New Insights into the Mechanism of Odorant Detection by the Malaria-transmitting Mosquito Anopheles gambiae

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Anopheles gambiae mosquitoes that transmit Plasmodium falciparum malaria use a series of olfactory cues present in human sweat to locate their hosts for a blood meal. Recognition of these odor cues occurs through the interplay of odorant receptors and odorant-binding proteins (OBPs) that bind to odorant molecules and transport and present them to the receptors. Recent studies have implicated potential heterodimeric interactions between two OBPs, OBP1 and OBP4, as important for perception of indole by the mosquito (Biessmann, H., Andronopoulos, E., Biessmann, M. R., Douris, V., Dimitratos, S. D., Eliopoulos, M., Guerin, P. M., Iatrou, K., Justice, R. W., Kröber, T., Marinotti, O., Tsitoura, P., Woods, D. F., and Walter, M. F. (2010) PLoS ONE 5, e9471; Qiao, H., He, X., Schymura, D., Ban, L., Field, L., Dani, F., Michelucci, E., Caputo, B., della Ade, A., Iatrou, K., Zhou, J. J., Krieger, J., and Pelosi, P. (2011) Cell. Mol. Life Sci. 68, 1799–1813). Here we present the 2.0 Å crystal structure of the OBP4-indole complex, which adopts a classical odorant-binding protein fold, with indole bound at one end of a central hydrophobic cavity. Solution-based NMR studies reveal that OBP4 exists in a molten globule state and binding of indole induces a dramatic conformational shift to a well ordered structure, and this leads to the formation of the binding site for OBP1. Analysis of the OBP4-OBP1 interaction reveals a network of contacts between residues in the OBP1 binding site and the core of the protein and suggests how the interaction of the two proteins can alter the binding affinity for ligands. These studies provide evidence that conformational ordering plays a key role in regulating heteromeric interactions between OBPs.

Anopheles gambiae mosquitoes are the primary vectors for malaria caused by Plasmodium falciparum and have an extremely high preference for feeding on human hosts (1, 2) and are attracted to odor molecules from incubated human sweat and other skin emanations (3–6). Disrupting the normal olfactory responses to these odors presents an attractive tool to combat transmission of malaria and other mosquito borne diseases, and a number of efforts are now under way to discover novel reagents for this purpose.

In insects the detection of odorants occurs primarily in the olfactory sensilla and involves the interplay of membrane-bound olfactory receptors (7) and odorant-binding proteins (OBPs), which are expressed into the lymph fluid that surrounds the olfactory dendrites (8) where they can reach concentrations in the millimolar range (9, 10). OBPs have multiple roles including protecting odors from degradation and transporting them to the olfactory receptors (11, 12). There is evidence that OBPs have two primary roles in odorant perception. In the first model a number of groups have proposed that OBPs act as passive carriers for the odorant, and pH changes in the vicinity of the dendritic membrane lead to conformational changes that stimulate ligand release, freeing the ligand to activate the receptor (13–17). In support of this, Carlson and co-workers (18, 19) have shown that in Drosophila, many odorant receptors, when expressed in “empty” neurons, exhibit the same response profile to odorants as the wild-type receptors, providing evidence that the odorant itself directly activates the receptor. In contrast, we and others have proposed that in some cases a specific complex between the OBP and odorant is required for odorant receptor activation (20–25). In support of this, we demonstrated that a key amino acid substitution in a critical loop of the OBP could activate pheromone receptors in the complete absence of pheromone (26, 27), providing direct evidence that the OBP is the ligand for the receptor complex. These differences in function most likely reflect the level of control, with responses to general odorants being less tightly regulated than other sexual or species-specific responses.

In An. gambiae there are 55 OBPs (28), and the expression profiles of a number of these correlate with changes in the host-seeking behavior of the mosquito (29, 30), suggesting that they may be involved in regulating olfactory responses to human specific odors. Recently it was demonstrated that the An. gambiae OBP1 is required for in vivo responses to indole and 3-methyl indole (31), both of which are major components of incubated human sweat that is attractive to female mosquitoes (3–6). Additional studies have proposed that this response requires the formation of heterodimeric complexes between OBP1 and OBP4 (32, 33). This was based on observations that...
Indole Is Required for Interactions between OBP1 and OBP4

(a) OBP4 has a higher affinity for indole than OBP1, (b) OBP1 and OBP4 co-localize within sensilla, and (c) OBP4 can cooperatively increase the binding of a fluorescent dye (1-NPN) to OBP1 or OBP3 (33). OBP4, OBP1, and OBP3 are the orthologues of the D. melanogaster OBPs LUSH, OS-E, and OS-F respectively, which are known to co-localize within the same sensilla (34).

