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Structure and mechanism in salivary proteins from blood-feeding arthropods

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Abstract

The saliva of blood-feeding arthropods contains rich mixtures of ligand binding proteins targeted at inhibiting hemostasis and inflammation in the host. Since blood feeding has evolved many times, different taxonomic groups utilize completely different families of proteins to perform similar tasks. Structural studies performed on a number of these proteins have revealed biologically novel and sophisticated mechanisms used to perform their functions. Here, the results of these structural and mechanistic studies are reviewed.

Keywords

Lipocalin; odorant-binding protein; D7; nitrophorin; tick; mosquito; Diptera; X-ray crystallography

Blood feeding has evolved many times in arthropods, and each time the blood sucker has been faced with an evolutionary battle to overcome the host defenses against blood loss (Francischetti, et al., 2009). Independent evolution has resulted in a huge diversity of biologically active proteins in the salivary secretions of these organisms. Often, proteins from different structural classes take on the same or similar functions in different taxa, implying that these are the result of independent recruitments into the salivary proteome of each (Francischetti, et al., 2009). Within a given species, additional diversity is created by gene duplication events that produce groups of related proteins expressed in a single salivary mixture. Members of these groups commonly diverge further to take on new functions in blood feeding. These changes can result in relatively small modifications of a single function or produce completely new functions. Diversification of single salivary protein families within a species has been described for many species. For a few groups such as the nitrophorins in *Rhodnius prolixus* (Andersen, et al., 2000, Ribeiro, et al., 1995), lipocalins in soft ticks (Mans, et al., 2008, Paesen, et al., 1999), and the D7 type ligand binding proteins in mosquitoes (Calvo, et al., 2006), the modification of existing functions and acquisition of new functions has been described.

While a few types of enzymes have been found in salivas of blood feeders, the majority of functionally characterized salivary proteins are inhibitors of host physiological processes that act by binding proteins or small molecules. The targets of these proteins include

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enzymes of the coagulation cascade, collagen (Calvo, et al., 2007), and small molecule agonists of platelet activation, inflammation and vasoconstriction / vasodilation (Andersen, et al., 2003, Francischetti, et al., 2000, Paesen, et al., 1999, Ribeiro and Walker, 1994, Sangamnatdej, et al., 2002). Blood-feeding Diptera, triatomine bugs, and ticks have all been shown to produce salivary proteins that bind agonists of hemostasis and inflammation. Among the molecules bound are serotonin (Andersen, et al., 2003, Calvo, et al., 2006, Sangamnatdej, et al., 2002), norepinephrine, epinephrine (Andersen, et al., 2003, Calvo, et al., 2006), histamine (Paesen, et al., 1999, Ribeiro and Walker, 1994), adenosine diphosphate (Francischetti, et al., 2000), cysteinyl leukotrienes (Calvo, et al., 2009, Mans and Ribeiro, 2008), leukotriene B₄, thromboxanes (Mans and Ribeiro, 2008), nitric oxide (Ribeiro, et al., 1993) and collagen (Calvo, et al., 2007). Two major structural families, the lipocalins and the odorant-binding proteins, have been extensively characterized in performing these tasks. Lipocalins serve this function in triatomine bugs, hard ticks and soft ticks, while odorant-binding proteins serve the function in mosquitoes.

This review will focus on structural studies of ligand binding proteins from the saliva of blood feeders. Most of the examples fall within the lipocalin and D7 (odorant-binding protein) families, and demonstrate the structural basis behind the remarkable functional diversity seen in these molecules. A number of coagulation inhibitors having Kunitz and Kazal domains have also been structurally characterized, but these studies have been recently reviewed (Corral-Rodriguez, et al., 2009, Koh and Kini, 2009) and other than the lipocalin thrombin inhibitor triabin, will not be covered here.

Nitrophorins: Lipocalin NO carriers from *R. prolixus*

Triatomine bugs are important as vectors of the new world trypanosomiasis known as Chagas' disease. While the pathogen is transmitted in the feces rather than in the saliva, host feeding is an important element of the transmission cycle. The salivas of these insects are extremely rich in proteins, with dozens of distinct secreted forms being described in the transcriptomes of *Rhodnius prolixus* or *Triatoma infestans* salivary glands (Assumpcao, et al., 2008, Ribeiro, et al., 2004). Lipocalins are by far the most abundant and diverse group of proteins in triatomine saliva (Andersen, et al., 2005). This structural diversity translates into functional diversity, with lipocalins serving as vasodilators, platelet aggregation inhibitors and anticoagulants. Some of the first studies of triatomine lipocalins were performed with the nitrophorins (NPs), an extremely interesting group of nitric oxide carrier proteins from *R. prolixus*.

