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Endogenous fatty acids in olfactory hairs influence pheromone binding protein structure and function in *Lymantria dispar* ☆

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ABSTRACT

The gypsy moth utilizes a pheromone, (7*R*,8*S*)-2-methyl-7,8-epoxyoctadecane, for mate location. The pheromone is detected by sensory hairs (sensilla) on the antennae of adult males. Sensilla contain the dendrites of olfactory neurons bathed in lymph, which contains pheromone binding proteins (PBPs). We have extracted and identified free fatty acids from lymph of sensory hairs, and we demonstrate that these function as endogenous ligands for gypsy moth PBP1 and PBP2. Homology modeling of both PBPs, and docking of fatty acids reveal multiple binding sites: one internal, the others external. Pheromone binding assays suggest that these fatty acids increase PBP-pheromone binding affinity. We show that fatty acid binding causes an increase in α-helix content in the N-terminal domain, but not in the C-terminal peptide of both proteins. The C-terminal peptide was shown to form a α-helix in a hydrophobic, homogeneous environment, but not in the presence of fatty acid micelles. Through partition assays we show that the fatty acids prevent adsorption of the pheromone on hydrophobic surfaces and facilitate pheromone partition into an aqueous phase. We propose that lymph is an emulsion of fatty acids and PBP that influence each other and thereby control the partition equilibria of hydrophobic odorants.

Introduction

Insects can communicate with members of their own species via pheromones. For example, the gypsy moth (*Lymantria dispar*) uses a hydrocarbon epoxide, (7*R*,8*S*)-2-methyl-7,8-epoxyoctadecane or (+)-disparlure, as a sex attractant. The females emit (+)-disparlure, and the males follow it to locate their mates. Pheromones and other odorants are detected by neurons in sensory hairs (sensilla), located on the antennae. The sensilla are hollow cuticular structures that are innervated by the dendrites of sensory neurons. The neuronal dendrites are bathed by sensillar lymph, an electrolyte solution rich in odorant-binding proteins (OBPs). Sensilla trichodea specialize in the detection of pheromones, and these contain OBPs that are also known as pheromone-binding proteins (PBPs).

Odorants from the air stream first adsorb on the cuticular structures of the insect. From there, the odorants are hypothesized to diffuse on the sensillar cuticle until they reach pores, through which the odorants enter the sensilla [1]. It has been proposed that PBPs desorb the hydrophobic pheromone from the pore canal [2] and ferry it through the lymph to the receptors on the neuronal dendrites in the sensillum, thereby protecting the pheromone from hydrolysis by a lymph esterase [3]. However, the kinetics of ligand binding and release are relatively slow (e.g., for PBP2: k_{on} $\sim 5 \times 10^2\ M^{-1}\ s^{-1}$ and k_{off} $\sim 5 \times 10^{-4}\ s^{-1}$) [4] for PBP-mediated transport to be the only mechanism of ligand delivery to the dendritic membrane. Furthermore, studies with insect odorant receptors/co-receptors (OR/Orco) in frog oocytes have clearly demonstrated that ligands can be delivered to OR/Orco directly through the bath solution, without a PBP [5,6]. Therefore, it has also been proposed that the PBPs contribute to pheromone scavenging, to prevent accumulation of the hydrophobic pheromone in the dendritic membrane of the sensillum [7].

We have observed moderate thermodynamic ligand binding selectivity for the two known PBPs of the gypsy moth toward various ligands [8,7,9,10]. Kinetic studies have shown that the modest selectivity detected in equilibrium binding assays arises in a multi-step ligand binding mechanism [4,11]. Although PBPs are essential for olfaction [12,13], their role and mode of action are still unclear.

A central question of PBP-ligand dynamics is whether PBPs have an empty binding site, when no pheromone has entered the lymph. Given that oleamide has been identified as an endogenous ligand

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for a locust chemosensory specific protein (CSP) [14] it is likely that the antennal lymph of other insect species contains a variety of potential endogenous ligands for PBPs, none of which have been identified. In addition, the presence of endogenous ligands may result in the PBP adopting a certain conformation, perhaps exposing residues involved in pheromone-specific interactions [15]. Lastly, endogenously bound ligands may act to synergize or antagonize pheromone binding, as has been seen with mixtures of odorants [7,15].

The two known gypsy moth PBPs (*Ldis*PBP1 and *Ldis*PBP2) belong to the class of "long" OBPs, with a hydrophobic, disordered C-terminal peptide of 10–18 amino acids in length [16]. Structures of "long" insect OBPs with six conserved cysteine residues reveal that these proteins have six α-helical segments, connected by flexible loops, and that their C-terminal peptide tends to be disordered (e.g., [17–19]. Based on sequence homology of *Ldis*PBPs to other OBP's of similar size, it is likely that *Ldis*PBPs are mostly α-helical with flexible loops, particularly between helices 2 and 3, and a non-structured, flexible C-terminus. Previous modeling and kinetic studies with gypsy moth PBPs suggest that the loop and C-terminus delineate an external site where ligands, such as the pheromone (+)-disparlure, can be captured as the first step of a multi-step binding mechanism [4,11,10]. How these structural features interact with other sensillar components, however, is not yet understood.

The goals of this work were: (1) to search for and identify endogenous ligands of PBPs, (2) to explore the binding of endogenous ligands to *Ldis*PBP1 and *Ldis*PBP2, (3) to assess the effect of endogenous ligands on PBP conformation and pheromone binding and (4) to assess the effect of endogenous ligands on pheromone and PBP partitioning among a solid surface, buffer and phospholipid vesicles.

