Inhibition of Hemostasis by a High Affinity Biogenic Amine-binding Protein from the Saliva of a Blood-feeding Insect*  

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The saliva of the blood-feeding insect Rhodnius prolixus contains numerous pharmacologically active substances. Included among these are a number of lipocalin proteins that bind various ligands important in hemostasis and inflammation. One such protein is a biogenic amine-binding protein (ABP) that binds serotonin, epinephrine, and norepinephrine. Based on amino acid alignments, it is most similar to the nitrophorin group of lipocalins found in the same insect species. Physiologically, this protein appears to act as both a vasodilator and platelet aggregation inhibitor. This protein inhibits smooth muscle contraction of the rat uterus in response to serotonin and of the rabbit aorta in response to norepinephrine. Platelet aggregation induced by a combination of low concentrations of ADP and either serotonin or epinephrine is inhibited because of the binding of serotonin and epinephrine. Potentiation of aggregation induced by low concentrations of collagen along with serotonin or epinephrine is also inhibited. Dissociation constants for biogenic amines were measured using isothermal titration calorimetry and the Hummel-Dreyer method of equilibrium gel filtration. In this manner, \( K_d \) values of 102, 24, and 345 \( \mu \)M were found for serotonin, norepinephrine, and epinephrine, respectively. Molecular modeling of ABP suggests that ligand binding is mediated by interaction with the side chains of aromatic amino acids and charged residues that line the binding pocket.  

A major function of the salivary secretion of blood-feeding arthropods is to inhibit hemostasis and inflammation in the host tissues and blood (1). In the case of Rhodnius prolixus, the saliva contains potent inhibitors of platelet aggregation, anticoagulants, vasodilators, antihistamines, and antiserotonins (2–5). Many of the active components of the saliva belong to the lipocalin protein family (6, 7). The structure of lipocalins is characterized by an 8-stranded \( \beta \)-barrel having a generally hydrophobic ligand-binding pocket in its interior (8). The entry to the binding pocket is surrounded by a number of flexible loops that can modulate binding through ligand-induced conformational changes (9, 10). A common mechanism of action for salivary lipocalins is the removal of small molecule receptor agonists from the feeding area (7, 11). High affinity binding of the pharmacologically active compounds histamine and ADP by the lipocalins nitrophorin and RPAI-1, respectively, has been demonstrated in R. prolixus (4, 7).  

Serotonin and catecholamines are important mediators of platelet aggregation, vasoconstriction, and inflammatory processes. Serotonin is contained in the dense granules of platelets and is released upon stimulation by various agonists. It acts to increase vascular permeability and is itself also a weak platelet agonist, leading to the activation of phospholipase C. The catecholamines norepinephrine and epinephrine, released by local nerves, cause vasoconstriction in response to bleeding. Epinephrine also potentiates platelet aggregation by a \( \mathrm{G}_\text{G2} \)-dependent signaling mechanism, leading to a decrease in adenyl cyclase activity (12).  

The inhibition of serotonin-inhibited smooth muscle contraction by R. prolixus salivary extracts has been shown. However, its mechanism remains to be determined. In this study, a lipocalin-encoding sequence that was encountered frequently during the random sequencing of a R. prolixus salivary gland library has been expressed in Escherichia coli. The protein was refolded and shown to be a biogenic amine-binding protein. It was found to bind serotonin as well as epinephrine and norepinephrine. It inhibits the serotonin-mediated contraction of the rat uterine horn, and the norepinephrine-mediated contraction of the rabbit aorta. During feeding, this protein also appears to function as a platelet-aggregation inhibitor by sequestering the agonists serotonin and epinephrine secreted by activated platelets and neural sources. It also may act to inhibit inflammatory responses at the feeding site by binding these proinflammatory molecules.  

EXPERIMENTAL PROCEDURES  

Materials—Serotonin, epinephrine, and norepinephrine were obtained from Sigma. The MicroFast Track mRNA preparation kit was from Invitrogen (San Diego, CA), and the SMART library construction kit from Clontech (Palo Alto, CA).  

