The Crystal Structure of D7r4, a Salivary Biogenic Amine-binding Protein from the Malaria Mosquito Anopheles gambiae*

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The D7-related (D7r) proteins of the malaria vector Anopheles gambiae have been shown to bind the biogenic amines serotonin, norepinephrine, and histamine with high affinity. One member of the group (D7r1 or hamadarin) has also been shown to have an anticoagulant/antikinin activity. To understand the mechanistic details of its antithrombotic/anti-inflammatory effects, we have determined the crystal structure of one member of this group, D7r4, along with the structures of ligand complexes with serotonin, tryptamine, histamine, and norepinephrine. The D7 fold consists of an arrangement of eight α-helices stabilized by three disulfide bonds. The structure is similar to those of the arthropod odorant-binding proteins, a relationship that had been predicted based on sequence comparisons. Although odorant-binding proteins commonly have six α-helices, D7r4 has eight, resulting in significantly different positioning and structure of the ligand binding pocket. The pocket itself is lined by hydrophobic side chains along with polar and charged groups oriented to form hydrogen bonds with the aliphatic amino group and with groups on the aromatic portions of the ligands. These structures, along with accompanying mutagenesis studies, have allowed us to identify critical residues for biogenic amine binding and to predict which members of the large D7 protein family found in blood-feeding nematicocerous Diptera will function as biogenic amine-binding proteins.

Anopheles gambiae is the major African vector of the malaria parasite Plasmodium falciparum that infects 300–500 million people and causes 1–2 million deaths annually. Malaria transmission occurs during ingestion of a blood meal by the female mosquito when the sporozoite stage of the parasite is passed to the host in the insect saliva. Blood feeding is facilitated by a salivary mixture of biologically active peptides and small molecules that target the processes of blood clotting, vasodilation, inflammation, tissue remodeling, and immunity (1). Inhibition of antithrombotic defenses by these components is essential for the efficient ingestion of blood and, consequently, for egg production and possibly parasite transmission.

Saliva from female A. gambiae contains a plethora of potent antithrombotic proteins, including the thrombin inhibitor anophepin (2), the platelet aggregation inhibitor apyrase (3, 4), and catechol oxidase-peroxidase, which serves as a vasodilator (5, 6). Among the most abundant salivary molecules are the D7-related (D7r) proteins (7–9), a group of five polypeptides (D7r1–D7r5) that interfere with various aspects of host physiology. D7r1 (also known as hamadarin) was initially shown to have an anticoagulant/antikinin activity resulting from strong binding interactions with high molecular weight kininogen and factor XIIa, two components of the contact activation system of coagulation (8, 10). More recently, D7r1–D7r4 were found to bind the biogenic amines serotonin, norepinephrine, and histamine, thereby reducing the concentrations of these effectors at the feeding site (8).

The process of blood clotting, the maintenance of vascular tone, and inflammatory responses are all regulated to some extent by biogenic amines. The widespread distribution of biogenic amine-binding lipocalin proteins in the saliva of tick and triatomine bug species suggests that inhibition of these processes is essential for efficient blood feeding (11–14). Interestingly, D7r5 does not bind biogenic amines but is also poorly expressed, suggesting that it may be nonfunctional and on an evolutionary path toward silencing (8).

Binding studies using recombinant D7r proteins have revealed significantly different ligand selectivities for the different forms, a possible driving force in the maintenance of this cluster of similar genes in the A. gambiae genome (8). All of the proteins, with the exception of D7r5, bind serotonin with high affinity and histamine with somewhat lower affinity. D7r2 and D7r3 bind epinephrine and norepinephrine with much higher affinity than D7r1 and D7r4 suggesting that these two members of the group may be specifically adapted for local catecholamine removal around a feeding site.

