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Structural Insights into the Ligand Binding and Releasing Mechanism of *Antheraea polyphemus* Pheromone-Binding Protein 1: Role of the C-Terminal Tail

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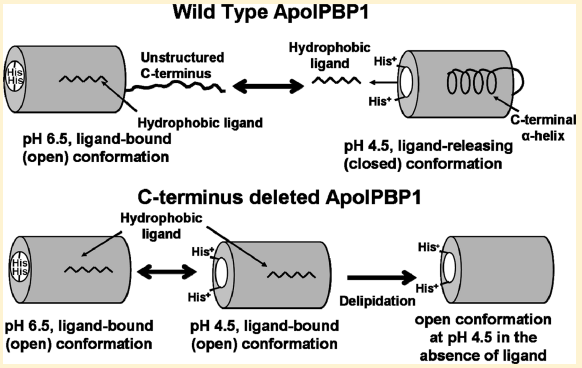
ABSTRACT: Pheromone-binding proteins (PBPs) in lepidopteran moths selectively transport the hydrophobic pheromone molecules across the sensillar lymph to trigger the neuronal response. Moth PBPs are known to bind ligand at physiological pH and release it at acidic pH while undergoing a conformational change. Two molecular switches are considered to play a role in this mechanism: (i) protonation of His^{70} and His^{95} situated at one end of binding pocket and (ii) switch of the unstructured C-terminus at the other end of the binding pocket to a helix that enters the pocket. We have reported previously the role of the histidine-driven switch in ligand release for *Antheraea polyphemus* PBP1 (ApolPBP1). Here we show that the C-terminus plays a role in the ligand release and binding mechanism of ApolPBP1. The C-terminus truncated mutants of ApolPBP1 (ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142) exist only in the bound conformation at all pH levels, and they fail to undergo pH- or ligand-dependent conformational switching. Although these proteins could bind ligands even at acidic pH unlike wild-type ApolPBP1, they had ∼4-fold reduced affinity for the ligand at both acidic and physiological pH compared to that of wild-type ApolPBP1 and ApolPBP1H70A/H95A. Thus, apart from helping in ligand release at acidic pH, the C-terminus in ApolPBP1 also plays an important role in ligand binding and/or locking the ligand in the binding pocket. Our results are in stark contrast to those reported for BmorPBP and AtraPBP, where C-terminus truncated proteins had similar or increased pheromone binding affinity at any pH.



Chemical communication in insects occurs via sensing of variety of small, volatile organic molecules called semiochemicals. Indeed, chemical sensing guides the most fundamental behaviors of insects, including feeding, mating, protection of sites of oviposition, and escape predation. Pheromones, a class of semiochemicals that elicit a behavioral response in the members of the same species, serve as chemical stimuli in intraspecies communication. In lepidopteran moths, sex pheromones secreted by females are detected with extreme selectivity and specificity by the males of the same species, initiating the mating process. Pheromone-binding proteins (PBPs) present in the antennae of male moths transport the hydrophobic pheromone molecules across the sensillar lymph to the membrane-bound receptors (ion channels)[1,2] while protecting them against the degrading enzymes to trigger the neuronal response. PBPs found in different moth species are highly similar, exhibiting >50% sequence identity.[3] These acidic proteins are very soluble in water and have molecular masses between 14 and 16 kDa. The six cysteine residues conserved in all moth PBPs form three disulfide bonds that hold six helices together forming the binding pocket.[3]

There are several reports suggesting the role of insect PBPs in either transportation and release of pheromone at the olfactory neuron site triggering neuronal response[4−10] or activation of the pheromone-sensitive neuron by the PBP−pheromone complex.[11−14] However, it is widely accepted that moth PBPs present in the sensillar lymph of male insect antenna bind ligand at physiological pH and release it at acidic pH near the site of the olfactory neuron. This ligand release is facilitated by a well-defined, dramatic conformational change in moth PBPs that is associated with a change in pH. Because the pheromone fails to bind PBP below pH 6.0,[4,6,9,10,15,16] the pH of the sensillar lymph has to be >6.0 to facilitate pheromone binding. Furthermore, release of pheromone driven by the pH-induced conformation change in PBP near the olfactory neuron is supported by the fact that the negatively charged dendritic membrane reduces the pH at the site of the pheromone-sensitive neuron.[17] Thus, the acidic pH at the site of the neuron facilitates the conformational change in PBP resulting in the release of the pheromone.[10]

Although a majority of the work on moth PBPs suggests that pheromone is released by PBP near the membrane while undergoing a pH-driven conformational change, it is still possible that the PBP−pheromone complex may activate the receptors directly. However, it is important to note that ligand-bound moth PBP undergoes a well-defined conformational

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change at acidic pH.[4] This conformational change should not be interpreted as acid denaturation of the protein, because ligand-free PBPs have a more organized conformation at any pH containing seven α-helices, including the structured internalized C-terminus.[4,6,15,18,19] In contrast, the bound conformation of PBP is composed of six α-helices with an unstructured exposed C-terminus at pH >6.0.[4,7,8,20] This pH-dependent conformational change might be important in PBP recycling if not for unloading the ligand at the site of receptor.[19] Therefore, it is important to study the different elements responsible for this switch to understand the role of PBPs in moth olfaction.