To understand the molecular events that occur in response to indole, we solved the crystal structure of An. gambiae OBP4 in complex with indole. Here we show that binding of indole to OBP4 induces a dramatic conformational change from a molten globule state to a well ordered conformation, and this is required to allow formation of a binding site for OBP1. Subsequently, we used NMR spectroscopy to show that the interaction of OBP1 and OBP4 occurs only in the presence of indole and to map the location of the binding site for OBP1 on OBP4. These results suggest that intrinsic disorder may be an important mechanism to regulate heterodimeric interactions between OBPs.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The sequences of OBP1 and -4 without the signal peptide, as predicted by SignalP (35), were PCR-amplified from an An. gambiae antennal cDNA library and subcloned into the Ndel/BamHI site of a pET13a vector (36). OBP1 primers used were (Ndel) 5’-ATAATATCATATGTATCCACGCCGAGTTGCTG-3’ and (BamHI) 5’-TTTTATGGATCCTTATCACACTAAGAAATAGTGCTTCGGATCCGGCTG-3’. OBP4 primers were (Ndel) 5’-CTAGCATATGACCATGAAACAGCTAACC-3’ and (BamHI) 5’-CTATGCATGACCATGAAACAGCTAACC-3’. OBP4 primers were (Ndel) 5’-CTAGCATATGACCATGAAACAGCTAACC-3’. In both cases the restriction sites are underlined. The final proteins contain 127 and 124 amino acids (OBP1 and -4, respectively). The resulting plasmids were transformed into Rosetta™BL21 cells (Novagen), and bacterial cultures were induced at A600 ~0.8 with 1 mM isopropyl-β-D-galactopyranoside, and both proteins were purified from inclusion bodies and refolded using a cysteine/cystine redox protocol in the presence of 1% n-butanol as previously described (22, 36, 37) and verified by mass spectroscopy. For NMR experiments, proteins were isotopically labeled by expression in minimal media containing 15N-labeled ammonium chloride (99 atom %) and/or 13C-labeled glucose (98 atom %).

**Biophysical Analysis**—Circular dichroism (CD) was performed on a Jasco-815 spectropolarimeter in the University of Colorado School of Medicine Biophysics Core using 5 μM protein samples at 25 °C using a 2-mm path length cell. Analytical ultracentrifugation was performed in the University of Colorado School of Medicine Biophysics Core using a Beckman Coulter XL-I analytical ultracentrifugation instrument with a titanium 4 place rotor (Beckman Coulter). Cells were fitted with 12-mm path length dual sector centerpieces and quartz windows. The reference sector of each cell was filled with 425 μl of sodium phosphate buffer, pH 7.3, whereas the sample sector contained 400 μl of OBP4 or OBP1 at 2 mg/ml in sodium phosphate at pH 7.3. All experiments were carried out at 25 °C using a rotor speed of 45,000 rpm. Absorbance was measured at a wavelength of 280 nm at intervals of 0.007 cm along the rotor radius. 300 scans were collected for each sample, and sedimentation velocity size-distribution analysis was carried out using SEDFIT (38).

**NMR Spectroscopy**—Samples for NMR were made in 90% H2O, 10% D2O containing 20 mM sodium phosphate at various pH values. NMR spectra were recorded on Varian 500, 600, or 900 MHz spectrometers at the University of Colorado School of Medicine. Backbone chemical shift assignments for OBP4 and OBP1 were made semi-automatically using the PINE server (39) followed by manual inspection from standard HNCA, CBC(CO)NH, HNCO, (HACA)CO(CA)NH, and heteronuclear single quantum coherence (HSQC)-NOESY-HSQC triple resonance experiments using the pulse sequences supplied by Varian. For assignments of OBP1, additional HNCA, CBC(CO)NH experiments were acquired at 15, 25, and 35 °C. NMR data were processed using NMRPipe (40) and visualized using CCPNMR (41). Normalized chemical shift differences were calculated as \( \Delta \delta = (\Delta \delta H^2 + 0.25 \Delta \delta N^2)^{1/2} \). All chemical shifts are reported relative to internal 40 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) with no correction for temperature.

