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Structure, Function, and Evolution of Biogenic Amine-binding Proteins in Soft Ticks*[S]

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Ben J. Mans, José M. C. Ribeiro, and John F. Andersen[1]

From the Laboratory for Malaria and Vector Research, NIAID, National Institutes of Health, Rockville, Maryland 20852

Two highly abundant lipocalins, monomine and monotonin, have been isolated from the salivary gland of the soft tick *Argas monolakensis* and shown to bind histamine and 5-hydroxytryptamine (5-HT), respectively. The crystal structures of monomine and a paralog of monotonin were determined in the presence of ligands to compare the determinants of ligand binding. Both the structures and binding measurements indicate that the proteins have a single binding site rather than the two sites previously described for the female-specific histamine-binding protein (FS-HBP), the histamine-binding lipocalin of the tick *Rhipicephalus appendiculatus*. The binding sites of monomine and monotonin are similar to the lower, low affinity site of FS-HBP. The interaction of the protein with the aliphatic amine group of the ligand is very similar for the all of the proteins, whereas specificity is determined by interactions with the aromatic portion of the ligand. Interestingly, protein interaction with the imidazole ring of histamine differs significantly between the low affinity binding site of FS-HBP and monomine, suggesting that histamine binding has evolved independently in the two lineages. From the conserved features of these proteins, a tick lipocalin biogenic amine-binding motif could be derived that was used to predict biogenic amine-binding function in other tick lipocalins. Heterologous expression of genes from salivary gland libraries led to the discovery of biogenic amine-binding proteins in soft (*Ornithodoros*) and hard (*Ixodes*) tick genera. The data generated were used to reconstruct the most probable evolutionary pathway for the evolution of biogenic amine-binding in tick lipocalins.

Hemostasis (blood clotting, platelet aggregation, and vasoconstriction) and immunity (inflammation, pain, and adaptive immunity) are the primary ways that the vertebrate host can respond against parasitic invasion and subsequent blood loss caused by blood-feeding arthropods. Hematophagous arthropods modulate these defenses by secreting a pharmacological mixture of bioactive components in their saliva during feeding. Most blood-feeders will have at least an anti-clotting, anti-platelet, and vasodilatory component in their salivary arsenal (1).

This classic paradigm of blood-feeding strategy can be extended to include anti-inflammatory proteins that scavenge biogenic amines, as their presence in all blood-feeding arthropods is becoming apparent. Thus far, scavengers of biogenic amines have evolved independently in assassin bugs, mosquitoes, and ticks, and this will probably hold for other blood-feeders as well (2–5). This is in part due to the myriad effects that biogenic amines have during host defenses against blood loss and parasite invasion.

5-Hydroxytryptamine (5-HT)[2] (or serotonin) is secreted by platelets and thrombocytes, as well as mast cells of many mammals, and functions to increase vascular permeability as well as inducing platelet aggregation and vasoconstriction (6). Histamine is released by activated mast cells and basophils and leads to vascular permeability and infiltration of monocytes and neutrophils into the feeding site (7). 5-HT and histamine also induce sensation of pain and itching in the host, thereby increasing the risk of tick rejection by host grooming (8). As such, evolution of the biogenic amine scavenging function might not only be a matter of chance in the evolution of blood-feeding behavior, but a prerequisite for successful feeding.

In some ticks, histamine and 5-HT are known to be scavenged by members of the lipocalin protein family (3, 9). Lipocalins are highly abundant proteins in both hard and soft tick salivary glands with large numbers of paralogous genes in each lineage (10, 11). Additional functions of lipocalins include inhibition of platelet aggregation and the complement cascade (12–14). Although the case for orthology of histamine-binding proteins from metastriate ticks is clear, the detection of orthologs in prostriates (*Ixodes* sp.) and soft ticks has been problematic due to the low sequence similarities observed between tick lipocalins (15). Whereas lipocalins are known to generally have low levels of sequence similarity (16), immune pressure from the host might play a major role as source of variability in tick lipocalins. The scavenging of biogenic amines is thus of particular interest, as binding occurs within the lipocalin barrel and should be unaffected by immune pressures. It could thus be expected that the molecular mechanisms of biogenic amine

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials, Figs. S1–S7, and Table S1.

The atomic coordinates and structure factors (codes 3BS2, 3BU1, 3BU9, and 3BRN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[1] To whom correspondence should be addressed: 12735 Twinbrook Parkway, Rockville, MD 20852. E-mail: jandersen@mail.nih.gov.

[2] The abbreviations used are: 5-HT, 5-hydroxytryptamine; BAB motif, biogenic amine-binding motif; MES, 4-morpholineethanesulfonic acid.

binding should be conserved even if orthologs are highly divergent.

It has been argued that because hard ticks remain attached to their hosts over a long period of feeding, anti-inflammatory mechanisms, such as histamine antagonism are necessary to prevent host rejection (3). However, blood-feeders with shorter feeding times also contain biogenic amine scavenging proteins, suggesting that this mechanism is generally essential for successful feeding (2, 4, 5). In the case of mosquitoes, the identity of the scavenging protein, known as D7, was predicted based on its abundance in saliva (17). Given the high abundance of lipocalins in the salivary glands of soft ticks (10), we considered it likely that some of these forms would act as biogenic amine scavengers.

We have isolated the two most abundant lipocalins, monomine (AM-10) and monotonin (AM-38), from the salivary glands of the soft tick *Argas monolakensis* and found them to bind histamine and 5-HT, respectively. The crystal structures of monomine and a paralog of monotonin, AM-182, have been determined, and the ligand binding modes observed. The conserved features of the ligand-binding pockets have been probed with site-directed mutagenesis, and used to derive a general predictive motif for the identification of potential biogenic amine-binding proteins among known tick salivary lipocalins. Characterization of lipocalins from representative genera allows us for the first time to derive a model for the evolution of biogenic amine binding in ticks.

EXPERIMENTAL PROCEDURES

Purification of Wild-type AM-10 and AM-38—AM-10 and AM-38 were previously identified from proteome and transcriptome analysis of salivary glands of the soft tick *A. monolakensis* (18). This included purification, identification, and quantification of AM-10 and AM-38 from crude salivary gland extract and the identification of their corresponding cDNA sequences from a cDNA library (18).

Bacterial Expression of Recombinant Proteins—AM-10 (GI number 114152936) and AM-38 (GI number 114152976) cDNA sequences were amplified using primers that allowed cloning into the pET17b expression vector (Novagen). For AM-10, the construct used (rAM-10) included a start codon at the 5′ end followed by the coding sequence for the mature protein. This yielded a protein with an extra methionine at the N-terminal end that was not processed (supplementary materials). The construct for AM-38 (rAM-38) lacked the 2 first N-terminal residues to obtain recombinant protein that was resistant to proteolytic degradation (supplementary materials).

In addition, AM-182 (GI number 114153056), a protein that shows high sequence similarity to AM-38 was produced using the same system. The cDNAs encoding IS-3 (GI 22164318), IS-4 (GI: 67083669), IS-14 (GI 67083266), and IS-15 (GI: 67083329) were obtained from a cDNA library of *Ixodes scapularis* previously constructed (11). The cDNA encoding for Arg r1 (GI number 58371884) and TSGP1 (GI 25991387) were chemically synthesized and cloned into the same expression system. All recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS cells (Novagen), inclusion bodies prepared, protein refolded, and purified as previously described (5). Based on electrospray mass spectrometry, Edman sequencing, and SDS-PAGE analysis, the recombinant proteins were pure and correctly expressed (supplementary materials).

Smooth Muscle Bioassay—Smooth muscle bioassays were performed as previously described (5). Briefly, the guinea pig ileum contractions to histamine and the rat uterus contractions to 5-HT were measured isotonically. A modified Tyrode solution (with 10 mM HEPES buffer, pH 7.4) was used for the ileum (19), whereas low calcium HEPES-buffered solution was used for the uterus assay (20). All solutions were kept oxygenated by bubbling air.

Isothermal Titration Calorimetry—Isothermal titration calorimetry was performed using a VP-ITC calorimeter (Microcal, Northhampton, MA) as previously described (5). Briefly, proteins were equilibrated in ITC buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl), which was also used to prepare all ligands tested. All solutions were degassed under vacuum for 4 min before use. Aliquots (5 μl) were injected every 120 s, and the syringe was stirred at 290 rpm while measuring heat of binding at 30 °C. After subtraction of the heats of dilution, the net enthalpy data were analyzed with a single or two-site binding model using the Microcal Origin software package. In most circumstances, the protein concentrations were 2–3 μM. In the case of some mutants that showed reduced binding affinity, the protein concentration was increased to 10 or 100 μM.

Structure Determination of Monomine—Monomine was expressed from 4 liters of culture to give a concentration of 32 mg/ml (final volume 400 μl). Screening of crystallization conditions showed that monomine crystallized in 100 mM Tris-HCl, pH 7.4, with 2 M NH_4SO_4 as precipitant using the hanging drop-vapor diffusion method. Co-crystallization with histamine (4 mM) also occurred under the same conditions. Crystals grew overnight into rod-like clusters, from which single crystals could be obtained by cluster break-up.