Lepiodopteran moth PBPs exist in two conformations: "open" or "bound" exists in the presence of ligand at high pH, and "closed" or "free" is observed in the absence of ligand and/or at low pH.[4] The open conformation is characterized by the unstructured C-terminal tail that extends to the solvent leaving the binding cavity open. In the closed conformation, the C-terminus occupies the binding cavity in the form of the seventh α-helix, thus closing the cavity. In our earlier work, we have shown that unliganded (delipidated) PBP from the giant silk moth, *Antheraea polyphemus* (ApolPBP1), is generally pH insensitive, existing in the closed conformation at acidic and physiological pHs, whereas the ligand-bound PBP undergoes a dramatic pH-dependent conformational change.[4] At low pH, the protein remains in the closed or free conformation regardless of whether the protein is undelipidated (ligand not removed by delipidation) or delipidated (ligand removed by delipidation).[4,21]

Two molecular switches have been proposed to play a role in the pH-dependent ligand release mechanism of moth PBPs: (i) protonation of His^{70} and His^{95} situated at one end of the binding pocket at pH <6.0 that opens the histidine gate via charge repulsion[9,16,20] and (ii) switch of the unstructured C-terminus to a helix at low pH that enters the binding pocket from the opposite end of the pocket as the ligand is released through the histidine gate.[16,19,22] Thus, the exit of the odor molecule is accompanied by the entry of the C-terminal tail to the binding pocket of PBP. We have previously demonstrated the role of the two histidine residues (His^{70} and His^{95}) in the ligand release mechanism of ApolPBP1.[4] At low pH, the repulsion between the two charged histidines opens the gate to unload the ligand.[4] When these two histidines were mutated to alanines, the ApolPBP1H70A/H95A double mutant remained in bound conformation at all pH levels as the alanines permanently shut the gate prohibiting ligand release. Thus, the neutral forms of His^{70} and His^{95} shut the gate at pH >6.0 to facilitate ligand binding, while the repulsion between these two charged histidines opens the gate at low pH, allowing ligand release.[4] Clearly, the exit of the ligand is controlled by the histidine gate located at one end of the binding pocket.

However, questions remain for the second molecular switch, the C-terminal tail of the protein. What is the role of this C-terminal switch? Is this C-terminal tail necessary for pheromone unloading at low pH? Can the ligand escape on its own when the histidine gate opens at low pH, or is it pushed out as the C-terminal helix occupies the binding cavity? It is now clear that the ligand-free ApolPBP1 is in the closed (or free) conformation at all pH levels, where the C-terminus is tucked inside the binding pocket as the seventh helix.[4] The C-terminus truncated PBPs from the silk moth *Bombyx mori* (BmorPBP) and navel orangeworm moth *Amyelois transitella* (AtraPBP) were able to bind the ligand at both acidic and physiological pH with affinities similar to that of the wild-type protein at physiological pH.[22,23] In contrast, the C-terminus truncated PBP of the gypsy moth *Lymantria dispar* (LdisPBP2) was found to have ~10-fold reduced affinity for ligand.[24] The moths *B. mori*, *A. transitella*, and *L. dispar* belong to the same insect order, Lepidoptera. Although these PBPs have sequences that are ~50% identical and retain many conserved features, including six strictly conserved cysteine residues, it is very interesting that their C-terminus truncated mutants behave differently with respect to ligand binding. Thus, it is clear from these contradictory reports that the general mechanism of odor perception varies even across the same insect order. The giant silk moth, *Antheraea polyphemus*, also belongs to the order Lepidoptera. However, there are no reports about the role of the C-terminus of ApolPBP1 in either ligand binding or release, although this is the first PBP on which most biochemical work has been conducted. Most importantly, no detailed investigation of the effect of pH and ligand on the conformational transition of a C-terminus truncated PBP or a PBP in which both molecular switches have been altered [C-terminus truncation along with mutation of both critical histidines (His^{70} and His^{95}) to alanines] has been conducted.

To investigate the role of the C-terminal tail along with the two critical histidine residues in the ligand binding and releasing mechanism in ApolPBP1, we initiated a comprehensive structural study of this protein. The 14 terminal residues (Pro^{129}–Val^{142}) were deleted from the C-terminus of ApolPBP1wt as well as ApolPBP1H70A/H95A. Two distinct hypotheses were tested here: (i) the C-terminus is essential for ligand release only, and its deletion would cause the protein to bind ligand freely at any pH because the binding pocket would be unoccupied at all pH levels: (ii) the C-terminus is essential for ligand release as well as binding, and its deletion would cause a reduction in the level of ligand binding at any pH. To test these hypotheses, we studied the effects of pH and ligand on the conformation of both C-terminus truncated and C-terminus truncated H70A/H95A mutants of ApolPBP1 by high-resolution solution nuclear magnetic resonance (NMR) spectroscopy. The effects of C-terminus deletion alone and that of both C-terminus deletion along with His^{70} and His^{95} mutations on the binding of ligand to these proteins at different pH values were also investigated by fluorescence spectroscopy. Our results suggest that regardless of whether C-terminus truncated ApolPBP1 is bound to a ligand or not, the protein is always in the open conformation without undergoing a pH- or ligand-dependent conformational switch. This means unloading of the ligand for a C-terminus truncated ligand-bound ApolPBP1 at low pH is not possible even when the histidine gate is opened. Thus, the ligand is not released through the opened histidine gate unless the C-terminal helix occupies the binding cavity. Therefore, the histidine gate at one end of the binding pocket and the C-terminal gate at the other end work hand in hand in ligand release. Additionally, the ligand-free forms of the C-terminus truncated proteins exist only in the open conformation at all pH levels as the unoccupied binding pocket remains open in the absence of the C-terminus. The important finding of our study is that the C-terminus truncated proteins have reduced affinity for the ligand as seen in a two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) NMR and fluorescence-based 1-aminoanthracene (AMA) binding assay. Thus, our study implies that the C-terminus not only is necessary in the ligand releasing mechanism but also plays an equally important role in