**Crystallization, X-ray Data Collection, and Refinement**—OBP4 crystals were grown in 100 mM MOPS, pH 6.8, 25.5% PEG 4000 at 18 °C, and the protein solution and well solution were saturated with indole before crystallization. X-ray diffraction data were collected at the Molecular Biology Consortium Beamline 4.2.2 (wavelength = 1.0 Å) at the Advanced Light Source at Lawrence Berkeley National Laboratory, Berkeley, CA, processed using D*Trek (42), and analyzed using Xtriage within Phenix (43). The crystal structure of OBP4 was solved by molecular replacement starting from the structure of LUSH (PDB code 1O0H) using Phaser (44) within the CCP4 suite (45) and refined using Refmac5 (46) followed by manual rebuilding in COOT (47).

**RESULTS**

**Crystal Structure of OBP4-Indole Complex**—The crystal structure of the OBP4-indole complex (PDB code 3Q8I) was solved to a 2.0 Å resolution (Fig. 1, A, B, D, and E, and Table 1) with final values of \( R_{work} \) and \( R_{free} \) of 20.0 and 24.0%, respectively. The structure of OBP4 is very similar to that of other medium length OBPs and consists of six main α helices stabilized by three disulfide bonds (Fig. 1B). Helix 5 breaks at Cys-92 and is followed by a short 3₁₀ helix (labeled α5b). OBP4 has the highest sequence similarity to LUSH (41% identity) (28), and the present structure is most similar to the LUSH-cVA complex (PDB code 2GTE), with a backbone RMSD of 1.36 Å (Fig. 1, D and E). There is a single protein molecule in the asymmetric unit. However, a crystallographic 2-fold axis generates a dimer interface formed by residues from helices 1, 3, and 4 that buries ~2800 Å² of total surface area with an estimated \( \Delta G_{diss} \) of ~9.3 kcal mol⁻¹ (48). Although sedimentation velocity analytical ultracentrifugation experiments show that under the conditions used for NMR, OBP4 exists as a monomer (supplemental Fig. S1, B and C).

There are a number of differences between OBP4 and LUSH. First, the conserved hairpin loop between α3 and α4 is rotated out from the body of the protein compared with LUSH (Fig.
Indole Is Required for Interactions between OBP1 and OBP4

Indole Binds at One End of the Central Pocket—In common with other OBPs, OBP4 has a large central cavity (−690 Å³) lined with mainly hydrophobic residues. In the LUSH cVA and BmorPBP1-bombykol complexes, the ligand completely fills this cavity and is almost entirely enclosed by the protein. In contrast, the cavity in OBP4 resembles that seen in OBP1 with two openings to the protein surface (supplemental Fig. S2). The main opening (−4.3 Å wide) is formed by the convergence of helices 1, 3, and 4 and is lined by residues Ser-9, Met-13, Ala-55, Gln-72, and Met-76. The second opening (4 Å wide) is formed by residues from the hairpin loop between helices 3 and 4 (Ile-64 and Phe-66) and residues in the 310 helix α5b (Lys-93 and Gln-96). In OBP1 this opening is located more toward the center of helices 4 and 5.

At one end of the central binding pocket there is a region of flattened density that refined well as indole (Fig. 1, A and C) (other components of the crystallization solution that we tested did not fit into this density). The lowest free R-factors are obtained by using an occupancy of 0.6 for the indole, which gives average B-factors of −23 Å². It is not possible to distinguish the location of the ring nitrogen atom from the carbon atoms in the ring. We have modeled the indole with the NH group oriented so that it points toward the hydroxyl of Thr-69 (dN-H 3.6Å), which allows the indole to fulfill its hydrogen bonding potential. However, it is entirely possible that the indole is flipped 180° around the long axis of the ring such that the positions of the N1 and C3 atoms within the pyrrole ring are swapped so that the NH group points into the central cavity. This would not affect other interactions between the indole and the protein. These include cation-π interactions with Thr-57 (2.9 Å) and van der Waals interactions with Ala-52 (3.6 Å), Ala-106 (3.8 Å), Ile-64 (3.7 Å), and Phe-123 (3.3 Å) (Fig. 1C).