Cloning of ABP  

A salivary gland cDNA library was prepared using the SMART cDNA library construction system (13, 14). Messenger RNA was prepared from 4 pairs of R. prolixus salivary gland pairs (5, 7, 10, and 14 days after blood feeding) using the MicroFast Track system. The cDNA for the ABP was cloned as part of an expressed tag sequencing project in which ~500 clones from the R. prolixus salivary gland library were partially sequenced. The ABP sequence was recognized as encoding a previously uncharacterized lipocalin and was well represented in the library.  

The abbreviations used are: ABP, amine-binding protein; ITC, isothermal titration calorimetry.

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The atomic coordinates and structure factors (code 1EUO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).  

The library has been expressed in Escherichia coli. The protein was refolded and shown to be a biogenic amine-binding protein. It was found to bind serotonin as well as epinephrine and norepinephrine. It inhibits the serotonin-mediated contraction of the rat uterine horn, and the norepinephrine-mediated contraction of the rabbit aorta. During feeding, this protein also appears to function as a platelet-aggregation inhibitor by sequestering the agonists serotonin and epinephrine secreted by activated platelets and neural sources. It also may act to inhibit inflammatory responses at the feeding site by binding these proinflammatory molecules.
**Protein Expression and Purification**—Using the ABP N-terminal sequence as a guide, the cDNA was modified to exclude the 5'-untranslated region and the region encoding the signal peptide. The modifications were performed using PCR with the primer CGGACCATATGGG-ATCTGGTTGTTCATGTTGAGATCTG. The resulting PCR product had a methionine (ATG) codon directly upstream of the first codon of the mature polypeptide. The PCR product was cloned into the vector PCR 2.1 and sequenced. An NdeI restriction site at the 5' end of the cDNA, added as part of the PCR mutagenesis, enabled its insertion into the expression vector pET 17b. The expression construct was then moved into the *E. coli* strain BL21(DE3) pLysE.

For the production of protein, 4 liters of LB broth were inoculated with the expression strain and shaken at 250 rpm and 37 °C until the absorbance at 600 nm was about 0.7. Isopropyl-1-thio-

In a 0.5-ml bath kept at 30 °C, the cells were harvested by centrifugation and washed with 20 mM Tris-HCl, pH 8.0.

The cell pellet was suspended in 100 ml of 20 mM Tris-HCl, pH 8.0, 0.2 mM phenylmethylsulfonyl fluoride, and the cells were lysed using a probe sonicator. The lysate was centrifuged at 10,000 × g and 10 °C for 20 min. Analysis by SDS-PAGE followed by staining with Coomassie Blue showed the protein to be present as inclusion bodies. The insoluble pellet was extracted with 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, followed by centrifugation at 10,000 × g. The extracted pellet was washed three times with 20 mM Tris-HCl, pH 8.0, with each wash followed by centrifugation.

The protein was solubilized in 20 ml of 20 mM Tris-HCl, pH 8.0, 6 M guanidinium hydrochloride, 10 mM dithiothreitol. This material was diluted to 250 ml in 20 mM Tris-HCl, pH 8.0, 0.4 mM arginine. After centrifugation, the supernatant was dialyzed against 2 changes of 20 mM Tris-HCl, pH 8.0. The dialyzed sample was concentrated by ultrafiltration, centrifuged at 100,000 × g, and purified by gel filtration chromatography on Sephacyr S-100 (16/60 column, Amersham Biosciences) using 20 mM Tris-HCl, pH 8.0, 0.2 mM NaCl for elution.

**Smooth Muscle Preparations**—Longitudinal contractions of isolated pieces of rat uterus horns (animals were primed 24 hr before dissection with estradiol) were recorded isotonically with transducers from Harvard Apparatus Inc. (Holliston, MA). Muscles were suspended in a 0.5-ml bath kept at 30 °C, using the solution recommended by Gaddum et al. (15). Rabbit aortic ring contractions were evaluated isometrically using transducers from the same company. The bath was kept at 37 °C, and Tyrode's solution was used (16). Additions to the bath were never greater than 5% of the volume of the bath.