NPs were originally described as four distinct heme proteins from the salivary gland extract of *R. prolixus* that were each found to carry a single molecule of Fe^{III}-coordinated nitric oxide (NO). (Ribeiro, et al., 1993). Further analysis of the salivary transcriptome of this species revealed the existence of a number of additional forms, some of which were subsequently isolated from salivary gland extracts.

NO is an endogenous effector in vertebrates produced by endothelial cells, macrophages, and in the nervous system. Its vasodilatory and antiplatelet effects are a result of cellular signaling involving the activation of soluble guanylate cyclase. Nitric oxide is labile in the blood and tissues where it is rapidly oxidized with a half life of < 1 s. Binding of NO with NPs protects it from oxidation, allowing its intact delivery to the host. When nitrophorins are injected during feeding, bound NO is released in a pH-dependent manner, making it available to affect host hemostatic and inflammatory processes. NO binds more tightly in the, acidic saliva (pH ~6.0) than at vertebrate physiological pH. When the protein is diluted in the host blood and tissues, and the pH is raised to 7.4, NO is released and causes vasodilation and inhibition of platelet aggregation. Interestingly, after release of NO,

nitrophorins are able to bind a molecule of histamine with high affinity in the distal pocket vacated by the NO molecule (Ribeiro and Walker, 1994). Binding of this mast cell-derived inflammatory mediator limits the itching response that results from recognition of salivary antigens by IgE.

The crystal structure of recombinant NO-free NP1 was solved using multiple isomorphous replacement methods and found to have an eight-stranded antiparallel β -barrel structure having a single heme moiety located in a central binding pocket (Fig. 1) (Weichsel, et al., 1998). The heme is tethered to the protein by coordination of the iron atom to the imidazole group of His 57 (Fig. 1A). Although sequence comparisons at the time did not reveal relationship with the lipocalin protein family, the structure clearly showed the protein to be identical in fold, and quite similar in structure to the prototypic insect lipocalins that bind the pigment biliverdin (Fig. 1A). Two disulfide bonds are present in the nitrophorins, with one linking the N-terminal portion to the β -barrel and the second linking the C-terminal segment to the barrel. Entry to the distal (ligand-binding) pocket of the protein is regulated by loops which surround the entry to the binding pocket and undergo conformational changes on NO binding (Fig. 1A,B) (Weichsel, et al., 2000). The NO ligand is observed in structures of the NP4 complex in a somewhat unusual (for an Fe^{III} complex) bent geometry (Roberts, et al., 2001). After release of NO, histamine enters the distal pocket where its imidazole group coordinates with the heme iron, forming a bisimidazole complex (Weichsel, et al., 1998). Histamine is also stabilized by interaction of its ammonium group with acidic and polar residues lining the distal pocket (Fig. 1C).

The structure of NP4 was critical to determining the ligand binding mechanism. It crystallizes under a wide range of pH conditions, diffracts to very high resolution, and unlike NP1 and 2, the recombinant form is free of an N-terminal methionine residue (Andersen, et al., 1998). Soaking of NP4 crystals in solutions of NO causes extensive ligand-dependent conformational changes involving the loops surrounding the distal pocket as well as the N-terminal segment of the protein (Fig. 1A,B) (Weichsel, et al., 2000). When NP4 binds NO, movements involving loop AB (connecting strands A and B of the β -barrel) and GH, act to close the binding pocket, expel ordered water, and form a new and extensive hydrogen bonding network. This results in a shift in the side chains of Val 36, Leu 130 and Leu 133 toward the lipophilic NO ligand essentially packing it in a hydrophobic environment (Fig. 1B). The hydrogen bonding network involving backbone and side chain atoms from Asp 129, Leu 130, Asp 35, Asp 30, Glu 32 and the N-terminal amino group is possibly of importance in the pH-dependence of the binding mechanism.

Unlike molecular oxygen, NO forms complexes with both the Fe^{II} and Fe^{III} oxidation states of heme. Binding of NO to Fe^{II} heme proteins is extremely tight and NO release is very slow, making them unsuitable to perform the NO-delivery function of the nitrophorins. In the saliva, nitrophorins are found in the Fe^{III} state, and are quite resistant to NO-dependent reductive reactions that would result in a poor NO-donating complex (Berry, et al., 2009, Ding, et al., 1999). High resolution structures of NP4 have been used to precisely determine the degree of distortion, or ruffling, of the planar heme structure. The highly ruffled heme of the nitrophorins, along with the presence of anionic amino acid residues in the distal pocket and the exclusion of water from the NO environment is thought to be important in stabilizing the Fe^{III} state of the protein in the presence of free NO (Roberts, et al., 2001).

The reason for maintenance of multiple nitrophorin forms in the genome is open to speculation. Comparison of the NO binding and release kinetics of four nitrophorin forms showed significant differences, particularly in the rates of release (Andersen, et al., 2000). This implies that multiple nitrophorin genes may be maintained due to adaptive differences

in function between them. Perhaps a mixture of fast and slow-releasing nitrophorin forms acts to optimize the duration of vasodilator and antiplatelet effects during feeding.

