The Crystal Structure of a Cockroach Pheromone-binding Protein Suggests a New Ligand Binding and Release Mechanism*

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Pheromone-binding proteins (PBPs) are small helical proteins found in sensorial organs, particularly in the antennae, of moth and other insect species. They were proposed to solubilize and carry the hydrophobic pheromonal compounds through the antennal lymph to receptors, participating thus in the peri-receptor events of signal transduction. The x-ray structure of Bombyx mori PBP (BmorPBP), from male antennae, revealed a six-helix fold forming a cavity that contains the pheromone bombykol. We have identified a PBP (LmaPBP) from the cockroach Leucophaea maderae in the antennae of the females, the gender attracted by pheromones in this species. Here we report the crystal structure of LmaPBP alone or in complex with a fluorescent reporter (aminophthalen sulfonate, ANS) or with a component of the pheromonal blend, 3-hydroxy-butan-2-one. Both compounds bind in the internal cavity of LmaPBP, which is more hydrophilic than BmorPBP cavity. LmaPBP structure ends just after the sixth helix (helix F). BmorPBP structure extends beyond the sixth helix with a stretch of residues elongated at neutral pH and folding as a seventh internalized helix at low pH. These differences between LmaPBP and BmorPBP structures suggest that different binding and release mechanism may be adapted to the hydrophilicity or hydrophobicity of the pheromonal ligand.

The sexual behavior of insects, and in particular the mate choice, is triggered by small volatile molecules called pheromones. In Lepidoptera, more than in other insect species, olfaction is preeminent compared with other senses. This feature has led to the development of large antennae, which allow the males to perceive the sexual pheromone blend emitted by females and to respond to it (1). In this family of insects, pheromone-binding proteins (PBPs)1 produced in males antennae and secreted in the antennal lymph have been proposed to accommodate pheromones and ferry them from the air/antenna interface to their receptors.

PBPs are acidic proteins containing an average of 120–150 amino acids with 6 conserved cysteines involved in three disulfide bridges (1–3). The crystal structure of a PBP from Bombyx mori (BmorPBP) has been solved in complex with bombykol (4), unique until now of this class of proteins. In addition, the structure of a functionally related chemosensory protein (CSP) has been reported recently (5, 6). BmorPBP three-dimensional structure revealed a six α-helices fold, which delineates a buried cavity containing the hydrophobic pheromone, bombykol, a C16 long chain alkene alcohol. Two three-dimensional structures of the apoprotein were further solved by NMR (7, 8). The one at low pH (pH 4.5) revealed that the C terminus (residues 125–137) has switched from an elongated stretch conformation to an α-helix. Amazingly, this seventh helix occupies the internal cavity filled with bombykol in the structure of the complex (7). In contrast, the other apoform, solved at neutral pH, exhibits a three-dimensional structure close to that observed by x-ray diffraction in the complex, confirming thus the importance of the pH in triggering the seventh helix formation and internalization (8). Since the pH is acidic near the membrane, the authors have proposed that the PBP may release the pheromone when reaching the membrane imbedded GPCR receptor using this mechanism (9). The direct involvement of PBPs in olfaction has been described for the first time by Krieger and Ross (10) who identified in fire ants two PBP alleles governing alternative social behaviors. This finding suggested that different receptors might be activated by a specific PBP allele-social pheromone complex.

In contrast with moths, in the cockroach Leucophaea maderae females perceive the sexual pheromone blend emitted by males (10). A protein of ~13 kDa has been isolated from the antennae of the females and characterized (11). Based on the dimorphism observed in its sex expression, its low, but significant, identity with moth PBPs (17% with BmorPBP, Fig. 1A) and its ability to bind some components of the pheromonal blend, this protein (LmaPBP) has been assigned to the PBP family (11).

The sexual pheromone blend of L. maderae is known and its components have been previously identified and behaviorally tested (10). The blend is composed by four compounds: the 3-hydroxy-butan-2-one (H3B2) is the long range attracting pheromone; the butane-2,3-diol seems to have no behavioral response and might be the precursor of H3B2; senecioic acid (3-methylbutenoic acid) and (E)-2-octenoic acid are the pheromone compounds that stop the female at a close distance of the male. In contrast with moth pheromones, which are highly hydrophobic, these compounds are all very hydrophilic. We

1 The abbreviations used are: PBP, pheromone-binding protein; BmorPBP, B. mori PBP; CSP, chemosensory protein; GPCR, G-protein-coupled receptor; LmaPBP, L. maderae; SAD, single wavelength anomalous diffraction; r.m.s.d., root mean square deviation; MbraCSP, M. brassicaceae chemosensory protein.

