Ligand Binding Turns Moth Pheromone-binding Protein into a pH Sensor

EFFECT ON THE ANtheraea polyphemus PBP1 CONFORMATION*□

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In moths, pheromone-binding proteins (PBPs) are responsible for the transport of the hydrophobic pheromones to the membrane-bound receptors across the aqueous sensillar lymph. We report here that recombinant Antheraea polyphemus PBP1 (ApolPBP1) picks up hydrophobic molecule(s) endogenous to the Escherichia coli expression host that keeps the protein in the “open” (bound) conformation at high pH but switches to the “closed” (free) conformation at low pH. This finding has bearing on the solution structures of undelipidated lepidopteran moth PBPs determined thus far. Picking up a hydrophobic molecule from the host expression system could be a common feature for lipid-binding proteins. Thus, delipidation is critical for bacterially expressed lipid-binding proteins. We have shown for the first time that the delipidated ApolPBP1 exists primarily in the closed form at all pH levels. Thus, current views on the pH-induced conformational switch of PBPs hold true only for the ligand-bound open conformation of the protein. Binding of various ligands to delipidated ApolPBP1 studied by solution NMR revealed that the protein in the closed conformation switches to the open conformation only at or above pH 6.0 with a protein to ligand stoichiometry of ~1:1. Mutation of His70 and His95 to alanine drives the equilibrium toward the open conformation even at low pH for the ligand-binding protein by eliminating the histidine-dependent pH-induced conformational switch. Thus, the delipidated double mutant can bind ligand even at low pH in contrast to the wild type protein as revealed by fluorescence competitive displacement assay using 1-aminoanthracene and solution NMR.

Chemoreception in insects is mediated via sensing of a variety of small, volatile organic compounds. Chemoreception plays a critical role not only in the regulation of the most fundamental chemosensory behaviors in insects but also for intraspecies communication. Pheromone is a chemical signal that triggers a natural behavioral response in another member within the same species. In lepidopteran insects, sex pheromones produced by females are detected by males of the same species with extreme sensitivity and selectivity. These hydrophobic compounds are transported to the membrane-bound receptors (ion channels) (1, 2) across the aqueous sensillar lymph by the pheromone-binding proteins (PBPs).2 PBPs are small, acidic proteins, highly soluble in water, with a molecular mass of 14–16 kDa. The first PBP to be identified, cloned, and expressed in the bacterial system was that from the giant silk moth Antheraea polyphemus (3, 4). Since then, PBPs have been isolated from at least eight moth species that share about 50% sequence identity, with six conserved cysteine residues forming three disulfide bridges that are important for the formation of the hydrophobic binding pocket (5).

Moth PBPs are known to undergo a dramatic conformational switch with a change in pH, which has been proposed to be necessary for the release of ligand at lower pH near the membrane-bound receptors (ion channels) (6–11). The current view on insect PBPs is that the unliganded protein exists in two conformations as follows: form A (PBP𝐴), the acidic or “ligand releasing” conformation that exists at low pH, and form B (PBP𝐵), the basic or “ligand binding” conformation that exists at high pH (7). The low and high pH conformations have been determined by solution NMR for PBPs from two representative moths, Bombyx mori (BmorPBP) and Antheraea polyphemus (ApolPBP1) (8–11), which are believed to represent the “free” or “unliganded” form of the two proteins. High pH conformations of both “ligand-bound” and free forms of BmorPBP have also been determined by x-ray crystallography (12, 13).

The conformation of BmorPBP in solution at pH 6.5 (9) consists of six helices that are arranged encasing a large hydrophobic cavity, with an unstructured C-terminal tail that extends out to the solvent. This conformation is otherwise called the “open” conformation because the C-terminal tail is out to the solvent leaving the hydrophobic pocket open. At a low pH of 4.5, BmorPBP undergoes a conformational switch to BmorPBP, resulting in the formation of a seventh C-terminal helix that occupies the ligand-binding cavity (8) in the “closed” conformation of the protein. It was proposed that the ligand is ejected by the C-terminal helix near the membrane-bound receptor as the pH is reduced due to membrane potential (8). ApolPBP1 has about 67% sequence identity with BmorPBP and shares a similar topology at a high pH, showing the presence of six α-helices held together by three disulfide bonds forming a hydrophobic cavity that can house the pheromone (10). How-