Other NP forms have evolved novel functions distinct from NO transport. In addition to its role as a NO carrier, NP2 is a potent inhibitor of the intrinsic factor Xase complex (Ribeiro, et al., 1995, Yuda, et al., 1997). Inhibition of this reaction prevents the massive accumulation of thrombin normally occurring at the site of a wound. NP2 acts by binding coagulation factors IX and IXa in a calcium-dependent manner (Isawa, et al., 2000). Another form, NP7, displays a positively charged surface that enables it to bind to anionic phospholipid membranes (Andersen, et al., 2004, Knipp, et al., 2007, Knipp, et al., 2007). This protein has been shown to block the assembly of the prothrombinase complex on vesicles and activated platelets, and the binding may also improve its ability to inhibit platelet aggregation by targeting it to the anionic surfaces of activated platelets.

Inhibition of the factor Xase complex by NP2 is due to features of the surface that are not related to heme or NO binding. NP3, the form most similar to NP2, is a much weaker inhibitor of coagulation, while NP1 and NP4 are completely inactive. In kinetic studies the inhibition was characterized as hyperbolic mixed type, as indicated by decreases in both $V_{\max}$ and K_m of factor X activation (Zhang, et al., 1998). Surface plasmon resonance studies indicated that an intact γ -carboxyglutamic acid-containing Gla domain of the coagulation factor is essential for interaction with NP2 (Isawa, et al., 2000). This observation is consistent with the calcium ion dependency of the binding reaction. Comparison of the NP4 and NP2 crystal structures shows that the surfaces of the two proteins are significantly different in shape, due mainly to an extended C-terminal region in NP4 (Andersen and Montfort, 2000). Site directed mutagenesis of the NP2 sequence, guided by comparisons between NP2, NP3 and NP4, identified an area of the protein surface formed mainly by residues of the EF loop that is critical to high affinity binding of NP2 with factors IX and IXa (Gudderra, et al., 2005).

Nitrophorin from *Cimex lectularis*

In a remarkable case of convergent evolution, the bedbug, *C. lectularis* produces a nitric oxide-carrying heme protein having a completely unrelated protein structure to the nitrophorins of *R. prolixus* (Valenzuela, et al., 1995, Weichsel, et al., 2005). Rather than belonging to the lipocalin family, this protein is clearly derived from the enzyme inositol polyphosphate 5-phosphatase. Critical residues responsible for the phosphatase reaction have been mutated causing a loss of enzymatic function, and a binding site for a single heme moiety is present that is not normally found in members of this family. The heme group in this case is bound to the protein through a thiolate linkage rather than through imidazole, a feature that is critical for its mechanism of NO binding and release.

The structure of *Cimex* nitrophorin consists of a β -sandwich structure with the heme moiety being inserted on the outside of the β -structure between the β -sheet and a peripheral α -helix (Fig. 1D) (Weichsel, et al., 2005). The helix contains Cys-60, the proximal ligand of the protein, while the residues lining the distal ligand binding pocket are located on the β -sheet structure (Fig. 1D).

Both the *Rhodnius* and *Cimex* nitrophorins bind NO in a reversible fashion, but the binding and release mechanisms of the two proteins are very different. *Cimex* nitrophorin binds two nitric oxide molecules in the fully loaded state, with one molecule being found as a 5-coordinate Fe^{II} complex, and the other as a nitrosothiol involving Cys-60 (Weichsel, et al., 2005). In the proposed mechanism of binding and release, nitric oxide first binds on the distal side of the heme to form a 6-coordinate Fe^{III} complex. A second molecule of NO then attacks the thiolate in the proximal pocket, breaking the thiolate linkage and forming a

nitrosothiol group. In this process the initial complex is reduced to a 5-coordinate Fe^{II} state. On dilution in the host blood and tissues, the process is reversed, with two molecules of NO being released and the Fe^{III} nitrophorin being regenerated (Weichsel, et al., 2005).

Some hints as to the evolutionary origin of this protein in the saliva can be found in the salivary proteomes of other hemipteran blood feeders. *R. prolixus* and *T. infestans* (Assumpcao, et al., 2008) contain proteins that are clearly homologous to *Cimex* nitrophorin in their salivas. In the case of *R. prolixus*, however, the protein was shown to be a true, enzymatically active inositol polyphosphate 5-phosphatase with selectivity for phosphoinositides having a 4,5-bisphosphate structure (Andersen and Ribeiro, 2006). The *T. infestans* protein has not been functionally evaluated, but residues essential for catalysis are conserved. The role of these proteins in blood feeding has not been shown, but their substrate selectivities and the fact that they are allosterically activated by phosphatidylserine and phosphoinositides suggests that act to regulate membrane phospholipid composition in platelets or other host blood cells.