At the time of crystallization of monomine, the closest related sequence for which a structure existed in the data base was that for female-specific histamine-binding protein (FS-HBP2) from the hard tick *Rhipicephalus appendiculatus* (3). Due to the low sequence similarity to FS-HBP2 (16% sequence identity) it was considered improbable that molecular replacement would work. However, AM-10 does not have any intrinsic methionine residues that could be used for the generation of selenomethionine derivatives. Four leucines were thus mutated to methionine (L57M, L63M, L87M, and L146M) using standard PCR-based mutagenesis techniques. This construct (AM-10SeMet) was transformed into BL21(DE3)pLysS cells and grown in SelenoMet™ (Molecular Dimensions Ltd., Apopka, FL) as per the manufacturer's instructions. Similar yields to rAM-10 were obtained and electrospray mass spectrometry analysis indicated 100% incorporation of selenomethionine into the recombinant protein. The selenomethionine derivative crystallized under the same conditions as rAM-10.

Prior to data collection, crystals were flash frozen after soaking the crystallization solution containing 15% glycerol. Data were collected at the Structural Biology Center, beamline 19-BM, and the Southeast Regional Collaborative Access Team (SER-CAT), beamline 22-BM at the APS, Argonne National

TABLE 1
Data collection, phasing, and refinement statistics for monomine (AM−10) and AM−182

	AM−10 Selenomethionine	AM−10 WT	AM−10 histamine	AM−182 5-HT
PDB code	3BU9	3BS2	3BU1	3BRN
Resolution (Å)	40.76-1.40	18.58-1.15	40.66-1.40	79.31-2.00
Beamline	19-BM	22-BM	19-BM	22-BM
Wavelength	0.97918	0.92000	0.97921	1.0000
Completeness (range)	99.80	99.73	99.38	99.69
Completeness (highest shell)	99.38	98.3	98.75	100
Average redundancy (total/high resolution shell)	3.9/3.5	6.0/3.8	7.6/7.1	7.8/6.8
R_{merge} (total/high resolution shell)	0.05/0.31	0.07/0.27	0.05/0.21	0.10/0.46
I/sigma (*I*) (total/ high resolution shell)	11.2/3.7	10.7/4.0	10.3/8.2	16.6/3.8
Observed reflections	181,485	264,343	184,438	235,821
Unique reflections	22,987	44,055	24,335	30,083
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P3_1$
Molecules in asymmetric unit	1	1	1	2
Unit cell dimensions (Å)				
a	36.281	36.399	36.418	91.694
b	56.547	56.592	56.324	91.694
c	58.747	59.033	58.774	37.836
α, β, γ	90	90	90	90, 90, 120
Phasing statistics				
Number of selenium sites	4			
FOM (Resolve)	0.83			
Root mean square deviations				
Bond lengths (Å)	0.011	0.007	0.006	0.013
Bond angles (degrees)	1.237	1.124	1.053	1.223
Ramachandran plot				
Favored/allowed	89.3/100	87.9/100	89.3/100	89.6/99.6
Mean *B* value for all atoms	12.23	12.77	11.12	43.95
R_{cryst}/R_{free}	17.7/21.5	16.8/19.2	15.1/18.0	22.2/26.1

Laboratory. Diffraction data for the selenomethionine derivative was collected at one wavelength near the selenium edge and scaled using HKL 3000. Initial phases were also obtained using the HKL 3000 package that combines SHELXD-E, MLPHARE, and DM for the location of selenium sites, solvent flattening, phasing, and density modification (21). A nearly complete model was built using ARP-WARP in conjunction with REFMAC refinement (22, 23). The remainder of the model was built manually using Coot and refined with REFMAC (22, 24). This model was used to determine the structure of wild-type liganded and unliganded monomine using difference Fourier methods. The resolution of all three structures (1.1–1.4 Å) allowed the refinement of anisotropic temperature factors in REFMAC.

Various manipulations of reflection and coordinate data during the course of structure determination were made using the CCP4 package (22). Model quality was checked using PROCHECK and MOLPROBITY web servers (25). Refined models for unliganded monomine (PDB code 3BS2) and the histamine complex (PDB code 3BU1) and their structure factors were deposited in the RCSB Protein Data bank. Statistics for data collection and refinement are presented in Table 1.

Determination of Monomine Residues Involved in Ligand Interaction—Residues that interact with the ligands were identified using the Ligand-Protein Contacts and Contacts of Structural Units server and confirmed manually in Coot (24, 26). Mutants of binding pocket residues were generated using standard PCR-based mutagenesis techniques. These included for monomine: Y21L, Y51L, Y51F, S83L, D94L, E103L, and W105L. For monotonin mutants constructed included F55Y and D101L. In the case of TSGP1 a series of mutants were constructed that included Y37L, E40L, Y61L, D76L, D88L, D90L, E92L, T98L, D110L, Q120L, Q124L, and E129L. Mutant constructs were confirmed by DNA sequencing. Recombinant proteins were expressed as described previously and molecular masses confirmed by electrospray mass spectrometry (supplementary materials).

Structure Determination of AM-182—AM-182 was expressed from 1 liter of culture to give a final concentration of 62 mg/ml (final volume 600 μl). Screening of crystallization conditions indicated that protein crystallized in 0.1 M MES, pH 6.5, with 25% (w/v) PEG 2000 MME as precipitant using the hanging drop-vapor diffusion method. Co-crystallization with 5-HT (4 mM) also occurred under the same conditions. Prior to data collection, crystals was flash frozen using the crystallization mother liquor with added 15% MPD. Data were collected at beamline 22-BM of SER-CAT. The structure was determined by molecular replacement using Phaser with a polyalanine variant of the monomine structure as a search model (27). The AM-182 model was built in Coot and refined using CNS and in REFMAC using a translation-libration-screw model (22, 28). The protein atoms of the two chains were treated as a single translation-libration-screw group. The quality of the model was evaluated with PROCHECK and the MOLPROBITY web server. The coordinates are deposited in the Protein Data Bank (PDB code 3BRN). Statistics for data collection and refinement are presented in Table 1.

Bioinformatical Analysis—The non-redundant databases were queried using either BLASTP or PSI-BLAST analysis (29, 30). Multiple alignments were obtained using ClustalX and manually checked and adjusted (31). Neighbor-joining analysis was performed using the Mega package (32). The Poisson model for amino acid substitution was used and 10,000 bootstraps were performed with complete deletion of gapped positions.

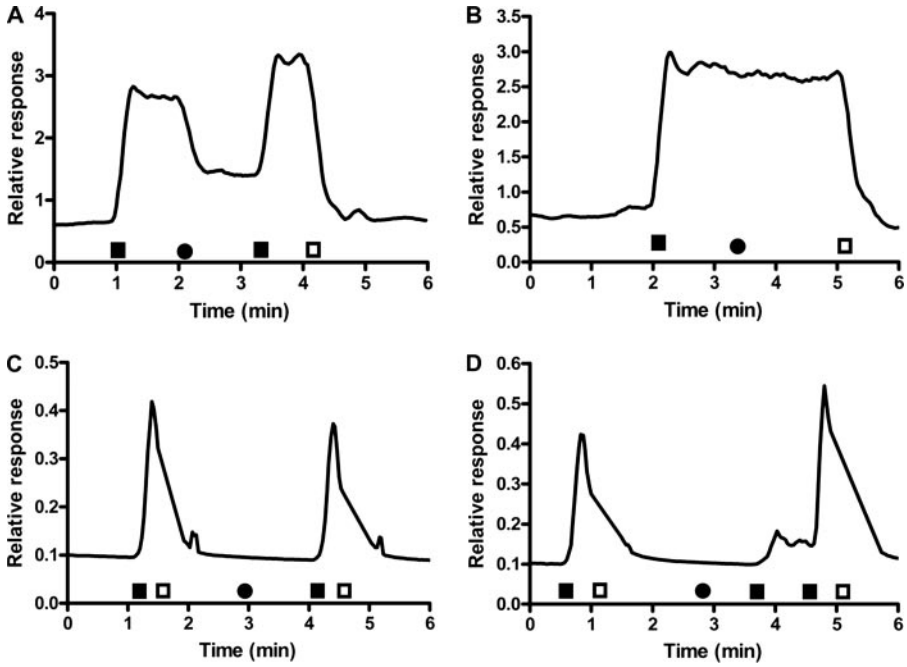

FIGURE 1. **Bio-assay of wild-type AM-10 and AM-38.** Guinea pig ileum (*A*) was contracted for 1 min with 1 μM histamine before addition of AM-10 (1 μM final concentration) or, AM-38 (*B*) (1.3 μM final concentration), respectively. Contraction could be rescued by saturation of AM-10 with histamine (1 μM final concentration). *C*, rat uterus was contracted using 0.5 μM 5-HT and contraction abolished by removal of 5-HT by washing with buffer. AM-10 (0.55 μM final concentration) or AM-38 (*D*) (0.55 μM final concentration) was added to the uterus preparation before the induction of subsequent contractions with 5-HT (0.5 μM aliquots). Contraction could be rescued by saturation of AM-38 with 5-HT (1 μM final concentration). *Filled rectangles* represent the time of addition of agonist, *open circles* represent time of addition of antagonists, and *open rectangles* represent times of washes.

