexadeциен-1-ацетат; E6,Z11-C16-Ac, (E6,Z11)-11-hexadeциен-1-ацетат; E4,Z9-C14-Ac, (E4,Z9)-tetradecadiен-1-ацетат; PAGE, polyacrylamide gel electrophoresis.
Fluorescence of bound AMA was used to follow and quantify the binding of several pheromonal compounds, taking advantage of the ability of MbraPBP1 and ApolPBP1 both for pheromones and fatty acids. The binding data reveal that the interaction of pheromones with binding sites of the two PBPs examined by AMA fluorescence shows little specificity.

**EXPERIMENTAL PROCEDURES**

**Pheromones and Analogues**

Pheromones and analogues were purchased from Chemtech B.V. (The Netherlands) and Sigma, and stored as specified by the manufacturers. AMA was from Fluka. All alcoholic solutions were freshly prepared.

**Subcloning in pET22b(+) Vector and Expression**

*Escherichia coli* strain XL1-Blue was used for DNA subcloning and propagation of the recombinant plasmid. The *MbraPBP1* gene was amplified by PCR using pQE30/PPB1 as template (25) with the following primers: Mbra1-MscI 5′-CGAGATTTACGCTACGCTAGC-3′; Mbra1-NotI 5′-CCGGGCGCGCGTACACGGCCGCTGCTACGCTGCTC-3′. The amplified PCR fragment was purified and digested with MscI and NotI before being cloned between the same restriction sites of the pET22b(+) vector (Novagen). Recombinant MbraPPB1 was produced by growing *E. coli* BL21(DE3) transformed with the recombinant pET22b(+)/MbraPBP1 plasmid at 37 °C in LB medium supplemented with 50 μg/ml carbenicillin. Cultures were induced by centrifugation harvesting of 0.5–4 liters of induced medium, and the temperature was decreased to 28 °C when *Aon* reached the value of 0.8.

The gene of *ApolPBP1* was amplified, subcloned, and expressed in a similar way. PCR was performed using the primers: Apol1PBP1-MscI 5′-CTTCCCGAGACATCAGTGAAGT-3′ and Apol1PBP1-XhoI 5′-AGA- CACTCGAGACATCAGTGAAGT-3′. An *ApolPBP1* cDNA clone (Apo-9 (27)) was used as template. The fragment obtained by PCR was purified, digested by Xhol, and cloned into the MscI and Xhol restriction sites of the pET22b(+) vector. The recombinant pET22b(+)/ApolPBP1 plasmid was transformed into *E. coli* BL21(DE3) and expression was performed following the protocol of Wojtasek and Leal (26) by growing the cells without induction at 28 °C in LB medium supplemented with 50 μg/ml carbenicillin until *Aon* reaches a value >2.5. The gene of BmorPPB was amplified, subcloned, and expressed as described by Wojtasek and Leal (26).

**Site-directed Mutagenesis of MbraPBP1**

Six residues of MbraPPB1, Ile-5, Met-8, Ala-56, Leu-61, Ile-62, and Met-68 were replaced by Met, Leu, Ser, Leu, and Leu, respectively, by PCR (Fig. 1). 5′ and 3′ regions of the cDNA of MbraPPB1 were amplified using pET22b(+)/MbraPBP1 as template and the following mutated primers (mutations are underlined, *NruI* site is in italic and encoded amino acids are indicated between parentheses). 5′ Region—M6-NruI, 5′-CCCGTCGGCAGTTAAAGGAATGACTG(M5)ACGAAACTGAGATGTTGATTCACGAAA-3′; 3′ Region—M6-int-forward, 5′-ATGTTGATGTGTATGACTGAATGGA-3′. M6 and M6-int were used as template. The fragment obtained by PCR was purified, digested by Xhol, and cloned into the MscI and Xhol restriction sites of the pET22b(+) vector. The recombinant pET22b(+)/MbraPPB1 plasmid was transformed into *E. coli* BL21(DE3) and expression was performed following the protocol of Wojtasek and Leal (26) by growing the cells without induction at 28 °C in LB medium supplemented with 50 μg/ml carbenicillin until *Aon* reaches a value >2.5. The gene of BmorPPB was amplified, subcloned, and expressed as described by Wojtasek and Leal (26).