Biogenic amine-binding proteins from *R. prolixus*

An additional group of two lipocalin proteins related to the NPs was identified in the *R. prolixus* salivary gland transcriptome. Although the sequence relationship with the NPs was clear, these proteins did not contain a histidine residue aligning with the proximal ligand His-59 of NP1 (Andersen, et al., 2003). This suggested that these were not heme binding proteins, but had a salivary function distinct from the NPs. Previous studies had suggested the presence of a serotonin-binding protein in the saliva based on the ability of salivary extracts to inhibit serotonin-induced contraction of the rabbit aorta. Heterologous expression of one of these proteins in bacteria, followed by ligand screening using bioassays and physical binding studies showed that these proteins specifically bind serotonin and the catecholamine norepinephrine. Because of this they were given the name amine binding protein or ABP. ABPs have no significant binding affinity for heme confirming that they do not function as nitric oxide carriers in the manner of the nitrophorins (Andersen, et al., 2003).

The crystal structure of ABP has been determined in the ligand-free form and in complex with the serotonin analog tryptamine (J.F. Andersen, unpublished observation). Its overall structure is quite similar to the nitrophorins, but changes in the amino acid composition of the binding pocket and the loops surrounding the pocket, have given the protein a completely different binding specificity. The residue corresponding to His-59, the proximal ligand of NP1 is mutated to asparagine, and acts in stabilizing the amino group of the ligand. The nitrophorin-ABP system represents a good example of diversification of a single protein type in the specialized tissue of the salivary gland to take on distinct and non-overlapping functions in blood feeding.

Triabin: a thrombin inhibitor from *T. pallidipennis*

Triabin was first identified as an inhibitor of thrombin-induced platelet aggregation in the saliva of *T. pallidipennis* (Noeske-Jungblut, et al., 1995). The protein inhibited clotting of plasma by thrombin, but did not inhibit hydrolysis of the small molecule thrombin substrate S2238, suggesting that it binds at the anion binding exosite of the enzyme rather than the catalytic site. On isolation and cloning, the active component was found to be a 17kDa protein having similarity to the lipocalin family. The crystal structure of the triabin-thrombin complex shows a unique modification of the eight-stranded β -barrel structure of the triabin molecule (Fig. 2B) (Fuentes-Prior, et al., 1997). In this structure, strands B and C of the β -barrel are twisted and reversed in position to give an up-up-down-down topology rather than the up-down-up-down topology seen in the lipocalins (Fig. 2B). The loop connecting β -

strands C and D closes the end of the barrel causing the central cavity of the barrel to be packed and no apparent ligand binding pocket to be present. A rearrangement of this type is very unusual, and is considered to have important implications regarding the evolution of new protein folds. The structure of the complex also confirms that triabin binds at the fibrinogen-recognition exosite with the interaction interface being made up mainly from elements of β -strands F, G and H, as well as the N-terminal segment of the triabin molecule (Fig. 2A) (Fuentes-Prior, et al., 1997). The area of Ser 195, His 57 and Asp 102, the catalytic triad of thrombin, is completely unoccluded, explaining the failure of triabin to inhibit hydrolysis of small molecule thrombin substrates.

The triabin sequence is similar to many other lipocalin forms found in the genera *Rhodnius* and *Triatoma*. Pallidipin and RPAI-1 are inhibitors of collagen mediated platelet aggregation (Francischetti, et al., 2000, Noeske-Jungblut, et al., 1994). The mechanism of action of pallidipin has not been clearly defined, but RPAI-1 is a binder of the dense granule component ADP (Francischetti, et al., 2000). Contact with collagen induces release of ADP which then potentiates platelet activation by stimulating G-protein-coupled receptors on the platelet surface. Binding of ADP by RPAI-1 has been shown to inhibit this response. No structures of this type of ligand-binding lipocalin have been determined, but the fact that RPAI-1 binds ADP suggests that, unlike triabin, it has a central ligand-binding pocket. It has also not been possible to determine the β -strand arrangement from sequence comparisons, and it is therefore not known if these proteins show the same modification of the β -barrel as triabin.

Biogenic amine-binding proteins from hard ticks

The salivary proteomes of hard and soft ticks are rich in members of the lipocalin family. Relatively few of these have been functionally characterized, but from those that have, a number of structures have been determined. The first reported structure of a tick lipocalin was the histamine binding protein (Ra-HBP2) from *Rhipicephalus appendiculatus* which binds histamine with a measured dissociation constant of 1.2 nM (Fig. 3A) (Paesen, et al., 1999, Paesen, et al., 2000). This structure was particularly noteworthy in that it identified two distinct ligand binding sites occupied by histamine while lipocalins with other binding specificities normally have only a single binding site (Fig. 3A). Overall, the protein is similar to other lipocalins in that it has an eight-stranded antiparallel β -barrel structure with a peripheral C-terminal α -helix. A helical segment of N-terminal portion of the molecule closes off one end of the barrel while the open end is surrounded by a series of flexible loops (Fig. 3A). One histamine binding site lies deep in the β -barrel, with the (probable) ammonium nitrogen and nitrogen ND1 of the imidazole group of histamine forming hydrogen bonds or ionic interactions with the side chain of Asp 24 which lies on the N-terminal segment of the protein. The ammonium group also interacts via an apparent cation- π interaction with the aromatic ring of Tyr 29, which also lies on the N-terminal segment. The ligand is further stabilized by hydrogen bonds with Ser 20 and Tyr 100 of the β -barrel (Paesen, et al., 2000).