RESULTS AND DISCUSSION

Isolation of A. monolakensis Lipocalins—Previous results have shown that AM-10 is encoded by ~5% (150/3087) and AM-38 is encoded by ~1.1% (34/3087) of randomly sequenced transcripts from a cDNA library (18). Chromatographic analysis of crude salivary gland extract indicated that AM-10 and AM-38 comprise ~23 and 8.8%, respectively, of the total salivary protein. Analysis by two-dimensional electrophoresis corroborated these results and indicated that they were major salivary gland extract proteins (18).

Edman degradation of purified AM-10 failed to yield a sequence, indicating possible modification of the N terminus. The sequence could, however, be obtained by peptide mass fingerprinting (18). Electrospray mass spectrometry of AM-10 showed a single peptide with a molecular mass of 15,707 Da. The observed deviation from the predicted mass of 15,725 Da suggests the presence of pyroglutamate (−18 Da difference) at the N terminus and that the protein is not post-translationally modified by glycosylation.

Unlike AM-10, AM-38 yielded a high quality N-terminal amino acid sequence after Edman degradation of protein separated by one-dimensional SDS-PAGE (18). Electrospray mass spectrometry of chromatographically purified AM-38 gave a molecular mass of 16,558.6 Da, which corresponds well with its calculated mass of 16,559.1 Da, indicating that no post-translational modifications are present.

Biological Activity of AM-10 and AM-38—Purified wild-type AM-10 inhibited tonic contraction of guinea pig ileum induced by histamine at equimolar concentrations of histamine and protein. The effect was abrogated by addition of excess histamine to the preparation (Fig. 1*A*). AM-10 did not inhibit rhythmic contraction of rat uterus induced by 5-HT (Fig. 1*C*).

When tested in an identical manner, AM-38 gave results opposite to those obtained with AM-10. It did not inhibit tonic contractions of guinea pig ileum induced by histamine (Fig. 1*B*), but did inhibit rhythmic contractions of rat uterus induced by 5-HT that could be abolished by addition of excess 5-HT (Fig. 1*D*).

Biogenic-amine Binding by Recombinant AM-10 and AM-38—To further investigate the modes of action of these proteins, AM-10 and AM-38 were expressed in recombinant form, and binding of biogenic amines was measured using isothermal microcalorimetry. AM-10 and AM-38 each possess a single binding site for their respective ligands as indicated by calculated binding stoichiometries (Fig. 2). We propose the name monomine for AM-10 to indicate binding of a single histamine molecule as well as being derived from *A. monolakensis*. For AM-38 we propose the name monotonin to indicate binding of a single 5-HT molecule as well as being derived from *A. monolakensis*.

Fitting of the experimentally determined binding enthalpies to a single-binding site model showed monomine to bind histamine ~295-fold more tightly than 5-HT (Fig. 2*A*, Table 2). Conversely, monotonin bound 5-HT at least 960-fold more tightly than histamine (Fig. 2*B*, Table 2).

Competition assays were used to determine whether 5-HT and histamine bind at the same site in each protein. Titration of monomine with histamine in the presence of an excess, fixed concentration of 5-HT resulted in an increase in the apparent dissociation constant for histamine (Fig. 2*A*), indicating that the two ligands occupy the same or overlapping binding sites. A similar result was obtained when monotonin was titrated with 5-HT in the presence of an excess, fixed concentration of histamine (Fig. 2*B*). No binding of norepinephrine or dopamine could be observed under the same conditions as used for 5-HT for each protein (results not shown). This indicates specific binding of their respective biogenic amines and suggests that these molecules will also be their physiological targets. Biological assay of the recombinant proteins using guinea pig ileum and rat uterus indicated that recombinants gave similar responses as was measured for the wild-type proteins (results not shown).

Biological Significance of Monomine and Monotonin—If it is assumed that only 50% of monomine is secreted during a

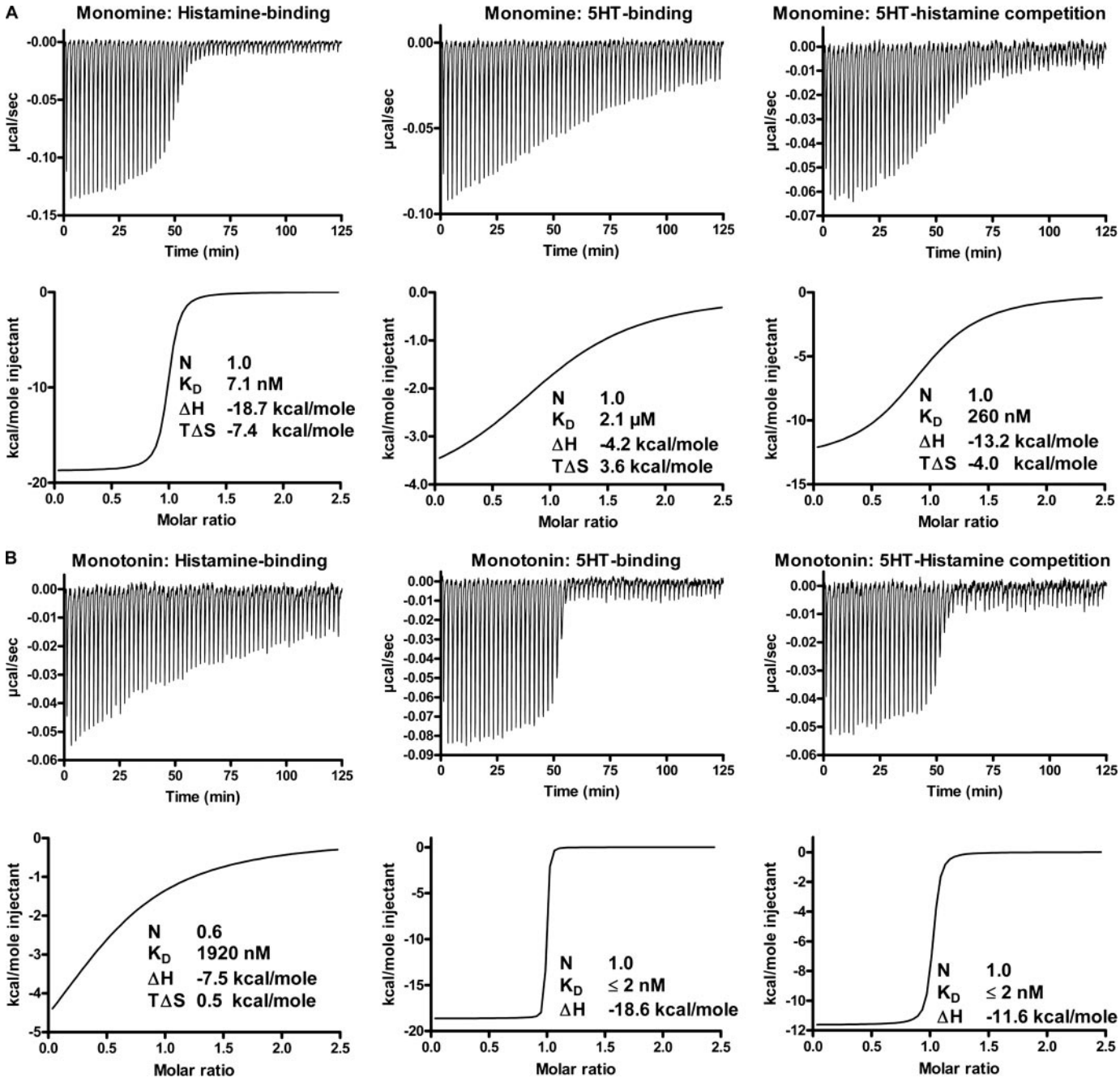

FIGURE 2. **Binding of biogenic amines by monomine and monotonin measured by microcalorimetry.** *A*, histamine binding was measured using 3 μM monomine and 30 μM histamine. 5-HT binding was measured using 10 μM monomine and 100 μM 5-HT, respectively. Competition of 5-HT and histamine was measured by preincubating 3 μM monomine with 100 μM 5-HT and titrating with 30 μM histamine. *B*, histamine binding was measured using 5 μM monotonin and 50 μM histamine. 5-HT binding was measured using 2 μM monotonin and 20 μM 5-HT. Competition of 5-HT and histamine was measured by preincubating 2 μM monotonin with 50 μM histamine and titrating with 20 μM 5-HT. Also indicated are thermodynamic parameters derived for each titration that includes the stoichiometry of binding (*N*), dissociation constant (K_D), change in enthalpy (ΔH), and change in entropy ($T\Delta S$) upon binding. K_D values less or equal than two have *c* values not in the 1–1000 range that can be accurately measured by isothermal titration calorimetry (43).

feeding session and that the feeding site is between 10 and 50 μl, concentrations of 2–15 μM may be reached. Applying the same argument, monotonin may reach concentrations of 1–5 μM in the feeding site. In both cases this is well within the concentrations that histamine and 5-HT need (1–10 μM) to attain saturation of their physiological receptors (33). Given the high affinity of AM-10 and AM-38 for histamine and 5-HT, respectively, this would suggest that they will be able to function as effective scavengers of histamine and 5-HT at the feeding site, thereby inhibiting inflammatory responses.