**Fluorescence Assays**

In the case of MbraPPB1, BmorPPB, and Mbra1-M6, the fluorescence spectra were recorded on a Jobin Yvon. Spectra were recorded in 10 mM Na phosphate, pH 7.0, at 20 °C between 178 and 280 nm, with a 30-s averaging.

**Intrinsic Fluorescence of PBPs**

The interaction of AMA and bombykol with PBPs was monitored by following the quenching of the intrinsic protein fluorescence excitation at 280 nm and emission 290–420 nm, as described above. Spectra were recorded with 1 μM protein in 20 mM Tris buffer, pH 8.0, 0.3% methanol and under the same conditions in the presence of different concentrations of AMA (0–10 μM).

**Fluorescence Emission Spectra Using AMA**

The effects of solvents, such as methanol, ethanol, and dimethyl sulfoxide on AMA binding have previously been tested with rat OBP (27). In that study, methanol was demonstrated to be less effective than ethanol in displacing the binding of AMA from the binding site. In our study, the displacement of AMA by ethanol and methanol has been determined by successively adding aliquots of solvent to the AMA/MbraPPB1 complex solution. As with rat OBP, ethanol was found to compete significantly with AMA bound to MbraPPB1, and methanol was found to have a lesser effect (data not shown). For this reason, methanol was used for dissolving AMA and its competitors in the fluorescence titrations.

**Fluorescence Binding Assay with Moths PBPs**

Purification of recombinant ApolPPB1 and BmorPPB proteins was performed by adjusting the periplasmic fraction to 10 mM Tris, pH 8.0, and loading it onto a 20-ml DEAE HR16/10 column (Toyopearl 650S, Tosoh, Japan) that was equilibrated with 10 mM NaCl in 10 mM Tris, pH 8.0. The column was washed with 100 ml of the same buffer. Elution was with a linear 0.1–2 M NaCl gradient in 10 mM Tris, pH 8.0. Fractions (0.5 ml each) were analyzed by SDS-PAGE. Both proteins were shown to be >95% pure in SDS and native PAGE. The integrity of the hexamutant was checked by mass spectrometry and N-terminal sequencing.

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**Mass Spectrometry and Circular Dichroism Analysis**

Mass analysis of recombinant PBPs was performed with a Voyager-DE EP spectrometer (PerSeptive Biosystems). Samples (0.7 μl containing 15 pmoles) were mixed with an equal volume of sinapinic acid matrix solution and spotted on the target, then dried at room temperature for 10 min. The mass standard was apomyoglobin.

The CD spectra were measured on a CD6 spectropolarimeter (Jobin Yvon). Spectra were recorded in 10 mM Na phosphate, pH 7.0, at 20 °C between 178 and 280 nm, with a 30-s averaging.

**Fluorescence Emission Spectra Using AMA**

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Fluorescence Binding Assay with Moths PBPs

ApollPB1, Mbra1-M6, and NmorPB

All the fluorescence experiments were carried out in 20 mM Tris, pH 8.0, at 20 °C (excitation 298 nm, emission 400–575 nm). The binding affinity for AMA was titrated by adding to the protein sample (1 mM) aliquots of a stock solution of AMA (10 mM) solubilized in 100% methanol. The fluorescence of AMA was recorded after stabilization of the signal (5–6 min) in the presence of AMA (5 μM) for 1 h at room temperature, the fluorescence signal of AMA was then monitored and the decrease in fluorescence intensity upon addition of different test compounds was recorded. The competition experiments were carried out at constant methanol concentration (0.3%) and with a test compound concentration between 0 and 10 μM.

ApollPB1

Binding and competition experiments were carried out in 20 mM Tris, pH 8.0, at 20 °C (excitation 256 nm, emission 440–600 nm). All values reported were obtained from three independent measurements. The curves for binding of AMA to ApollPB1 were obtained by titration of 2 μM protein with increasing concentrations of chromophore dissolved in methanol. In competition assays, we monitored the fluorescence signal of AMA (2 μM) equilibrated with ApollPB1 (2 μM) upon addition of increasing amounts of competitor. Fluorescence intensities at the maximum of emission (487 nm) were determined for different concentrations of competitor and were corrected before further data analysis by the extent of AMA fluorescence decrease due to the methanol present in the cuvette.

