Sexual attraction in the silkworm moth: structure of the pheromone-binding protein-bombykol complex

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Introduction

Olfaction is the primary sensory mode of insects and the way they locate mates, food, and oviposition sites. Electrophysiological recordings indicate that male *Bombyx mori* antennae can detect single molecules of its sex pheromone, bombykol. Furthermore, the moth must be able to recognize bombykol with a high degree of specificity: saturating a single double bond decreases its effectiveness as a pheromone by a factor of a thousand. Such a high level of olfactory function is provided by the complex biochemical system of cooperating olfactory proteins in the antenna, in which odorant binding proteins (OBPs) play an important, yet poorly understood, role.

The primary function of the OBPs is to solubilize hydrophobic odorants in the aqueous lymph which surrounds olfactory dendrites, delivering the odorant molecules to downstream odorant receptor proteins. Although genetic evidence strongly suggests that insect OBPs play a role in odorant discrimination, it is not known how strongly OBPs discriminate in binding to ligands, nor it is clear what features of an odorant molecule (for example size, functionality, or the locations and conformations of double bonds) are involved in that stage of discrimination by the olfactory system. Biochemical efforts to elucidate the binding specificities of these proteins have been disappointing, hampered by the extreme hydrophobicity of their ligands, leading us to attempt a structure-function approach to the problem.

Also poorly understood is the fashion in which OBPs deliver ligand to odorant receptor proteins. The *B. mori* pheromone binding protein (PBP) is known to undergo a pH-dependent conformational change from a "closed" to an "open" conformation when the environment drops below pH 5.8. Experiments with model membranes have indicated that the pH drop near a cell membrane is sufficient to trigger this change, and it has been suggested that the PBP could switch to an open conformation as it approaches the neuron, thus releasing pheromone in the immediate vicinity of waiting olfactory receptor proteins.

Using multiwavelength anomalous diffraction (MAD) phasing and data collected at the Advanced Photon Source (APS), we were able to determine the first three-dimensional structure of an OBP.

Methods and Materials

B. mori PBP was overexpressed in *E. coli* as described previously [1]. Crystals were obtained by the hanging drop method: drops of 2 µl protein complex and 2 µl reservoir solution were equilibrated at room temperature with a reservoir solution of 50% (w/v) PEG 20,000 and 100 mM Tris buffer, pH 8.2. Typical crystal dimensions were 0.06 x 0.06 x 0.5 mm^3. Native data were collected to 1.8 Å at MacCHESS station A-1 using an ADSC Quantum-4 detector. Attempts to solve the structure through molecular replacement failed due to the protein's novel fold; strongly diffracting, strongly derivatized crystals could not be obtained from heavy-atom soaks for MIR. The high proportion of methionine residues (seven out of 142 residues total) suggested MAD phasing, and selenomethionyl protein was prepared and crystallized under identical conditions to native protein. Selenomethionyl crystals were then taken to beamline 14-BM-D at the APS, and data collected to 2.8 Å using an ADSC Quantum-4 detector. All data were processed using the DPS/Mosflm/CCP4 graphical interface. Selenium positions were determined using SHELXS; selenium positions were refined and phases calculated using SHARP. Model building was carried out in the program O, and the structure was refined in CNS and REFMAC of the CCP4 suite. Protein geometry was assessed using PROCHECK.

Results

The overall fold of PBP is roughly conical, with four antiparallel helices converging to a point and enclosing the hydrophobic pocket with the broad end of the cone capped by a fifth helix. A sixth, smaller helix flanks the cone (see Figure 1). This fold has been seen previously only in THP12, a protein of unknown function from the mealworm *Tenebrio molitor*. Significantly, most of the residues that form the N-terminal helix of PBP are partially unwound in THP12, and the latter protein therefore has a hydrophobic groove open to solvent rather than PBP's enclosed pocket.

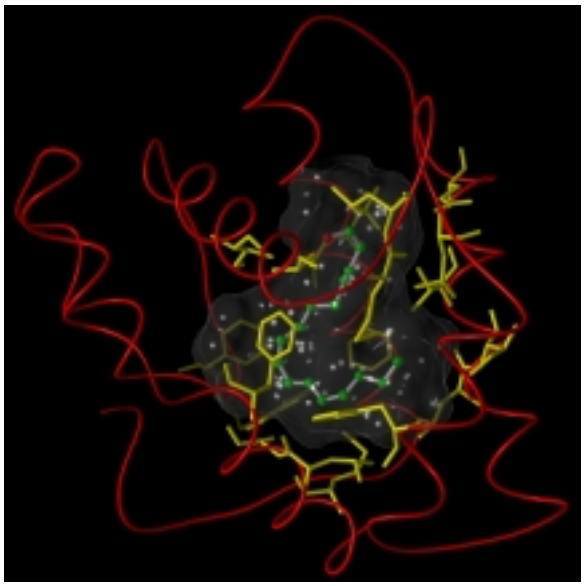

Figure 1: Overall fold of PBP.

PBP's hydrophobic pocket is flat and roughly triangular in shape, fitting closely to the curved conformation of bound bombykol. The residues lining the pocket are nearly all hydrophobic, with the notable exception of Ser56, which hydrogen bonds to the hydroxyl group of bombykol.

