Conformational Change in the Pheromone-binding Protein from *Bombyx mori* Induced by pH and by Interaction with Membranes*

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Hubert Wojtasek‡ and Walter S. Leal§

From the Laboratory of Chemical Prospecting, National Institute of Sericultural and Entomological Science, 1-2 Ohwashi, Tsukuba 305-8634, Japan

The pheromone-binding protein (PBP) from *Bombyx mori* was expressed in *Escherichia coli* periplasm. It specifically bound radiolabeled bombykol, the natural pheromone for this species. It appeared as a single band both in native and SDS-polyacrylamide gel electrophoresis and was also homogeneous in most chromatographic systems. However, in ion-exchange chromatography, multiple forms sometimes appeared. Attempts to separate them revealed that they could be converted into one another. Analysis of the protein by circular dichroism and fluorescence spectroscopy demonstrated that its tertiary structure was sensitive to pH changes and that a dramatic conformational transition occurred between pH 6.0 and 5.0. This high sensitivity to pH contrasted markedly with its thermal stability and resistance to denaturation by urea. There was also no significant change in CD spectra in the presence of the pheromone. The native protein isolated from male antennae displayed the same changes in its spectroscopic properties as the recombinant material, demonstrating that this phenomenon is not an artifact arising from the expression system. This conformational transition was reproduced by interaction of the protein with anionic (but not neutral) phospholipid vesicles. Unfolding of the PBP structure triggered by membranes suggests a plausible mechanism for ligand release upon interaction of the PBP-pheromone complex with the surface of olfactory neurons. This pH-linked structural flexibility also explains the heterogeneity reported previously for *B. mori* PBP and other members of this class of proteins.

Recognition of chemical signals in insects takes place in their olfactory sensilla. A class of small proteins (~13–17 kDa), odorant-binding proteins (OBPs), is believed to facilitate the passage of hydrophobic odorant molecules from the environment to the surface of olfactory receptor neurons (1–3). These proteins are present in the sensillar lymph at an enormous 10–20 mM concentration (3). In Lepidoptera, two classes of OBPs have been distinguished: one involved in the recognition of general odorants (general odorant-binding proteins (GOBPs)) (4). Since the identification of the first such proteins in *Antheraea polyphemus* (5), homologous proteins have been detected and characterized in many species from several insect orders. A number of OBPs have been cloned (6–14), and a few of them have been expressed (15–18). Sequences of lepidopteran PBPs are highly conserved, but their homology to GOBPs and OBPs from other insect orders is only moderate. The whole family, however, shows highly conserved motifs, among them the six cysteine residues thought to participate in formation of disulfide bonds (6). Circular dichroism measurements and theoretical structure prediction have revealed that these proteins are in a large part α-helical (15, 19). However, despite substantial efforts in the past several years, their three-dimensional structure remained elusive. Also, details of their function are still not fully understood. Although candidate pheromone receptors have just been identified in *Drosophila* (20, 21), the mechanism of their stimulation and the postulated participation of PBPs in this process (19, 22) still remain to be established.

Here we report expression of the pheromone-binding protein from *Bombyx mori* in *Escherichia coli*. Circular dichroism and fluorescence spectroscopy showed that this protein is very sensitive to pH changes. This phenomenon has been most likely responsible for the failures in structural studies of this class of proteins. We also demonstrate that the protein undergoes partial unfolding upon interaction with model membranes, analogous to the conformational change observed at low pH.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning and Preparation of Expression Vectors—RNA was isolated by a single-step acid/guanidinium/phenol/chloroform extraction (23) from 20 male antennae. PolyATtract (Promega) was used to purify mRNA, and the first cDNA strand was synthesized with avian myeloblastosis virus reverse transcriptase (Promega) and an oligo(dT) primer. Polymerase chain reactions were carried out in a MiniCycler (PTC-150, MJ Research, Inc.) using Pfu DNA polymerase (Stratagene) in 50 mM Tris-HCl, pH 8.5, 15 mM (NH₄)_2SO₄, and 1.5 mM MgCl₂ with annealing at 50 °C. The following primers were designed based on the published sequence of *B. mori* PBP (10): CGTCTCAAGAAGTCATGA (5'-primer; blunt-end ligation into the *McoI* site) and AGACACTCGAGATCCTCAAAAATCGCT (3'-primer; the *Xho*I site is underlined). Polymerase chain reaction products were cloned into the *McoI* and *Xho*I sites of the pET22b expression vector (Novagen). Ligation reaction was used for transformation of *E. coli* MRF³ Kan cells (Stratagene). Plasmids were isolated, and the constructs were sequenced with T7 promoter and T7 terminator primers using Dye Terminator Reaction Ready kits on an ABI PRISM Model 373A automated DNA sequencer (PE Applied Biosystems).