ligand binding in the case of ApolPBP1. This is the first report detailing the effect of pH and ligand on the conformation of the truncated C-terminus and when both switches (C-terminal gate and histidine gate) are eliminated in a moth PBP.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification. Truncation of 14 residues (Pro^{129}–Val^{142}) from the C-terminus of ApolPBP1wt and ApolPBP1H70A/H95A was achieved using a polymerase chain reaction-based approach. *ApolPBP1wt* and *ApolPBP1H70A/H95A* genes, cloned into pET-21a vector, were amplified with forward (5′ GGAATTCCA|TATGTCG-CCAGAGATCATGAAG 3′) and reverse (5′ GCG|GATCC-CTAAACCCAGTTCAGCTTATGGATCTC 3′) (restriction sites underlined) primers and subcloned between the restriction sites of *Nde*I and *Bam*HI of the pET-21a vector. The correct orientations of both constructs were determined by DNA sequencing. All plasmid constructs were transformed into *Escherichia coli* Origami cells and expressed as described previously.[4] M9 minimal medium supplemented with $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) was used for the expression of isotope-labeled recombinant proteins. Unlabeled and ^{15}N-labeled proteins were expressed, purified, and delipidated as described previously.[4] The purity of the proteins was assessed by liquid chromatography and electrospray ionization mass spectrometry (Figure 1A,B). Protein concentrations were determined spectrophotometrically using the theoretical E_{280} of 14230 M^{-1} cm^{-1}.

NMR Measurements. All NMR data were collected at 35 °C on a Bruker Avance 600 MHz spectrometer at the Department of Chemistry and Biochemistry of Auburn University. pH titrations were conducted on 400 μL of uniformly ^{15}N-labeled proteins (0.3 mM) in 50 mM sodium phosphate buffer (pH 6.5 or 4.5), 1 mM EDTA, 0.01% NaN_3, and 5% D_2O (used as a lock solvent) in a Shigemi tube. Two-dimensional {^1H–^{15}N} HSQC spectra were collected for ^{15}N-labeled undelipidated and delipidated ApolPBP1ΔP129–V142 and ApolPBP1H70A/H95AΔP129–V142 samples at pH 4.5 and 6.5. Ligand titration studies were conducted with ^{15}N-labeled delipidated ApolPBP1ΔP129–V142 and ApolPB-P1H70A/H95AΔP129–V142 at pH 4.5 and 6.5 with palmitic acid as a ligand. The proteins [310 μL of a 220 μM sample in 50 mM phosphate buffer (pH 6.5 or 4.5) containing 5% D_2O, 1 mM EDTA, and 0.01% (w/v) NaN_3] were titrated with increasing concentrations of palmitic acid (0–2.2 mM), and the corresponding two-dimensional HSQC spectra were recorded at each titration point.

Fluorescence Spectroscopy. Fluorescence experiments were conducted on a 55B spectrofluorimeter (PerkinElmer Life Sciences) as described previously.[4] All experiments were repeated at least twice to confirm reproducibility.

AMA Binding Studies. The binding of AMA to delipidated ApolPBP1ΔP129–V142 and ApolPBP1H70A/H95AΔP129–V142 at pH 6.5 and 4.5 was assessed by monitoring the increase in the AMA fluorescence at 480 nm as described previously.[4] Phosphate buffer with the appropriate amount of AMA served as control for each data point. All PBP mutants showed maximal fluorescence intensity at 5 μM AMA; hence, this data point was chosen to compare the relative binding affinities of all proteins. The fluorescence intensity of ApolPBP1wt with a 1:5 protein/AMA mixture was considered as 100% binding affinity, and relative binding affinities for other proteins were calculated accordingly.

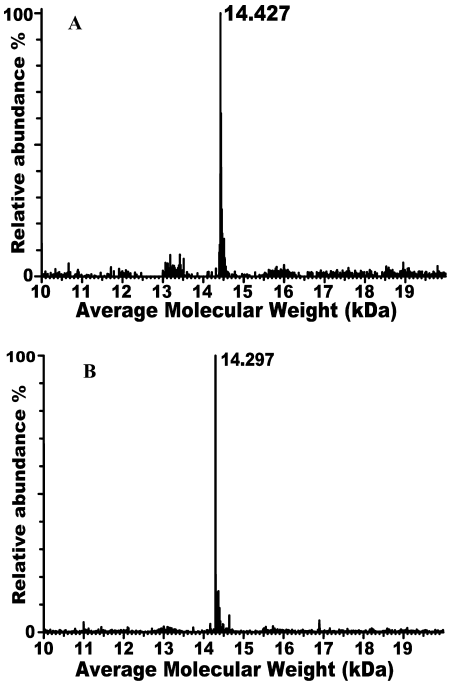

Figure 1. (A) Deconvoluted mass spectrum of the delipidated unlabeled ApolPBP1ΔP129–V142 showing a peak at 14.427 kDa. The theoretically calculated molecular mass is 14.433 kDa. (B) Deconvoluted mass spectrum of the delipidated unlabeled ApolPB-P1H70A/H95AΔP129–V142 showing a peak at 14.297 kDa. The theoretically calculated molecular mass is 14.301 kDa. The difference between the masses of these two proteins corresponds to the substitution of two histidines with alanines. The electrospray ionization-mass spectrometry was carried out on a Q-Tof Premier (Waters) mass spectrometer at the Department of Chemistry and Biochemistry of Auburn University.