**Preparation of Human Washed Platelets and Platelet Aggregation Assays**—Platelet-rich plasma was obtained by platelet pheresis from medication-free platelet donors at the DTM/NIH blood bank under the direction of Dr. S. Leitmann, as described (17). Briefly, after addition of 1 ml of stilbestrol (0.5 mM, 30 min at 37 °C to lyse adherent cells), the platelet-rich plasma was added to a 1 ml of 0.5 mM Tris-HCl, pH 8.0, 0.25% bovine serum albumin, pH 7.4). Platelets were resuspended in 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl for elution.

**Modeling of ABP**—A homology-based molecular model of ABP was constructed based on the coordinates of the nitrophorin 2 crystal structure (19), with all water and heme atoms removed (Protein Data Bank accession number 1EU0). The model was constructed by an automated procedure using the web-based SwissModel (20). Equivalent positions in nitrophorin 2 and ABP were determined by alignment. The resulting model was minimized by 200 cycles of steepest descents followed by 300 cycles of conjugate gradient minimization. Serotonin coordinates were added to the model and positioned manually using the Insight II (MSI, San Diego, CA) modeling software.

**RESULTS**

**Detection and Cloning of ABP**—ABP was first detected in a survey of expressed tag sequences from a *R. prolixus* salivary gland cDNA library. It was part of a group of related clones encoding putative lipocalins of unknown function (Fig. 1). Members of the group were encountered frequently, suggesting that the mRNAs were present in high abundance. Analysis of the cDNA by alignment with the nitrophorin sequence did not exceed 23%. This is consistent with the low identity levels commonly seen among members of the lipocalin group. The alignment of all four cysteine residues in ABP with their nitrophorin counterparts suggests that two disulfide bonds are present that

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*2 J. M. C. Ribeiro, unpublished observation.*
correspond in position to those seen in crystal structures of nitrophorins 1, 2, and 4. A second group of *R. prolixus* salivary lipocalins, typified by the ADP-binding protein RPAI-1, is less similar to ABP, having six cysteines and three apparent disulfide bonds. The ABP shares only 11% identity with a serotonin-histamine-binding lipocalin recently isolated from the saliva of the tick *Dermacentor reticulatus*, with a different distribution of cysteine residues, suggesting that the functional relationship between these two molecules is because of convergence rather than common lineage (22).

**Heterologous Expression of ABP**—ABP was expressed at high levels in *E. coli* as insoluble inclusion bodies. After solubilization in guanidine and reduction with dithiothreitol, the protein was folded by dilution in arginine-containing buffer, followed by dialysis. Gel filtration chromatography produced purified ABP as a soluble monomer. Because the protein is related to the heme-containing nitrophorins, heme was added to a portion of the refolded ABP followed by gel filtration chromatography. Monitoring of the eluent at both 280 and 405 nm indicated that no heme was associated with the monomeric protein (data not shown).

**Inhibitory Effects of ABP on the Rat Uterus**—Serotonin is a known mediator of mammalian uterine contraction via interaction with the 5-HT2A receptor. *R. prolixus* salivary homogenates and a partially purified salivary protein have been shown to inhibit this activity. In this study, both the *R. prolixus* salivary gland homogenate (results not shown) and the purified, reconstituted recombinant ABP completely abrogated the response of the rat uterus to serotonin when supplied in sufficient quantity (Fig. 3). When 25 μg of ABP was added to the uterine preparation, no response to incremental additions of serotonin were seen until the cumulative concentration of agonist in the bath just exceeded the concentration of ABP (Fig. 3). The approximate 1:1 protein-agonist stoichiometry required for minimal inhibition suggests that each molecule of ABP binds a single molecule of serotonin (Fig. 3). Replacement of the saline solution containing ABP with fresh solution caused the preparation to regain its normal response to serotonin, indicating that the inhibition is reversible (Fig. 3).