Monomine Is Homologous to Arg r1—The most closely related protein sequence retrieved in BLAST data base searches using monomine as the query is Arg r1, a potent allergen from the pigeon tick *Argas reflexus* (34). Monomine and Arg r1 show 47% sequence identity, indicating that they may be orthologous. Phylogenetic analyses of the tick lipocalins support orthology of monomine and Arg r1 and indicate

TABLE 2

Thermodynamic parameters for binding of histamine, 5-HT, or tryptamine by various *Argas* and *Ixodes* lipocalins and binding pocket mutants

Protein	n^a	ΔH^b	$T\Delta S^b$	$K_D{}^c$	ΔG^b
Histamine					
Monomine	1.0	−18.7 ± 0.1	−7.4	7.1 ± 1.0	−11.3
Monomine_Y21L	0.8	−4.1 ± 0.5	3.3	4484 ± 859	−7.4
Monomine_Y51L	1.0	−14.3 ± 0.2	−6.4	2159 ± 76	−7.9
Monomine_Y51F	1.0	−11.1 ± 0.2	−3.6	3580 ± 184	−7.5
Monomine_S83L	1.0	−16.7 ± 0.1	−8.0	535 ± 15	−8.7
Monomine_D94L	NB[d]	NB[d]	NB[d]	NB[d]	NB[d]
Monomine_E103L	1.2	−13.6 ± 0.6	−4.6	309 ± 11	−9.0
Monomine_W105L	1.1	−13.9 ± 0.2	−5.6	1096 ± 60	−8.3
rArg r1	1.0	−19.0 ± 0.1	−7.9	10.3 ± 1.2	−11.1
Monotonin	0.6	−7.5 ± 1.5	0.5	1920 ± 446	−8.0
Monotonin_F55Y	1.0	−7.6 ± 0.1	0.6	1300 ± 64	−8.2
Monotonin_D101L	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
rAM−182	1.2	−15.3 ± 1.0	−6.2	246 ± 12	−9.1
rIS−14	1.0	−14.9 ± 0.7	−6.0	427 ± 66	−8.9
rIS−15	0.9	−12.7 ± 0.3	−4.2	746 ± 66	−8.5
5-HT					
Monomine	1.0	−4.2 ± 0.2	3.6	2180 ± 277	−7.8
Monomine_Y51L	0.9	−11.2 ± 0.4	−3.9	4980 ± 339	−7.3
Monomine_Y51F	1.1	−13.4 ± 0.1	−4.2	249 ± 7	−9.2
rArg r1	1.0	−10.6 ± 0.1	−1.7	407 ± 25	−8.9
Monotonin	1.0	−18.6 ± 0.1		≤2[c]	≤−12.1
Monotonin_F55Y	1.0	−18.7 ± 0.1		≤2[c]	≤−12.1
Monotonin_D101L	NB[d]	NB[d]	NB[d]	NB[d]	NB[d]
rAM−182	1.3	−20.3 ± 0.1		≤2[c]	≤−12.1
rIS−14	1.0	−16.0 ± 0.2		≤2[c]	≤−12.1
rIS−15	1.0	−19.0 ± 0.2		≤2[c]	≤−12.1
Tryptamine					
Monomine	0.7	−7.8 ± 0.3	1.4	227 ± 39	−9.2
Monomine_Y51L	1.0	−15.3 ± 0.1	−4.2	9.6 ± 0.8	−11.1
Monomine_Y51F	1.0	−12.3 ± 0.1	−1.8	23.9 ± 2.4	−10.5
Monomine_D94L	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
rArg r1	1.1	−9.3 ± 0.1	1.4	18.8 ± 3.2	−10.7
Monotonin	1.0	−15.9 ± 0.1		≤2[c]	≤−12.1
Monotonin_F55Y	1.0	−16.9 ± 0.1		≤2[c]	≤−12.1
Monotonin_D101L	0.7	−4.3 ± 1.0	2.9	6670 ± 1690	−7.2
rAM−182	1.0	−20.1 ± 0.1		≤2[c]	≤−12.1
rIS−14	0.9	−14.0 ± 0.2		≤2[c]	≤−12.1
rIS−15	1.0	−18.0 ± 0.2	−6.3	3.6 ± 0.7	−11.7

[a] Values indicate the stoichiometry of ligand binding.
[b] Values are given as kcal/mol. $\Delta G = -RT \ln K$, where K is the association equilibrium constant. Values for $T\Delta S$ ($=\Delta H - \Delta G$) are omitted in cases where the equilibrium constant could not be precisely determined.
[c] Values are given as nanomolar. A value of ≤ 2 indicates that a protein concentration of 2 μM was used and the measured K_D value was less than or equal to 2 nM (43). Standard errors were calculated from regression residuals.
[d] Mutant tested at 50 μM protein and 500 μM ligand.
[e] Mutant tested at 10 μM protein and 100 μM ligand.

that monotonin groups as a sister clade with other *Argas* lipocalins (18).

To confirm that monomine and Arg r1 are functional orthologs, we produced Arg r1 in *E. coli* (rArg r1) and tested the purified protein for binding with histamine, 5-HT, and other biogenic amines (Table 2). rArg r1 bound histamine with similar affinity to monomine ($K_D \sim 10$ nM) and bound 5-HT with lower affinity ($K_D \sim 407$ nM). 5-HT also competed with histamine as a ligand, in a manner similar to that seen with monomine (results not shown). Bioassays also gave results similar to those obtained with monomine and suggest that the scavenging of histamine as well as 5-HT could be physiological functions for this allergen (results not shown). Together, these results strongly suggest that Arg r1 is an ortholog of monomine and also suggest that monotonin is paralogous to this group.

The Structure of Monomine—The crystal structure of monomine was determined by single wavelength anomalous dispersion methods using the selenomethionine derivative of the protein. The structure of the wild-type protein without ligands and in complex with histamine was also determined by difference Fourier methods (Fig. 3, *A* and *B*, and Table 1). The unliganded structure and histamine complex were nearly identical with a root mean square deviation (for all C-α positions) of 0.15 Å, suggesting that ligand binding does not induce significant conformational changes.

Monomine has the eight-stranded antiparallel β-barrel structure characteristic of the lipocalin protein family. Its structure is similar to that of OMC1, a recently described complement inhibitor from a soft tick *Ornithodoros moubata* (root mean square deviation = 1.32 Å for all aligned C-α positions) and also to the hard tick histamine-binding protein FS-HBP2 (root mean square deviation = 1.97 Å) (3, 35). The monomine structure is stabilized by three disulfide bonds linking Cys-4 near the N terminus with Cys-120 in the second α-helical region, Cys-93 of β-strand G with Cys-121 of the second α-helix, and Cys-34 of β-strand B with Cys-142 near the C terminus. This disulfide bonding pattern is also seen in OMC1, and sequence alignments suggest that it occurs in other soft tick lipocalins (10, 35).

FS-HBP2 differs functionally from monomine in binding two molecules of histamine at distinct binding sites (3), whereas monomine binds a single molecule of histamine. Comparison of the two structures reveals the basis for this observation (Fig. 3, *C* and *D*). The high affinity histamine-binding site (H-site) of FS-HBP2 sits close to the opening of the β-barrel and is formed by the folding of mainly loop 1 (connecting β-strands A and B) so that Tyr-36 and Asp-39 can interact with the amine group of histamine (3). In monomine this site is not present. Loop 1 is shorter and does not fold over the opening of the β-barrel, whereas loops 5 and 7 are also shorter leading to a wide opening into the cavity of the β-barrel (Fig. 3*C*).

Structure of the Binding Pocket of Monomine—In the monomine-histamine complex, the ligand is oriented similarly to that found in the low affinity lower (L)-site of FS-HBP2 (Fig. 3*C*). The amine group from the ligand forms hydrogen bonds or ionic interactions with the carbonyl group of Ser-12 and the carboxylate of Asp-94, which correspond to Ser-20 and Asp-120 in FS-HBP2, respectively (Fig. 4). The amino group also contacts the side chain of Tyr-21 in a manner similar to Tyr-29 in the structure of FS-HBP2 (Figs. 3*E* and 4). The positively charged amino group is positioned 3.3 Å from the centroid of the aromatic ring of Tyr-21 making this a cation-π interaction (Fig. 3*E*), which would contribute strongly to the overall ligand binding energy (36).

In addition to the hydrogen bonding residues listed above, a number of hydrophobic side chains line the binding pocket of monomine and make van der Waals contact with the ligand. These include Val-37, Ile-76, Val-96, and Trp-105 (Fig. 4*A*).