There are two other regions of weak density within the pocket that have a flatter and more elongated aspect ratio than water and could represent additional molecules of indole or PEG from the crystallization precipitant. In previous structures of OBP1 from An. gambiae, Aedes aegypti, and Culex quinquefasciatus, a continuous hydrophobic tunnel is observed that runs between the two protein molecules in the asymmetric unit.

1D). This may be due to Met-58, which packs between the loop and α6, whereas in LUSH this residue is a less bulky valine. In addition, crystal-packing contacts occur with the top of α6 and α3 and may distort the structure of this region. Second, the loop between α4 and α5 that lines the main opening to the ligand binding pocket is rotated down compared with the same region in LUSH (Fig. 1D) and makes the opening to the pocket wider (−4.3 Å compared with 3.9 Å in LUSH). The third difference is in the loop between α6 and the C-terminal tail (Fig. 1E). In LUSH substitution of Asp-118 with alanine in this loop generated a constitutively active protein (26). In OBP4 (and OBP1) the homologous residue is a proline, and this loop adopts the same conformation seen in OBP1. The remainder of the C-terminal tail packs into the core of the protein, as seen in OBP1 and LUSH, and forms one edge of the ligand binding pocket. The position of the C-terminal carboxyl group in the core is stabilized by electrostatic interactions with Arg-32 and Arg-14, which are bridged on the outside of the protein by Asp-11, forming a tetrad of charged residues that neutralize each other.

A hydrogen bond between Tyr-47 and the C-terminal carboxyl group additionally helps to stabilize the C-terminal tail in the center of the protein.

TABLE 1

X-ray data collection and refinement statistics

Data are from a single crystal.

|             | P2₁ 2₁ 2₁ |
|-------------|-----------|
| Space group | P2₁ 2₁ 2₁ |
| Cell dimensions |
| a, b, c (Å) | 55.24, 66.36, 33.67 |
| α, β, γ (°) | 90.0, 90.0, 90.0 |
| Resolution (Å) | 14.88-2.00 (2.051-2.00)† |
| Completeess (%) | 100 (100) |
| Redundancy | 7.03 (7.17) |
| Rmerge | 15.8 (6.6) |
| Rwork (%) | 20.0/24.0 (26.1/36.8) |
| Ramachandran (plot) % (favorable, additional, generous, allowed) | 93.7,6.3,0,0 |

| Root mean square deviations |
| Bond lengths (Å) | 0.0144 |
| Bond angles (°) | 1.4651 |

† Values in parentheses are for highest resolution shell.
In AgOBP1 and AegOBP1 this is bridged by a single molecule of PEG. In OBP4 no such tunnel exists as the main entrance to the binding pocket is blocked by helix-1 from a symmetry-related molecule, whereas in the second opening the electron density clearly defines a series of water molecules rather than PEG (Fig. 1A). Therefore, it appears more likely that this weak density originates from additional poorly ordered indole molecules. However, because of its uncertainty and low intensity, we have not modeled anything in this density.

**Indole Stabilizes the Structure of OBP4**—Studies of LUSH revealed that it exists as a molten globule in the absence of ligand (22, 53). Therefore, we postulated that a similar situation might exist with OBP4. CD spectra show the α-helical secondary structure of OBP4 is well formed in the absence of ligand even at low pH values (supplemental Fig. S1A). To probe tertiary structure formation, we recorded two-dimensional $^1$H,$^{15}$N HSQC NMR spectra. In the spectrum of apoOBP4 we observe only 35 intense, well resolved peaks from backbone amides of an expected 114 (Fig. 2A). The remaining peaks are broadened or not detectable, indicative of significant conformational averaging occurring on the micro- to millisecond timescales. The addition of saturating amounts of indole produces a dramatic improvement in the appearance of the spectrum, with 107 of the expected 114 peaks now observable (Fig. 2B). Many of the intense peaks (25 of 35) observed in the spectrum of the apoprotein show little or no chemical shift change on binding indole, indicating that they originate from residues that are in well ordered regions of the apoprotein. We then determined the backbone chemical shift assignments for the OBP4-indole complex (106 of 107 peaks assigned) and mapped the location of these ordered residues onto the crystal structure. These residues cluster onto one face formed by the loop between α1 and α2, the N terminus of α3, and the N terminus of α6 (Fig. 3A). This region contains two of the three disulfide bridges and so is one of the most stable regions of the protein. In contrast, the majority of the other residues in the apoprotein exhibit significant conformational averaging (gray in Fig. 3A).