RESULTS

Role of the C-Terminus in Ligand Binding and Release. Lepidopteran moth PBPs are believed to release ligand at low pH near the membrane-bound receptors (ion channels) by undergoing a conformational change, which is facilitated by two molecular switches: the histidine switch and the C-terminal switch. In this work, we have studied the role of the C-terminal switch in the ligand binding and release of ApolPBP1. The two-dimensional {^1H–^{15}N} HSQC spectrum is considered to be the fingerprint of a protein, as it is very sensitive to environmental changes like pH, temperature, substrate binding, mutations, etc. Local or global conformational changes occurring in the protein are reflected in the HSQC spectrum as the changes in the chemical shift positions of resonances of the amino acid residues involved. The HSQC spectra of delipidated ApolPBP1ΔP129–V142 and ApolPB-P1H70A/H95AΔP129–V142 at pH 6.5 exhibited well-dispersed resonances, indicating that both proteins were properly folded. These spectra largely resembled that of the open conformation of ApolPBP1wt, with marked disappearances of the resonances belonging to the residues in the C-terminal tetradecapeptide segment (Figure 2A,B). Using the assignment of undelipidated ApolPBP1wt at pH 6.5 (19), ∼85 and ∼81% of the original peaks could be located in the HSQC spectra of delipidated ApolPBP1ΔP129–V142 and ApolPB-P1H70A/H95AΔP129–V142, respectively. Very interestingly, these delipidated proteins, in which the endogenous ligand of the expression system has been removed through the

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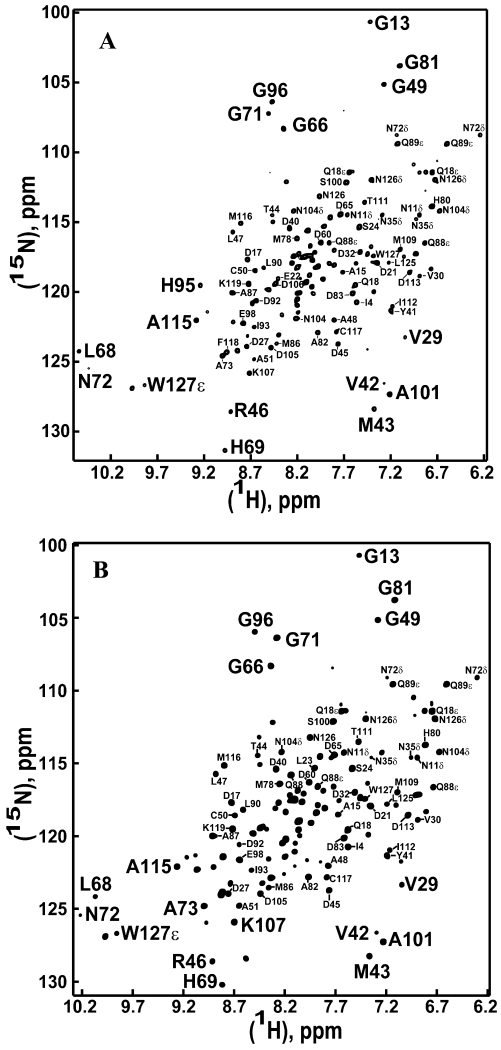

Figure 2. Two-dimensional {^{1}H−^{15}N} HSQC spectra of delipidated 0.3 mM C-terminus deleted mutants of ApolPBP1 and ApolPBP1H70A/H95A in 50 mM sodium phosphate buffer (pH 6.5) containing 5% D_2O, 1 mM EDTA, and 0.01% sodium azide. Most of the peaks are labeled on the spectra except for a few from the crowded region. Some of the peaks from the periphery are labeled in a larger font: (A) ApolPBP1ΔP129−V142 and (B) ApolPBP1H70A/H95AΔP129−V142.

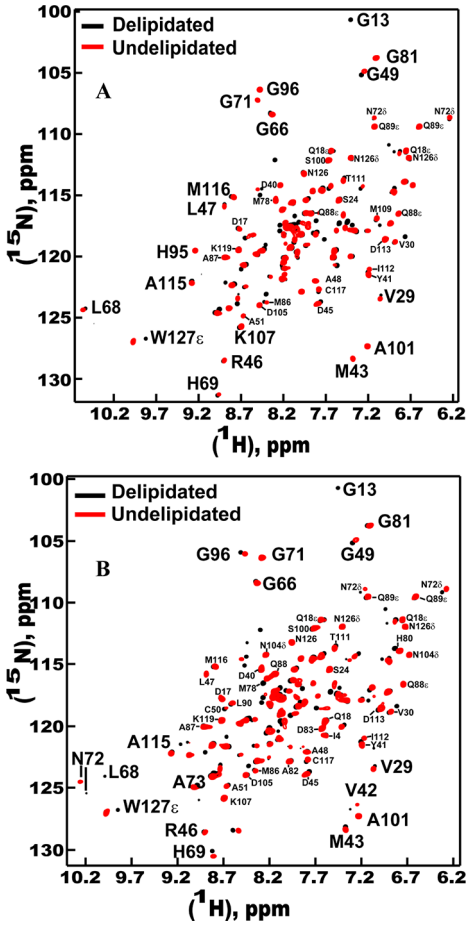

Figure 3. Effect of delipidation on ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 at pH 6.5. The figures represent an overlay of two-dimensional {^{1}H−^{15}N} HSQC spectra of 0.3 mM delipidated (black) and undelipidated (red) proteins in 50 mM sodium phosphate buffer (pH 6.5) containing 5% D_2O, 1 mM EDTA, and 0.01% sodium azide. Note the disappearances of resonances such as those belonging to G13, W127ε, and several others in the spectrum of undelipidated proteins: (A) ApolPBP1ΔP129−V142 and (B) ApolPBP1H70A/H95AΔP129−V142.

delipidation procedure, are still in the open (bound) conformation, although the hydrophobic cavity is empty. These data suggest that for the C-terminus truncated mutants, the binding pocket is no longer closed by the C-terminus. Thus, the conformation of the protein is open even when no ligand is bound to the pocket.