**Inhibitory Effects of ABP on the Rabbit Aortic Ring**—Norepinephrine is important in the maintenance of vascular tone by inducing the contraction of smooth muscle via interaction with α-adrenergic receptors. The release of norepinephrine at sympathetic presynaptic membranes in response to wounding
leads to rapid vasoconstriction (23). Scavenging of norepinephrine would benefit a feeding insect by inhibiting vasoconstriction, thereby allowing unimpeded blood flow to the feeding site. The effect of ABP on norepinephrine-induced vasoconstriction was evaluated using a rabbit aortic ring preparation. Addition of ABP (to a concentration of 2 μM) to a preparation stimulated to contract with 1 μM norepinephrine caused relaxation that began immediately after addition of protein (Fig. 4). When the protein was removed from the bath, the preparation could again be induced to contract by addition of agonist. This reversible antagonism of norepinephrine is consistent with binding of this compound by ABP.

**Inhibition of Platelet Aggregation by ABP**—Serotonin and epinephrine are secreted by platelets upon activation as constituents of the dense granules. Serotonin is an agonist of platelet aggregation acting through activation of the 5-HT2A receptor (12). Binding to this receptor activates phospholipase C, resulting in platelet shape change, but not aggregation. Serotonin alone has the effect of potentiating aggregation induced by subthreshold levels of other agonists such as collagen and ADP. Given in combination with epinephrine, an agonist of α2-adrenergic receptors, serotonin induces an aggregation response. Epinephrine alone does not produce a detectable effect on platelet aggregation, but like serotonin it potentiates the effect of other agonists (12).

We evaluated the effects of ABP on platelet aggregation in the presence of serotonin and epinephrine. When platelets are treated with the cyclooxygenase inhibitor indomethacin, the effects of added serotonin and epinephrine on platelet activation can be evaluated in the absence of thromboxane A2 secretion. This limits the contribution of secreted agonists, such as ADP to platelet activation. ABP was found to completely eliminate, in a concentration-dependent manner, the shape change response of washed, indomethacin-treated human platelets to serotonin, consistent with the observed serotonin-binding activity of the protein (Fig. 5A). Conversely, ABP had no effect on platelet shape change induced by low concentrations of ADP or on the aggregation response induced by higher agonist concen-
trations, indicating that ADP does not bind with ABP (Fig. 5B).

Serotonin and epinephrine are important as potentiators of platelet agonists such as ADP and collagen. When ADP is administered to indomethacin-treated platelets at concentrations below the threshold for platelet aggregation, only shape change is seen (Fig. 5C). At higher concentrations of ADP, an aggregation response is observed (Fig. 5C). However, when low concentrations of ADP are added along with serotonin or epinephrine, a full aggregation response is seen, indicating that serotonin and epinephrine potentiate the response to ADP. In the presence of ABP, no potentiation of aggregation by serotonin or epinephrine is observed (Fig. 5, C and D). This suggests binding of these two compounds by ABP eliminates any potentiation of the ADP response. A similar effect is observed on the potentiation by serotonin or epinephrine of collagen-mediated aggregation of nonindomethacin-treated platelets (Fig. 6, A and B). The results indicate that in the natural physiological system, where thromboxane A2 synthesis is not suppressed, ABP increases the threshold concentration of collagen necessary for an aggregation response.

**Determination of ABP Ligand Binding Affinities**—The binding affinity of ABP for serotonin was determined using both the Hummel-Dreyer method of equilibrium gel filtration and ITC. Fig. 7 shows a modified Hummel-Dreyer analysis of serotonin binding when the ligand concentration changed from 10 nM to 3 μM. The changes in optical properties were saturable with increasing ligand concentration, and allowed determination of a dissociation constant of 201 ± 7 M.