Proteins showing sequence similarity to the D7r proteins occur in other blood-feeding Diptera, including mosquito species in the genera Anopheles (15, 16), Aedes (17), and Culex (18), sand flies in the genera Lutzomyia and Phlebotomus (19) and Culicoides sp. (20). Some of these, including “long form” D7, the first described member of the group from the yellow fever mosquito Aedes aegypti, are larger proteins that contain two domains, each being comparable with that of the entire D7r protein (9). Sequence comparisons have suggested a distant...
relationship between the D7/D7r proteins and the arthropod odorant (or pheromone)-binding protein (OBP) family (8).

In this study, we describe the three-dimensional structure of D7r4 along with the structures of a number of ligand complexes. Details of the structures reveal how the D7r proteins act to inhibit hemostatic and inflammatory processes by binding a diverse set of ligands with high affinity. The study also confirms the relationship of the D7r proteins with the OBPs, suggesting a possible origin for the group.

**EXPERIMENTAL PROCEDURES**

**Materials**—Crystallization reagents were obtained from Hampton Research. The selenomethionine media kit (SelenoMet) was obtained from Molecular Dimensions Ltd. Serotonin, tryptamine, and histamine were obtained from Sigma as hydrochloride salts. l-Norepinephrine bitartrate salt was also obtained from Sigma.

**Preparation of Protein and Crystallization**—The D7r4 cDNA, cloned into the expression vector pET 17b, was used to produce recombinant protein in *Escherichia coli* as described previously. Inclusion bodies were prepared 3 h after induction, solubilized, and refolded as described by Calvo et al. (8). The refolded proteins were purified by a combination of gel filtration and cation exchange chromatography. Selenomethionine-containing protein was produced in *E. coli* using SelenoMet medium (Molecular Dimensions Ltd.) according to the manufacturer’s instructions. The protein was refolded and purified in a manner similar to the wild type protein. Crystals were obtained using the hanging drop-vapor diffusion method with 20% polyethylene glycol 6000, 100 mM Tris-HCl, pH 8.0, as a precipitant. Prior to data collection, crystals were flash-frozen after a short soak in 30% PEG 6000, 100 mM Tris-HCl, pH 8.0, containing 10% glycerol. Co-crystals were grown by adding serotonin or tryptamine to the precipitant solution at a concentration of 1 mM. Ligands were soaked into crystals by placing a crystal into a 5-μl drop of cryoprotectant solution containing a 20–100 μM ligand concentration and allowing it to soak for several minutes before flash freezing.

**Data Collection and Structure Determination**—Data were collected at beamlines 19-ID and 19-BM at the Structural Biology Center, APS, Argonne National Laboratory. Diffraction data for the selenomethionine derivative was collected at two wavelengths near the selenium edge and integrated and scaled using HKL 3000 (21). Initial phases were also obtained using the HKL 3000 package which combines SHELXD-E (22, 23), MLPHARE (24), and DM (25) for the location of selenium sites, whereas the remainder of the protein backbone structure and the side chains were built manually using a native D7r4 data set. Numerous cycles of rebuilding and refinement were performed with Coot (27) and REFMAC (28) to obtain the final structure of the unliganded protein. Various manipulations of reflection and coordinate data during the course of structure determination were made using the CCP4 package (29). Model quality was checked using the MOLPROBITY web server (30). The structural figures presented here were produced with PyMOL (DeLano Scientific).

In the histamine and norepinephrine complexes, where the crystals were soaked with ligand-containing cryoprotectant solutions, a change in unit cell dimensions was observed that resulted in two molecules of D7r4 in the asymmetric unit rather than one. The space group of both crystals was *P*4*3* and trans. The two molecules in the asymmetric unit of the large unit cell were positioned by molecular replacement using PHASER (31), and the structures were rebuilt and refined as described above. The crystallographic and phasing data for all structures are given in Table 1.