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2 The abbreviations used are: PBP, pheromone-binding protein; ApolPBP1, A. polyphemus PBP1; BmorPBP, B. mori PBP; HSQC, heteronuclear single quantum correlation; AMA, 1-aminoanthracene.
ever, at low pH there are differences in the ApolPBP1 and BmorPBP conformations. Although BmorPBP<sup>A</sup> at pH 4.5 forms the closed conformation (8), ApolPBP1 is devoid of the C-terminal helix at pH 5.2 and exists in more than one conformation (11). These differences led to the suggestion of a different ligand release model at low pH for ApolPBP1 (11). In a recent report (14), the delipidated ApolPBP1 at pH 4.5 was shown to have the same closed conformation as that of BmorPBP<sup>A</sup> with the C-terminal dodecapeptide segment tucked into the binding cavity after forming the seventh α-helix. However, there was a significant difference in the ApolPBP1 sample preparation in this study (14), which included a delipidation step to remove the endogenous hydrophobic molecule(s) picked up by the protein from the expression host (Escherichia coli). It is noteworthy here that many previous biochemical (4, 6, 15) and biophysical investigations (7–11) on either BmorPBP or ApolPBP1 have been conducted on the undelipidated protein, i.e. without the removal of the hydrophobic ligand from the protein. The x-ray analysis of BmorPBP at pH 7.5 (13) is the first report of a delipidated (unliganded) protein structure followed by the more recent report on the delipidated (unliganded) ApolPBP1 solution NMR structure (14) at pH 4.5. Interestingly, similar to the structural differences between the undelipidated and delipidated ApolPBP1 at low pH (11, 14), the structures of the undelipidated and delipidated BmorPBPs at high pH were significantly different as far as the seventh C-terminal helix is concerned (9, 13). Although the delipidated unliganded BmorPBP at pH 7.5 was in the closed conformation (13), the structure of the undelipidated BmorPBP at pH 6.5 was in the open conformation (9). All the above observations for both ApolPBP1 and BmorPBP contradicted each other, implying that the same protein at a low or high pH may or may not have the internalized C-terminal helix (9, 11, 13, 14).

To understand the mystery underlying the differences between structures of these undelipidated (9, 11) and delipidated moth PBPs (13, 14) at different pH values, we carried out a thorough investigation to clarify the effect of delipidation, pH, and ligand on the conformation of ApolPBP1 as a representative member of the moth PBP family using high resolution solution NMR and fluorescence spectroscopy. Moreover, the proposed ligand release model involving the pH-induced histidine-driven conformational switch at low pH (11–12, 22) was verified by the mutation of His<sup>70</sup> and His<sup>95</sup> to alanine in ApolPBP1. To gain insight into the role of pH and ligand on the conformational switch of both undelipidated and delipidated ApolPBP1, ligand binding studies monitored by fluorescence and NMR spectroscopy revealed that although the delipidated ApolPBP1 is able to bind a number of hydrophobic compounds, binding of pheromone is preferred over its analogs. Furthermore, the wild type protein can bind ligands only at a pH above 6.0, whereas the ApolPBP1H70A/H95A double mutant is able to bind ligands even at a pH below 5.0, thus providing evidence for the low pH-induced conformational switch driven by protonated histidines.

**EXPERIMENTAL PROCEDURES**

**Cloning, Mutagenesis, Overexpression, and Purification**—The ApolPBP1 gene, previously cloned into pHN1<sup>1</sup> vector (4), was amplified using the following primers: forward, 5′-GGGATTCCTGATATGCAGAGATCATGAG-3′, and reverse, 5′-GCGGATCCCTAAACTTCAGCTAAGACCC-3′ (restriction sites underlined) and cloned into the NdeI and BamHI sites of PET-21a vector. The orientation and sequence of the ApolPBP1 gene in the expression vector were confirmed by restriction analysis and DNA sequencing. ApolPBP1H70A and ApolPBP1H70A/H95A mutant plasmids were constructed from the ApolPBP1-pET-21a vector by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit of Stratagene. Native as well as mutant plasmids were transformed into E. coli origami cells, and the expression was carried out as reported previously (10, 11). The isotope-labeled proteins were expressed in M9 minimal medium with [15N]<sub>2</sub> ammonium chloride (Cambridge Isotope Laboratories, MA). The expression and lysis of cells were followed as reported previously (10, 11). After centrifugation of the cell lysate, the recombinant proteins were purified by a combination of dialysis, anion exchanger DEAE-chromatography, and finally twice on size exclusion chromatography using a Superdex 75 column fitted to ÅKTA FPLC (GE Healthcare). Protein concentrations were determined spectrophotometrically using the theoretical 

**Delipidation**—The delipidation of native and mutant ApolPBP1 was performed according to Bette <em>et al.</em> (17). The protein was first concentrated to 500 μl using a Millipore ultrafiltration concentrator (capacity 15 ml, molecular weight cutoff of 5000), and 4.5 ml of 50 mM sodium citrate buffer pH 4.5 (buffer A) was added. The procedure of concentration and the addition of buffer were repeated twice, and the final volume of the protein was adjusted to 2 ml with buffer A. The protein was then incubated overnight with Lipidex<sup>TM</sup>-1000 equilibrated with buffer A, in a column shaking continuously. The temperature of incubation did not have any effect on the efficiency of delipidation as same results were obtained for the protein delipidated at
4°C, 22°C or 37°C. The protein was eluted from Lipidex-1000 with buffer A. The eluted protein was concentrated to 2 ml, incubated again overnight with fresh Lipidex-1000, and eluted with buffer A. The eluted and delipidated ApoLPBP1 was concentrated and purified again by size exclusion chromatography on Superdex 75 column using 20 mM sodium citrate buffer, pH 4.5, with 150 mM NaCl, 1 mM EDTA, and 0.01% NaN₃ as the mobile phase. To prepare NMR samples, the purest fractions were exchanged to 50 mM sodium phosphate buffer, pH 6.5 or pH 4.5, with 1 mM EDTA and 0.01% sodium azide, containing 5% D₂O, and concentrated using the Millipore ultrafiltration concentrator as per the protocol mentioned above. The purity of the sample was assessed by electrospray ionization-mass spectrometry (supplemental Fig. 1).

