Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2

Structural and Inhibitory Effects of Hinge Loop Mutagenesis in Serpin-2 from the Malaria Vector *Anopheles gambiae*

Xin Zhang¹, David A. Meekins⁵, Chunju An¹,², Michal Zolkiewski³, Kevin P. Battaile⁴, Michael R. Kanost¹, Scott Lovell⁵, and Kristin Michel¹

From the ¹Division of Biology, Kansas State University, Manhattan, KS 66506, the ²Department of Entomology, College of Agriculture and Biotechnology, China Agricultural University, Beijing, China, the ³Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS 66506, ⁴IMCA-CAT, Hauptman-Woodward Medical Research Institute, APS Argonne National Laboratory, Argonne IL 60439, and the ⁵Protein Structure Laboratory, Del Shankel Structural Biology Center, University of Kansas, Lawrence, KS 66407

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To whom correspondence should be addressed: Kristin Michel, Kansas State University, Division of Biology, 267 Chalmers Hall, Manhattan, KS 66506, USA; Tel: 785-532-0161, Fax: 785-532-6653, email: kmichel@ksu.edu

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**Background:** Serpin-2 (SRPN2) is a key regulator of mosquito immunity and contains an inserted hinge region linked to activation in other serpins.

**Results:** Structure/function analyses of hinge mutations refute a hypothesized activation mechanism in SRPN2.

**Conclusion:** SRPN2 hinge insertion provides a thermodynamically stable conformation without restricting inhibitory capability.

**Significance:** To effectively utilize SRPN2 for vector control, its mode of action must be understood.

**ABSTRACT**

Serpin-2 (SRPN2) is a key negative regulator of the melanization response in the malaria vector *Anopheles gambiae*. SRPN2 irreversibly inhibits CLIPB9, which functions in a serine proteinase cascade culminating in the activation of pro-phenoloxidase (proPO) and melanization. Silencing of SRPN2 in *An. gambiae* results in spontaneous melanization and decreased lifespan and is therefore a promising target for vector control. The previously determined structure of SRPN2 revealed a partial insertion of the hinge region of the reactive center loop (RCL) into β sheet A. This partial hinge insertion participates in heparin-linked activation in other serpins, notably antithrombin III. SRPN2 does not contain a heparin binding site and any possible mechanistic function of the hinge insertion was previously unknown. To investigate the function of the SRPN2 hinge insertion, we developed three SRPN2 variants in which the hinge regions are either constitutively expelled or inserted and analyzed their structure, thermostability, and inhibitory activity. We determined that constitutive hinge expulsion resulted in a 2.7-fold increase in the rate of CLIPB9Xa inhibition, which is significantly lower than previous observations of allosteric serpin activation. Furthermore, we determined that stable insertion of the hinge region did not appreciably decrease the accessibility of the RCL to CLIPB9. Together, these results indicate the partial hinge insertion in SRPN2 does not participate in the allosteric activation observed in other serpins and instead represents a molecular trade-off between RCL accessibility and efficient formation of an inhibitory complex with the cognate proteinase.
Anopheles gambiae mosquitoes are dominant insect vectors for the most virulent species of human malaria parasites, Plasmodium falciparum in Africa (1-3). Malaria continues to be a devastating disease, responsible for over 800,000 deaths in 2013 mostly among children in sub-Saharan Africa (4). The lack of vaccines and substandard medical resources in infected areas, coupled with drug resistance, complicate successful treatment of infected patients (5,6). Vector control remains the foremost method for controlling the spread of malaria, but resistance to all four classes of insecticides has been reported in malaria vectors (4,7,8). This highlights the need for new insecticides that are less susceptible to the selective pressures that drive resistance. Theoretical studies have suggested that late life acting (LLA) insecticides that target females would be the most effective measure to impact vector populations without facilitating resistance (9,10). The serine proteinase inhibitor (serpin) SRPN2 is a particularly promising potential LLA insecticide (11,12). SRPN2 is a negative regulator of the mosquito melanization response, and depletion of SRPN2 in An. gambiae causes spontaneous melanization and significantly shorts the life span of adult female mosquitoes (11). Chemically targeting SRPN2 in field mosquito populations therefore shows promise in limiting the spread of malaria in endemic areas.