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### Table 1

Data collection, phasing, and refinement statistics for D7r4 and ligand complexes

| Crystal | SeMet-int (SeMet-hr) | Unliganded | Serotonin | Tryptamine | Histamine | Norepinephrine |
|---------|---------------------|------------|-----------|------------|-----------|----------------|
| Resolution (Å) | 30.0-2.50 | 62.6-2.00 | 50.0-2.00 | 63.1-2.20 | 88.3-2.00 | 87.1-2.20 |
| Space group | *P*4*3* | *P*4*3* | *P*4*3* | *P*4*3* | *P*4*3* | *P*4*3* |
| Unit cell dimensions (Å) | 62.652 (62.733) | 62.651 | 63.358 | 63.101 | 88.392 | 87.807 |
| Completeness (%) | 99.7/97.9 (99.7/98.8) | 98.1/85.8 | 99.6/98.5 | 99.3/92.8 | 99.5/99.7 | 99.8/99.7 |
| Resolution (Å) | 62.652 (62.733) | 62.651 | 63.358 | 63.101 | 88.392 | 87.807 |
| Observed reflections | 56,172 (34,578) | 48,906 | 78,485 | 68,162 | 99,119 | 92,717 |
| Unique reflections | 11,400 (11,430) | 10,657 | 9,797 | 8,194 | 21,619 | 13,953 |
| Data collection, phasing, and refinement statistics for D7r4 and ligand complexes |

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2 The abbreviation used is: OBP, odorant-binding protein.
Site-directed Mutagenesis and Binding Studies—Site-directed mutagenesis was performed using a PCR-based procedure and verified by DNA sequencing. Protein expression and purification were performed using the same methods as for wild type D7r4. Binding studies were performed by isothermal titration calorimetry using a Microcal VP-ITC microcalorimeter at a temperature of 30 °C. Protein and ligand solutions were prepared in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl. The protein solution was added to the microcalorimeter sample cell, and ligand solutions were injected in 5–10-μl aliquots. The measured heats were converted to enthalpies (per injection) and analyzed using the Microcal-Origin software package.

RESULTS AND DISCUSSION

Structure of D7r4—The structure of D7r4 without ligands was determined by multiple anomalous dispersion methods using a selenomethionine derivative of the protein. Additionally, the structures of ligand complexes containing serotonin, tryptamine, norepinephrine, and histamine were determined using molecular replacement or difference Fourier methods. The serotonin and tryptamine complexes were obtained by co-crystallization of protein and ligand, whereas the norepinephrine and histamine complexes were obtained by soaking crystals with ligand-containing solutions. In all cases, interpretable electron density was observed for the ligand (Fig. 2).

The D7r protein fold consists of an arrangement of eight helices stabilized by three disulfide bonds (Fig. 1). The helices surround a small, centrally located ligand binding pocket. A narrow channel bounded by helices B, G, and H leads to the binding pocket and is the most likely ligand entry path. In both the ligand-free and ligand-bound structures, the loops connecting helices A-B and B-C are more mobile than the rest of the structure, exhibiting larger temperature factors and poor electron density for the side chains. This suggests that flexibility in these areas of the protein may play a role in ligand entry.

Despite being only 12–15% identical in amino acid sequence (by alignment with ClustalW), D7r4 is similar in structure to the arthropod OBPs, a group of all-helical proteins that putatively function in shuttling odorant molecules from the surface of hair-like antennal olfactory sensillae, through the sensillar lymph to olfactory receptors at the neural membrane. The OBP group also contains THP12, a protein of unknown function found in the hemolymph of the beetle Tenebrio molitor (32). Arthropod OBPs and THP12 are hexahelical proteins, lacking the C-terminal helices G and H of D7r4 (Fig. 1 and Fig. 2A).

The Bombyx mori pheromone-binding protein (BmPBP) and other olfactory OBPs contain three disulfide bonds connecting helix A with C, helix E with F, and helix C with F. The former two bonds are conserved in D7r4, linking Cys-6 with Cys-38 and Cys-77 with Cys-96 (Fig. 1). A third disulfide bond in D7r4 is unique to the group and links Cys-19 of helix B with Cys-144 of helix H (Fig. 1). THP12 retains the conserved disulfide bonds connecting helix A with C and helix E with F, but it is missing the bond linking helix C with F that is seen in BmPBP (Fig. 1).