**Mass Spectrometry**—Mass spectrometry was carried out at the Department of Chemistry and Biochemistry, Auburn University Mass Spectrometry Facility. The delipidated ApoLPBP1 was analyzed by electrospray ionization-mass spectrometry on a Q-Tof Premier™ (Waters) mass spectrometer.

**NMR Measurements**—All NMR data were collected at 35°C on a Bruker Avance 600-MHz spectrometer equipped with a triple resonance ¹H/¹³C/¹⁵N TCI cryoprobe at the Department of Chemistry and Biochemistry, Auburn University. NMR samples used for pH titrations consisted of 400 μl of 0.8–1.1 mM uniformly ¹⁵N-labeled ApoLPBP1wt or ApoLPBP1H70A/H95A in 50 mM sodium phosphate buffer, pH 6.5 or pH 4.5, containing 1 mM EDTA, 0.01% NaN₃, and 5% D₂O (used as a lock solvent) in a Shigemi tube. Two-dimensional ¹H,¹⁵N heteronuclear single quantum coherence (HSQC) spectra were collected for unliganded and delipidated and delipidated ApoLPBP1wt and ApoLPBP1H70A/H95A double mutant samples at pH 4.5 and 6.5.

The pH titration of delipidated ApoLPBP1wt was carried out in the pH range of 4.5 to 6.5 while following the movement of resonance peaks in two-dimensional ¹H,¹⁵N HSQC spectra. The protein at pH 4.5 was titrated to pH 6.5 using 1 μM NaOH. HSQC spectra were recorded at each titration point, and the movement of peaks was followed using the assignment published previously (14). For the pH titration of ligand-bound protein, the delipidated ApoLPBP1wt and ApoLPBP1H70A/H95A samples were first incubated with an excess of ligand (1:5 palmitic acid) prior to titration from pH 6.5 to 4.5. The two-dimensional ¹H,¹⁵N HSQC spectra were collected at each titration point.

For ligand titration experiments, uniformly ¹⁵N-labeled delipidated ApoLPBP1wt (310 μM of 220 μM in 50 mM phosphate buffer, pH 6.5, containing 5% D₂O, 1 mM EDTA, and 0.01% (w/v) NaN₃) was titrated with increasing concentrations of various ligands (0–22 mM), and the corresponding two-dimensional ¹H,¹⁵N HSQC spectra were recorded. Titration studies were carried out using eight different ligands as follows: 6E,11Z-hexadecadienyl acetate (A. polyphemus pheromone); 6E,11Z-hexadecadienyl diazocacetate and 6E,11Z-hexadecadienyl alcohol (A. polyphemus pheromone analogs); fatty acids (palmitic acid, palmitoleic acid, and oleic acid); and hydrophobic fluorescent probes (1-aminoaanthracene (AMA) and 1-anilino-8-naphthalene sulfonic acid). Control experiments involved titration of ¹⁵N-labeled delipidated ApoLPBP1wt with the same volumes of deuterated methanol as used for ligand titrations. Ligand titration studies were also carried out for the ¹⁵N-labeled delipidated ApoLPBP1H70A/H95A double mutant with palmitic acid at pH 4.5 and 6.5. All data were processed using NMRPipe (18) and analyzed using NMRView (19).

**Fluorescence Spectroscopy**—Fluorescence spectra were recorded on a 55B spectrofluorimeter (PerkinElmer Life Sciences) at a right angle configuration using a quartz cuvette of 1-cm path length. Silt widths of 7.5 and 7.0 nm were used for excitation and emission, respectively, and each spectrum was an average of 15 scans recorded at a speed of 500 nm/min. All fluorescence measurements were carried out in 20 mM phosphate buffer, pH 6.5 or pH 4.5, in the presence of 0.3% methanol at 22°C. Methanol at this concentration had the maximum effect on the AMA fluorescence. All experiments were repeated at least twice to confirm reproducibility.

**AMA Binding Studies**—The binding of AMA to the undelipidated and delipidated ApoLPBP1wt and ApoLPBP1H70A/H95A at pH 6.5 and 4.5 was studied by monitoring the increase in the AMA fluorescence at 480 nm. To 2 ml of a 1 μM protein sample, small aliquots of 2 mM AMA solution in methanol were added to a final concentration of 0–20 μM. After incubation for 10 min, the fluorescence spectra were recorded at the excitation wavelength of 256 nm, and emission of 400–600 nm. Appropriate controls were used to correct the spectra. Binding constants were determined from the nonlinear regression of the data with an equation corresponding to a single binding site.

**Competitive Displacement of AMA by Various Ligands**—The competitive displacement of AMA from the delipidated ApoLPBP1wt by various ligands was studied by monitoring the decrease in AMA fluorescence with increasing ligand concentrations. 1 μM delipidated ApoLPBP1wt was equilibrated overnight with 1 μM (5 μM for the titrations at pH 4.5) AMA at 4°C in dark. AMA fluorescence was recorded with an excitation wavelength of 256 nm and the emission of 400–600 nm. Ligands were added as aliquots of 2 mM stock solutions. After each addition, the complex was incubated for 10 min before recording the spectrum. The fluorescence spectra of ligands added to 1 μM AMA solution in the absence of the protein served as controls.