**Purification, Characterization, and Modification of the Recombinant Protein**—Recombinant vectors were transferred into BL21(DE3) expression hosts (Novagen). Expression was performed in LB medium with 50 μg/ml carbenicillin in 1-liter flasks (12 × 330 ml, 4-liter total culture volume) at 28 °C without induction. Cultures were grown to A₆₀₀nm > 5; cells were harvested by centrifugation; and the periplasmic proteins were released by osmotic shock as described by the manufacturer (Novagen).

The periplasmic fraction was loaded onto a 20-ml DEAE HR 16/10
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Insect odorant-binding proteins have been previously expressed both in bacterial and eukaryotic systems. PBP from *Antheraea pernyi* was expressed in the baculovirus system (16), but a low yield prevented the production of sufficient amounts of the protein for structural studies. Intracellular expression of the same protein in *B. coli* gave a higher yield, but the majority of the recombinant protein was produced in the insoluble form and required refolding for structural and functional analysis (18).

We have selected the pET22b vector, which allows expression of the recombinant proteins fused to the pelB signal peptide, directing the proteins to the *E. coli* periplasm. This provides an appropriate oxidative environment for the formation of disulfide bonds. Insect PBPs are secreted proteins, and the existence of two to three disulfide bonds has been postulated (6). By measuring the molecular weight of the carboxymethylated protein, we determined that, in *B. mori* PBP, indeed all six cysteines form disulfide bridges. The molecular weight of the protein after alkylation with iodoacetamide was 15,877 ± 2, identical to the unreacted protein. This value was the same for the recombinant and native proteins and corresponded well with the molecular weight obtained from translation of the cDNA (15,84,4 uncovalent; 15,81,2 molar ratios of the six cysteines are in disulfide bonds), which demonstrates that *B. mori* PBP is not post-translationally modified. When alkylation of the protein was performed after reduction with 50 mM dithiothreitol, the molecular weight increased to 16,230 ± 3, which corresponds exactly to incorporation of six carboxamidomethyl groups.
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Using the periplasmic expression system, we routinely obtained 6–10 mg of pure protein/liter of culture. The highest yield was obtained without induction, when the protein was expressed slowly at low temperature (28 °C). This provided optimal conditions for protein translocation into the periplasm and its enzymatic processing. Although induction with isopropyl-β-D-thiogalactopyranoside significantly increased the amount of total recombinant protein, it reduced the amount of the soluble protein translocated into the periplasm. Periplasmic localization also circumvented the problem of degradation of recombinant proteins experienced, for example, in the case of Manduca sexta GOBP1 (15).