ITC was used to evaluate the binding of serotonin, norepinephrine, and epinephrine (Fig. 8). The results show that the primary amine norepinephrine binds with the highest affinity (Kd = 24 ± 6 nM) whereas the secondary amine epinephrine binds with a 10-fold lower affinity (Kd = 345 ± 67 nM). The affinity for serotonin is intermediate with a Kd value of 102 ± 35 nM, agreeing reasonably well with the result of the Hummel-Dreyer experiment. Analysis of the ITC data using a single-site binding model gave binding stoichiometries (N) of 0.85 ± 0.07, 0.80 ± 0.01, and 1.28 ± 0.03 for serotonin, norepinephrine, and epinephrine, respectively; this further strengthened the evidence for a single binding site for each ligand. To determine whether serotonin and the catecholamines bind to separate sites, the heats generated on titration of ABP with norepinephrine were measured in the presence of saturating (1 mM) concentrations of serotonin. After correcting for heats of dilution, no significant binding of norepinephrine could be detected, suggesting that identical, or considerably overlapping, binding sites are used for both serotonin and the catecholamines.

**Modeling of ABP**—The ABP sequence was aligned with that of nitrophenol 2 and modeled using the SwissModel automated program with the coordinates for the nitrophorin 2 crystal structure (Fig. 9). The resulting model shows the seventh strand of the β-barrel moved toward the center of the protein relative to the template, reducing the volume of the binding pocket. A molecule of serotonin, manually inserted into the protein, is surrounded by the side chains of the aromatic residues Phe59, Tyr111, and Tyr113, which stabilize the ligand via pi-pi interactions. Situated in this manner the aliphatic amino group of the serotonin molecule would be further stabilized by electrostatic interactions with the carboxylates of Glu57 and Asp113 as well as the hydroxyl group of Tyr88 (Fig. 9).

**DISCUSSION**

The Lipocalin Structure and Ligand Binding Function—The lipocalin fold is employed in many salivary functions that involve ligand binding and protein-protein interactions (24, 25). Three major *R. prolixus* salivary lipocalin groups have been characterized to date. The nitrophorins are a group of heme-binding nitric oxide transporters that also bind histamine with high affinity (3). RPAI-I is a high affinity ADP-binding protein that also binds a variety of other adenosine derivatives (7). Finally, ABP binds the biogenic amines serotonin, epinephrine, and norepinephrine.
On a structural level, the propensity of lipocalins to bind so many ligand types derives from the flexibility of the \(-\text{barrel}\) structure and its ability to tolerate considerable amino acid side chain variability while maintaining its fold (8). Sequence comparisons with other \(R.\ prolixus\) lipocalins show that ABP is most closely related to the nitrophorins (6). The positions of the cysteine residues are equivalent in the two groups, suggesting that the disulfide bonding pattern is the same in the two (Fig. 2). However, the proximal histidine residue (His\(^{59}\) of nitrophorin 1), which coordinates with the heme iron atom and is located on the third \(\beta\)-strand of the nitrophorins, is replaced with Asn\(^{61}\) (in the fully processed protein) in ABP. Consequently, ABP shows no ability to bind heme. ABP and the nitrophorins are apparently derived from a common ancestor through gene duplication events. Divergence of the two protein forms has resulted in highly different ligand-binding specificities and, consequently, very different functional roles.

The high affinity binding of two structurally different amine types, having either hydroxyindole or catechol nuclei in the same binding site, suggests flexibility in the ABP-binding pocket. In the crystal structure of a histamine-binding lipocalin from saliva of the tick \(Rhipicephalus appendiculatus\), pi-pi stacking interactions are important for the binding of aromatic ligands. In each of the two histamine-binding sites, the planar imidazole ring of histamine is stabilized by aromatic amino acid side chains (11). The aliphatic amino group is also stabi-
lized by electrostatic interactions with a number of polar and acidic side chains, as well as ordered water molecules. Like the tick proteins, ABP contains numerous aromatic side chains that would be expected, from the results of alignments and modeling studies, to pack the ligand binding pocket. These side chains could potentially stabilize any appropriately sized aromatic group, and allow for the relatively broad specificity for various aromatic ligands displayed by ABP.