The decrease in the fluorescence intensity of AMA at the λₘₐₓ (480 nm) was calculated as (Fₐ − Fₘᵢₙ)/(Fₐ − Fₘᵢₙ), where Fₐ is the initial fluorescence intensity of AMA-ApoLPBP1wt complex; Fₐ is the corrected fluorescence intensity at a ligand concentration [C], and Fₘᵢₙ is the fluorescence intensity at the saturating concentration of the competitor. The data were fit to a nonlinear regression of the plot of (Fₐ − Fₘᵢₙ)/(Fₐ − Fₘᵢₙ) against [C] with the equation corresponding to a single binding site. From these graphs, the IC₅₀ values were determined at the ligand concentrations where the AMA fluorescence was quenched to half of its maximal intensity. Kₐ values were calculated using Equation 1,

\[ K_a = \frac{[C]}{1 + [AMA]/K_{AMA}} \]  

where [AMA] is the free AMA concentration, and K_{AMA} is the dissociation constant for AMA-ApolLPBP1wt complex determined from the AMA fluorescence measurements (20). To compare the ligand binding affinities of different protein
samples, AMA displacement assays were carried out for the undelipidated and delipidated forms of ApolPBP1wt and ApollPBP1H70A/H95A at pH 6.5 and 4.5, using a single ligand, the acetate pheromone.

RESULTS

Effect of Delipidation and pH on the Conformation of ApolPBP1wt—The two-dimensional $\text{^{1}H,^{15}N}$ HSQC spectrum represents the fingerprint region of a protein. Thus, this spectrum is very sensitive to any environmental changes such as pH, temperature, substrate binding, salt, etc. The changes in chemical shift positions in the HSQC spectrum may indicate a conformational change in the protein that can be either local or global. The two-dimensional $\text{^{1}H,^{15}N}$ HSQC spectrum for the undelipidated and delipidated ApolPBP1wt at pH 4.5 and 6.5 (Fig. 1, A–D) revealed that although the undelipidated protein underwent a dramatic pH-dependent change (Fig. 1, A–D), the delipidated protein did not sense the effect of pH (Fig. 1, E and F). The HSQC spectrum at pH 6.5 (Fig. 1C) of the undelipidated ApolPBP1wt matches the previously reported open conformation (10), and at pH 4.5 (Fig. 1D), it corresponds to the previously reported closed conformation (14). In contrast, the delipidated protein did not undergo any conformational switch from pH 6.5 (Fig. 1C) to pH 4.5 (Fig. 1D) as indi-
cated by the nearly identical HSQC spectra. Thus, the delipidated protein remained in the closed conformation at both high and low pH values. However, the peak intensities of a few residues are affected by the local dynamics and/or $pK_a$ values that are close to the experimental pH. For example, in the HSQC spectrum of the delipidated protein at pH 6.5, several peaks such as Lys20, Ser28, His70, Ala73, Val42, etc. appear to have less intensity than those at pH 4.5. The switch from the closed to the open conformation in the undelipidated protein is indicated by large changes in chemical shift positions for several residues, including those lining the binding pocket and the C-terminal dodecapeptidyl segment (Met131–Val142).

It is important to note that although the undelipidated ApolPBP1wt at pH 6.5 shows a single set of peaks corresponding to the open conformation in the HSQC spectrum (Fig. 1A), at pH 4.5 the major conformation is closed, but a few weak peaks belonging to the open conformation are observed at lower contour levels. Similarly, the HSQC spectrum of the delipidated ApolPBP1wt at pH 4.5 shows a single set of peaks corresponding to the closed conformation (Fig. 1D), and at pH 6.5 the major conformation is still closed, but certain weak peaks corresponding to the open conformation are observed at very low contour levels (Fig. 2). These weak peaks, corresponding to a very small population of the open conformation, appear even after repeated delipidation of ApolPBP1wt. This indicates that the equilibrium shifts very slightly toward the open conformation for both the undelipidated PBP at low pH and the delipidated PBP at high pH (where undelipidated PBP is PBP bound to the endogenous hydrophobic ligand from the host expression system, and delipidated PBP is PBP after the removal of hydrophobic ligand from the host expression system by delipidation).

These observations demonstrate that the presence or absence of a ligand favors the open or closed conformation, respectively, whereas high pH favors the open conformation and low pH favors the closed conformation (Fig. 3).

Indeed, the above observation that the delipidated ApolPBP1 at pH 6.5 is mostly in the closed conformation is consistent with the crystal structure of the delipidated BmorPBP at pH 7.5 (13), which is also in the closed conformation. This closed conformation of the delipidated BmorPBP crystal structure at pH 7.5 (13) is quite different from the open conformation of the undelipidated BmorPBP NMR structure at pH 6.5 (9). The differences in the observed structures of

FIGURE 2. Titration of delipidated ApolPBP1 from pH 4.5 to 6.5. The figure represents an expanded section of the two-dimensional $\{1^H, 15N\}$ HSQC spectrum of 0.9 mM protein in 50 mM sodium citrate buffer containing 5% D$_2$O, 1 mM EDTA, and 0.01% sodium azide at various pH points as indicated in the spectrum. Peaks labeled b represent the bound conformation.