NMR studies of the *Bombyx mori* PBP showed that the protein exists in slow conformational exchange between two states at pH values between 4.9 and 6.0 (54). To ensure that the conformational exchange we observe in apoOBP4 is not due to the effects of pH, we recorded HSQC spectra of the protein from pH 4.6 to 7.4. At all pH values tested, the apoprotein exhibits significant conformational averaging (supplemental Fig. S2D).
Over the same pH range, CD spectroscopy indicates that the \( \alpha \)-helical content of the apoprotein does not change significantly (supplemental Fig. S1A). When we tested the effect of adding indole at different pH values we found that indole induced a conformational shift to a more ordered structure even at pH 4.6 (supplemental Fig. S2, A and C). There are differences in the NMR spectrum of the indole-bound protein at pH 4.6 compared with higher pH values, indicative of some conformational differences, but in general it does not appear that lowering the pH has prevented binding of indole, which is in contrast to other OBPs/PBPs.

**OBP1 Is Folded in the Absence of Ligand**—We then tested if indole has an effect on the structure of OBP1. The \(^{1}H, {^{15}}N\) HSQC spectrum of apoOBP1 recorded at pH 7.4 contains 100 of an expected 116 peaks, indicating that the protein is relatively well ordered (supplemental Fig. S3A). The addition of indole induces a number of chemical shift changes (supplemental Fig. S3B) but does not result in the dramatic changes seen with OBP4, with only an additional 3 peaks observed (103 of 116). Interestingly, analytical ultracentrifugation experiments recorded for apoOBP1 reveal that the protein exists predominantly as a dimer under the conditions used for the NMR experiments (supplemental Fig. S1, B and C). However, OBP1 was purified as a monomer at lower concentrations as judged by size exclusion chromatography. This observation is in partial agreement with previous reports that OBP1 is predominantly monomeric but slowly interconverts to dimers (49). We could not analyze the effects of indole on OBP1 dimerization using analytical ultracentrifugation because of the strong UV absorbance of the indole.

**Interactions between OBP1 and -4 Require Indole**—We next tested if OBP4 and OBP1 interact in the absence of indole by recording NMR spectra of \(^{15}\)N-labeled OBP4 with increasing amounts of unlabeled OBP1. These spectra are essentially unchanged compared with the spectrum of apoOBP4 (not shown). A caveat is that the extensive exchange broadening may obscure the effect of any interaction between the two proteins. Therefore, we recorded spectra using \(^{15}\)N-labeled OBP1 while adding unlabeled OBP4. Here again we saw no significant chemical shift changes in the absence of indole (Fig. 4, A and C). However, when spectra were recorded in the presence of indole, the addition of OBP4 led to specific chemical shift changes in a number of residues of OBP1 (Fig. 4, B and D).

To verify that the interaction between OBP1 and OBP4 required indole, we prepared fresh samples of \(^{15}\)N-labeled OBP1 in the presence of unlabeled OBP4 but without indole. As before, we saw no difference in the chemical shifts of OBP1 (supplemental Fig. S4A). Upon the addition of indole to this sample, we saw chemical shift changes in OBP1 (supplemental Fig. S4B), but these chemical shift changes are different from those induced by the addition of indole to OBP1 alone (supplemental Fig. S4C). The end point of each of these experiments is essentially identical and shows that OBP1 and OBP4 interact only in the presence of indole. There is no evidence for formation of a significant population of an OBP1-OBP4 complex before indole binds. Therefore, we conclude that at pH 7.4 interactions between OBP1 and -4 require the presence of indole.

**Binding of Indole to OBP4 Leads to Formation of a Binding Site for OBP1**—We hypothesized that the conformational changes in OBP4 induced by the binding of indole must be required to allow binding of OBP1. Therefore, we measured chemical shift changes in the OBP4-indole complex upon the addition of OBP1 (Fig. 5) to identify the location of the binding site for OBP1. The addition of OBP1 induces chemical shift changes in OBP4 that are different from those induced by the addition of indole alone (Figs. 3 and 5). Those residues most affected by binding of OBP1 cluster predominantly on the front face of the protein formed by helices 1, 3, and 4 (Figs. 3, B and C, and 5C and supplemental Movie 1) and so are most likely to be those residues that form the binding site for OBP1. As anticipated, we find there is almost no overlap between those residues that are well ordered in apoOBP4 and those residues that interact with OBP1 in the presence of indole. This indicates that conformational changes in OBP4 are required to allow binding of OBP1.