SRPN2 is part of a complex regulatory pathway that modulates the insect immune response (13-15). Insects lack an adaptive immune system and must rely solely upon innate immune reactions, including melanization, to combat infectious organisms (14). Melanization is employed to encapsulate and kill invading pathogens and is initiated upon pathogen detection (16). Recognition proteins in the insect hemolymph recognize non-self biomolecules and activate a clip-domain serine proteinase cascade (17-20). This cascade culminates in the activation of prophenoloxidase activating proteinases (PAPs), which convert prophenoloxidase (PPO) to phenoloxidase (PO) (21-23). PO hydroxylates monophenols to catechols and oxidizes catechols to quinones, which polymerize to form eumelanin (24,25). Although melanization is an efficient mechanism for targeting foreign pathogens, it adversely affects insect longevity (11). Uncontrolled melanization is likely to be physiologically detrimental due to the production of reactive and toxic byproducts (16), and thus the probable cause of the decreased lifespan of SRPN2 depleted mosquitoes.

The specific serpins (SRPNs) and cognate functional clip-domain serine proteinases (CLIPBs) that interact to regulate the melanotic response in An. gambiae are still largely uncharacterized (26). However, SRPN2 inhibits CLIPB9 both in vitro and in vivo and is the most well characterized regulatory interaction in the mosquito melanization pathway (12). CLIPB9 contains a single N-terminal clip domain and a C-terminal serine proteinase catalytic domain and is synthesized as a zymogen, becoming activated upon proteolytic cleavage at the beginning of the catalytic domain (12). Serpins inhibit proteinases via a suicide inhibitory mechanism that results in permanent inactivation of both the serpin and its cognate proteinase (27,28). Serpins generally contain 350-400 amino acids and adopt a conserved native fold consisting of three β-sheets (A, B, and C) surrounded by up to nine α-helices (A through I) with a reactive center loop (RCL) that acts as bait for target proteinases. This native serpin fold exists in a stressed, metastable form. Upon cleavage of the RCL scissile bond (P1-P1') by a target proteinase, the acyl-intermediate undergoes a remarkable 70Å translocation whereby the RCL is inserted into β-sheet A as an additional β-strand (29). This conformational change is achieved because the relaxed, cleaved form is more thermodynamically stable than the native fold. The translocation disrupts the integrity of the proteinase active site, rendering it inactive and covalently linked to the serpin in an SDS-stable complex (30). SRPN2 uses this mechanism to permanently inactivate CLIPB9, thereby limiting PO activation and the melanotic immune response (12).

The crystal structure of An. gambiae SRPN2 was previously determined to a resolution of 1.75Å in its native, active form (31). As expected, SRPN2 adopts the conserved serpin fold. The most notable difference between SRPN2 and most other native serpins is the conformation of the N-terminal region of the RCL, the hinge region, comprised of residues L356 to A360. The SRPN2 hinge region is partially inserted between strands 3 and 5 of β-sheet A (βA3 and βA5). A similar partial hinge insertion has only been found in a small number of inhibitory serpins: non-heparin bound antithrombin III (ATIII) (32,33), heparin cofactor II (HCII) (34), murine...
antichymotrypsin (mACT) (35), and Spn48 from Tenebrio molitor (36). In ATIII, the partial hinge insertion is linked to heparin-mediated activation. This insertion in ATIII restricts the flexibility of the RCL, which limits accessibility of the P1-P1’ bond to the target proteinase (37-39). Binding of heparin pentasaccharide (H5) to helix D induces significant conformational changes including c-terminal helix D extension and expulsion of the hinge region resulting in extension of the RCL. As a result of these conformational changes ATIII activity dramatically increases against factors IXa and Xa (34,37,40,41). Further evidence for H5-mediated activation of a hinge-inserted serpin is reported for Spn48, in which heparin binding significantly increases inhibition against its target proteinase (36). However, SRPN2 does not contain a heparin binding site and H5 does not increase the activity of SRPN2 against CLIPB9Xa (31). Therefore, although SRPN2 contains a partial hinge insertion linked to allosteric inactivation of some other serpins, its mechanistic role in SRPN2, if any, is entirely unknown.

In previous studies, mutagenesis was employed to either constitutively expel or stabilize the hinge insertion of ATIII, to determine the concomitant effect on inhibitory activity (40,42-44). Mutation-induced expulsion of the hinge region mimicked heparin activation in ATIII and mutation-induced restriction of the hinge region significantly diminished its inhibitory capability (42,44). In the current study, we used equivalent mutations in SRPN2 to elucidate the influence of the partial hinge insertion on SRPN2 inhibition of CLIPB9Xa. We created SRPN2 mutations that constitutively expelled (S358E) or restricted (S358W, E359C/K198C) the hinge region, investigated the structural and thermodynamic effects of these mutations, and determined the mutants’ ability to inhibit CLIPB9Xa. The results indicate that the partial hinge insertion in SRPN2 is not a structural regulatory mechanism as observed in ATIII. Instead, the data suggests that the partial hinge insertion in SRPN2 is thermodynamically stable, permits P1-P1’ accessibility to CLIPB9Xa, and maintains the ability for efficient inhibitory complex formation.