Data Analysis

The affinity of different compounds for MbraPB1 and ApollPB1 was estimated by plotting the decrease of intensity of AMA fluorescence at the emission maximum, calculated as (I – I_min)(I_max – I_min) against the competitor concentration; I is the maximum of fluorescence intensity of the complex AMA-MbraPB1 and AMA-ApollPB1, at 492 and 487 nm, respectively. I is the fluorescence intensity after addition of an aliquot of competitor, and I_min is the fluorescence intensity at saturating concentration of the competitor. The IC_{50} values were estimated on the direct plot by non-linear regression with equation corresponding to a single inhibition curve.

RESULTS

Design of the Mutant of MbraPB1

Residues were chosen using two criteria: first, they are localized in the binding pocket defined in the three-dimensional structure of B. mori PBP (17) and second, they are not conserved among the PBPs. Six residues of MbraPB1 were thus replaced by their counterparts in B. mori PBP (Fig. 1). Three primers were designed to introduce these mutations by PCR. The final DNA sequence, subcloned in pET22b(+) vector, was verified by automated DNA sequencing to check the introduction of mutations and PCR fidelity.

Subcloning, Expression, and Purification

After PCR amplification, gel purification, digestion, and precipitation, the MbraPB1, hexamutant Mbra1-M6, NmorPB, and ApollPB1 cDNAs were subcloned into the pET22b(+) vector. This vector leads to expression of a pelB leader/PBP fusion protein in E. coli, which targets the recombinant proteins from

The cyttoplasm to the periplasm of the bacteria. Upon translocation, the pelB signal peptide is cut off and the PBP is released into the oxidative environment of the periplasm, favorable to the formation of disulfide bonds. This system has been successfully used for the expression of the pheromone-binding protein of Bombus mori (26), which contains 3 disulfide bridges (28), and in the case of a chemosensory protein from M. brassicae.²

² V. Campanacci, A. Mosbah, O. Bornet, R. Wechselberger, E. Jacquin-Joly, H. Darbon, C. Cambillau, and M. Tegoni, submitted for publication.
Evidence for tryptophan-sensitized fluorescence, which argues against a Förster resonance energy transfer mechanism, but rather for local environmental effects.

**Bombykol**—To confirm the proper folding of BmorPBP, we have used bombykol as an intrinsic fluorescent probe, exploiting its conjugated double bond. Bombykol produced an increase in the intrinsic fluorescence of BmorPBP, and saturation was reached after addition of 5 equivalents. A very similar result was observed with Mbra1-M6 (Table I).

**AMA Displacement by Pheromones or Fatty Acids**

To estimate the binding affinities of MbraPBP1 and ApolPBP1 for a variety of different ligands, we monitored the decrease of AMA fluorescence resulting from the ability of different pheromones and fatty acids to displace AMA, and determined the $K_{\text{diss}}$ values for the different compounds.

For MbraPBP1, the three specific *M. brassicae* pheromones, Z11-C16-aldehyde, alcohol, and acetate, exhibit very similar AMA displacement properties with $K_{\text{diss}}$ values of 0.29, 0.17, and 0.20 $\mu M$, respectively (Table I). However, these species-specific, and physiologically relevant, compounds are less efficient than bombykol, the pheromone specific to the moth *B. mori* ($K_{\text{diss}} = 0.13 \mu M$; Table II; Fig. 4A). The best AMA competitor was found to be cetyl alcohol (C16-OH), the saturated equivalent of the Z11-C16-OH ($K_{\text{diss}} = 0.09 \mu M$). Fatty acids also bind well to MbraPBP1, especially palmitic acid (C16-COOH) which exhibits a $K_{\text{diss}}$ (0.12 $\mu M$) very close to the $K_{\text{diss}}$ of bombykol. The $K_{\text{diss}}$ values determined for the compounds tested in competition with AMA binding to MbraPBP1 cluster in a narrow range, between 0.09 and 0.63 $\mu M$ (7-fold), irrespective of their behavioral specificity (Table II).