The protein purified from the periplasm appeared as a single band both in native and SDS-polyacrylamide gel electrophoresis (Fig. 1A) and comigrated with the protein isolated from male antennae (data not shown). It specifically bound bombykol, its natural ligand, in the native polyacrylamide gel electrophoresis assay (Fig. 1B). However, the far-UV CD spectra of the recombinant and native proteins were not always identical. Preliminary NMR experiments performed by Wüthrich and co-workers3 (ETH, Zürich, Switzerland) at pH 6.2 indicated the existence of at least two conformations. Originally, we considered this to be a misfolded form. Later, however, we were able to obtain this low-salt eluting form by incubation with ammonium sulfate and sucrose (Fig. 1C). This unexpected conformational flexibility led us to a thorough spectroscopic analysis of both the recombinant and native proteins under various conditions. We analyzed the effect of temperature, salts, pH, denaturing agents, and the pheromone on the structure of the protein by near- and far-UV CD and by fluorescence spectroscopy. The most dramatic structural changes were observed under the influence of pH (Figs. 3–5). Changes in the near-UV CD and fluorescence spectra indicated a great conformational transition between pH 6.0 and 5.0. Near-UV CD spectra, reflecting mostly packing constraints of aromatic residues, demonstrated that these side chains became much more flexible at lower pH; thus, the protein underwent partial unfolding, and most of the tertiary contacts were broken. The decrease in the intrinsic fluorescence at low pH also indicated that the aromatic residues became more exposed to solvent (Fig. 5). The secondary structure (far-UV CD) was also affected, but only slightly (Fig. 4A). The decrease in the spectrum intensity at 222 nm, corresponding to the decrease in helicity, indicated that some unwinding of helices occurred. A plot of the dependence of the intrinsic fluorescence on pH indicated that this conformational transition was mediated by protonation of residue(s) with an apparent pK_a of ~5.7 (Fig. 5B). The presence of an equimolar concentration of bombykol (0.6 μM) did not prevent this transition. However, we observed a substantial enhancement of fluorescence at neutral and basic pH values (i.e. in the closed conformation), which we interpret as an indication of ligand binding. It is consistent with increased hydrophobicity of the environment around the tryptophan residues. At low pH (5.0 and below), there was a slight quenching of the fluorescence signal. Since such quenching also occurs at neutral pH when a large excess of bombykol is added, we attribute this effect to the presence of free ligand, which would imply that at low pH it is not bound.

We also tested the effect of the pheromone on the protein tertiary structure by near-UV CD spectroscopy under conditions close to native (sensillar Ringer solution, pH 6.5). However, changes in the protein spectra at a pheromone concentra-

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tion close to stoichiometric (0.5–2 molar ratio of the ligand to the protein) were minimal (data not shown).

The limited availability of the protein from the natural source prevented us from performing a comparable analysis with the native protein. However, we have taken fluorescence spectra at selected pH points (Fig. 5 A) and far-UV CD spectra at neutral (7.0) and acidic (4.5) pH values (Fig. 4). The native protein showed the same changes in its spectroscopic properties (decrease in fluorescence intensity and helicity at low pH) as the recombinant material.

This opening of the PBP structure was confirmed by binding of 1-anilino-8-naphthalenesulfonic acid. The fluorescence intensity of this hydrophobic probe was substantially enhanced at pH 4.5 compared with pH 6.5 or denaturing conditions (Fig. 6). Such enhancement of ANS fluorescence has been considered as an indication of the formation of the molten globule state (28). However, we observed much stronger ANS fluorescence in the presence of PBP at pH <4.5. Therefore, it is conceivable that the conformation at pH 4.5 is not the molten globule state, but a distinct unfolding intermediate.

We have considered the possibility that the pH-induced conformational transition may lead to changes in the protein quaternary structure. We have used gel filtration chromatography, electrospray ionization mass spectroscopy, and chemical cross-

Fig. 3. Near-UV CD spectra of the recombinant protein at various pH values. Spectrum 1, 20 mM sodium phosphate, pH 6.0, and 20 mM sodium acetate; spectrum 2, pH 5.5; spectrum 3, pH 5.0; spectrum 4, pH 4.5. Mol. Ellip., molar ellipticity.

Fig. 4. Far-UV CD spectra of the recombinant (A) and native (B) proteins. Spectra for recombinant PBP were taken in the same buffers as described in the legend to Fig. 3. Spectra of the native protein were taken in 50 mM sodium phosphate, pH 7.0 (spectrum 1), and 50 mM sodium acetate, 4.5 (spectrum 2). Mol. Ellip., molar ellipticity.

Fig. 5. Effect of pH on the intrinsic fluorescence of native (A) and recombinant (B) B. mori PBPs. Spectra of the native protein were taken in 50 mM Tris-HCl, pH 8.0 (spectrum 1); 50 mM sodium phosphate, pH 7.0 (spectrum 2) and 6.0 (spectrum 3); and 50 mM sodium acetate, pH 5.5 (spectrum 4) and 4.5 (spectrum 5). Spectra of the recombinant protein were taken in 20 mM buffers: sodium borate (pH 9.0), Tris-HCl (pH 8.0), sodium phosphate (pH 7.0 to 6.0), sodium acetate (pH 5.5 to 4.0), and sodium formate (pH 3.5 and 3.0) without ligand or with bombykol (0.6 µM; 1:1 molar ratio). Each data point in B represents a mean of at least three measurements. S.E. values for most points were too small to be shown in the graph.