2D HSQC spectra of undelipidated ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 at pH 6.5 matched very well with those of their delipidated counterparts (Figure 3A,B) and exhibited the characteristic open conformation pattern. A similar phenomenon was observed at pH 4.5 as well. Interestingly, several resonances such as those belonging to Gly^{13}, Asn$^{53}\delta$, Trp$^{127}\varepsilon$, etc., were found to disappear in the spectra of undelipidated proteins because of line broadening. Such resonances could be readily located in the spectra of delipidated counterparts of the same proteins, indicating that these residues must be in the intermediate exchange regime on the NMR time scale in the ligand-bound protein.

Effect of pH and Ligand on the Conformation of ApolPBP1 C-terminus Deleted Mutants. Delipidated ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 at pH 4.5 (Figure 4A,B) showed the same open conformation in HSQC spectra that was observed at pH 6.5. The undelipidated proteins at pH 4.5 also exhibited the same characteristic open conformation as the delipidated proteins at pH 4.5. Thus, these proteins did not undergo the pH-dependent conformational change like that of the undelipidated ApolPBP1 wild-type protein reported previously.[4] These results are in agreement with those reported for the BmorPBP(1−128) variant.[25] However, several resonances (for example, those belonging to Gly^{13}, Gly^{49}, Gly^{81}, Gly^{96}, etc.) displayed a moderate to large change in their chemical shift positions (Figure 5A,B) as a result of the change in pH. This indicated that although the conformation remains open at both pH values, local environmental changes caused by a change in pH affect chemical shifts of these residues (Figure 5C).

To investigate the effect of ligand on the conformations of delipidated forms of ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142, ligand titration studies were conducted at pH 6.5 and 4.5 using palmitic acid (a fatty acid

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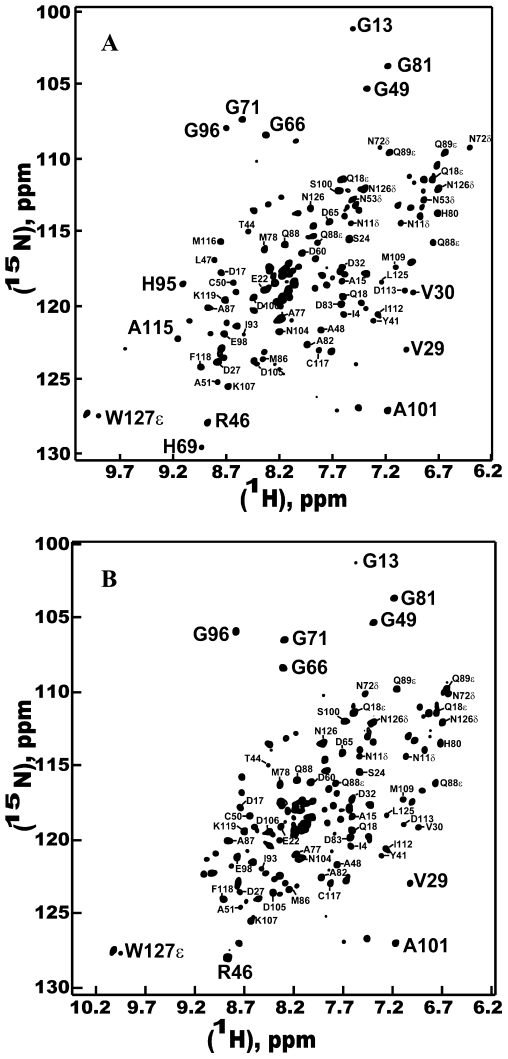

Figure 4. Two-dimensional {^{1}H−^{15}N} HSQC spectra of delipidated 0.3 mM C-terminus deleted mutants of ApolPBP1 and ApolPBP1H70A/H95A in 50 mM sodium phosphate buffer (pH 4.5) containing 5% D_2O, 1 mM EDTA, and 0.01% sodium azide: (A) ApolPBP1ΔP129−V142 and (B) ApolPBP1H70A/H95AΔP129−V142.