The second binding site is formed in large part by the loops surrounding the entry to the β -barrel (Fig. 3A). Hydrogen bonds or ionic interactions with Tyr 36 (phenolic hydroxyl), Asp 110 (carboxylate) and an ordered water molecule stabilize the ammonium group of the ligand. Additionally, the side chains of Asp 39 and Glu 82 form hydrogen bonds with the imidazole group. Phe 108 and Trp 42 are also positioned to form stacking interactions with the planar imidazole ring, further stabilizing this molecule (Paesen, et al., 2000).

The relative affinities of the two binding sites were determined by soaking crystals in ligand-free solutions after crystallization. In this way it was determined that of the two, the

upper binding site showed the higher-affinity for histamine and is now referred to as the H-site. The lower site loses the ligand on soaking, and is referred to as the L-site. The binding specificity of Ra-HBP2 was further investigated using mass spectrometry. Histamine was shown to be the preferred ligand for these proteins although much lower-affinity binding to serotonin and dopamine was also detected (Oldham, et al., 2003). A second two-site binding protein, known as SHBP, has been isolated from the hard tick *Dermacentor reticulatus* and shows a high degree of homology to Ra-HBP (Sangamnatdej, et al., 2002). Unlike Ra-HBP2, however, this protein exhibits high-affinity binding for histamine and serotonin, with a single binding site for each. A molecular model was constructed with serotonin positioned in the site corresponding to the low-affinity site of Ra-HBP and histamine in the site corresponding to the high-affinity site. Support for the binding site assignments was drawn from the degree of sequence conservation relative to Ra-HBP, and from the fact that the less-bulky residues lining the low-affinity site created space for the larger serotonin ligand (Sangamnatdej, et al., 2002).

Biogenic amine-binding lipocalins from soft ticks

Several biogenic amine-binding lipocalins were identified in the saliva of the soft tick species *Argas monolakensis* and *Ornithodoros savignyi*. Two of the *Argas* proteins, monomine and monotonin, were crystallized and their structures determined (Fig. 3B,D,E) (Mans, et al., 2008). Unlike the Ra-HBP and SHBP proteins, these lipocalins bind a single ligand molecule, with monomine being specific for histamine (Fig. 3D) and monotonin for serotonin (Fig. 3E). Although they show only 16 % sequence identity, monomine and Ra-HBP are quite similar in overall structure and the histamine binding site of monomine is equivalent to the L or low affinity site in Ra-HBP2 (Mans, et al., 2008). The affinity of monomine for histamine is quite high, however, being measured at 7.1 nM using isothermal titration calorimetry. Many elements of the binding sites from the two proteins are conserved, but a number of significant differences are observed. Hydrogen bonding/ionic interactions with the histamine amino group are similar in the two with Ser 12 and Asp 94 in monomine being equivalent to Ser 20 and Asp 120 in Ra-HBP2. Also, Tyr 21 in monomine serves the function of Tyr 29 in Ra-HBP2 in forming a cation- π interaction with the apparent quaternary amino group of the ligand (Fig. 3D). The imidazole binding environment differs significantly between the two proteins. In Ra-HBP2 the imidazole is stabilized by hydrogen bonding with Asp 24 and Tyr 100, while in monomine Tyr 51 and a hydrogen bonding network involving ordered water, Ser 83 and Glu 103 serve similar functions.

Monotonin did not crystallize readily, so a closely-related protein from the same species, AM-182, was expressed and its structure determined (Mans, et al., 2008). Like monotonin, AM-182 binds serotonin with high affinity and specificity. In the structure of the AM-182-serotonin complex, the ligand is positioned very similarly to the histamine in monomine (Fig. 3E). When the complexes are superimposed, the pyrrole portion of the serotonin indole moiety and the imidazole ring of histamine overlap almost perfectly (Mans, et al., 2008). The side chains of the two ligands are also very similarly positioned. No direct hydrogen bonding interactions occur between the protein and the indole portion of serotonin, but the hydroxyl group participates in hydrogen bonding network involving an ordered water molecule (Fig. 3E).