As a rule, members of the OBP family are either truncated at the end of helix F or have a nonhelical C-terminal portion. A functionally important exception is BmPBP that forms an additional helix in a pH-dependent conformational change. The ligand-bound protein found at pH 6.5 exhibits an extended conformation at the C terminus, which forms a seventh helix when the pH is lowered to 4.5 (33–35). The C-terminal helix inserts into the pheromone binding pocket and presumably displaces the pheromone ligand (33). The pH dependence of this process is potentially important because the pH at the receptor membrane surface, where ligand release occurs, is apparently
lower than the pH of 6.5 measured for the bulk sensillar lymph (33).

Helix A of the OBPs is longer than that of D7r4 and contributes significantly to the structure of the binding pocket (Fig. 1B). THP12 also has an extended N terminus (Fig. 1), but it forms a disordered coil giving rise to an open binding groove, rather than the closed ligand-binding pocket of the OBPs (32). D7r4 has helices A–F arranged in a similar manner to THP12, but the binding pocket is closed by the additional C-terminal helical segments G and H (Fig. 1A and 2A). These differences significantly change the position and structure of the binding pocket in D7r4 relative to the sensory OBPs. In the latter, helices D–F make up a large part of the pocket structure, whereas in D7r4 they are largely displaced by helices G and H, and, with the exception of Tyr-94, do not contribute to the structure of the pocket.

Details of the Ligand Binding Pocket and Ligand Complex Structures—D7r proteins bind a variety of structurally distinct biogenic amines with high affinity, yet employ only a single binding site. This broad ligand specificity is accomplished via a generally hydrophobic binding pocket with polar or charged side chains placed at various locations where they can form hydrogen bonds with ligand functional groups in different arrangements (Fig. 4). The apparent entry path to the binding pocket is surrounded by the anionic side chains of Asp-111, Glu-114, and Asp-139, which act to stabilize the aliphatic amino group of the bound ligand (Fig. 4). The interior of the pocket is lined by aromatic and hydrophobic residues, including Tyr-24 and Phe-110, which contact the aromatic rings and aliphatic side chain of biogenic amine ligands (Fig. 3). At the closed end of the pocket, opposite the ligand entry channel, Glu-7 and His-35 are positioned to form hydrogen bonds with hydroxyl groups found on the aromatic portions of serotonin and norepinephrine (Figs. 3 and 5). In the serotonin complex, the ligand is oriented with its indole nucleus directed toward the closed end of the pocket and the aliphatic amino group toward the apparent access channel. The 5-hydroxyl group forms hydrogen bonds with the side chains of Glu-7 and His-35 (Fig. 3).
The side chain of Arg-22 extends from the protein backbone parallel to the plane of the indole ring system and crosses over it (Fig. 3). It is bent 90° at C-δ allowing the guanidino moiety to contact the edge of the ring as well as its face. The side chain of Tyr-24 forms a third side of the box-like pocket holding the ligand (Fig. 3). An apparent salt bridge between Arg-22 and Glu-114 positions the latter to form a hydrogen bond with the amino group of serotonin (Fig. 3). Also interacting with the amino group are Asp-111, which forms a hydrogen bond with the amino group of serotonin (Fig. 3). Also interacting with the amino group are Asp-111, which forms a hydrogen bond with the amino group of serotonin (Fig. 3). Also interacting with the amino group are Asp-111, which forms a hydrogen bond with the amino group of serotonin (Fig. 3). Also interacting with the amino group are Asp-111, which forms a hydrogen bond with the amino group of serotonin (Fig. 3).

Not surprisingly, tryptamine and serotonin occupy nearly identical positions in the binding pocket despite the absence of a 5-hydroxyl group in tryptamine (Fig. 4B). All of the hydrogen bonding interactions with the aliphatic amino group seen in the serotonin complex are maintained in the tryptamine complex, as is the long hydrogen bond between the Tyr-94 hydroxyl and the indole nitrogen of the ligand (Fig. 4B). The near equivalence of the serotonin and tryptamine binding modes allowed us to estimate the energetic contributions of hydrogen bonding interactions with the 5-hydroxyl group of serotonin using isothermal titration calorimetry. The 4.7 kcal/mol difference in the binding enthalpy (ΔH) seen between the two ligands is apparently because of the loss of hydrogen bonding interactions with Glu-8 and His-35 in the D7r4-tryptamine complex (Table 2). The more favorable entropy change exhibited in the tryptamine binding reaction is probably due to the more hydrophobic nature of this compound.