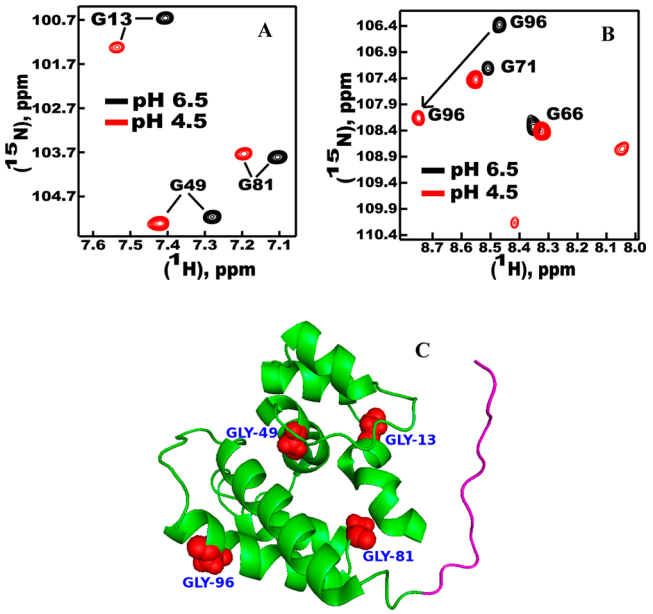

Figure 5. (A and B) Expanded regions of the two-dimensional {^{1}H−^{15}N} HSQC spectra of delipidated ApolPBP1ΔP129−V142 (0.3 mM) in 50 mM sodium phosphate buffer containing 5% D_2O, 1 mM EDTA, and 0.01% sodium azide, showing movements of certain peaks as a result of pH. The pH of the buffer is indicated on the spectra. (C) Residues that undergo a moderate to large change in their chemical shift positions as a result of a change in pH are shown as red spheres in the three-dimensional structure of ApolPBP1 (Protein Data Bank entry 1QWV). The C-terminal tail (residues 131−142) is colored magenta. The three-dimensional structure representation was prepared using the PyMOL molecular graphics system.

that binds to ApolPBP1). Protein:palmitic acid ratios were varied from 1:0 to 1:10. Ligand titration studies revealed that the C-terminus truncated proteins did not undergo a conformational change upon the addition of ligand. These delipidated proteins remained in the open conformation before and after the addition of excess ligand. However, some of the resonances (for example, Gly^{13}, Leu^{68}, etc.) exhibited line broadening and/or disappearance, indicating that they were in the intermediate exchange regime on the NMR time scale (Figure 6A−C). Several other resonances were seen to be in the fast exchange regime on the NMR time scale, characterized by gradual changes in their chemical shift positions. These observations were in stark contrast to those observed earlier in the ligand titration assay of delipidated ApolPBP1wt and ApolPBP1H70A/H95A, in which almost all resonances were seen to be in slow exchange regime characterized by two sets of peaks for ligand-free (closed) and ligand-bound (open) conformations.[4] Thus, while delipidated ApolPBP1wt and ApolPBP1H70A/H95A exhibited very strong (nanomolar) affinities for ligands, their C-terminus deleted counterparts had much reduced affinities in the micromolar to millimolar range.

AMA Binding Studies by Fluorescence. The hydrophobic fluorescent dye AMA fluoresces weakly in the aqueous environment after excitation at 256 or 298 nm with a λ_{max} of 563 nm. In hydrophobic environments such as the binding cavity of ApolPBP1, the fluorescence of AMA is enhanced considerably with a λ_{max} of ∼480 nm. We have already used AMA titration studies to compare the binding affinities of various proteins under different conditions.[4] In this work, we conducted titrations of delipidated ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 with AMA at pH 6.5 and 4.5. Our studies revealed that the affinity of the C-terminus truncated proteins toward AMA at pH 6.5 was greatly reduced in comparison to that of ApolPBP1wt and ApolPBP1H70A/H95A. Similarly, C-terminus truncated proteins could bind AMA at low pH unlike ApolPBP1wt, although the affinities were greatly reduced compared to that of the ApolPBP1H70A/H95A mutant (Figure 7). The maximal fluorescence intensity observed for all C-terminus deleted proteins was approximately 23−28% of what was observed for the wild-type protein, under the identical experimental conditions (Figure 7). These results are consistent with the ligand titration studies monitored by NMR.

DISCUSSION

In this work, we are reporting for the first time the importance of the C-terminus in the ligand binding and releasing mechanisms of ApolPBP1. Truncation of the C-terminus of ApolPBP1wt and ApolPBP1H70A/H95A resulted in proteins

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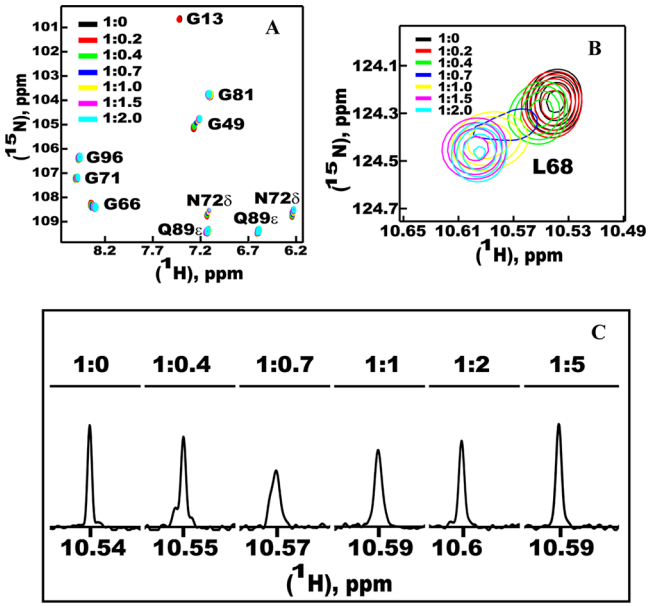

Figure 6. (A) Expanded region of the two-dimensional {^{1}H−^{15}N} HSQC spectra of delipidated ApolPBP1ΔP129−V142 (220 μM) in 50 mM phosphate buffer (pH 6.5) upon titration with palmitic acid. Protein:ligand ratios are given. Note the disappearance of the resonance belonging to Gly^{13} due to line broadening. Some other resonances are in the intermediate to fast exchange regime. (B) Resonance corresponding to Leu^{68} showing the phenomenon of intermediate exchange, from the same spectrum as in panel A. (C) One-dimensional slices from the ^{1}H axis taken in the midpoint of the resonance corresponding to Leu^{68} (same as in panel B) showing the intermediate exchange phenomenon. Protein:ligand ratios are indicated atop each slice. All slices are scaled relative to the same *y* axis.