* This work was supported in part by the Provence, Alpes, Cote d’Azur (PACA) region (number 2011/2052) and by the Conseil Général des Bouches-du-Rhône. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1ORG, 1OW4, and 1IP28) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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have shown recently, using ANS as a fluorescence reporter, that H3B2 and butane-2,3-diol are able to displace ANS and bind to LmaPBP, while the two acidic compound are not (11).

Based on these binding results, we have crystallized LmaPBP alone or in complex with H3B2 (12) and in the presence of the fluorescent reporter ANS, which was used to test pheromone binding (11). We report here the high resolution crystal structures of the apo-LmaPBP and of the two complexes, which suggest a mechanism of ligand binding and release differing drastically from that of BmorpB.

MATERIALS AND METHODS

**LmaPBP Cloning, Expression, and Purification**—LmaPBP was cloned from female adult antennae and subcloned in PET-22b+ as described elsewhere (11) and for other PBP s or CSPs (14, 15). In brief, expression was carried out in the periplasm of Escherichia coli Bi21(DE3) at 18°C after induction by 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. The periplasmic proteins were released by osmotic shock and purified by anion exchange on ResourceQ column (Amer sham Biosciences) and gel filtration (Superdex 200, Amersham Biosciences). Expression of selenomethionine substituted LmaPBP was performed using the method of methionine biosynthesis pathway inhibition (16). Purification of the selenomethionine protein was performed as described above.

**Crystalization of LmaPBP**—Single crystals of LmaPBP were obtained at 20°C by the sitting drop vapor-diffusion method using nano drops (17) in two different crystal forms as reported elsewhere (12). The crystals of form I, that of the apo-protein and the complex with ANS, belong to space group P1, with cell dimensions a = 43.2 Å, b = 45.1 Å, c = 45.7 Å, α = 118.6°, β = 93.0°, and γ = 106.9°, and contain two molecules in the asymmetric unit (Vₐ = 2.6 Å³/Da, 52% solvent). A second crystal form was obtained with the pheromone component H3B2, with space group P2₁, cell dimensions a = 38.2 Å, b = 62.2 Å, c = 45.1 Å, and β = 93.0° and two molecules in the asymmetric unit (Vₐ = 1.9 Å³/Da, 35% solvent).

**Diffraction and Data Collection**—All data sets were collected at European Synchrotron Radiation Facility (Grenoble, France). Diffraction images were indexed and integrated with DENZO (18) and scaled with SCALA (19). A Se-Met-substituted crystal of form I was exposed at ID29 beamline at three wavelengths tuned at the peak, inflection, and remote energies. The crystal used was cryocooled at 100 K within its crystallization mother liquor and with 25% of glycerol. Images were collected on an ADSC-Q4 detector, with a crystal to detector distance of 200 mm, using a 1° oscillation with 1 s exposure time. A complete data set has been carefully collected at 1.9 Å resolution using 528 images for the peak wavelength data set; the data at other wavelengths were useless for structure resolution. The diffraction data have a R-value of 4.7% and completeness of 89% (Table I). In addition, a second SAD data set, a second SAD data set has been recorded on BM14 beamline at the selenium peak energy. 720 images were collected at 1.7 Å resolution on a CCD camera with a crystal detector distance 100 mm and the same oscillation range as above. The R-value and the completeness are 4.5 and 96%, respectively (Table I).

The crystal form I of LmaPBP obtained in presence of ANS was exposed at BM14 beamline. Two data sets were recorded for the high and low resolution, consisting of 180 images (1° oscillation) and 90 images (2° oscillation), respectively. The distance crystal detector was fixed to 150 and 250 mm. The crystal diffracts to 1.6 Å resolution, and the R-value is of 5.7% with a completeness of 85% (Table I).

The crystal form II obtained in the presence of H3B2 was collected at European Synchrotron Radiation Facility BM14 beamline, using a procedure similar to that of form I. Between 30- and 1.7 Å resolution, the R-value is 4.6%, and the completeness was 97.0% (Table I).