Although monomine and FS-HBP2 bind the aliphatic side chain of histamine in much the same manner, they bind the imidazole portion of the molecule differently. In FS-HBP2, the carboxylate of Asp-24 hydrogen bonds to both the amine group as well as nitrogen ND1 of the imidazole ring, whereas Tyr-100 hydrogen bonds with the ND2 atom of the imidazole ring (Fig. 4*B*). No water molecules participate in the interaction with histamine. In the case of monomine, Tyr-51 forms a hydrogen bond with the ND1 atom of the imidazole ring and with the

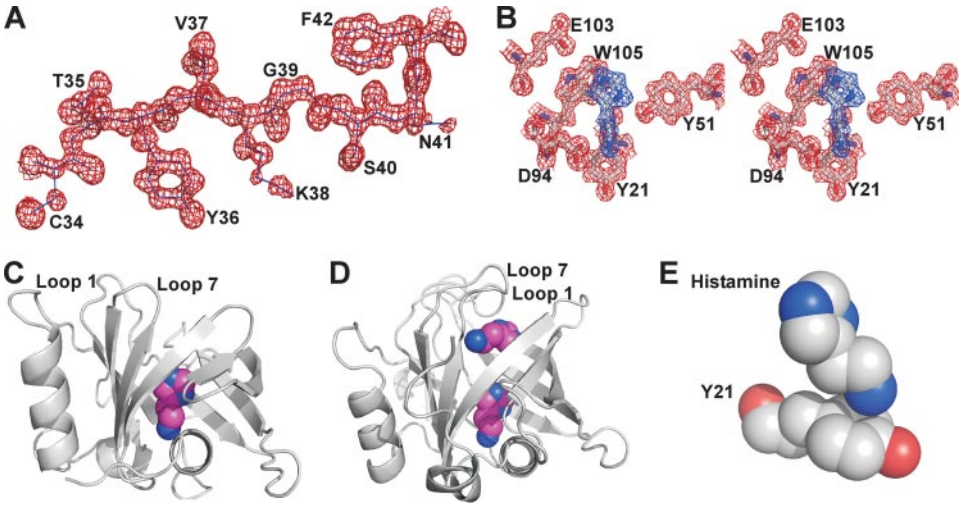

FIGURE 3. **Structures of monomine and FS-HBP2 in complex with histamine.** *A*, $2F_o - F_c$ electron density of wild-type monomine structure without ligands. *B*, $2F_o - F_c$ electron density for the ligand binding pocket of monomine in complex with histamine. *C*, monomine with the single histamine presented as a space filling model viewed down the axis of the N-terminal helix. *D*, FS-HBP2 in complex with 2 histamine molecules presented as space-fill models viewed down the axis of the N-terminal helix. Indicated are loops 1 and 7 located at the open end of the β-barrel. *E*, the cation-π interaction of the aliphatic amino group of histamine with Tyr-21 as indicated by space filling models.

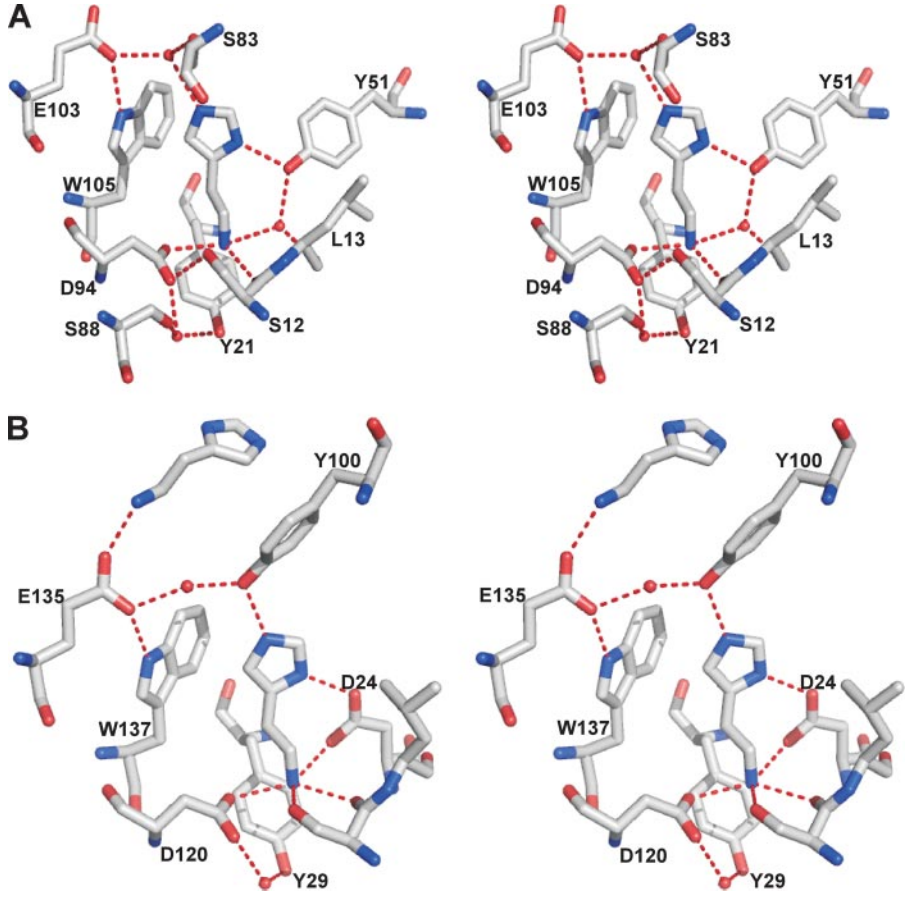

FIGURE 4. **Interaction of monomine and FS-HBP2 with histamine.** *A*, a stereo image of the ligand binding pocket of monomine in complex with histamine. *B*, a stereo image of the L-site of FS-HBP2 in complex with histamine. Also indicated is the histamine found in the H-site. Hydrogen bonds are indicated with *red* and *magenta dashed lines*. Water molecules are shown as *red spheres*.

amine group via a water molecule that is stabilized by hydrogen bonding with the carbonyl oxygen of Leu-13. The ND2 atom of the imidazole ring forms a hydrogen bond with a water molecule, which is stabilized by Ser-83 and Glu-103 (Fig. 4*A*).

Determining the Relative Contribution of Binding Site Residues in the Interaction with Histamine—Site-directed mutagenesis was used to evaluate the contributions of individual residues involved in ligand binding for monomine. As expected, residues forming hydrogen bonds with histamine contribute most significantly to the binding interaction (Table 2). Mutation of Asp-94 to leucine eliminates the hydrogen bond or salt bridge stabilizing the aliphatic amino group of histamine. This mutant showed no detectable binding even at elevated ligand concentrations. Likewise, mutation of Tyr-51 to leucine removes the hydrogen bond between the phenolic hydroxyl of tyrosine and nitrogen ND1 of histamine. This change resulted in a 303-fold decrease in affinity. The importance of the cation-π interaction between the amino group of histamine and Tyr-21 was demonstrated with the Y21L mutant that reduced the affinity for histamine 628-fold due to large, unfavorable changes in both the binding enthalpy and entropy (Table 2).

Two residues, Ser-83 and Glu-103, stabilize the imidazole group of histamine through hydrogen bonds involving bridging water molecules. Mutation of Ser-83 to leucine resulted in a 75-fold decrease in binding affinity, whereas the E103L mutation gave a 43-fold decrease, indicating that the hydrogen bonding network involving ordered water in the binding pocket contributes significantly to ligand binding.

The bulky side chain of Trp-105 forms one side of the ligand-binding pocket and contacts the imidazole portion of histamine. Mutation of this residue to leucine results in a large reduction in affinity (153-fold decrease), which may be attributable to changes in the topology and hydrophobicity of the binding site.

Structure of AM-182 in Complex with 5-HT—Attempts to crystallize monotonin were unsuccessful, but AM-182, a closely related paralog from *A. monolakensis,* was expressed and crystallized readily. As in monotonin, this protein possesses phenyl-

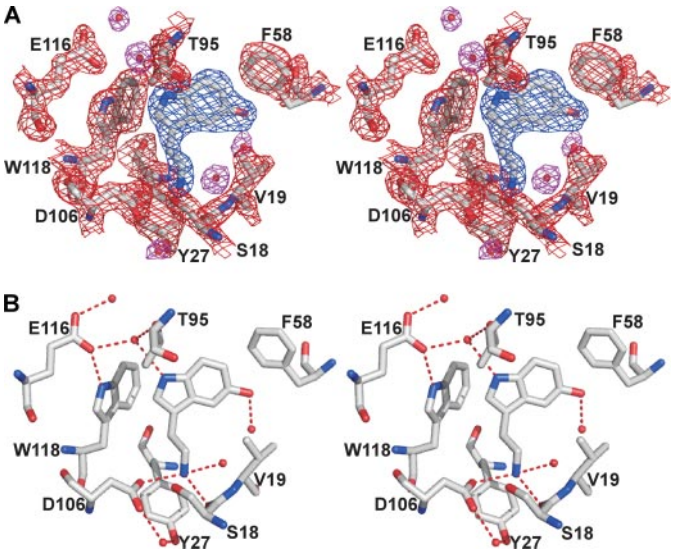

FIGURE 5. **Interaction of AM-182 and 5-HT.** *A*, stereo view of the $2F_o - F_c$ electron density map of the ligand-binding pocket of AM-182 in complex with 5-HT. *B*, stereo view of the ligand-binding pocket of AM-182 in complex with 5-HT. Hydrogen bonds are indicated with *red dashed lines* and water molecules as *red spheres*.

alanine at position 58 and, like monotonin, bound 5-HT with high affinity (0.2 nM). The protein differs from monotonin in that it also binds histamine with detectable affinity (246 nM) (Table 2).

The structure of AM-182 is similar to that of monomine with a root mean square deviation of all Cα positions of 1.11 Å. The 5-HT ligand is oriented with the pyrrole portion of the indole nucleus occupying a nearly identical position to the imidazole ring of histamine in monomine. The amino group of the ligand forms direct hydrogen bonds or ionic interactions with the side chain of Asp-106 and the carbonyl of Ser-18 (Fig. 5).