The catecholamine ligand norepinephrine has a single six-membered aromatic ring with 3,4-dihydroxy substitution rather than the indole or 5-hydroxyindole ring systems of serotonin and tryptamine. It differs from other ligands tested here in having a hydroxyl substituent at the asymmetric side chain (C-7) position. These features produce a different pattern of hydrogen bonding interactions with the protein, which may explain the significantly lower binding affinity of this compound (Fig. 5A). In the norepinephrine complex, the C-7 hydroxyl lies in the position occupied by the amino group in the other complexes and forms hydrogen bonds with Glu-114 and Asp-111 (Fig. 5A). The amino group occupies a similar position to the bridging water molecule in the serotonin structure, bringing it into proximity to the carboxylate of Asp-139 (3.14 Å) and the carbonyl oxygen of Arg-22 (3.10 Å). Previous measurements of norepinephrine binding with D7r proteins showed D7r2 and D7r3 to have over 100-fold greater affinity than D7r1.

### Table 2: Thermodynamic parameters for binding of serotonin (top) and tryptamine (bottom) with D7r4 and various binding pocket mutants

| Mutant        | Ligand     | ΔH° | ΔAΔH° | TΔS° | K° | ΔG° | ΔAΔG° |
|---------------|------------|-----|-------|------|----|-----|-------|
| Wild type     | Serotonin  | −21.1| −9.2  | 2.4  | −11.9|     |
|               | Tryptamine | −16.4| −6.3  | 53   | −10.1|     |
| E7L           | Serotonin  | −14.8| 6.3   | −4.9 | 84  | −9.8 | 2.1   |
|               | Tryptamine | −10.0| 6.4   | −1.7 | 980 | −8.0 | 1.8   |
| H35L          | Serotonin  | −30.6| −9.5  | 16.5 | 0.1 | 14.0 | −2.1  |
|               | Tryptamine | −28.3| −11.9 | 15.6 | 0.6 | 12.7 | −2.6  |
| E7L/H35L      | Serotonin  | −15.9| 5.2   | −7.0 | 360 | −8.9 | 3.0   |
|               | Tryptamine | −29.8| −13.4 | 18.6 | 8.5 | −11.2| −1.1  |
| Y94L          | Serotonin  | −23.7| −2.6  | −12.4| 6.4 | −11.3| 0.6   |
|               | Tryptamine | −13.9| 2.5   | −5.9 | 1700| −8.0 | 2.1   |
| D111L         | Serotonin  | −16.7| 4.4   | −7.6 | 270 | −9.1 | 2.8   |
|               | Tryptamine | ND   |       |     |     |     |       |
| E114L         | Serotonin  | −9.8 | 11.3  | −2.7 | 7800| −7.1 | 4.8   |
|               | Tryptamine | ND   |       |     |     |     |       |
| D139L         | Serotonin  | −13.4| 7.7   | −4.3 | 270 | −9.1 | 2.8   |
|               | Tryptamine | ND   |       |     |     |     |       |

*Values are given as kcal/mol.
ΔAΔH° indicates ΔH°mutant − ΔH°wild type.
ΔAΔG° indicates ΔG°mutant − ΔG°wild type.
No binding was detectable.
and D7r4 (8). The reason for this remains unclear. The major difference in the putative binding pockets of the two groups is at position 110 where D7r1/D7r4 contains phenylalanine, whereas D7r2/D7r3 has valine. However, construction of the F110V mutant in D7r4 resulted in no change in the affinity for norepinephrine reported previously (data not shown).