**Structure Solution and Refinement**—All attempts to solve the LmaPBP structure by molecular replacement using AMoRe (20) with BmorpB as a search model failed. This is certainly due to the low sequence identity between the two proteins (Fig. 1A). The structure of apo-LmaPBP was finally solved from a Se-Met-substituted crystal (form I) using SAD method at the Se-K edge (λ = 0.9792 Å, f = –8.3; Table I). Initial phases were obtained at 2.5 Å with a figure of merit of 0.468 using SOLVE (21). A total of 9 sites out of the 12 selenium atoms per asymmetric unit were located. Phases were improved by solvent flattening using Resolve (19) and led to a figure of merit of 0.707. The experimental density map after solvent flattening was clear enough to manually build two-thirds of the model using Turbo-Frodo (22) and to locate the three disulfide bridges. Phase improvement was carried out at 1.7 Å using phases coming from the constructed model with ARP—ARP (23). The good quality of the resulting map made it possible to finish building all missing parts of the model. The model was refined using Refmac (24). The structure of the complex with ANS was obtained by isomorphous replacement, while the complex with the H3B2 was solved by molecular replacement with AMoRe (20). The two models were also refined using Refmac (22). Statistics of refinement and model quality are reported in Table I. Protein geometry of the native, ANS complex, and H3B2 complex was assessed using Procheck (25) showing 95.7, 94.8, and 95.7% residues in the most favorable region, respectively. The coordinates and structure factors have been deposited in the Protein Data Bank at the Research Collaboratory for Structural Bioinformatics as entries 1ORG, 1OW4, and 1P28, respectively.

**RESULTS**

**Overall Structures of LmaPBP Alone or in Complex**

**Crystallographic Results**—The apo-LmaPBP and the ANS complex belong to the same crystal form (Form I) and contain two monomers in the asymmetric unit (12). The small interaction surface observed between the two monomers is in favor of a non-biological assembly, as expected from gel filtration results where a monomer was observed. The LmaPBP-H3B2 pheromone complex belongs to crystal form II, which also contains a non-biological dimer in the asymmetric unit (12). The complete polypeptide chain of the apoprotein was built in the electron density map between residues 1 and 118 for chains A and B. In addition, 186 water molecules and two glycerol molecules were identified. In the complex with ANS, besides the residues from the native gene sequence, two and one extra residues from the expression vector (11) were visible in the electron density map, at the N terminus, for chains A and B, respectively. A molecule of ANS was readily identified in the electron density map, one per monomer. A positive residual electron density remained after refinement, which could be interpreted by fitting glycerol molecules on top of the ANS molecule, with a 30:70 occlusion ratio (glycerol:ANS). A total of 243 water molecules were also added in the electron density map. In the complex with the H3B2 pheromonal compound, the polypeptide chain is visible between residues –1 to 117 and 2 to 118 in chains A and B, respectively. 235 water molecules were added. A pheromone molecule was clearly visible in both monomers. Overall, the B-factors are quite low in the three structures (24.4, 19.5, and 16 Å², respectively) taking into account their high resolution (Table I).

**LmaPBP Fold**—LmaPBP is formed of six helices comprised between residues 1–16 (A), 24–31 (B), 39–52 (C), 68–74 (D), 80–96 (E), and 103–117 (F) (Fig. 1B). Three disulfide bridges are established between cysteine pairs 16/47, 43/104, and 92/113 and bridge together helices A/C, C/F, and E/F, respectively. They are located at the positions expected from the sequence alignment with BmorpB (Fig. 1, A and B). The total helical amount of 65% observed in the three-dimensional structure is significantly higher than the values from secondary structure prediction obtained from Jpred (45%) or those from circular dichroism (40%) (11). Amazingly, helices are not connected by turns but by extended stretches of 5–7 residues in length (Fig. 1B). An exception occurs between helices C and D, where a segment of 15 non-helical residues forms a quasi-hairpin structure (residues 54–62) between two turns (Fig. 1B).

**Deviations between Monomers**—The r.m.s.d. values calculated between each pair of monomer (A and B) in the three structures range between 0.40 and 0.84 Å for all Cα atoms. The apoprotein and the ANS complex present minor differences compared with those observed between their monomers A and B. In contrast, the complex with H3B2 exhibits slightly larger differences between monomers, 0.8 Å r.m.s.d. versus 0.48 Å and 0.40 Å for the two former structures. Monomer B seems to be responsible for this larger deviation, since its r.m.s.d. value
calculated against monomers A and B of the apo and H3B2 structures are 0.1 Å higher in average than those originating from monomer A. Excluding monomer B of the H3B2 complex from the r.m.s.d. calculations lead to an average value of 0.55 Å between the eight couples.