In the case of ApolPBP1, three types of test ligands were used in AMA competition assays: the three species-specific pheromones, pheromones from other species, and fatty acids. The $K_{\text{diss}}$ values observed for all compounds were between 0.48 and 1.36 $\mu M$ (Table II, Fig. 4B). The three components of the pheromonal blend released by *A. polyphemus* females, E6,Z11-C16-Ald, E6,Z11-C16-Ac, and E4,Z9-C14-Ac, display similar $K_{\text{diss}}$ values of 0.50, 0.48, and 0.51 $\mu M$, respectively (Table II). The two former compounds are the best AMA competitors of this series; however, like MbraPBP1, a low $K_{\text{diss}}$ value, indicative for a high affinity to ApolPBP1, has also been determined for bombykol (0.54 $\mu M$). Indeed this $K_{\text{diss}}$ value is very close to that obtained for the components of the specific pheromonal blend. As seen for MbraPBP1, fatty acids are also very efficient in displacing AMA from ApolPBP1; $K_{\text{diss}}$ values in this case ranged between 0.56 and 1.36 $\mu M$, indicating an affinity of fatty acids to ApolPBP1 similar to that of specific pheromones.

**DISCUSSION**

Fluorescence of probes like AMA, 1-anilino-8-naphthalenesulfonic acid, or 1,1'-bi(4-anilino)naphthalene-5,5'–disulfonic acid have already been used to study the affinity constants of ligands to lipocalins, such as rat OBP (32, 33), porcine OBP (34), apolipoprotein D, insecticyanin, and bovine β-lactoglobulin (35). Although fluorescence spectroscopy has proved its worth in probing the binding of odorous compounds to vertebrate OBPs, this technique has rarely been applied to the study of the interaction between insect PBPs and pheromones. Intrinsically fluorescence and 1-anilino-8-naphthalenesulfonic acid fluorescence have previously been used, however, but only to check for structural changes of *B. mori* PBP at different pH values and for its binding to bombykol (26, 36). In the present study, we successfully reproduced the effect of bombykol on the BmorPBP intrinsic fluorescence. The behavior of Mbra1-M6, designed to mimic BmorPBP, with AMA and bombykol used as fluorescence quenchers was found to be similar to that of the authentic
Fluorescence Binding Assay with Moths PBPs

B. mori PBP. The intrinsic fluorescence of MbraPBP1 and ApolPBP1 decreased upon increasing the concentration of AMA, which indicates an interaction between AMA and tryptophans of the protein.

The three-dimensional structure of the B. mori PBP (17) displays a buried cavity surrounded by the 6 helices constituting the PBP fold. The sequences of MbraPBP1 and ApolPBP1 are, respectively, 44 and 67% identical to B. mori PBP (Fig. 1) and show no insertions or deletions. Furthermore, the CD spectra indicate a very similar overall fold for the four PBPs under study. It seems therefore reasonable to speculate on the monitored at 490 nm with excitation at 298 nm. Data represent the mean of three independent measurements. Standard deviations are indicated by error bars. The curve corresponds to the theoretical binding curve for $n = 1$ and $K_{diss} = 4.5 \mu M$. Inset, fluorescence emission spectra of AMA in the presence of MbraPBP1. Recombinant MbraPBP1 (1 \mu M in 20 mM Tris, pH 8.0) was titrated with increasing amounts of AMA (0–9 \mu M). Excitation wavelength was 298 nm. Fluorescence emission spectra were recorded at 20 °C between 440 and 600 nm. The emission maximum was at 492 nm.

**TABLE I**

| Ligand   | MbraPBP1, $K_{diss}$ | ApolPBP1, $K_{diss}$ | BmorPBP, $K_{diss}$ | Mbra1-M6, $K_{diss}$ |
|----------|----------------------|----------------------|---------------------|----------------------|
| AMA$^a$  | 4.5 (6.0)$^b$       | 0.95                 | NB$^c$              | NB$^c$               |
| Bombykol | 0.13$^b$            | 0.54$^c$            | 1.1$^b$            | 0.93$^b$            |

$^a$ AMA fluorescence.

$^b$ Tryptophan fluorescence.

$^c$ NB, no binding.