Sequence comparisons among the known biogenic amine-binding proteins from different tick species allowed the formulation of a sequence motif that can be used to identify potential biogenic amine binders in the transcriptomes of new species of both hard and soft ticks. The motif sequence CD[VIL]X(7,17)EL[WY]X(11,30)C was successfully used to

identify biogenic amine-binding proteins in the hard tick species *Ixodes scapularis* and the soft tick *Ornithodoros savignyi* (Mans, et al., 2008).

OmC1: a lipocalin complement inhibitor from soft ticks

OmC1, a protein having a structure similar to the biogenic amine-binding lipocalins and the ability to inhibit the complement system has been identified in the saliva of *Ornithodoros moubata* (Fig. 3C,F) (Nunn, et al., 2005, Roversi, et al., 2007). The protein binds the C5 component of complement and prevents its cleavage by the C5 convertase. Determination of the crystal structure of this protein showed that it, like other tick lipocalins, has a ligand binding pocket located in the center of a β -barrel structure (Roversi, et al., 2007). In this structure, a ligand apparently derived from the *Pichia methanolica* expression strain was present in the pocket, and the shape of its electron density suggested that the ligand was a fatty acid derivative. Mass spectral analysis confirmed that the major small molecule component in preparations of the protein was ricinoleic acid, (*R*)-12-hydroxy-cis-9-octadecenoic acid (Roversi, et al., 2007). The double bond and substituent positions of this compound suggested that the natural ligand may be an eicosanoid compound, since the ligand structure is particularly reminiscent of the fatty acid chains of the leukotrienes or thromboxanes. Lipocalins related to OmC1 were subsequently cloned from *Ornithodoros savignyi* and *O. moubata*, expressed in bacteria, and shown to bind leukotrienes and thromboxanes with high affinity (Mans and Ribeiro, 2008, Mans and Ribeiro, 2008).

Lipocalin structures are present in the complement system as the C8 γ domain of C8 and the C345C domains of C3, C4 and C5. Roversi et al. (Roversi, et al., 2007) hypothesized that OmC1 recognizes the C345C binding site on C5 and displaces the natural ligand. C345C is a necessary component in the C5 cleavage reaction. A molecular model of the putative OmC1-C5 complex shows that the lipocalin can be oriented to make similar contacts to the C345C. The model suggests that in OmC1 loops BC (connecting β -strands B and C), DE and EF as well as the N-terminal portion of the protein make important contacts with C5. Subsequent SAXS, surface plasmon resonance and electrophoretic studies indicate that while OmC1 does interact with the core structure of C5, it stabilizes the interaction of C345C with the core rather than displacing it (Fredslund, et al., 2008).

D7 proteins: small molecule binders from mosquitoes

The small-molecule binding functions performed by lipocalins in triatomines and ticks are taken on by members of the D7 family in mosquitoes (Calvo, et al., 2002, James, et al., 1991, Malafronte, et al., 2003, Valenzuela, et al., 2002). The first member of the family (D7 or AeD7) was identified as a major antigen in the saliva of the yellow fever mosquito *Aedes aegypti*. Since then, many genes encoding similar proteins have been described in other dipterans. Sequence comparisons suggested a distant similarity of AeD7 with the all-helical arthropod odorant-binding protein (OBP) family, a group of proteins normally found in sensory organs. In the antennae of insects, these proteins bind odorant molecules dissolved in the receptor lymph that fills the hair-like sensillae. In some cases the OBP may be needed to shuttle the protein to the neural surface where odorant receptors are located, although this has not been clearly determined. OBP family members are also found in the hemolymph of insects where their functions are not known. The AeD7 sequence encodes a protein having two OBP-like domains, and similar two-domain forms are found in *Culex* and *Anopheles* sp. Mosquitoes, sand flies and other blood-feeding dipterans, also have a variety of single-OBP domain proteins in their salivas.

Like the lipocalins in triatomines and ticks, the D7s have been shown to bind biogenic amines and, in some cases, act as anticoagulants (Calvo, et al., 2006, Isawa, et al., 2002). Single-domain D7 proteins from *An. gambiae* are referred to as D7-related proteins in order

to distinguish them from the two-domain forms, and are given the names D7R1 through D7R5 (Calvo, et al., 2006). Members of this cluster have been individually expressed in bacteria and of the five proteins evaluated, four were found to bind biogenic amines, with one poorly expressed form showing no binding activity. In addition to binding biogenic amines, D7R1 is also an inhibitor of the contact activation system of coagulation and of bradykinin release through its binding with coagulation factor XII and high molecular weight kininogen (Isawa, et al., 2002). This situation is reminiscent of the nitrophorin system where NP 1–4 all bind NO and histamine, but NP2 acts as an inhibitor of coagulation by binding factor IX/IXa. The two-domain form AeD7 also binds biogenic amines, but binds only a single ligand molecule per molecule of protein, suggesting that one of the domains may have a different function (Calvo, et al., 2006). The C-terminal domain of AeD7 is quite similar in sequence to the D7R of *Anopheles sp.*, implying that it is responsible for the biogenic amine binding and leaving the function of the N-terminal domain unresolved.