FIGURE 3. Closed (ligand-free) and open (ligand-bound) conformations of ApolPBP1wt adopted from the Protein Data Bank files 2JPO and 1QWV, respectively. The equilibrium between the closed and open conformations shifts depending upon pH and the presence or absence of a ligand. N and C termini are indicated in the figure. The chemical structure of the true ligand for ApolPBP1, 6E,11Z-hexadecadienyl acetate (A. Polyphemus pheromone), is shown in the figure. However, ApolPBP1 can bind a number of hydrophobic ligands, which cause the same conformational change from closed to open. The protein structure representations were prepared using Protein Data Bank ProteinWorkshop 3.5.
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FIGURE 4. A, expanded region of the two-dimensional \(^{1}H^{,15}N\) HSQC spectra of delipidated ApolPBP1 (220 \(\mu M\)) in 50 mm phosphate buffer, pH 6.5, upon titration with \(6\%\) 11,12-hexadecadienyl acetate (A. polyphemus pheromone) showing the decrease in intensities of resonances corresponding to the "unbound" conformation and increase in intensities of those corresponding to the "bound" conformation labeled as B. Protein:ligand ratios are indicated in the figure with corresponding colors. B, one-dimensional slices from the \(^{1}H\) axis of the two-dimensional \(^{1}H^{,15}N\) HSQC spectra of 220 \(\mu M\) ApolPBP1 upon titration with \(6\%\) 11,12-hexadecadienyl acetate, taken in the midpoint of the resonances corresponding to Gly\(^{96}\) in the free as well as the bound form. Protein:ligand ratios are indicated on top of each pair of slices. All slices are scaled relative to the same \(y\) axis.

BmorPBP at high pH are therefore due to the absence (13) or presence (9) of the hydrophobic ligand from the bacteria.

Effect of Ligands on the Conformation of Delipidated ApolPBP1wt—To investigate the effect of different ligands on the conformation of ApolPBP1wt, ligand binding studies were carried out on the delipidated protein at pH 6.5 with eight different ligands. The protein:ligand ratio was varied from 1:0 to 1:10. The ligand binding experiments could not be carried out beyond the protein:ligand ratio of 1:10 because heavy precipitation occurred in the protein sample, and almost all peaks in the HSQC spectrum showed changes in chemical shift positions indicating nonspecific binding (21). Methanol had a negligible effect on the HSQC spectrum of delipidated ApolPBP1wt up to a 5% v/v concentration, after which it also caused changes in chemical shifts as well as intensity of peaks.

The effects of all eight ligands studied here on the conformation of delipidated ApolPBP1wt were more or less the same. Resonances belonging to the free form (closed conformation) of the protein became gradually weak and finally disappeared, whereas those belonging to the bound form (open conformation) appeared and strengthened with each addition. At a certain point during the ligand binding studies, the free form (closed conformation) of the delipidated ApolPBP1wt was completely converted into the bound form (open conformation) with an approximate protein:ligand ratio of 1:1 (Fig. 4, A and B). Interaction of ApolPBP1 wt with all these ligands was seen to be in slow exchange on the NMR time scale as two different sets of peaks were observed for free and bound states, indicating very high affinities of ligands toward the protein. Because the delipidated ApolPBP1wt has very high affinities toward various ligands, we did not attempt to determine the dissociation constants from the NMR titration data. Although NMR spectroscopy has been used efficiently to determine dissociation constants in the micromolar to millimolar range, strong binding with a dissociation constant in the nanomolar range cannot be determined accurately due to the inherent sensitivity issue. The higher protein concentration (which is 220 \(\mu M\) in the present case) necessary for the NMR studies prohibits the accurate calculation of dissociation constants for high affinity ligands.

Effect of pH on the Conformation of Ligand-bound ApolPBP1wt—To investigate the effect of pH on the ligand-bound conformation, two-dimensional \(^{1}H^{,15}N\) HSQC spectra of ligand-bound ApolPBP1wt were recorded over the pH range of 6.5 to 4.5. The pH titration carried out on the delipidated ApolPBP1wt in the presence of excess ligand (1:5 palmitic acid, which has a similar effect on the delipidated ApolPBP1wt as that of the pheromone) revealed that above pH 6.0 the HSQC spectrum resembled that of the previously reported undelipidated ApolPBP1wt high pH open conformation (10). However, at pH levels below 5.0 the HSQC spectrum resembled that of the recently reported low pH closed conformation (14), whereas in the pH range between 5.0 and 6.0, the open conformation exists in equilibrium with the closed conformation showing two sets of peaks (supplemental Fig. 2). This observation supports the earlier reports of conformational heterogeneity seen for the undelipidated ApolPBP1 at pH 5.2 (11) and undelipidated BmorPBP at pH 5.5 (7). Even though the protein exists primarily in the closed conformation below pH 5.0, a few peaks corresponding to the open conformation could be observed at a very low contour level.

Effect of Histidine Mutations—To investigate the proposed role of histidines in the pH-induced conformational switch (11, 12, 22), His\(^{70}\) and His\(^{95}\), located at one end of the binding pocket, were mutated to alanine. The HSQC spectrum of the undelipidated ApolPBP1H70A/H95A at pH 6.5 (Fig. 5A) largely resembled that of the wild type protein, which is in the open conformation. However, at pH 4.5 it remained in the open conformation (Fig. 5B) rather than switching to the closed conformation as in the wild type protein. The spectrum displayed some changes due to the difference in pH (Fig. 5, A and B) as expected.