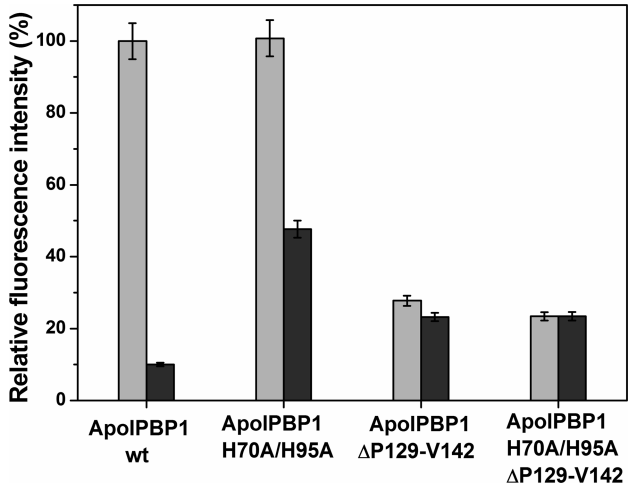

Figure 7. Relative fluorescence intensities of delipidated ApolPBP1 mutants upon addition of 5 μM AMA to 1 μM protein samples: gray for pH 6.5 and black for pH 4.5. Error bars indicate the standard deviation of the data. ApolPBP1wt and ApolPBP1H70A/H95A show similar binding at pH 6.5. ApolPBP1wt has negligible binding at pH 4.5, while ApolPBP1H70A/H95A shows considerable binding at pH 4.5. Both ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 show a reduced level of binding (23−28% of that of the wild-type protein at pH 6.5) at both pH 6.5 and 4.5.

that exhibited the open (bound) conformation regardless of the pH and the presence or absence of ligand (Figures 1A,B, 2A,B, and 3A,B). Although the change in pH did not cause any drastic conformational change in these proteins from the open to closed conformation as was observed for undelipidated (ligand-bound) ApolPBP1wt,[4] it did affect chemical shifts of several resonances, indicating that local changes occurred in the protein because of the effect of pH (Figure 5A−C). Delipidation also did not produce any major conformational change in these proteins (Figure 3A,B) in a striking contrast to what was seen earlier for ApolPBP1wt and ApolPBP1H70A/H95A at pH 6.5.[4] Thus, the C-terminus truncated ApolPBP1 stays in the open form at all pH values regardless of the presence or absence of a ligand as the unoccupied binding pocket remains open in the absence of the C-terminus (Figure 8).

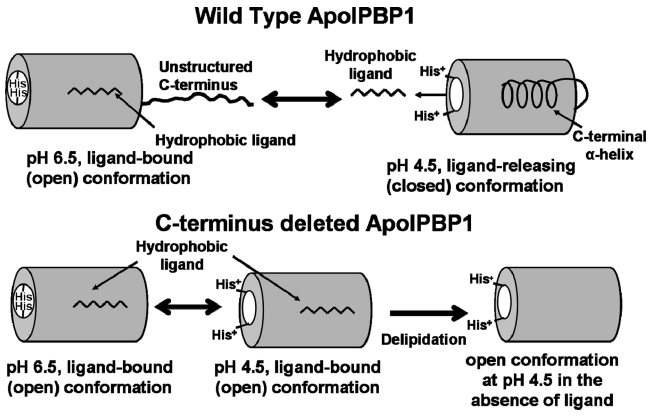

Figure 8. Schematic representation of the role of the C-terminus in the ligand binding and release mechanism in ApolPBP1. The ligand-bound wild-type protein (in the open conformation) at physiological pH undergoes a pH-dependent conformational change to release ligand at acidic pH (closed conformation). The C-terminus truncated protein, however, remains in the open conformation at both physiological and acidic pH, in the presence or absence of ligand.

Several resonances that had disappeared in the spectra of undelipidated proteins could be easily located in the spectra of delipidated proteins. These resonances again underwent line broadening and/or disappearance during ligand titration (Figure 6A). Most of the resonances in all C-terminus truncated proteins exhibited intermediate to fast exchange phenomena on the NMR time scale during ligand titration (Figure 6A−C), implying micromolar to millimolar affinities for the ligand. Thus, while ApolPBP1wt and ApolPBP1H70A/H95A had nanomolar affinities for ligands (characterized by the slow exchange seen for almost all resonances in HSQC during ligand titration), their C-terminus truncated counterparts had much lower affinities. In fluorescence spectroscopic studies, ApolPBP1ΔP129−V142 and ApolPBP1H70A/H95AΔP129−V142 showed the ability to bind AMA even at low pH, unlike the wild-type protein (Figure 7). However, their binding affinities at both pH 6.5 and 4.5 were greatly reduced (by ∼4-fold) compared to those of ApolPBP1wt and ApolPBP1H70A/H95A (Figure 7). Our results are in contrast to those reported for BmorPBP and AtraPBP using the cold binding assay.[22,23] For BmorPBP, the C-terminus deleted mutant retained its binding ability at pH 7 that was nearly the same as that of the wild type and displayed significant binding at pH 5.[22] In the case of AtraPBP, the C-terminus truncated mutant showed a 1.5-fold increase in the level of binding at pH 7 and retained the binding at pH 5, albeit a lower level than at pH 7.[23] However, C-terminus truncated LdispPBP2 showed a higher K_d (and,

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hence, lower affinity) than the wild-type protein in a GC assay,[24] which is consistent with our observations.