EXPERIMENTAL PROCEDURES

Cloning, expression, and purification of recombinant proteins - Ag-SRPN2-WT was cloned previously into a pET-28a vector to encode the full-length mature protein and an N-terminal His6 tag (12). Ag-SRPN2-S358W was generated using the QuickChange Multi Site-Directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. The following mutagenic primers were used to mutate S358 to a tryptophan: 5’-ATCACCATCAATGAGCTGGGCTGGGAGGCTACGCTGCTACCGAATTCAACTCG-3’, Fs: 5’-TGCTACCGAAATTCAACCTCG-3’, Rt: 5’-GGTGAAGCCTCCTCGCCCAGCTCATGCCTGATGATGCC-3’, Rs: 5’-AGCTCATTGATGATGATGCC-3’. Ag-SRPN2-K198C/E359C was generated using the QuickChange Multi Site-Directed mutagenesis kit (Stratagene) to first generate the K198C mutation with the following mutagenic primer: 5’-CTTCATTGATGATGATGCC-3’, followed by generation of the E359C mutation using the SLIM method with the following primers: Ft: 5’-GGGCAGTTGCGCTACGCTGCTACCGAAATTCAACCTCG-3’, Fs: 5’-TGCTACCGAATTCAACCTCG-3’, Rt: 5’-GGTGAAGCCTCCTCGCCCAGCTCATGCCTGATGATGCC-3’, Rs: 5’-AGCTCATTGATGATGATGCC-3’. All mutagenesis was confirmed by DNA sequencing (Genewiz).

All SRPN2 variant proteins were expressed and purified as previously described with the following modifications (12,45). The plasmid constructs were used to transform E. coli strain BL21(DE3) competent cells (Invitrogen). Bacteria were grown in 1L of LB medium at 37°C until an optical density (O.D._600) of 0.6-0.8 was reached. 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added and the cells were shaken at 20°C and 150 rpm for 16hr. The cells were then lysed by sonication and purified by NTA agarose chromatography (Qiagen) followed by ion-exchange chromatography on a Q-Sepharose column (GE Healthcare). The fractions were examined by 10% SDS-PAGE and Coomassie blue staining to confirm homogeneity. Fractions containing sufficiently pure recombinant protein were pooled for later use.
The cloning, expression, and purification of recombinant \textit{Ag-proCLIPB9\textsubscript{Xa}} was performed as previously described with the following modifications. Full length \textit{Ag-proCLIPB9} was cloned into a pFastBac1 (Life Technologies) vector and the activation cleavage site (IGMR) was mutated (IEGR) via a QuickChange Multi Site-Directed Mutagenesis kit (Stratagene) to generate \textit{Ag-proCLIPB9\textsubscript{Xa}} and permit controlled activation by factor Xa. Recombinant baculoviruses were generated by using the resulting plasmid according to the manufacturer’s instructions (Invitrogen). Recombinant protein was expressed in Sf9 cells following the manufacturer’s protocol and purified by NTA agarose chromatography (Qiagen) followed by ion-exchange chromatography on a Q-Sepharose column (GE Healthcare). The fractions were examined by 10% SDS-PAGE and Coomassie blue staining to confirm homogeneity. Fractions containing sufficiently pure recombinant protein were pooled for later use.

\textbf{Determination of SRPN2 mutant crystal structures} - All crystallization screening was conducted in Compact Jr. (Rigaku Reagents) sitting drop vapor diffusion plates at 20°C using equal volumes or protein and crystallization solution equilibrated against 75 \(\mu\)L of the latter. Protein samples were concentrated to: SRPN2-S358E (15.5 mg/mL), SRPN2-S358W (12.1 mg/mL), SRPN2-K198C/E359C (9.8 mg/mL) in 150 mM NaCl, 20 mM Tris pH 8.0. Prismatic crystals of SRPN2-S358E were obtained in 1-2 days from the Wizard 3-4 screen (Rigaku Reagents) condition G6 (20\% (w/v PEG 3350, 100 mM bis-Tris propane pH 8.5, and 200 mM sodium malonate). SRPN2-S358W crystals displaying a prismatic morphology were obtained in 1-2 days from the Index HT screen (Rigaku Reagents) condition E6 (1.0 M sodium phosphate monobasic monohydrate, potassium phosphate dibasic, pH 6.9). All sample crystals were transferred to a solution containing 80\% crystallization solution and 20\% glycerol as a cryoprotectant before freezing in liquid nitrogen except for SRPN2-K198C/E359C crystals which utilized a cryoprotectant of 70\% crystallization solution and 30\% glycerol. X-ray diffraction data were collected at the Advanced Photon Source IMCA-CAT beamline 17-ID using a Dectris Pilatus 6M pixel array detector.