Some of the largest chemical shift changes in OBP4 are seen for residues that make direct contact with the indole, including Ala-52 and Thr-57. Interestingly, changes are observed for several residues that are not directly accessible from the protein surface, including Met-10, Ala-52, Thr-57, Thr-69, Ile-73, Ala-110, Lys-111, Ala-114, and Phe-121, and a number of these (Ala-110, Lys-111, Ala-114, and Phe-121) are located a significant distance away from the proposed OBP1 binding site. These distal changes can be rationalized through a network of allosteric interactions that directly link the proposed OBP1 binding site to the C-terminal tail (Fig. 1C). Binding of OBP1 on the “front” face of the protein (Fig. 3B) induces conformational changes that lead to changes in Ala-52, Thr-57, and Thr69 that contact indole (Fig. 1C). This could result in a shift in the position of the indole, which makes contact with residues on the “back” face of the protein, including Phe-123 (Fig. 1C), which is in close proximity to Phe-121 (4.0 Å). We have not been able to...
obtain assignments for residues 122–124, as these peaks are missing in all NMR spectra that we have recorded to date. Phe-121 in turn contacts Ala-114 (3.7 Å), which is hydrogen-bonded to Ala-110 (Fig. 1C). That interactions with OBP1 are transmitted to residues that directly interact with the ligand may explain previous observations that the presence of OBP1 and OBP4 can lead to an increase in the binding affinity for a fluorescent dye (33). We conclude that binding of indole to OBP4 results in a significant increase in the conformational stability of residues that are required to allow interactions with OBP1.

We next examined the interactions of indole and OBP4 with OBP1. We obtained backbone chemical shift assignments for all but three of the major peaks in the 1H,15N HSQC spectrum of OBP1 recorded in the presence of indole. Residues that could not be assigned are located predominantly in helices 5 and 6 (blue, Fig. 6, B and C). Most of the peaks from these residues are completely absent and are not observed in any spectra collected in the presence of indole or OBP4 and over the temperature range of 10–35 °C. This region of the protein forms the homodimeric interface in the crystal structure (Fig. 6C), although the binding energy for this interface is predicted to be relatively weak in solution. This observation in combination with our analytical ultracentrifugation results (supplemental Fig. S1) leads us to hypothesize that this dimer interface is also likely to be maintained at the concentrations used for NMR spectroscopy and that conformational exchange at the dimer interface may lead to exchange broadening of the peaks from residues in this interface.

Analysis of the chemical shift changes upon the addition of indole to OBP1 revealed that the residues affected by indole are distributed relatively uniformly throughout the protein (supplemental Fig. S5, B and C) and with no one region exhibiting dramatically larger chemical shift changes than another. Such a result is more reflective of a global change in the protein structure, consistent with indole inducing a change in the overall conformational stability of the protein but without producing the dramatic change seen with OBP4.

In contrast, the largest chemical shift changes observed upon the addition of OBP4 to the OBP1-indole complex cluster into two regions. The first is on the face that includes helix 1 (residues 14–23) and the C-terminal end of helix 3 (residues 58–64) (left panel of Fig. 6, A and B, supplemental Fig. 5A, and supplemental Movie 2). The second is in the extended region immediately after helix 5 and includes residues 95–97. These residues are potentially allosterically coupled to the C terminus of helix 3 through an interaction with Leu-110 and Trp-114, both of which are in close proximity to Cys-95 and Phe-59. Other interactions may also exist but cannot be detected at this stage because of missing assignments as discussed above. It is possible that OBP4 interacts with both regions or that it interacts with only one region and allosterically transmits the effects of binding to the other. Future work will aim to discriminate between these possibilities.

It is notable that binding of indole and OBP4 to OBP1 both induce chemical shift changes to a number of the same residues, albeit with different magnitudes. This may indicate...
binding of OBP4 to OBP1 also requires a conformational change in OBP1 that is induced by the binding of indole, in which case residues that are involved in the interactions with OBP4 are most likely to be those that are structurally regulated by binding of indole.