When ABP is modeled based on the nitrophorin 2 crystal structure, the putative ligand binding pocket is seen as a cleft lined with the side chains of Phe, Tyr, and Tyr. When serotonin is manually placed in the cleft, its indole nucleus is surrounded by three aromatic side chains (Fig. 9) that form both stacking and end-on interactions between the protein and ligand. The acidic side chains of Glu and Asp are positioned to stabilize the aliphatic amino group of the ligand. The model is, of course, hypothetical, but these types of binding interactions may explain the combination of submicromolar binding affinity with the relative lack of specificity indicated by the ability to accommodate both hydroxyindole and catechol-type amines in the same binding site.

**Antihemostatic Functions of ABP**—Serotonin and catecholamines play important roles in numerous biologic processes. Inhibition of many of these would be of obvious benefit to a blood-feeding insect. The release of norepinephrine from the sympathetic nervous system occurs on wounding and results in the constriction of blood vessels to prevent blood loss. The highly abundant nitrophorins shuttle the vasodilator nitric oxide from the *R. prolixus* salivary gland to the host skin, and are apparently the primary mechanism for increasing blood flow around the feeding site. However, the binding of norepinephrine by ABP may complement the effect of nitric oxide by preventing an antagonistic constriction response. A functionally similar role is played by the salivary catechol oxidase of the mosquito *Anopheles albimanus*. This enzyme destroys norepinephrine oxidatively and apparently serves as a vasodilator (26).

Activation of platelets by strong agonists such as thrombin or collagen induces the release of dense granules that contain the weak agonists ADP, serotonin, and epinephrine. Aggregation induced by weak agonists requires activation of phospholipase C and inhibition of adenyl cyclase via a G-coupled receptor pathway. At high concentrations, ADP stimulates both pathways sufficiently to induce aggregation. At low concentrations, only shape change is seen because of stimulation of phospholipase C via the P2Y1 receptor. Serotonin activates the phospholipase C pathway via the 5-HT1A receptor but not the G, dependent pathway, and consequently induces only shape change when administered alone (12). Epinephrine binds with G-coupled adrenergic receptors, inhibiting adenyl cyclase, and eliciting no detectable aggregation response by itself (12).

Administration of serotonin or epinephrine, along with low concentrations of ADP, induces a complete aggregation response, apparently by increasing the level of stimulation to the necessary signal transduction pathways (Fig. 5). Notably, adhesion of serotonin or epinephrine along with subthreshold levels of collagen produces a full aggregation response, indicating that these molecules are active in reducing the concentration threshold for collagen-mediated aggregation (Fig. 6). The presence of ABP in the circulation would therefore cause a localized increase in the agonist threshold concentration for platelet aggregation. This protein would act in concert with RPAI-1, the ADP-binding protein, and salivary aminopeptidase to reduce the concentration of weak agonists in the vicinity of the feeding site, thereby attenuating the overall stimulus for platelet aggregation.

Hemostasis and inflammation are complex and redundant host reactions against which *Rhodnius* has evolved a complex salivary mixture of antigens. ABP is the latest such component to be described. Many of these antagonists act on the same physiologic processes, such as platelet aggregation, but are aimed at different biochemical pathways. Based on the properties of individual components of *R. prolixus* salivary secretion, an increasingly complex model is emerging to describe the interactions between the insect and the host hemostatic system. Nitrophorins released into the blood and skin provide a burst of nitric oxide that dilates blood vessels and inhibits platelet aggregation (3, 10). Nitrophorin 2 acts as a specific inhibitor of coagulation by interacting with the proteolytic factor Xase complex (28). ABP binds norepinephrine and inhibits the host vasoconstriction response to blood loss. When platelets are activated and the contents of dense granules are released, ABP binds the agonists serotonin and epinephrine, whereas salivary aminopeptidase degrades the agonist ADP (29). Trace amounts of ADP are removed by binding with lipocalin RPAI-1 (7). Additionally, any inflammation that may induce a defensive response from the host is inhibited via histamine binding by the four nitrophorins (4).

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