The pH-dependent conformational change is thought to occur at the loop from residues 60-69, which covers a large gap between α-helices at the hydroxyl end of bombykol. This loop is stabilized by an approximate antiparallel β-sheet arrangement of three hydrogen bonds. Three histidine residues (His69, His70, and His95) are clustered in the area and are strictly conserved across all known lepidopteran PBPs. A drop in pH below 6 would likely result in the protonation of these residues, causing electrostatic repulsion between His69 and His70 on the loop and His95 on the main body of PBP, which would move the loop away from the entrance of the binding pocket. Additionally, salt bridges between His69 and His70 and two residues on the loop, Asp63 and Glu65, could strain the loop and further destabilize it.

A second possible mechanism for the conformational change is suggested by the aforementioned THP12. The N-terminal helix, partially unwound in THP12, has few interactions with the rest of the protein and is interrupted by a short stretch that is not perfectly helical. Protonation of His80 could suffice to disrupt this helix, thereby opening the hydrophobic pocket to solvent. Either or both mechanisms, opening a lid formed by the loop or unwinding the N-terminal helix, would suffice to permit bombykol to enter or leave the pocket.

Interestingly, two molecules of PBP were present in the asymmetric unit. Although PBP has been observed to form a dimer in a pH-dependent fashion and would be expected to form a dimer at the pH of crystallization, it is unlikely that any dimer interface seen in the crystal structure is physiologically relevant. The largest contact area between molecules is 221Å^2. This interface involves only a small hydrophobic patch, consisting of Pro64 from one monomer and Met131, Val133, and Lys38 from another, and is not symmetric and would lead, in the limit, to aggregation rather than dimerization. The largest symmetric protein–protein contact occurs at a crystallographic two-fold axis with a contact area of 190Å^2, but involves no hydrophobic surface and only one salt bridge (Lys38 and Asp132) and one hydrogen bond (Ser130 and Lys6) for each monomer.

Discussion

The strong structural conservation of lepidopteran OBPs, as indicated by sequence identity (60% among PBPs in SWISS-PROT) and strict conservation of cysteine residues, means that the *B. mori* PBP structure is an important benchmark in understanding the binding specificity of these proteins. Comparison of binding site residues indicates that the majority of residues are highly conserved across lepidopteran PBPs and OBPs (displayed in red in Figure 2) and therefore are involved in nonspecific interactions with odorants. Leu8, Ser9, Leu68, and Leu90 (orange) are highly conserved across lepidopteran PBPs but not OBPs and are most probably the specificity determinants for the sixteen-carbon straight-chain motif common to the pheromones of all lepidopteran species used in this comparison. Met61, Leu62, Ile91, and Val114 (yellow) are variable across lepidopteran PBPs and are all located near the ends of the bombykol molecule. Although it is possible that these residues enable the PBP to discriminate between pheromones with different arrangements of double bonds, their placement

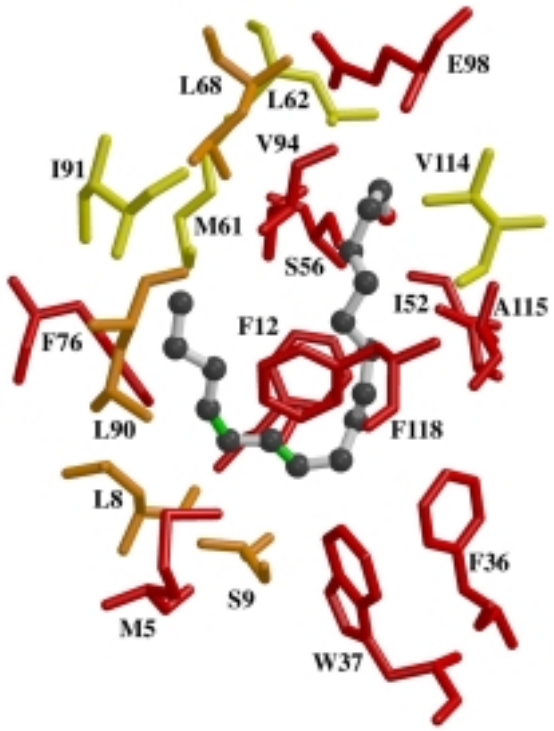

Figure 2: Comparison of binding site residues.

and the conservative nature of the substitutions make this unlikely. More significantly, Ser56, which is hydrogen bonded to the hydroxyl of bombykol, is mutated to alanine in those species that utilize acetyl ester pheromones (*A. pernyi* and *A. polyphemus*) and thus should serve as a specificity determinant for the functionality of the oxidized end of the ligand. The PBP would thus serve as a filter for molecules of the appropriate size and functionality, leaving the odorant receptor protein to perform the finer discrimination between molecules with different arrangements of double bonds.

The absence of the physiologically relevant dimer from the crystal structure of the PBP-bombykol complex and its presence in biochemical studies on the native protein suggest that dimerization takes place only in the absence of ligand. Such a result indicates that dimerization could play an important role in the transfer of pheromone from air to the odorant receptor proteins, possibly by maintaining the native protein in an open conformation (and therefore able to accept bombykol) when the PBP is not in the immediate vicinity of the neuron.

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Reference

[1] B.H. Sandler *et al*., *Chem. Biol*. **7 (2)**, 143–151 (2000), and references therein.