Fig. 6. Fluorescence spectra of ANS in the presence of the recombinant B. mori PBP. Spectra were taken in 20 mM sodium phosphate, pH 6.5 (spectrum 1); 20 mM sodium phosphate, pH 6.5, and 6 M guanidine HCl (spectrum 2); and 20 mM sodium acetate, pH 4.5 (spectrum 3) and 4.0 (spectrum 4).
linking to test this hypothesis, but we have obtained no evidence for formation of dimers or oligomers at pH values between 4 and 8. It has been shown in a number of cases that pH-linked unfolding of proteins reflects their interaction with membranes (29–31). We therefore examined the structural changes in B. mori PBP in the presence of phospholipid vesicles. Unfolding of the protein, analogous to that observed at low pH, indeed occurred in the presence of anionic (DMPC) or mixed (DMPC + DMPG) vesicles. The decrease in the intensity of the near-UV CD spectrum in the presence of pure anionic vesicles was even stronger than at low pH when the experiments were performed in a low ionic strength buffer. The presence of a physiological salt concentration were indistinguishable. Without vesicles, the spectra in the presence or absence of a physiological salt concentration were indistinguishable. Mol. Ellip., molar ellipticity.

We have also examined the sensitivity of recombinant PBP to thermal and chemical denaturation. As indicated by the changes in the CD spectra, B. mori PBP was very stable at high temperatures (Fig. 8), contrasting with other examples of pH-sensitive proteins such as α-lactalbumin (30) and apolipoproteins (32, 33), which showed heat-induced denaturation midpoints at $-50$–$60 \degree C$. This stability was lost, however, after breaking the disulfide bonds with 20 mM dithiothreitol (data not shown). B. mori PBP was also very resistant to denaturation by urea (almost no structural changes up to 6 M; data not shown).

**DISCUSSION**

The number of proteins that show pH-linked structural changes has been increasing in recent years. Examples include transferrins (34), influenza hemagglutinin (35), ferricytochrome c (36), and retinol-binding protein (37). Such conformational transitions are physiologically significant, mediating, for example, ligand release or fusion with membranes. In the case of transferrins, translocation across the membranes, from the neutral pH of the bloodstream to the acidic intracellular pH, leads to opening of the protein structure and iron release, apparently with the active participation of the receptor (38). Unfolding of the retinol-binding protein to the molten globule state at low pH has been shown to trigger retinol release (37).

pH-linked conformational transitions frequently reflect interactions of proteins with membranes. This is due to the fact that the membrane potential decreases the local pH as compared with the bulk of the solution (39). The difference has been determined experimentally to be 1.6 units, and the calculated value reaches 2.7 units (29). Interaction of proteins with membranes is also frequently associated with partial unfolding to a state referred to as the molten globule (30, 40–42). A similar phenomenon has also been observed for apolipoproteins (both insect and mammalian) upon binding to the lipoprotein surface (33, 43) and to model membranes (44). We have now shown that the structural changes in B. mori PBP follow the same pattern. Loss of a rigid tertiary structure demonstrated by near-UV CD spectra and the enhancement of ANS fluorescence at pH 4.5 indicate that the protein undergoes partial unfolding under these conditions. It has been demonstrated recently that the molten globule, or the A-state, of cytochrome

**FIG. 8. Effect of temperature on the structure of B. mori PBP.**

The near-UV CD spectra (A) were taken in the sensillar Ringer solution, pH 6.5, at 25 °C before heating (spectrum 1), at 85 °C (ascending temperature; spectrum 2), and at 95 °C (spectrum 3). The far-UV CD spectra (B) were taken in 20 mM sodium phosphate, pH 6.5, at 25 °C before heating (spectrum 1), at 55 °C (ascending temperature; spectrum 2), and 95 °C (spectrum 3). Spectra at 25 °C before heating and after cooling down were indistinguishable. Mol. Ellip., molar ellipticity.
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c is stabilized by high concentration of sugars (45). We have observed a similar phenomenon for one of the PBP conformations, which could be separated chromatographically only in the presence of 20% sucrose (Fig. 2C).