Here we have identified several free fatty acids, present in high concentration in the lymph of sensilla trichodea of male gypsy moths. Furthermore, we show that *Ldis*PBP1 and *Ldis*PBP2 associate with these endogenous lipids. The impact of PBP-endogenous ligand interaction on protein secondary structure, on disparlure binding affinity and on partition between aqueous, lipid bilayer and solid phases were also investigated. We show that sensillar lymph in *L. dispar* s. trichodea is a basic emulsion of free fatty acids, and that the fatty acids affect PBP conformation and partition of pheromone between aqueous and hydrophobic phases.

Materials and methods

All reagents used were of the highest purity available. Ethyl acetate and hexane were distilled in glass prior to use. Linoleic acid, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), bis-trimethylsilyltrifluoroacetamide (BSTFA)[2] and dimethyldichlorosilane were from Sigma–Aldrich (St. Louis, MO). The pheromone, (+)-disparlure, was synthesized in-house [10]. Cholesterol was from Fisher Scientific (Fair Lawn, NJ), stearic acid was from Matheson Coleman & Bell (Cincinnati, OH) or from Anachemica Chemicals (Rouses Pt, NY), palmitic acid was from Fisher Scientific (Fair Lawn, NJ), oleic acid was from Mallinckrodt Chemical Works (St. Louis, MO), potassium hydroxide was from BDH Inc. (Toronto, ON), sodium chloride and Tris were from Caledon (Georgetown, ON). Phosphatidylcholine and phosphatidylethanolamine were purchased from Sigma–Aldrich (St. Louis, MO). The N-acetylated-C-terminal peptides for *Ldis*PBP1 and *Ldis*PBP2 were from Biomatik (Cambridge, Ontario, Canada). The sequence of CPBP1 was: Ac-WAPTLDVAVGELLADT-OH, and the sequence of CPBP2 was: Ac-WAPDVELLVADFLAESQ-OH.

Gas chromatography was done on a Perkin Elmer Clarus 500 GC, fitted with a temperature-programmable injector, a 30 m long SPB5 column (0.2 mm i.d., 0.25 μm film thickness) and a flame ionization detector. Gas chromatography-mass spectrometry (GC–MS) was performed on a Varian CP3800 GC with a temperature-programmable split/splitless injector, interfaced with a Varian Saturn, 2000 ion trap MS. The GC–MS was fitted with the same type of column as the GC and was operated in electron impact mode (30 μamp emission current) with automatic gain control on. All gas chromatography was done in splitless mode.

Extraction of endogenous lipids from aqueous whole antennal homogenate

Please see Supplemental information for the preparation of a homogenate of 492 antennae from male gypsy moths in 100 mM Tris buffer (pH 7.5).

PBP-bound lipids

Extraction of the antennal homogenate, followed by GC–MS analysis of trimethylsilyl derivatives of extracted lipids revealed C16 and C18 fatty acids and cholesterol (Supplemental Fig. S1).

FPLC of the antennal homogenate (Supplemental Fig. S2) provided PBP1 and PBP2 fractions which were analyzed by 16% native PAGE and were Coomassie and silver stained, as well as Western blotted and detected with either PBP1 or PBP2 antibodies. The corresponding PBP1 (1.5 mL) and PBP2 (2 mL) fractions were pooled in silanized glass vials. An equivalent amount of freshly distilled ethyl acetate was added to the protein solution, mixed (vortex) for 2 min and allowed to stand for 10 min before the organic layer was removed. The procedure was repeated once more, and the combined organic solvent was concentrated by rotary evaporation to about 0.25 mL. The solution was then transferred to a glass ampoule and evaporated under vacuum to 25 μL for GC–MS analysis. Buffer controls were prepared by subjecting buffer to the same procedures, including passage through the FPLC system.

To confirm the identity of the acids in the extracts, the extract was silylated using BSTFA, which reacts with hydroxyl groups to form trimethylsilyl derivatives. BSTFA samples were prepared by taking 3–5 μL of the sample into a glass ampoule and evaporating to dryness under a vacuum. The sample was then resuspended in 5 μL of neat BSTFA and sealed for 1 h, followed by dilution with 45 μL of ethyl acetate and analysis by GC–MS (Supplemental Table S2) [7].

Sensillar extract

Please see Supplemental information for the extraction with methanol of 89 mg of sensilla, sheared from 28 male gypsy moth antennae, and analysis of this extract with BSTFA.

Isolation of fatty acids from lymph droplets extruded from individual severed sensilla

To verify that the fatty acids found in the antennal and sensillar extracts came mainly from sensillar lymph, we immobilized individual antennae on glass slides, using Scotch tape, and severed the tips of sensilla along selected branches, using a scalpel mounted on a micromanipulator (Syntech, Kirchzarten, Germany). Gentle pressure was then applied manually to the base of the antenna, which causes lymph droplets to extrude from the severed tips [20]. A sensillum with a droplet was then selected

[2] *Abbreviations used:* BSTFA, bis(trimethylsilyl)trifluoroacetamide; Tris, Tris(hydroxymethyl)aminomethane; GC–MS, gas chromatograph/mass spectrometer; MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometer; PBP, pheromone binding protein; FPLC, fast protein liquid chromatography; CD, circular dichroism, CSP, chemosensory specific protein.