**TABLE II**

Inhibition of AMA binding to MbraPBP1 (A) and ApolPBP1 (B) by pheromones and fatty acids

Competitor concentrations causing a decay of fluorescence to half-maximal intensity were determined as IC$_{50}$ values from curves resulting from competition assays as shown in Fig. 4. $K_{diss}$ values were calculated according to $K_{diss} = [IC_{50}]/(1 + [AMA]/K_{AMA})$. [AMA] = free AMA concentration; $K_{AMA}$ = dissociation constant for PBP/AMA.

**Fluorescence Binding Assay with Moths PBPs**

**Fig. 3.** AMA fluorescence. A, AMA fluorescence emission spectra. MbraPBP1 (---), free AMA (--), MbraPBP1 + AMA (--). Excitation wavelength was 298 nm; MbraPBP1 was 1 \mu M, and AMA was 6 \mu M. B, titration of MbraPBP1 with AMA. Relative fluorescence intensity is plotted as a function of AMA concentration. Conditions were as follows: 1 \mu M MbraPBP1 in 20 mM Tris, pH 8.0, at 20 °C. AMA fluorescence was monitored at 490 nm with excitation at 298 nm. Data represent the mean of three independent measurements. Standard deviations are indicated by error bars. The curve corresponds to the theoretical binding curve for $n = 1$ and $K_{diss} = 4.5 \mu M$. Inset, fluorescence emission spectra of AMA in the presence of MbraPBP1. Recombinant MbraPBP1 (1 \mu M in 20 mM Tris, pH 8.0) was titrated with increasing amounts of AMA (0–9 \mu M). Excitation wavelength was 298 nm. Fluorescence emission spectra were recorded at 20 °C between 440 and 600 nm. The emission maximum was at 492 nm.

**C**

$K_d = 0.95 \mu M$

$[AMA] \mu M$

$0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8$

Relative fluorescence intensity 300

$250$

$200$

$150$

$100$

$50$

$0$

wavelength (nm)

$[AMA] \mu M$

$0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8$

Relative fluorescence intensity 300

$250$

$200$

$150$

$100$

$50$

$0$

wavelength (nm)

$[AMA] \mu M$

$0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8$

Relative fluorescence intensity 300

$250$

$200$

$150$

$100$

$50$

$0$

wavelength (nm)
three-dimensional structural properties of MbraPBP1 and ApolPBP1 based on those of the B. mori PBP. In such a model, the two conserved tryptophans (at the same position in the three sequences) have been localized relative to the cavity; Trp-37 is buried in the PBP and is an integral part of the cavity wall, whereas Trp-127 is far from the cavity, and not fully exposed to the solvent (Fig. 1). In this context, the decrease in intrinsic fluorescence in the presence of AMA for two PBPs is likely to be mediated by the interaction between the fluorophore and Trp-37 as suggested by the following arguments: first, upon binding to the protein, the maximum emission wavelength of AMA is blue-shifted and the quantum yield is substantially increased, indicating a significant modification of the environment of AMA, which inside the protein becomes protected from the solvent. Second, the intrinsic fluorescence can be quenched only if the tryptophan is close enough to AMA (a few Å) to interact electrostatically, and this excludes Trp-127. In the three-dimensional model, AMA fits well into the cavity, positioned about 5 Å from Trp-37 (Fig. 5). The $K_{\text{diss}}$ of AMA for MbraPBP1 was found to be $6.17 \pm 0.3$ nM by intrinsic fluorescence and AMA fluorescence, respectively. Finally, the AMA binding assays with MbraPBP1 and Mbra1-M6, where the first PBP binds AMA and its variant not, indicate that mutations in the internal cavity have impeded AMA binding, and, therefore, that AMA very likely binds to the internal cavity in the native protein.