As in monomine, Tyr-27 participates in a cation-π interaction with the protonated amino group of 5-HT. In a similar fashion to Leu-16 of monomine, the carbonyl group of Val-19 also interacts with the amino group of 5-HT via a bridging water molecule. Other protein-ligand contacts include a hydrogen bond between the indole nitrogen of 5-HT and the side chain of Thr-95 as well as a hydrogen bond between the indole nitrogen and the carboxylate of Glu-116 via a bridging water molecule. This is similar to the interaction of ND2 of histamine with the homologous Ser-83 and Glu-103 residues of monomine.

Tyr-51 of monomine is substituted by Phe-58 in AM-182. The side chain of this residue is rotated away from the ligand relative to its position in monomine, creating additional space in the binding pocket for the larger hydroxyindole portion of 5-HT. In this orientation, the hydroxyl group of 5-HT forms a hydrogen bonding network with the carbonyl group of Leu-44 via an intervening water molecule. Tryptamine, which lacks a phenolic hydroxyl group, showed a 2.7 kcal/mol loss of binding enthalpy as measured by isothermal titration calorimetry (Table 2), demonstrating the importance of this hydrogen bonding interaction.

Specificity of Monomine and Monotonin—The major structural difference in the binding pockets of monomine and AM-182 is in the identity and positioning of a single aromatic residue identified as Phe-58 in AM-182 and Tyr-51 in monomine. In monomine, the hydroxyl group of Tyr-51 forms a hydrogen bond with the imidazole ring of histamine, whereas in AM-182, the side chain of Phe-58 is rotated away from the ligand, resulting in a larger binding pocket (Fig. 5). We tested the importance of this residue as a specificity determinant by mutating Tyr-51 of monomine to phenylalanine. The expectation was that histamine binding would be reduced and 5-HT binding enhanced. This proved to be the case, as histamine affinity was decreased 500-fold due to loss of hydrogen bonding interactions with the imidazole ring, whereas the affinity for 5-HT increased 8.7-fold, presumably due to a loss of steric hindrance in the interaction of the hydroxyindole ring with the protein (Table 2).

Interestingly, the mutation of Phe-58 in monotonin to tyrosine did not have the inverse effect. Neither 5-HT nor histamine binding were significantly affected. This suggests that factors other than the side chain structure of residue 58 are playing a role in specificity.

Conservation of Binding Site Residues in Argas Lipocalins—Recently, the salivary gland transcriptomes from a number of tick species have been described and include the hard tick *I. scapularis* and the soft ticks, *A. monolakensis* and *Ornithodoros parkeri* (11, 18, 37). The transcriptomes of all tick species are rich in lipocalins and current estimates on tick lipocalin sequences present in the non-redundant data base approaches 300.[3] In most cases, these lipocalins show low sequence identity. In this regard it can be observed that most tick-derived lipocalins are identified by a "His_binding" signature in the Pfam data base (PF02098) and many have been annotated as being histamine-binding proteins. Given the low sequence identities and similarities observed for tick lipocalins, it stands to reason that not all of these annotated proteins will have biogenic amine-binding functions. As such, we attempted to identify a motif that can be used to classify lipocalins as potential biogenic amine binders.

The conservation of the ligand-binding aspartate in monomine (Asp-94), monotonin (Asp-101), and FS-HBP2 (Asp-120) suggests that it could be a marker for biogenic amine binding in general. In addition, this residue is located next to a cysteine that forms a conserved disulfide bond with a cysteine that brackets the conserved ELW sequence, yielding a conserved motif (biogenic amine-binding motif, BAB motif) that contains both functional as well as structural determinants (Fig. 6). The more degenerated pattern CD[VIL]X(7,17)EL[WY]X(11,30)C was used to search available databases for possible histamine/5HT-binding proteins. Given the low sequence conservation among lipocalins, this motif could be useful to identify other potential biogenic amine-binding proteins in ticks. As such, the conserved aspartate will indicate general binding activity, but will not give any information on the specificity of binding, as this is mainly conferred via residues that interact with the ring moieties of the biogenic amines.

[3] B. Mans, personal observation.

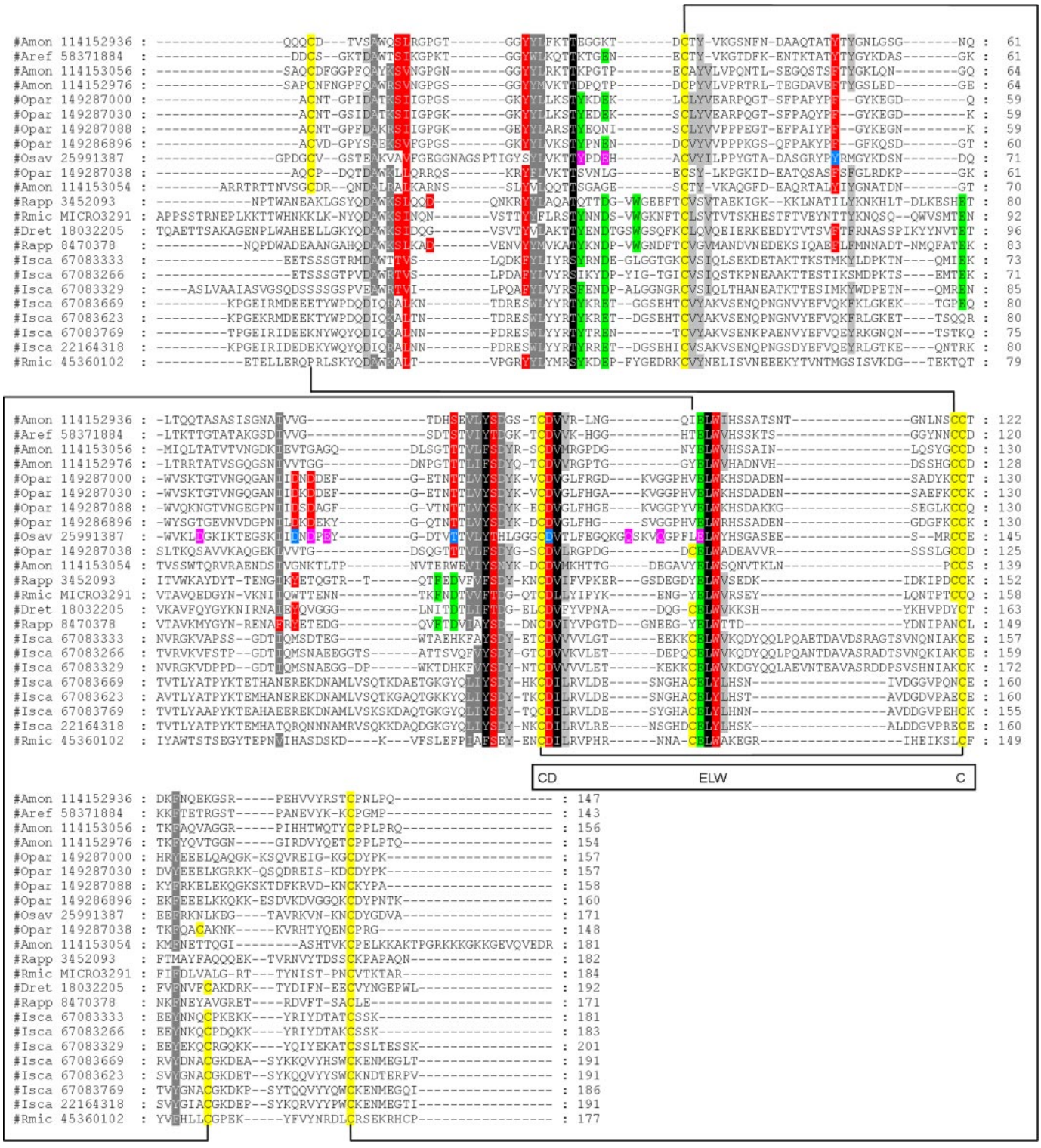

FIGURE 6. **Tick biogenic amine-binding proteins.** Alignment of tick lipocalins that possess the BAB motif. Conserved disulfide bonds as derived from the structures of AM-10 and FS-HBP2 are indicated with *lines*. Residues involved in histamine binding in the L-site of FS-HBP2 and AM-10 are colored in *red*, whereas residues involved in histamine binding in the H-site of FS-HPB2 are colored in *green*. Residues mutated for TSGP1 are colored in *blue* (L-site) and *purple* (H-site). The BAB motif is *boxed*.

Analysis of all tick lipocalins currently in the data base indicates that 22 possess the BAB motif (Fig. 6). Of these, biogenic amine-binding function has been confirmed for the hard ticks, *R. appendiculatus* and *Dermacentor reticulatus* (3, 9) and in this study for the soft ticks *A. monolakensis* and *A. reflexus*. No data are, however, available on the biogenic amine-binding capabilities of lipocalins from prostriate ticks (*Ixodes* sp) and the *Ornithodoros* genus of the soft tick family.

Biogenic Amine-binding Proteins in the Hard Tick Genus Ixodes—Six different genes from *I. scapularis* possess the biogenic amine-binding motif. Phylogenetic analysis indicates that they group into two distinct but well supported clades (Fig. 7).