The five-membered imidazole ring of histamine contains no hydroxyl substituent and is smaller than the aromatic portions of the other ligands. This leaves space in the binding pocket for a water molecule that forms hydrogen bonds with Glu-7 and His-35 (Fig. 5B). This water is positioned to form a hydrogen bond with the nitrogen atom NE2 of the imidazole portion of histamine. As in the serotonin complex, the aliphatic amino group forms hydrogen bonds with Asp-111 and Glu-114. No bridging water molecule was detected between the amino group and Asp-139, however. This is possibly due to the fact that the crystals of this complex were obtained by soaking in cryoprotectant solution containing histamine and show weaker ligand electron density (lower ligand occupancy) than the other complexes that were obtained by co-crystallization.

In the absence of ligands, the structures of the binding pocket and that of the protein as a whole are nearly unchanged, with functionally important residues occupying essentially the same positions as in the complexes. Superposition of C-α atoms between the serotonin complex and the unliganded protein showed a root mean square deviation of only 0.21 Å, suggesting that no major conformational changes take place during ligand binding. The pocket of the unliganded structure contains several poorly ordered solvent molecules that were modeled as water during refinement.

**Site-directed Mutagenesis and Binding**—A panel of mutants was constructed to test the importance of binding site residue structure on overall affinity using serotonin and tryptamine as ligands (Table 2). The most dramatic effects were obtained by mutating the acidic residues forming hydrogen bonds with the aliphatic amino group. Mutation of Asp-111 or Asp-139 to leucine produced increases in the dissociation constant of ~100-fold over the wild type protein for serotonin, whereas binding of tryptamine was completely eliminated at the ligand concentrations tested (Table 2). These decreases in affinity are due mainly to reductions in binding enthalpy (for serotonin, \( \Delta \Delta H = 4.4 \text{ kcal/mol for D111L and 7.7 kcal/mol for D139L} \) as a consequence of disruption of the hydrogen bonding network stabilizing the aliphatic amino group (Table 2). Mutation of Glu-114 showed a more dramatic effect on serotonin binding than either Asp-111 or Asp-139, with an increase in the dissociation constant of 3000-fold over the wild type protein, corresponding to a \( \Delta \Delta H \) of 11.3 kcal/mol (Table 2). Large decreases in affinity were also seen with tryptamine. In addition to disrupting interaction of the side chain with the ligand, mutation of Glu-114 may cause more extensive changes in the binding pocket structure by eliminating the salt bridge between Glu-114 and Arg-22, which appears to stabilize the bent conformation of the C-terminal side chain (Fig. 3).

The importance of the possible hydrogen bonding interaction between the phenolic hydroxyl of Tyr-94 and the indole nitrogens of serotonin and tryptamine was assessed by mutation of this residue to leucine. This change resulted in a modest 0.6 kcal/mol loss of binding free energy (\( \Delta \Delta G \)) for serotonin (Table 2). Interestingly, the same mutation had a much larger effect on tryptamine binding (\( \Delta \Delta G = 2.1 \text{ kcal/mol} \)), suggesting that in the absence of stabilization via the aromatic hydroxyl group, mutation of Tyr-94 may result in a change in the position of the ligand in the binding pocket.

Changes at the closed end of the binding pocket also had significant effects on ligand affinity. Mutation of His-35 to leucine resulted in a decrease in the dissociation constant for serotonin of 7.5-fold, whereas mutation of Glu-7 reduced binding affinity 35-fold (Table 2). Surprisingly, the same effect was seen with tryptamine despite the absence of the 5-hydroxyl group, indicating that the effect is not simply because of addition or loss of hydrogen bonds with the ring hydroxyl (Table 2).