At pH 6.5 the delipidated ApolPBP1H70A/H95A displayed a spectrum that had a mixture of peaks representing both the closed and open conformations (Fig. 5C) in contrast to what was observed for the wild type protein (Fig. 1C), which was predominantly in the closed conformation. This observation indicates that the equilibrium slightly shifts toward the open conformation at high pH even in the absence of a ligand when the histidines are substituted by alanine.

The HSQC spectrum of the delipidated ApolPBP1H70A/H95A at pH 4.5 was similar to the closed conformation of the wild type protein with some changes affected by the mutation (Fig. 5D). Most notable among them was the disappearance of the resonances corresponding to Gly\(^{71}\) and Gly\(^{96}\).
residues that follow the mutated histidines, i.e. His70 and His95).

To further confirm that the hydrophobic ligand from the bacterial system is still bound to the undelipidated Apol-PBP1H70A/H95A at pH 4.5, keeping it in the open conformation, we carried out NMR titration studies of the delipidated double mutant at pH 4.5 with palmitic acid as the ligand. The closed conformation of the delipidated double mutant switched to the open conformation upon binding to the ligand (Fig. 6), thus confirming the ability of the double mutant to bind the ligand even at low pH. The binding affinity at pH 4.5 was lower than at pH 6.5, as the complete conversion of the closed form to the open form occurred at a protein:ligand ratio of 1:2.5. With the above results, we have successfully decoupled the effect of pH on ligand binding.

Moreover, we have demonstrated here that His70 and His95 are responsible for the pH-induced conformational switch of the ligand-bound protein from open to closed at low pH, which is likely the mechanism of ligand release near the membrane-bound receptor.

AMA Binding Studies by Fluorescence—AMA in aqueous environments displays a weak fluorescence after excitation at 256 or 298 nm with a λmax of 563 nm. The fluorescence of AMA is considerably enhanced in a hydrophobic environment like the binding cavity of PBPs with a blue shift in the λmax. Titration of the wild type and the double-mutated ApolPBP1 (in both the undelipidated and delipidated form) with AMA at pH 6.5 and 4.5 revealed that AMA did not bind to the wild type protein at pH 4.5. On the contrary, AMA was able to bind to ApolPBP1H70A/H95A even at pH 4.5. However, the maximum fluorescence intensity reached at saturation was about half of what it was at pH 6.5 (Fig. 7). The delipidation also had an effect on the maximum fluorescence intensity reached at saturation; the delipidated proteins had higher intensity than their undelipidated counterparts at both pH levels (Fig. 7). The dissociation constants (Kd) for both proteins in different conditions are listed in Table 1. In general, the delipidated proteins had higher affinities than their undelipidated counterparts, and the double mutant proteins had lower affinities at pH 4.5 than at pH 6.5.

Binding Affinities Determination by Competitive Displacement of AMA—Fluorescence assays involving competitive displacement of AMA from the protein-AMA complex by various ligands have frequently been used to determine the Kd values (20, 23, 24). The binding of delipidated ApolPBP1wt with various ligands was investigated by the competitive displacement assay using AMA fluorescence. The IC50 and Kd values obtained from the competitive displacement of AMA by various ligands have been listed in Table 2. The three fatty acids (Fig. 8A) palmitic acid, palmitoleic acid, and oleic acid showed very similar Kd values (80, 70, and 60 nM, respectively). In the case of A. polyphemus pheromone and its analogs (Fig. 8B), the pheromone showed the highest affinity (Kd = 50 nM), whereas the alcohol and diazocacetate pheromone analogs displayed lower binding affinities (Kd = 430 and 120 nM, respectively).

The IC50 and Kd values for the titration of the wild type and the double-mutated ApolPBP1 (in both the undelipidated and delipidated form) at pH 6.5 and 4.5 with acetate pheromone monitored by the competitive displacement assay are shown in Table 1. In general, the undelipidated proteins have lower binding affinities than the delipidated ones.
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FIGURE 6. Expanded region of the two-dimensional \(^{(1\text{H},^{15}\text{N})}\) HSQC spectra of delipidated ApolPBP1H70A/H95A (220 \(\mu\text{M}\)) in 50 mM phosphate buffer, pH 4.5, upon titration with palmitic acid showing the disappearance of resonances corresponding to the bound conformation labeled as b. Protein:ligand ratios are indicated on the figure with corresponding colors.

DISCUSSION

We have shown here that ApolPBP1, when expressed heterologously in \(E.\ coli\), binds to a hydrophobic ligand that is endogenous to the host cells. This ligand-bound protein is in the open conformation at pH above 6.0 unless the ligand is removed by a delipidation procedure. Our observations demonstrate that all undelipidated PBPs (8–11) are in fact the closed and open conformations, respectively. Furthermore, in the intermediate pH range of 5.0 to 6.0, the ligand-bound or undelipidated protein exists in equilibrium between open and closed conformations (supplemental Fig. 2). This observation explains the previously reported conformational heterogeneity for both undelipidated ApolPBP1 at pH 5.2 (11) and the undelipidated BmorPBP at pH 5.5 (7). It is interesting to note that the ApolPBP1 used in the above study (11) was produced as inclusion bodies in \(E.\ coli\). Surprisingly, the hydrophobic ligand remained bound to the protein even after the inclusion bodies were solubilized in 6 \(M\) guanidine hydrochloride, reduced with 10 mM dithiothreitol, refolded, and purified through multiple steps (4, 10, 11). This implies that the hydrophobic ligand from the bacterial system is able to bind to the protein molecules even during refolding and purification processes. It is likely that picking up the hydrophobic molecule(s) from the host expression system could be a common feature for many lipid-binding proteins contrary to the belief that these proteins were unliganded. Moreover, the so-called “low pH or acidic (A) form” and the “high pH or basic (B) form” conformations reported for undelipidated PBPs (7–11) are in fact the closed and open conformations, respectively.