With the aim of determining the specificity of MbraPBP1 and ApolPBP1, competitive binding experiments with several aliphatic ligands were performed. The data indicate that pheromones as well as fatty acids can displace AMA from its binding site. Furthermore, there was no obvious correlation between the presence, the position, and the number of double bonds, and $K_{\text{diss}}$, nor were there any obvious effects of the functional group of the first carbon and the chain length (C14, -16, or 18). These results are in contrast with previous studies suggesting that pheromone discrimination might occur not only at a receptor level, but also in the first molecular step, i.e. binding of pheromones to PBPs (8, 21–24, 37–39). Concerning MbraPBP1, migration on native gels and autoradiography indicated that the antennal protein was able to bind the tritiated major pheromonal compound, Z11-C16-Ac (38). However, since this was the only compound tested, the result should be taken with caution. Furthermore, autoradiography studies with the recombinant PBP indicated an affinity not only for the Z11-C16-Ac, but also for the Z11-C16-TFMK (trifluoromethyl ketone), but not for the saturated C16-Ac (25). Indeed, the literature is punctuated with reports misgiving the specificity of PBPs. AperPBP1, expressed in insect cells, has been shown to bind three different compounds, E6,Z11-C16-acetate, -alcohol, and -aldehyde (22), while the PBP of A. polyphemus has been shown to bind compounds other than the specific pheromone, namely decyl-thio-trifluoropropanone, an inhibitor of pheromone receptor neurons of the moth (40). On this basis it has been suggested that decyl-thio-trifluoropropanone, like pheromones, could be carried through the sensillum lymph by PBP and compete with the pheromone-PBP complex at the receptor. Moreover, previous binding experiments of ApolPBP1 with two different photoactivable compounds, (E6,Z11)-[3H]hexadecadienyl diazoacetate and (E4,Z9)-[3H]tetradecadienyl diazoacetate, led to the
proposition that two residues, Thr-44 and Asp-32, were part of the pheromones-binding site (41). However, based on the three-dimensional structure of B. mori PBP, these residues and the covalently modified regions do not belong to the binding cavity (17).

Our present results raise two important structural questions: 1) does AMA bind to the same internal binding site as pheromones, the site identified in the x-ray structure (17) of BmorpBP? 2) Do the various recombinant PBPs have a three-dimensional structure identical to that of the natural antennal PBPs? With regards to question 1, AMA binding to both Mbra and Apol PBPs yields a significant decrease of intrinsic fluorescence (see above), suggesting a close approach between AMA and a tryptophan. Since, as deduced from the PBP structural model, the external surface of both PBPs contains only a few hydrophobic residues and no hydrophobic patches, it is not very likely that AMA binds at the PBP's surface near the second tryptophan (Trp-127). Furthermore, Mbra1-M6, in which only residues located within the cavity have been changed to mimic the cavity of BmorpBP, loses its ability to bind AMA thus resembling BmorpBP. With respect to the second question, due to the lack of a direct comparison of three-dimensional structure or binding data for natural and recombinant PBPs, no direct evidence for the correct folding of recombinant PBPs is available. However, several lines of evidence suggest these proteins fold correctly. (i) Recombinant PBPs are expressed in E. coli periplasm, in conditions allowing disulfide bridge formation; (ii) no post-translational modifications have been identified in natural PBPs, therefore bacterial expression should be adequate for obtaining a native fold; (iii) the CD spectra are all very similar and in agreement with the secondary structure predictions; (iv) BmorpBP generated by the same procedure resulted in crystalline PBP leading to the three-dimensional structure and binding bombykol (26).

CONCLUDING REMARKS

Based on a fluorophore displacement assay, two recombinant PBPs are shown not to display significant discriminatory capacity between different pheromones, while two others do not bind the fluorophore AMA, precluding displacement studies. The observed lack of discrimination brings into question the hypothesis that PBP subtypes may be specifically tuned to distinct species-specific pheromones. One way to reconcile our data with previous results, is to postulate that among the several PBPs found in a single moth species (often three different PBPs), some might be nonspecific while others are more specific. Since AMA is a bulky compound, very different from the insect pheromone structures, it might be possible that PBPs able to bind AMA are rather nonspecific. Among the three PBPs studied here, MbraPB1 and ApolPB1 would be nonspecific, while BmorpBP might be specific. Indeed current studies indicate that some other recombinant PBPs bind AMA and others do not. While the existence of several PBPs, each specific for a distinct component of the pheromonal blend, would be conceivable in a molecular and evolutionary context, the co-existence of several nonspecific PBP subtypes retained throughout evolution is a challenging question. Alternatively, one could imagine that compounds may be readily bound by the proteins without being able to activate them, thus reflecting the situation of receptor proteins which are able to interact with a large variety of pharmacological compounds, some of which activate the receptor (agonists) whereas other do not activate (antagonists), although the latter often display a higher binding affinity.

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