**DISCUSSION**

In *An. gambiae* it was recently demonstrated that perception of indole and 3-methyl-indole requires OBP1 (31), and this may involve the formation of interactions between OBP1 and OBP4 (32, 33). This study reveals that binding of indole to OBP4 results in a dramatic conformational ordering that is required to form a binding site for OBP1. No significant interactions are observed between the two proteins in the absence of indole. The crystal structure of the OBP4-indole complex demonstrates that, in common with other OBPs, the ligand binds in a central hydrophobic pocket. In contrast to OBP4, OBP1 is significantly more ordered in the absence of ligand at pH 7.4, and indole has relatively little effect on the overall structure. However, at present we cannot rule out that conformational changes in OBP1 are also required for the interaction between the two proteins to occur.
Indole Is Required for Interactions between OBP1 and OBP4

Our results contrast with previous work which demonstrated that OBP1 and OBP4 could form heterodimers in vitro in the absence of ligand (32). However, these prior studies used proteins that contained additional residues at the C terminus to aid in purification and detection. In medium length OBPs, including OBP1 and -4, the C-terminal tail folds into the center of the protein where it makes a number of specific contacts that stabilize its position in the core. Therefore, inclusion of additional residues at the C terminus could disrupt the native structure leading to non-native interactions, including with other OBP molecules.

A number of studies have provided evidence that pH-dependent changes regulate the ligand binding affinity of a number of OBPs (13–15, 50, 55), including AgOBP1 (49), and this appears to be highly prevalent for the longer classical type of OBPs typified by BmorPBP1 (13, 14). However, medium length OBPs like AgOBP4, LUSH (22, 26, 37), or honey bee PBP Asp-1 (56) do not undergo any pH-dependent change in the C-terminal tail that affects ligand binding. In this study we saw no change in the structure of apoOBP4 at low pH values, and further indole could still bind and induce the structural ordering of OBP4 even at pH 4.6. However, it is possible that changes in pH may affect protein–protein interactions.

The effect of indole on the structure of OBP4 correlates well with our previous findings for the Drosophila OB P LUSH, where binding of ligand induces a significant conformational ordering that is required to regulate its interactions with other components of the olfactory signaling cascade including sensory neuron membrane protein (26, 53, 57, 58). Conformational heterogeneity has been observed in several other OBPs (14, 17, 59, 60) and is most often associated with residues that regulate access to the ligand binding pocket. However, this heterogeneity is not nearly as extensive as that seen with OBP4 and LUSH. In addition, for OBP4 and LUSH the high degree of intrinsic disorder occurs over a wide range of pH values and exists even at the neutral pH values found in the bulk sensillar lymph. Therefore, we propose that the presence of high levels of intrinsic conformational disorder represents a mechanism to regulate the activity of OBPs and their interactions with other components of the olfactory system.

Intrinsic disorder as a mechanism for regulating protein activity is well established in many areas, including transcriptional regulation, cell signaling, and disease states (61–63). The fact that this has not been observed for more OBPs may be because most structures of invertebrate OBPs have been solved by X-ray crystallography that necessarily selects against the conformational heterogeneity present in solution. Indeed, our previous crystal structure of apoLUSH showed very few differences compared with the ligand-bound state (37). Alternatively, some OBPs appear well ordered in the absence of ligand, such as OBP1, but their interacting partner may require ligand to induce the appropriate conformational changes to allow formation of heterodimeric complexes.

In the present study we found that OBP1 exists predominantly as a dimer in the absence of indole. In contrast, OBP4 exists predominantly as a monomer. Therefore, one possible model is that indole induces a conformational change in OBP4 that leads to formation of a binding site for OBP1 (or other OBPs), whereas indole may cause dissociation of OBP1 dimers to allow binding of OBP4. Alternatively, multiple molecules of OBP4 may interact with OBP1 dimers. A recent study showed that OBP4 could synergistically increase the binding affinity of a fluorescent dye to either OBP1 or OBP3 (33). However, combining OBP1 with OBP3 had no effect on binding (33). This opens up the possibility that OBP4 may act as a central regulator of OBP interactions and the high degree of conformational disorder seen for OBP4 may be required to limit its interactions with other olfactory proteins that could occur in a constitutive manner if OBP4 was well structured. If interactions between multiple OBPs depend on the specific conformational changes induced by binding of a ligand, this would provide an additional level of selectivity in regulating behavioral responses to specific olfactory cues.

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