In contrast to the undelipidated ApolPBP1wt (Fig. 1, A, B, and E), the delipidated protein (free form) does not undergo the drastic conformational switch from low to high pH or vice versa indicating that the protein primarily exists in one conformation at both high and low pH (Fig. 1, C, D, and F). Thus, the delipidated protein is not a pH sensor and exists primarily in the closed conformation. This closed conformation readily converts into the open conformation upon addition of ligand at pH 6.0 or higher (Fig. 4). Ligand binding to ApolPBP1 is reversible and drastically reduced below pH 5.0, which might be important for the release of pheromone near the membrane receptors (ion channels) to trigger the neuro-

**TABLE 1**

Dissociation constants \((K_{d})\) for the binding of AMA to different ApolPBP1 samples, determined using the increase in AMA fluorescence at 480 nm, and the \(K_{d}\) values of the 6\(E\),11Z-hexadecadienyl acetate pheromone determined from the competition assay.

| Protein                        | \(K_{d}(\text{AMA})\) | IC\(_{50}\)(AMA) | \(K_{d}\) for acetate pheromone |
|-------------------------------|------------------------|-----------------|---------------------------------|
| Undelipidated ApolPBP1wt, pH 6.5 | 880 ± 100              | 620             | 1000                            |
| Delipidated ApolPBP1wt, pH 6.5 | 500 ± 30               | 160             | 1000                            |
| Undelipidated ApolPBP1H70A/H95A, pH 6.5 | 990 ± 70 | 230             | 1000                            |
| Undelipidated ApolPBP1H70A/H95A, pH 4.5 | 1060 ± 220 | 1160           | 5000                            |
| Delipidated ApolPBP1H70A/H95A, pH 6.5 | 620 ± 30 | 130             | 1000                            |
| Delipidated ApolPBP1H70A/H95A, pH 4.5 | 1290 ± 360 | 360             | 5000                            |

**TABLE 2**

Dissociation constants \((K_{d})\) for the binding of various ligands to ApolPBP1 determined from the competition assay.

| Ligand                                              | IC\(_{50}\)AMA | \(K_{d}\) |
|-----------------------------------------------------|---------------|----------|
| 6\(E\),11Z-Hexadecadienyl acetate                  | 160           | 50       |
| 6\(E\),11Z-Hexadecadienyl diacetoacetate           | 370           | 120      |
| 6\(E\),11Z-Hexadecadienyl alcohol                  | 1290          | 430      |
| Palmitic acid                                       | 230           | 80       |
| Palmitoleic acid                                    | 210           | 70       |
| Oleic acid                                          | 190           | 60       |
Furthermore, the undelipidated ApolPBP1wt or the delipidated protein switching the closed to the open conformation. HSQC, revealed that all ligands induced similar changes in conformational states of ApolPBP1wt and ApolPBP1H70A/H95A, pH 6.5 or 4.5, in the presence or absence of ligand as observed in the two-dimensional $^{1}H,^{15}N$ HSQC NMR titration studies of the delipidated double mutant using palmitic acid at pH 4.5. Our hypothesis was that the delipidated double mutant, which is in the close conformation at pH 4.5 (Fig. 5D), should switch to the open conformation if it binds the ligand. The NMR titration studies of the delipidated double mutant with palmitic acid revealed that the protein could indeed bind the ligand at pH 4.5 and switch from the closed to the open conformation (Fig. 6). Thus, His70 and His95, located at one end of the binding pocket, act as a gate that is shut at high pH but opens at low pH to release the ligand. It is important to note that although the double mutant can bind a ligand at low pH, delipidation of this protein is still possible at pH 4.5 using the Lipidex resin, which possibly has higher affinity to the ligand than the protein.

Contrary to the undelipidated double mutant at pH 4.5 (Fig. 5B), the delipidated double mutant is in the closed conformation (Fig. 5D) similar to the delipidated wild type protein at the same pH (Fig. 1D). However, at pH 6.5 the delipidated double mutant exists as a mixture of closed and open conformations (Fig. 5C) in contrast to the delipidated wild type protein, which remains in the closed conformation at both high and low pH levels (Fig. 1, C and D). These results demonstrate that the equilibrium shifts toward the open conformation for the delipidated double mutant at high pH even in the absence of a ligand contrary to what is observed for the wild type protein (Table 3).