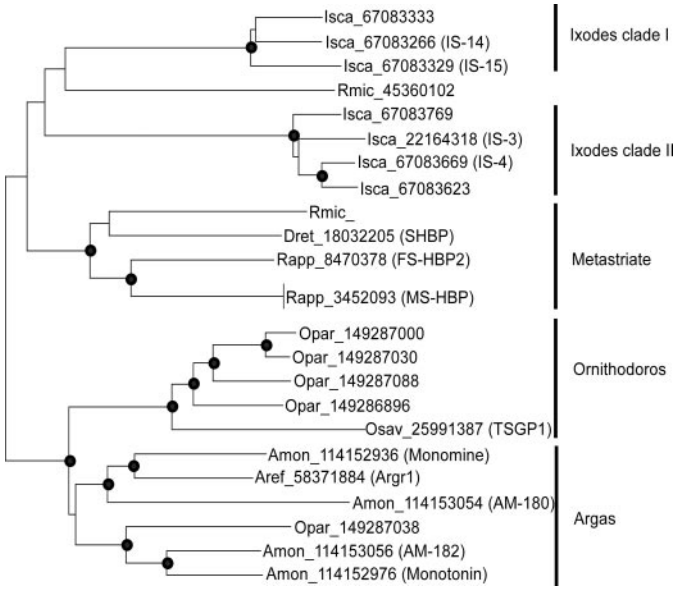

FIGURE 7. **Neighbor-joining analysis of lipocalins that possess the BAB motif.** Branch support (10,000 bootstraps) above 80% is indicated with *black circles*. Sequence names include 4 letter species abbreviations: *I. scapularis* (*Isca*), *R. appendiculatus* (*Rapp*), *D. reticulatus* (*Dret*), *O. parkeri* (*Opar*), *O. savignyi* (*Osav*), *A. reflexus* (*Aref*), and *A. monolakensis* (*Amon*), followed by their GenBank™ accession number and common name.

We produced protein for two representatives from each clade and included for clade I, IS-3 and IS-4 and for clade II, IS-14 and IS-15, respectively.

IS-3 and IS-4 failed to bind histamine or 5-HT (results not shown). This is most likely due to the loss of the amine stabilizing cation-π interaction through substitution of Tyr-21 for serine, or by substitution of Trp-105 for tyrosine in the binding site (Fig. 6). IS-14 and IS-15 bound 5-HT with high affinity (~2 nM) with a stoichiometry that indicates a single binding site (Table 2). Histamine was also bound, but at lower affinity ($K_D \sim 400$–800 nM), which would probably not be physiologically significant. Competition of histamine with 5-HT indicates that they bind to the same site (results not shown). Bioassay confirmed the results from isothermal microcalorimetry, in that contraction of rat uterus induced by 5-HT was inhibited by IS-14 and IS-15, whereas contraction of guinea pig ileum by histamine was not (results not shown).

Biogenic Amine-binding Proteins in the Genus Ornithodoros—Salivas from the soft tick genus *Ornithodoros* are also rich in lipocalin proteins (10, 37). TSGP1 was previously identified as the most abundant lipocalin in the salivary glands of *Ornithodoros savignyi* (10) and its sequence contains the BAB motif described above. We produced this protein in recombinant form and found it to bind 5-HT ($K_D \sim 6$ nM) with a stoichiometry of ~1, suggesting the presence of a single binding site (Fig. 8*B*; Table 3). The protein was also found to bind histamine, but in this case the data were best fitted to a two-binding site model (Fig. 8*A*). One site shows high affinity for the histamine ligand ($K_D \leq 3$ nM), and the other site has lower affinity ($K_D \sim 680$ nM) (Table 3).

Titration of TSGP1 with histamine (60 μM final concentration) in the presence of saturating (60 μM) 5-HT eliminated histamine binding at the low affinity binding site (Fig. 8*C*). Only the high affinity ($K_D \leq 3$ nM) binding site was observable, suggesting that 5-HT was bound at the low affinity histamine-binding site.

To confirm this, TSGP1 was titrated with an approximately equimolar mix of 5-HT and histamine. The data were fit to a single binding site model (Fig. 8*D*), and the apparent enthalpy change ($\Delta H \sim -27$ kcal/mol of total ligand) was found to be approximately equal to the sum of that seen for 5-HT binding alone and that obtained for histamine binding at the high affinity site ($\Delta H \sim -31$ kcal/mol) and the apparent dissociation constant was ≤3 nM. This result give additional support to the idea that TSGP1 possesses two binding sites with one showing specificity for histamine and a second being highly selective for 5-HT.

Site-directed mutagenesis based on sequence alignments of TSGP1 with monomine, monotonin, and the two-binding site histamine-binding protein FS-HPB2 was used to probe the structure and specificity of the two ligand-binding sites. A number of residues found in the upper ligand-binding site of FS-HBP2 are conserved in TSGP1, as are residues found in the lower binding sites of FS-HBP2, monomine and monotonin (Fig. 6, Table 3).

When Asp-110 of TSGP1 was mutated to leucine, binding of 5-HT and the low affinity binding of histamine were completely abolished, whereas the high affinity binding of histamine was not affected (Table 3). This residue corresponds to the aspartic acid in the lower binding pocket of FS-HBP2, which forms an ionic interaction with the amine group of the ligand. This result indicates that the lower binding site of TSGP1, corresponding to the binding sites of monomine and monotonin, is specific for 5-HT. Mutation of two other residues (Y61L and T98L) also predicted to be of importance in the lower binding site supported this conclusion (Table 3).

Mutants predicted to participate in the upper ligand binding pocket were found to almost exclusively affect histamine binding. These include E40L, D88L, D90L, and E129L. Of these, D88L was the only mutant that also affected 5-HT binding. This residue corresponds to Tyr-100 of FS-HBP2, a residue that plays a role in both the upper and lower ligand-binding pockets of this protein.

Of the residues affecting histamine binding, Glu-40 of TSGP1 corresponds to Asp-39 of FS-HBP2, a residue that interacts with the ND1 atom of the imidazole ring of histamine. Glu-129 of TSGP1 corresponds to Glu-135 of FS-HBP2, a residue that interacts with the amine group of histamine in the upper binding site, and Asp-88 aligns with Tyr-100 of FS-HBP2, a residue involved with binding at both ligand-binding sites.

Asp-90 of TSGP1 did not align with any residue that contacts the ligand in the upper binding pocket of FS-HBP2, but due to its conservation in all *Ornithodoros* lipocalins (Fig. 6) and its close proximity to Asp-88 was also mutated to leucine. This mutation had a large effect on the high affinity binding of histamine, suggesting that the upper ligand-binding sites in FS-HBP2 and TSGP1 may differ significantly in structure.

In summary, the mutation studies with TSGP1 strongly support the lower site as being specific for 5-HT and the upper site being specific for histamine. It should be noted,