Anopheles gambiae D7R4 has been crystallized, and its structure determined in the unliganded form and in complex with a number of biogenic amines (Fig. 4C) (Mans, et al., 2007). As expected, the protein has an arthropod OBP fold, but with eight α -helices rather than the usual six or seven. The first six helices are positioned similarly to their counterparts in the sensory OBPs, but the two most C-terminal helices contribute significantly to the binding pocket and give it a unique structure (Fig. 4C) (Mans, et al., 2007). The structure is stabilized by three disulfide bonds, two of which are conserved in OBPs (Fig. 4C). In the sensory OBPs the binding pocket is made up largely of residues from helices D, E and F, while in D7R4, the two terminal helices, G and H displace these, and make up much of the binding pocket themselves (Fig. 4C,D). Structures of D7R4 complexes show that the ligand is stabilized in a similar manner to the tick biogenic amine binding proteins (Mans, et al., 2007). The amino group of the ligand interacts with the acidic side chains of Asp-111 and Glu-114, while the aromatic nuclei of norepinephrine, and serotonin are situated in a hydrophobic pocket, and the side chains of Glu-7 and His-35 form hydrogen bonds with the phenolic hydroxyl groups (Fig. 4D).

Analysis of the D7R proteins did not clarify the role of the two-domains in AeD7. Both anopheline and culicine mosquitoes have single- and two-domain proteins in their salivas, suggesting that the two must be functionally distinct. The sequences of the two domains of AeD7 are only distantly similar, sharing about 15% amino acid identity, while the C-terminal domain more similar to D7R4 (26 %) of *An. gambiae*. Also, most of the residues involved in ligand binding in D7R4 are conserved in the C-terminal domain of AeD7, suggesting that the C-terminal domain of AeD7 is responsible for biogenic amine binding.

To verify the ligand binding specificity of the C-terminal domain and to understand the role of the N-terminal domain in blood feeding, the crystal structure of AeD7 was determined (Calvo, et al., 2009). As expected, the C-terminal domain is quite similar in structure to D7R4, but surprisingly, the eighth helix (H_2), is not formed despite the sequence similarity with D7R4 (Fig. 4A). Also, the side chains of two important binding pocket residues, Arg-174 and Glu-268, take on a conformation that creates a more open binding pocket than in D7R4 (Calvo, et al., 2009). When norepinephrine is soaked into the crystal, however, Arg-174 and Glu-268 adopt the closed conformation of D7R4, and helix H_2 becomes ordered giving the C-terminal domain of AeD7 a very similar overall structure to D7R4 (Fig. 4B).

The conformational change binding mechanism exhibited by AeD7 may help to explain its higher affinity for norepinephrine relative to D7R4, as well as the difference in the norepinephrine binding mode observed between the two proteins. Catecholamines have a

secondary hydroxyl on the aliphatic side chain that must be accommodated differently than serotonin (Calvo, et al., 2009).

Determination of the AeD7 structure led directly to discovery of the apparent natural ligand for the N-terminal domain. The binding pocket of this domain is narrow and lined mainly with hydrophobic amino acids, suggesting that it may bind a fatty acid ligand, possibly an eicosanoid. Screening of potential ligands with isothermal titration calorimetry revealed that the cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ were bound with high affinity. The structure of the AeD7-LTE₄ complex showed that the fatty acyl portion of the ligand extends into the hydrophobic channel of the protein (Fig. 4A,E). The amino acid portion of the ligand was poorly ordered and not visible in the crystal. This observation is consistent with calorimetry data showing similar thermodynamic parameters for the three compounds. Since they are equally effective in producing wheal and flare reactions and itching responses in the skin, a binding mechanism that does not discriminate between LTC₄, LTD₄ and LTE₄ would seem to be adaptive.

Conclusions

The diversity of salivary components in blood-sucking arthropods has resulted in many novel mechanisms for the inhibition of host physiological processes. Increasingly, X-ray crystallography is being used to determine the structures of these proteins and uncover important details of their mechanisms. Structural results have demonstrated the occurrence of stabilizing conformational changes in the nitrophorins and D7 proteins, have uncovered a novel fold abnormality in triabin, a lipocalin-like protein, have been used to elucidate the mechanism of NO binding in Cimex nitrophorin, and have allowed the prediction of binding functions in tick lipocalins and mosquito D7 proteins. Many potentially interesting proteins remain uncharacterized, and still, a large number of salivary proteins show no sequence homologs in database searches. Future structural studies are certain to tie together many aspects of the evolution of blood feeding in arthropods, and lead to an understanding of mechanism in these proteins.

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Abbreviations

NO	nitric oxide
NP	nitrophorin
OBP	odorant-binding protein

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Fig 1.