To investigate the effect of delipidation, mutation (H70A and H95A), and pH on ligand binding affinity of ApolPBP1, fluorescence titration studies were carried out using AMA as a ligand. The titration data revealed that delipidation affects the binding affinity of ApolPBP1 toward ligands. At pH 6.5 the undelipidated ApolPBP1wt has about 1.5-fold lower binding affinity ($K_d = 880$ nM) than the delipidated protein ($K_d = 500$ nM) clearly showing that the hydrophobic ligand from the expression host competes with the added ligand (Table 1). At pH 6.5 the delipidated forms of both the wild type and ApolPBP1H70A/H95A show similar binding affinities (Table 1). However, at pH 4.5 the wild type protein in both the undelipidated and delipidated forms showed a very small increase in the fluorescence intensity upon titration with AMA indicating an extremely low binding affinity consistent with what was observed previously using a cold binding assay for the undelipidated ApolPBP1 (25). In contrast to the wild type protein, the

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Competitive binding of fatty acids, *A. polyphemus* pheromone, its alcohol metabolite, and diazoacetate analog to ApolPBP1wt, displacing AMA. A, fatty acids are as follows: ■, oleic acid (--); ●, palmitic acid (- - -); ▲, palmitoleic acid (--). B, pheromone and pheromone analogs are as follows: ■, 6E,11Z-hexadecadienyl acetate (--); ●, 6E,11Z-hexadecadienyl alcohol (- - -); ▲, 6E,11Z-hexadecadienyl diazoacetate (—). Standard deviations are indicated by error bars in all plots.

### Table 3

Conformational states of ApolPBP1wt and ApolPBP1H70A/H95A, pH 6.5 or 4.5, in the presence or absence of ligand as observed in the two-dimensional $^{1}H,^{15}N$ HSQC NMR.

| Protein       | Ligand | pH   | Conformational state |
|---------------|--------|------|----------------------|
| ApolPBP1wt    | -      | 6.5  | Open                 |
| ApolPBP1wt    | +      | 4.5  | Closed               |
| ApolPBP1wt    | -      | 6.5  | Closed               |
| ApolPBP1wt    | -      | 4.5  | Closed               |
| ApolPBP1H70A/H95A | +  | 6.5  | Open                 |
| ApolPBP1H70A/H95A | +  | 4.5  | Open                 |
| ApolPBP1H70A/H95A | -  | 6.5  | Closed + open        |
| ApolPBP1H70A/H95A | -  | 4.5  | Closed               |
double mutant shows considerable binding affinity toward AMA at pH 4.5 ($K_d = 1290$ nM), which is only 2-fold lower than at pH 6.5 ($K_d = 620$ nM) (Table 1). This observation clearly demonstrates that mutation of these two histidines to alanine enables the protein to bind the ligand even at low pH.

The effect of delipidation was also studied by competitive displacement of AMA. Competitive displacement of a fluorescent probe has proved to be a valuable tool for determining the binding constants of various ligands toward odorant-binding proteins/PBPs (20, 23, 24). In the competitive displacement assay using AMA as a fluorescent probe with $6E,11Z$-hexadecadienyl acetate pheromone as the competing ligand, we observed that the delipidated ApolPBP1wt at pH 6.5 ($K_d = 50$ nM) has about 6-fold higher affinity than the undelipidated protein ($K_d = 290$ nM) at the same pH (Table 1). Similar but less pronounced effects were observed for the undelipidated ($K_d = 110$ nM) and delipidated ($K_d = 50$ nM) ApolPBP1H70A/H95A at pH 6.5 (Table 1).

In the fluorescence spectroscopic studies, we observed that the intrinsic tryptophan fluorescence of the delipidated ApolPBP1wt responds differently to different ligands. This observation suggested that the mode of interactions of the protein to various ligands is different, similar to what was reported previously (17). Thus, determination of binding constants using intrinsic fluorescence was impractical for these ligands. Hence, we have determined the binding affinities of pheromone, its corresponding alcohol metabolite, diazoacetate analog, and three fatty acids (palmitic, palmitoleic, and oleic acids) by competitive displacement of AMA. The $K_d$ values obtained are in the nanomolar range, indicating strong affinity of all these ligands toward delipidated ApolPBP1. The binding affinities of the three fatty acids toward the delipidated ApolPBP1 are similar to that of $A. polyphemus$ pheromone (Table 2). However, the alcohol metabolite and diazoacetate analog have about 8.5- and 2.5-fold lower affinities, respectively, than the pheromone (Table 2) confirming that the binding of the pheromone to the delipidated ApolPBP1 is preferred over the pheromone metabolite and/or analogs.

In conclusion, we have shown here that ApolPBP1, when expressed in $E. coli$, picks up hydrophobic molecule(s) from the host system that keeps it in the open conformation at high pH. The recombinant ApolPBP1 remains in the open conformation at pH above 6.0 unless the hydrophobic ligand is removed by delipidation. This open conformation is pH-sensitive although the closed conformation of the delipidated protein is generally pH-insensitive. High pH and/or the presence of ligand favor the open conformation of wild type ApolPBP1, whereas low pH and the absence of ligand favor the closed conformation. Our data also confirm the role of His$^{70}$ and His$^{95}$ in the pH-induced conformational switch, which releases the pheromone at low pH. Mutation of these two histidines to alanine drives the equilibrium toward
the open conformation, which allows the protein to bind ligand even at low pH.

An understanding of the molecular mechanisms of olfactory perception is essential if disruption of mating via sensory inhibition is to be achieved. The result of this study may have a far-reaching impact beyond sensory neurobiology to insect control, through pheromone-based integrated pest management, to control the olfactory behavior of deleterious insect pests. Such detailed mechanistic studies will help to biorationally design pheromone mimetics or “anti-pheromones” for an effective pest management.

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