**D7-like Proteins from Other Blood-feeding Diptera**—D7-like proteins are found in numerous species of blood-feeding nematocerous Diptera, but their functional significance is largely unknown. An alignment of single domain D7-like proteins from culicine mosquitoes (36), sand flies (19), and Culicoides sp. (20) shows a lack of conservation of residues responsible for stabilizing bound ligands in D7r4 (Fig. 6). None of the three acidic residues (Asp-111, Glu-114, and Asp-139) involved in stabilizing the amino group of the ligand can be clearly identified in any of the non-anopheline forms, nor can His-35 that hydrogen bonds with aromatic hydroxyl groups of serotonin and norepinephrine. These results strongly suggest that “short” D7-like proteins do not have a biogenic amine binding function in culicines, Culicoides or sand flies (Phlebotomus). In anophelines, a high degree of conservation of binding pocket residues is seen in both old world and new world species, suggesting that the biogenic amine-binding function evolved prior to the radiation of this group (Fig. 6). The absence of detectable binding by the poorly expressed A. gambiae protein D7r5 is explained by the lack of conservation of Glu-7, His-35, Tyr-94, Asp-111, and Asp-139. It has been proposed that this gene may be nonfunctional and in the process of being silenced.

In Aedes and Culex sp., amine binding appears restricted to the long form D7 proteins that have been shown previously to bind biogenic amines in a similar manner to D7r1–D7r4. Although alignments indicate two D7-type domains in the long form of Aedes sp., the binding stoichiometries determined with isothermal titration calorimetry show only a single ligand molecule bound per molecule of protein (8). Amino acid sequence alignments reveal a high degree of similarity between the putative C-terminal domain of the Ae. aegypti long form D7 and the D7r proteins of A. gambiae. This strongly suggests that it is the C-terminal portion of the long form protein that binds biogenic amines.

**Evolution of Ligand Binding in Salivas of Blood Feeders**—The salivary D7 protein family is structurally related to the large, diverse arthropod OBP family, suggesting that the ancestral D7 originated from recruitment of an OBP adapted for binding small hydrophobic ligands. Subsequently the protein was modified, perhaps through duplication of a portion of the molecule, resulting in the addition of two C-terminal helices that contribute significantly to the binding pocket structure. Further duplication resulted in a cluster of related genes that diverged and acquired additional functions and altered binding specificities. 
Although sequence similarity between OBPs and D7 proteins is detectable using position-specific data base searching techniques (37), the degree of sequence conservation is low. This suggests that the OBP-D7 proteins can tolerate a considerable diversity of amino acid composition without disruption of the protein fold.

In many arthropod salivas, proteins belonging to the lipocalin family serve a biogenic amine-binding role despite having no structural similarity to the D7s (38). Like the OBP-D7 group, the sequence of the eight-stranded $\beta$-barrel structure of the lipocalins is highly variable. For example, the biogenic amine-binding protein of Rhodnius prolixus and the histamine-binding protein of the tick Rhipicephalus appendiculatus are salivary lipocalins adapted to bind biogenic amines with high affinity (11, 12). The proteins function in a similar manner but are dissimilar at the sequence level. Phylogenetic analyses and analyses of binding pocket structure indicate that the two lipocalins have evolved their ligand-binding functions independently. Apparently, members of the lipocalin and OBP protein families are particularly suited to adopt these functions. It is interesting to note that mammalian odorant-binding proteins belong to the lipocalin family, but they function in a manner similar to the all-helical arthropod OBPs (39). Perhaps the structural plasticity required for binding of a diverse set of odorant molecules is an evolutionary preadaptation for nonolfactory ligand binding functions.

Conclusions—As predicted from sequence comparisons, D7r4 shows structural similarity to members of the arthropod OBP family. However, it contains two additional C-terminal helices, which make up a major portion of the ligand-binding pocket. The likely entry path for the ligand lies between helices B, G, and H and is bordered by three acidic residues, Asp-111, Glu-114, and Asp-139, which stabilize the aliphatic amino group of the bound ligands. Sequence comparisons of short salivary D7-like proteins from mosquito species, Culicoides sp., and sand flies suggest that only the anopheline forms have the biogenic amine-binding function and that the non-anopheline forms most likely serve different functions in feeding biology. Studies of hematophagous arthropod salivas have uncovered a large variety of proteins that bind biologically active small molecule ligands with high affinity. These proteins may have potential practical utility as therapeutic agents (40) or as biosensors (41).

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