Structures of nitrophorins. A: Ribbon diagram of the NP4-ammonia complex (PDB accession number: 1D2U, (Roberts, et al., 2001)). The lipocalin β -barrel is colored green: heme is a stick diagram with carbon colored white, oxygen colored red, nitrogen colored blue, and iron colored orange. Coordination bond of the proximal imidazole of histidine (His, in the same color scheme as heme) with the heme iron is shown. Ammonia occupies the distal pocket. The side chain of Leu 30 is colored red, and is shown in its position in the absence of NO. B: The NO-complex of NP4 (1ERX (Weichsel, et al., 2000)). In this case the lipocalin structure is colored cyan. The rest of the complex is colored as in A. Note the conformational changes in loops surrounding the binding pocket and resulting in a shift in the position of Leu 130. These changes act to bury the NO ligand (in the distal pocket) in a hydrophobic environment. C: Detail of the NP4-histamine complex (1IKE, (Roberts, et al., 2001)). Carbon atoms from protein residues are shown in cyan, from heme are shown in white, and from histamine are shown in red. Atom colors are as in A, and hydrogen bonds/ionic bonds are shown as red dashed lines. D: Structure of *Cimex lectularis* nitrophorin (1YJH, (Weichsel, et al., 2005)). The protein structure is shown in cyan, with heme as a stick diagram. Heme coloring is the same as in A.

Fig. 2. Structure of the triabin-thrombin complex (1AVG, (Fuentes-Prior, et al., 1997)). A: Ribbon diagram of the triabin-thrombin complex, with thrombin (light) and triabin (dark) labeled. B: Detail of the triabin fold showing rearrangement of strands A-D from the up-down-up-down arrangement of a true lipocalin to up-up-down-down.

Fig. 3.

Structures of tick lipocalins. A: Ribbon diagram of the Ra-HBP2 structure with bound histamine ligands shown as a stick diagram (1QFT, (Paesen, et al., 1999)). The position of histamine bound in the low-affinity site is marked L and that in the high affinity site is marked H. B: For comparison, a ribbon diagram of the structure of monotinin with histamine ligand (Hist) bound (3BU1, (Mans, et al., 2008)). Note that the position of the ligand corresponds to the L site in Ra-HBP2. C: Ribbon diagram of the OmC1 structure with the ligand (*R*)-12-hydroxy-cis-9-octadecenoic acid (Ric) bound (2CM4, (Roversi, et al., 2007)). D: Detail of histamine binding in monotinin. Amino acid residues are labeled. Histamine is shaded darker than the protein. Water molecules are indicated by spheres, and hydrogen / ionic bonds are indicated by dashed lines. E: Serotonin binding in AM182 (3BRN, (Mans, et al., 2008)). Stick diagram is colored as in D. Note the similarity in positioning of the ligand between D and E, as well as the conserved nature of interactions involving the ammonium group. F: Detail of the interaction of (*R*)-12-hydroxy-cis-9-octadecenoic acid with OmC1 (Roversi, et al., 2007). Hydrogen bonding / ionic interactions of the carboxyl and hydroxyl groups of the fatty acid ligand are shown as dashed lines.

Fig. 4.

Structures of D7 type proteins from mosquitoes. A: Ribbon diagram of the AeD7-leukotriene E₄ complex (3DZT, (Calvo, et al., 2009)). The protein structure is shown in cyan, and the fatty acid portion of LTE₄ is shown as a stick diagram with carbon colored blue, and oxygen red. Note that the C-terminal portion of the C-terminal domain is a partially disordered coil. B: Ribbon diagram of the AeD7-norepinephrine complex (3DYE, (Calvo, et al., 2009)). The protein portion is colored green and norepinephrine is shown as a stick diagram with carbon and oxygen colored red and nitrogen colored blue. Note that norepinephrine binding causes the C-terminal coil to order into an eighth α -helix, helix H₂. C: Ribbon diagram of the D7R4-serotonin complex (2QEH, (Mans, et al., 2007)). The protein portion of the molecule is shown in white and the serotonin ligand is shown as a stick diagram with carbon and oxygen colored red and nitrogen blue. Disulfide cysteines are shown in yellow. The helices are labeled A-H with A being the most N-terminal. Helix H occupies a nearly identical position to helix H₂ in the norepinephrine complex of AeD7. D: Stick diagram showing binding pocket detail of the D7R4 complex. Protein residues are shown with carbon in green, oxygen in red and nitrogen in blue. Serotonin is shown with carbon and oxygen in red and nitrogen in blue. The large number of hydrogen bonds and ionic interactions stabilizing the ligand are shown as dashed red lines. E: Stick diagram showing binding pocket detail of the AeD7-LTE₄ complex. Protein residues are shown as in D. LTE₄ is shown with carbon as blue and oxygen as red. Hydrogen bonds and ionic interactions are shown as in D.