This conformational flexibility, although unexpected, explains the microheterogeneity of pheromone-binding proteins in B. mori and other insect species. Recently, several isoforms of PBP, differing in their isoelectric points and mobility in native polyacrylamide gel electrophoresis, have been reported in B. mori (46). The proteins were isolated by isoelectric focusing and collected in buffers with pH values between 4.6 and 5.2. As we have shown, the most dramatic structural changes occur in B. mori PBP in this pH region, and the isoforms detected in that study may well represent different conformations of the same protein. Nagnan-le Meillour and co-workers (17) have also observed several bands in native gel electrophoresis for the same protein. Nagnan-le Meillour and co-workers (17) have proposed that study may well represent different conformations of the same protein. Nagnan-le Meillour and co-workers (17) have observed several bands in native gel electrophoresis for the bacterially expressed GOBP from Mamestra brassicae. They attributed this heterogeneity to either partial proteolysis or chemical modifications of amino acid side chains (e.g. deamidation). They also considered the possibility of protein misfolding during the refolding procedure. We now have evidence that a pH-induced conformational transition occurs also in odorant-binding proteins from scarab beetles. It may therefore be a general property of the whole OB family.

This fact would have deep consequences for the current model of the perireceptor events in insect olfaction. The details of ligand transport, receptor stimulation, and pheromone degradation in the sensillar lumen are still a matter of considerable debate (1, 3). Vogt et al. (47) proposed a kinetic equilibrium model in which the pheromone interacted dynamically with the three physiologically relevant protein components in the sensillar lumen: the pheromone-binding protein, the membrane receptor, and the pheromone-degrading enzyme. That model, however, had difficulty explaining how the pheromone survived the passage through the sensillar lumen in the presence of a very aggressive enzyme. The discrepancy between the rates of pheromone degradation in vivo (48–50) and in vitro (47) also led to the questioning of the role of pheromone-degrading enzymes in signal inactivation. A mechanism involving a redox shift of PBPs, catalyzed by the receptor protein, has even been proposed (51, 52). We think that our data provide a mechanism complementing the kinetic equilibrium model of Vogt et al. and suggest a new view of the perireceptor events in the insect olfactory system. We have demonstrated that at high pH the protein remains mostly in the closed conformation. At high pH, the protein binds the pheromone (most of the binding studies have been done with the native polyacrylamide gel electrophoresis assays, thus at pH 8.8, as in Fig. 1). The changes in fluorescence in the absence and presence of the pheromone (Fig. 5B) suggest that the ligand is not bound to the open conformation at low pH. The conformations of B. mori PBP interconvert slowly in solution at physiological pH, but the opening is promoted by membranes. If we adopt the mechanism proposed for the binding of retinol-binding protein to PBPs, the pheromone would remain bound to the PBP during its passage through the sensillar lumen and would be protected from degradation. Upon encountering the membrane, the protein would unfold, releasing the ligand, which would now become available to the receptor (and later degradation). This mechanism offers a dramatic enhancement in efficiency and is consistent with the extraordinary sensitivity of the insect olfactory system.

A detailed analysis of unfolding of the retinol-binding protein on the surface of membranes identified conserved charged residues located on the retinol-binding face that are involved in formation of salt bridges. At low pH, these bridges are broken, and a number of positively charged residues become exposed, facilitating interaction with membranes and ligand release (53). However, in the case of retinol-binding protein, the difference between the native pH and the pH required for the protein unfolding is too large to be explained only by the local pH decrease, created by the membrane potential, and other factors (e.g. lower dielectric constant or interaction with the receptor) had to be taken into consideration (54). In the case of B. mori PBP, there is no such discrepancy. The protein unfolds 2 units below the native pH and at native pH in the presence of anionic phospholipids. Sequence alignment of the nine PBPs cloned has revealed a striking conservation of charged amino acids (one arginine, all five histidines, three lysines, and nine acidic residues are preserved in all species). Only the six cysteines and very few hydrophobic residues are also conserved in all of these proteins. This striking homology to the retinol-binding protein model indicates that the mechanism of ligand release may indeed be similar. Although we have demonstrated that the interaction of PBP with model anionic or mixed membranes is sufficient to trigger the conformational transition, participation of other factors, e.g. the pheromone receptors or the olfactory membrane protein Snmp-1 (55), should also be taken into consideration.

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