Comparison with the BmorBPB Structures—Although sharing only 17% identity (Fig. 1A), LmaPBP and BmorPBP present similar folds (Fig. 2). We have compared the structure of apo-LmaPBP with the x-ray structure of Bmor-PBP bound to its pheromone, bombykol, at neutral pH (4). The r.m.s.d. value involving 118 Cα atoms aligned in the two x-ray structures is 2.9 Å. Indeed, the six helices have similar orientations (Fig. 2A); helices A, C, D, and F have their main axes co-linear, whereas helices B and E diverge. Consequently, the two disulfide bridges established between the closely aligned helices A/C and C/F are within 0.8 Å (Cys Cα atom), whereas the third one,
between helices E and F, has the Cα atoms of the Cys at a 1.4–1.9-Å distance. LmaPBP is 19 residues shorter than BmorPBP: 4 residues at the N terminus and 15 at the C terminus; the small differences in length observed in loops or helices, a total of 4 residues, cancel each other. The NMR structure of the apo-BmorPBP at acidic pH (7) is also well superposed to LmaPBP, with a r.m.s.d. value of 2.60 Å. Indeed, most of the differences between the two BmorPBP structures occur between residue 122 and the C terminus, a segment of residues absent in LmaPBP (Fig. 2A). The apo-LmaPBP structure presents an open pocket delineated by its six helices (Fig. 3A). This cavity is wide open to the bulk solvent (Fig. 3A) and is much smaller than that observed in BmorPBP where the cavity communicate with the solvent through a small opercula (Fig. 3B). In the apoprotein, the cavity contains a serendipitous glycerol molecule. Such molecules are often observed in small crevices at protein surfaces. A glycerol molecule, introduced as cryoprotectant, has also been observed nested in the pheromone binding cavity of a porcine PBP (28). The glycerol molecules B-factors are higher than average, indicating a high positional mobility, as expected for weak ligands (Table I). In the apo-PBP, the cavity is about 15 Å deep and ~10 × 15 Å in diameter. Its walls are covered by hydrophobic residues (Leu residues at positions 36, 45, 49, and 54, Ala46, Val89, Ile107, and Phe110), as in BmorPBP, but also by polar non-charged residues (Tyr residues at positions 5 and 75 and Thr111). The mouth of the cavity is an oval of 10 by 8 Å formed by 6 residues (Arg33, Asn44, Pro65, Lys85, Val114, and Arg115), among which three are positively charged. These charged side chains form the lips of the cavity and can provide potential binding sites for ligands.

The Complex with H3B2—Amazingly, despite the considerable amount of glycerol brought into the crystal for cryocooling (25%, 2.7 M), the H3B2 molecule was not displaced as a result of its good affinity with LmaPBP ($K_d = 3.8 \mu M$ (11)). Indeed, the H3B2 pheromonal ligand being smaller than glycerol has room enough to bind in the LmaPBP cavity without perturbing the cavity wall side chains (Fig. 3C). The H3B2 electron density map was clearly defined. Some positive difference density remained after fitting the S-enantiomer, however, and the R-enantiomer was subsequently introduced to account for it. After refining the positions and the occupancies (50:50), no significant residual electron density was present. This indicates that LmaPBP does not select one of the H3B2 R- or S-enantiomer from the commercial racemic mixture, but binds both (Fig. 3D). The H3B2 molecule establishes interactions with polar, charged, and hydrophobic residues, which point inside the cavity: Tyr5 and Tyr75, Lys89 (lips), and Phe110 (Table II). As in the case of glycerol, interactions are mainly mediated by electrostatic forces.

The Complex with ANS—In contrast with the H3B2 ligand, the much larger fluorescent probe ANS (Fig. 3E) modifies largely the LmaPBP cavity yielding a volume increase of ~50% (Fig. 3F). The ANS molecule contacts many more residues than the H3B2 ligand (Table II). The molecule is positioned with its hydrophobic phenyl and naphthyl groups imbedded within the cavity, the sulfate group emerging at the level of the cavity mouth (Fig. 3F). If positioned in the apoprotein cavity, ANS clashes severely ($d < 1 \AA$) with 2 residues: its naphthyl moiety with the Met71 side chain and its sulfate group with Lys85, an opercula residue. Consequently, in the complex the χ1 angle of Met71 has changed by 120°, yielding a larger space, but provoking an overlap of the rotated atoms with the ring atoms of Phe77. The χ1 angle of Phe77 had to change by 130°, bringing the phenyl ring in a position totally exposed at the protein surface. At the cavity mouth, Lys85 side chain rotates far from...
ANS, to a position 5.0 Å away from it. Meanwhile, Arg33, another mouth residue, has moved slightly in the direction of the ANS sulfate group, establishing two ion bonds (3.0 Å) through its N/H92572 and N/H9280 atoms with two sulfate oxygens. A hydrogen bond (2.5 Å) is also established between Tyr75 OH and a sulfate oxygen. The Lys85 short contact together with another short contact with Val89 (2.8 Å), perpendicular to the naphthyl ring, have been released by a main chain movement of the residue stretch 81–89, with a maximum displacement of 1.8 Å for the C/H9251 atom of Thr82.