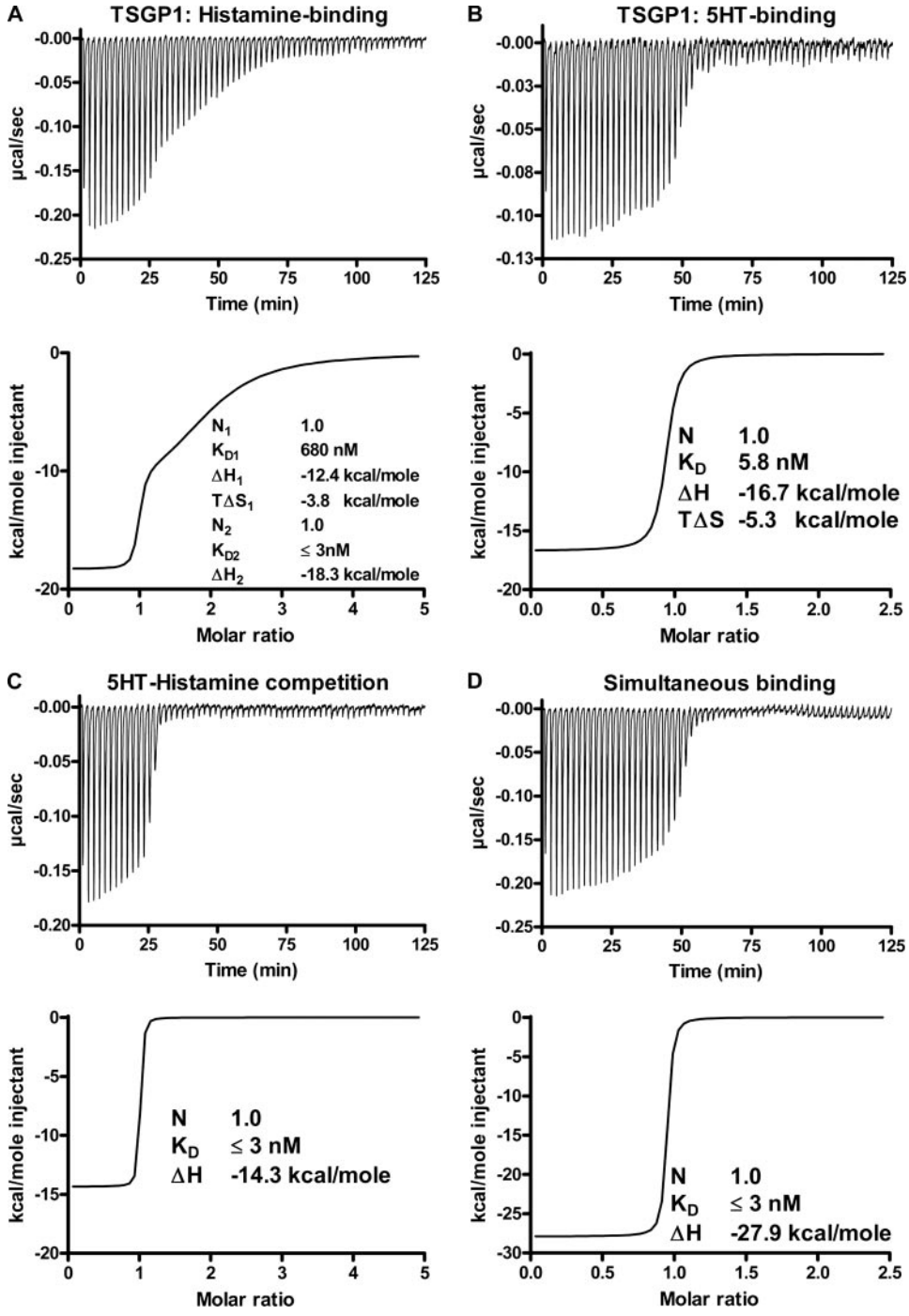

FIGURE 8. **Binding of biogenic amines by TSGP1 as measured by microcalorimetry.** *A*, histamine binding was measured using 3 μM TSGP1 and 60 μM histamine. *B*, 5-HT binding was measured using 3 μM TSGP1 and 30 μM 5-HT. *C*, competition of 5-HT and histamine were measured by preincubating 3 μM TSGP1 and 60 μM 5-HT and titrating with 60 μM histamine. *D*, simultaneous binding of 5-HT and histamine to TSGP1 were measured by titrating 3 μM TSGP1 with 30 μM 5-HT and 30 μM histamine at the same time. Also indicated are thermodynamic parameters derived for each titration that includes the stoichiometry of binding (*N*), dissociation constant (K_D), change in enthalpy (ΔH), and change in entropy ($T\Delta S$) upon binding. K_D values less or equal than 3 have *c* values not in the 1–1000 range that can be accurately measured by isothermal titration calorimetry (43).

Evolution of Biogenic Amine-binding Functions in Tick Lipocalins—The results thus far generated on the specificity of tick lipocalins for biogenic amines indicate that the L-site seem to be conserved in regard to 5-HT binding and would support it as being an ancestral site. This implies that histamine binding evolved independently at the L-site in *Argas* and *Rhipicephalus* genera.

Parsimonious arguments would suggest that the high affinity histamine-binding H-site evolved in the ancestral lineage. This implies loss of the site in both *Argas* and *Ixodes* genera. On the other hand, the H-site could have evolved independently in both *Ornithodoros* and rhipicephaline lineages. The mutagenesis data on TSGP1 suggest that the binding site residues and hence mechanism of binding is not completely conserved between TSGP1 and FS-HBP2. As such, the possibility that this site evolved more than once cannot be discarded.

The final possibility is that an ancestral lipocalin existed that bound a different biogenic amine, such as tyramine or octopamine, as housekeeping function. In such a case, the mechanism of interaction with the aliphatic amine group would have been conserved, but interaction with the aromatic ring structure would have differed. Gene duplication after divergence of the main tick families then led to independent evolution of histamine and the 5-HT binding function. This could be possible if it is found that the mechanisms of 5-HT binding differ between hard and soft ticks. It should be noted that this alternative scenario fits with independent adaptation of the major tick families to a blood-feeding lifestyle (38, 39).

Biogenic Amine-binding Proteins as Allergens—Many of the molecules that have been identified to date as being biogenic-amine scavengers are also allergens. This includes tick lipocalins Arg r1 and TSGP1 homologs and D7 proteins from mosquitoes (34, 40–42). It should not be surprising that these molecules will be allergenic, given the fact that they need to be highly abundant in

that this same partitioning of ligand specificities has been proposed for a 5-HT/histamine-binding protein found in saliva from the hard tick *D. reticulatus* (9). The data also suggest that some degree of similarity in binding mechanism exists between hard and soft tick lipocalins for the upper site.

TABLE 3

Thermodynamic parameters for binding of histamine, 5-HT, or tryptamine by TSGP1

Protein	n[a]	ΔH[b]	$T\Delta S$[b]	K_D[c]	ΔG[b]
Histamine					
TSGP1	1.0	−18.3 ± 0.1		≤3[c]	≤−11.8
L-site[d]	1.0	−12.4 ± 0.6	−3.8	680 ± 72	−8.6
Upper site					
TSGP1_Y37L	1.0	−12.2 ± 0.1		≤10[c]	≤−11.1
L-site[d]	1.0	−9.9 ± 0.4	−1.2	538 ± 44	−8.7
TSGP1_E40L	1.0	−19.9 ± 0.5	−11.8	1390 ± 89	−8.1
L-site[d]	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
TSGP1_D76L	1.0	−17.3 ± 0.1		≤10[c]	≤−11.1
L-site[d]	1.0	−11.4 ± 0.2	−2.5	380 ± 22	−8.9
TSGP1_D88L	1.0	−16.2 ± 0.1	−6.6	128 ± 8.9	−9.6
L-site[d]	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
TSGP1_D90L	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
L-site[d]	1.0	−13.9 ± 0.2	−5.2	602 ± 36	−8.7
TSGP1_E92L	1.0	−15.5 ± 0.2	−5.0	23 ± 6.0	−10.5
L-site[d]	1.1	−12.2 ± 0.4	−3.4	439 ± 17	−8.8
TSGP1Q120L	1.0	−18.1 ± 0.1		≤3[c]	≤−11.8
L-site[d]	1.1	−11.4 ± 0.2	−2.7	568 ± 42	−8.7
TSGP1_Q124L	1.0	−18.2 ± 0.1		≤3[c]	≤−11.8
L-site[d]	1.1	−12.6 ± 0.2	−4.0	637 ± 33	−8.6
TSGP1_E129L	1.0	−16.0 ± 0.1	−7.2	442 ± 20	−8.8
Lower site					
TSGP1_Y61L	0.9	−15.9 ± 0.2	−7.8	1540 ± 58	−8.1
L-site[d]	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
TSGP1_T98L	1.0	−12.7 ± 0.2	−4.3	926 ± 55	−8.4
L-site[d]	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
TSGP1_D110L	1.0	−15.2 ± 0.7		≤10[c]	≤−11.1
L-site[d]	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
5-HT					
TSGP1	0.9	−16.7 ± 0.2	−5.3	5.8 ± 1.5	−11.4
Lower site					
TSGP1_Y61L	0.9	−18.0 ± 0.1	−8.3	94 ± 5.0	−9.7
TSGP1_T98L	1.0	−16.3 ± 0.1	−6.2	55 ± 2.0	−10.1
TSGP1_D110L	NB[e]	NB[e]	NB[e]	NB[e]	NB[e]
Upper site					
TSGP1_Y37L	1.0	−17.9 ± 0.1		≤10[c]	≤−11.1
TSGP1_E40L	1.0	−16.7 ± 0.1		≤10[c]	≤−11.1
TSGP1_D76L	1.0	−16.5 ± 0.1		≤10[c]	≤−11.1
TSGP1_D88L	1.0	−13.8 ± 0.1	−3.9	67 ± 3.9	−9.9
TSGP1_D90L	1.0	−16.8 ± 0.1		≤10[c]	≤−11.1
TSGP1_E92L	1.0	−17.3 ± 0.1		≤10[c]	≤−11.1
TSGP1_Q120L	1.0	−14.9 ± 0.1		≤10[c]	≤−11.1
TSGP1_Q124L	1.0	−15.9 ± 0.1		≤10[c]	≤−11.1
TSGP1_E129L	1.0	−16.6 ± 0.1	−5.5	11 ± 0.6	−11.1

[a] Values indicate the stoichiometry of ligand binding.
[b] Values are given as kcal/mol. $\Delta G = -RT \ln K$, where K is the association equilibrium constant. Values for $T\Delta S$ ($=\Delta H - \Delta G$) are omitted in cases where the equilibrium constant could not be precisely determined.
[c] Values are given as nanomolar. A value of ≤10 indicates that a protein concentration of 10 μM was used and the measured K_D value was less than or equal to 10 nM, while a value of ≤3 indicates a protein concentration of 3 μM and a K_D value of less than or equal to 3 nM (43). Standard errors were calculated from regression residuals.
[d] The L-site refers to the second low affinity histamine-binding site.
[e] Mutant tested at 50 μM protein and 500 μM ligand.

saliva to act as scavengers of biogenic amines. This has obvious negative implications for their use in the pharmaceutical industry (15).

Conclusion—The binding mechanisms that confer ligand specificity for histamine differs between soft and hard ticks, implying that they evolved this function independently during adaptation to a blood-feeding lifestyle. Even so, the mechanism of interaction with the aliphatic amine group is conserved, suggesting that all biogenic amine-binding lipocalins from ticks evolved from a common biogenic amine-binding ancestor. Whether biogenic amine-binding evolved in this ancestral lipocalin as adaptive response to the vertebrate defense mechanisms of the host remain to be determined.

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