The affinity of AMA for ApolPBP1wt and various mutants was compared under identical experimental conditions. While ApolPBP1H70A/H95A retained 100% of the affinity for AMA at pH 6.5 and nearly 50% affinity at pH 4.5 (Figure 7), the C-terminus truncated ApolPBP1 and C-terminus truncated ApolPBP1H70A/H95A lost 75% of their affinity for AMA at both pH 6.5 and 4.5 (Figure 7). Thus, on the basis of the AMA binding assay data, we can conclude that the C-terminus truncated proteins have much less affinity for ligands at any pH values in comparison with that of ApolPBP1wt and ApolPBP1H70A/H95A under identical experimental conditions.

The reduced binding affinities of the delipidated C-terminus truncated mutants of ApolPBP1 can be attributed to inefficient delipidation because of the absence of the C-terminus that helps in ejecting the ligand at low pH. It is possible that the delipidation is not as effective in C-terminus truncated proteins as in ApolPBP1wt and ApolPBP1H70A/H95A. As a result, the endogenous ligand could compete with the added ligand, resulting in a relatively lower binding affinity compared to that of ApolPBP1wt and ApolPBP1H70A/H95A. However, on the basis of the following observations, we conclude that this is not the case: (i) Even undelipidated proteins exhibited much reduced ligand binding affinity at any pH compared to their counterparts with intact C-termini. (ii) We see the disappearance of certain resonances in the undelipidated samples in the HSQC spectrum. (iii) Addition of palmitic acid to the delipidated proteins caused the same resonances to broaden and disappear, resulting in the spectra that matched that of undelipidated proteins. This indicates that the delipidation of these proteins was effective and complete.

Thus, it is important to note that even when the C-terminus is missing, ligand removal is possible at low pH via the delipidation procedure. This is quite surprising especially for ApolPBP1H70A/H95AΔP129−V142 where both molecular switches forming the ligand release mechanism (histidines as well as the C-terminus) are removed. Therefore, even though these two switches are very important for ligand release, there might be some other residues playing a minor role in this mechanism. It is also possible that the delipidation is effective because the protein loses its overall binding affinity at acidic pH or the affinity of the Lipidex resin for the hydrophobic ligand is greater than that of the protein at low pH. This phenomenon was observed even in the case of ApolPBP1H70A/H95A, where the affinity of the protein at pH 4.5 was lower than that at pH 6.5.[4]

The histidines are important only in the ligand releasing mechanism. As reported in our earlier work, their substitution with alanines confers the protein the ability to bind ligand even at low pH.[4] This substitution does not affect the ligand binding affinity of the protein at high pH either because the delipidated forms of both ApolPBP1wt and ApolPBP1H70A/H95A have similar binding affinities for AMA and acetate pheromone at pH 6.5.[4] Unlike ApolPBP1wt and ApolPBP1H70A/H95A, the C-terminus truncated mutants behave very differently. Although C-terminus truncated proteins can bind the ligand at low pH, their affinities at low pH are much lower than that of ApolPBP1H70A/H95A. Additionally, the affinities of the C-terminus truncated proteins for a ligand at high pH are also drastically reduced compared to those of wild-type and double-mutant ApolPBP1. Indeed, one would expect a considerably tight protein−ligand association in the case of ApolPBP1H70A/H95AΔP129−V142 even at low pH, where both molecular switches have been removed, as compared to that of ApolPBP1wt or ApolPBP1H70A/H95A. Our results, on the other hand, indicate reduced ligand binding affinities for both C-terminus deleted proteins under all of the conditions tested. Thus, it is evident that the C-terminus plays a major role in the ligand release mechanism at low pH as well as in ligand binding in the case of ApolPBP1. It is also possible that in addition to helping with the binding of the ligand in the binding pocket, the C-terminus also acts as a gate to "lock" the ligand in the binding pocket,[24] until the ligand is released by the protonation of histidines at low pH. Hence, removal of the C-terminus might be hampering effective association resulting in faster dissociation of the ligand from the protein, reducing the overall affinity.

CONCLUSION

We are reporting here for the first time the effects of ligand and pH on the C-terminus truncated and C-terminus truncated with histidine gate mutated PBPs. Both NMR and fluorescence data suggest that unlike the highly homologous C-terminus of BmorPBP, the C-terminus of ApolPBP1 is involved in both ligand binding and release. It is clear from this work that even though PBPs from moths belonging to the same insect order have a very high level of sequence identity, the C-terminus deleted proteins behave differently under identical conditions. Thus, the mechanistic details of the role of ligand binding or release of moth PBPs can not be generalized across an entire insect order; rather, there are subtle species-specific differences.

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Author Contributions

S. Mohanty conceived and designed the strategies and techniques employed, supervised the research, and analyzed the data. U.V.K. designed the primers. S. Mazumder cloned the genes. U.V.K. and S. Mazumder performed protein expression, purification, NMR sample preparation, and delipidation. S. Mazumder collected 2D HSQC data. U.V.K. performed NMR data processing and analysis. U.V.K. and S. Mazumder performed fluorescence experiments and analysis. S. Mohanty and U.V.K. wrote the paper. U.V.K. prepared all figures.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

PBP, pheromone-binding protein; ApolPBP1, *A. polyphemus* PBP1; BmorPBP, *B. mori* PBP; LdisPBP2, *L. dispar* PBP2; HSQC, heteronuclear single-quantum coherence; AMA, 1-aminoanthracene; undelipidated ApolPBP1, ApolPBP1 bound to the hydrophobic endogenous ligand from the host

expression system; delipidated ApolPBP1, ApolPBP1 after the removal of the hydrophobic ligand by delipidation.

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