The strength of the ANS interaction with LmaPBP seems to result from two antagonist features: the positive one is the strong electrostatic and hydrophobic interaction with the cavity, and the negative one is the strain induced by this large ligand on side-chains and partly on the main chain. The relatively low $K_d$ of 2.1 M observed (11) reflects well the partial cancellation of these antagonist forces. The sulfate group appears to be a key element for the binding. It is not surprising therefore that the fluorescent reporter AMA bearing a NH$_2$ group could not bind to LmaPBP (11).

**DISCUSSION**

The conformations of LmaPBP unbound and bound to its pheromone are very close. Similarly, the BmorPBP pheromone complex (x-ray) and the apoprotein (NMR), both obtained at neutral pH, do not reveal significant changes considering the different techniques of determination. The helical structure of PBPs is probably rigidified by three interhelical disulfide bridges, the positions of which are remarkably conserved between Lma- and BmorPBP and very probably with all other classical hexacysteine PBPs. Despite its backbone rigidity, LmaPBP presents a cavity flexible enough to bind ANS, a ligand much larger than the pheromone; this flexibility is mainly due to the fluidity of the side chains. This contrasts with the drastic backbone conformational changes resulting upon binding of three surrogate 12-bromododecanol molecules.
in *Mamestra brassicae* chemosensory protein (MbraCSP; Refs. 5, 6, and 29). The flexibility of MbraCSP may be due to the position of the disulfide bridges, located within loops, which provide thus little constraint to the whole protein structure (5, 6, 29).

The ANS-LmaPBP complex structure is the first example of a fluorescence reporter observed in the binding site of an insect PBP. In a previous work, the binding site of fluorescent probes introduced to assess PBP binding capacity was questioned (15). We indirectly demonstrated that the binding site was unique and was the internal cavity, as expected from structural considerations. The complex LmaPBP-ANS demonstrates that more generally, as for mammalian PBPs/OBPs (30), the fluorescent reporter binds in the internal ligand cavity of PBPs.

LmaPBP cavity walls and mouth, although containing a majority of hydrophobic side-chains, contain also a significant amount of polar or charged residues. These polar/charged residues make it possible to bind small hydrophilic ligands such as H3B2. The presence of polar and charged side chain forming the walls of the LmaPBP cavity is in contrast with the cavities of BmorPBP (4, 7, 8) or MbraCSP (5, 6, 29), which are almost exclusively formed of hydrophobic residues. Furthermore, sequence comparisons among PBPs from moth confirm the hydrophobic nature of their cavity, in agreement with the largely hydrophobic nature of their pheromonal compounds: long alkyl chains (C14–C18) alcohols, aldehydes, or acetyl esters. However, the low sequence similarity observed between PBPs from moth and those from other classes of insects prevents any clear identification of the nature of their binding cavity; this should await the resolution of more three-dimensional structures.

Indeed, the fact that LmaPBP has a cavity including several polar side chains fits nicely with the hydrophobic nature of *Leucophaea maderae* pheromonal blend. Interestingly, both insect or mammal PBPs have been proposed to ferry hydrophobic compounds, mostly insoluble in the peri-receptor fluid, from air to the GPCR receptors. The fact that LmaPBP binds H3B2, a compound highly soluble in water, raises doubts about a unique role of PBPs as transport proteins and reinforces the hypothesis of a wider function in which they would trigger their receptors once bound to the pheromone (9, 15, 31), in line with the functional finding of Krieger and Ross (15). In this context, the crown of positive residues around the cavity mouth of LmaPBP might interact with a negatively charged GPCR receptor. It has been postulated that bombykol, the very hydrophobic pheromone of BmorPBP, would interact with the cell membrane around the receptor. It has also been proposed that the formation of a seventh internalized helix at low pH (near the membrane) might be used to expel the pheromone in the vicinity of the receptor. In LmaPBP the absence of the amino acid stretch corresponding to this seventh helix together with the evidence of an hydrophilic cavity binding an hydrophilic ligand suggest that another, more classical, mechanism of direct ligand release might be used.

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J. Biol. Chem. 2003, 278:30213-30218.
doi: 10.1074/jbc.M304688200 originally published online May 23, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304688200

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