Intensities were integrated using XDS (46) and the Laue class check and data scaling were performed with Aimless (47). Structure solution was conducted by molecular replacement with Phaser (48) via the Phenix (49) or Molrep (50) interface using the SRPN2-WT structure (PDB: 3PZF) (31) as the search model. Refinement and manual model building were carried out with Phenix and Coot (51), respectively. TLS refinement (52) was incorporated in the later stages to model anisotropic atomic displacement parameters. Structure validation was conducted with Molprobity (53). Disordered side chains were truncated to the point where electron density could be observed. Figures were prepared using Pymol (54) and the CCP4MG package (55). Relevant crystallographic data are provided in Table 1. The structures were deposited in the Protein Database (PDB) with reference codes: SRPN2-S358E (4RO9), SRPN2-S358W (4ROA), and SRPN2-K198C/E359C (4RSQ).

The diffraction data for SRPN2-K198C/E359C were initially indexed in an orthorhombic \(P\) lattice \(a=97, b=164, c=186.23\) and displayed a large pseudo-translation peak approximately 30\% of the origin at 0.263, 0.5, 0.456 and the self-rotation function indicated 3-fold non-crystallographic symmetry parallel to the crystallographic \(b\)-axis. The two NCS trimers were positioned by molecular replacement with Phaser and Molrep using a single chain from PDB 3PZF as the search model. The same solutions were obtained with both programs with the top score in the space group \(P2_2_2_1\). However, following refinement, the electron density was clear for one trimer (chains A-C) but poor for the second trimer (chains D-F) and the \(R\)-factors converged at \(R/\text{R}_{\text{free}}=29%/36\%\). The molecules were arranged in rows that stacked along the crystallographic \(b\)-axis and consisted of alternating sets of two A-C trimers rows and two D-F trimer rows. Inspection of the crystal packing revealed chain D of the D-F trimer overlapped a symmetry mate related by a crystallographic 2-fold axis along \(b\). We considered that this might be a case of lattice-translocation disorder given that the diffraction spots were streaked along the \(b^*\) reciprocal lattice direction. However, this condition is typified by alternating strong and diffuse spots in the diffraction pattern (56) which was not observed.
for the SRPN2-K198C/E359C crystals. The data were reprocessed in a P1 unit cell and a molecular replacement solution consisting of 24 molecules (8 trimers) was obtained with Molrep. This allowed us to determine the packing arrangement of the trimers and subsequently apply the results of this solution to a higher symmetry monoclinic $P$ lattice ($P_2_1$, $a=97.93$, $b=164.39$, $c=186.18$, $\beta=90.04^\circ$) containing 12 molecules (4 trimers) in the asymmetric unit. Further confirmation was obtained with the program Zanuda (57) which yielded the same top solution for the monoclinic $P_2_1$ lattice. Twin refinement with Refmac (58) using the pseudo-merohedral twin law (h, -k, -l), determined by Xtriage within the Phenix package, yielded a final model with $R/R_{\text{free}}=19.4%/25.5\%$ and a twin fraction of 45%. Thus it appeared that the crystal structure was best modeled as a monoclinic $P$ lattice with a $\beta$-angle near 90$^\circ$ which “mimicked” a higher symmetry orthorhombic $P$ lattice.

Reducing/nonreducing SDS-PAGE analysis - To analyze the presence of a disulfide bond in SRPN2-K198C/E359C proteins were boiled at 95°C for 10 min in SDS-PAGE loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, and 10% glycerol) with and without 100mM dithiothreitol (DTT). SDS-PAGE gel electrophoresis was then performed and stained with Coomassie blue.

Differential Scanning Calorimetry (DSC) - To determine the thermal stability of the SRPN2 mutants, purified protein (0.5 mg/mL) was dialyzed in 10 mM sodium phosphate pH 7.6 and 150 mM NaCl overnight, degassed, and loaded into a VP-DSC MicroCalorimeter (MicroCalTM Inc.). The temperature was scanned at 1°C/min from 20°C to 80°C. Heat Capacity, $C_p$ (kcal/mol/K), was plotted after subtraction of a blank experiment without protein. A second scan for each protein was performed but excluded from final data due to aggregation. To compare $T_m$ of each protein the baseline $C_p$ was normalized to SRPN2-WT.

Activation of proCLIPB9$_{xa}$ - The activation of proCLIPB9$_{xa}$ was performed as described previously (12) with the following modifications. 5 μg of purified proCLIPB9$_{xa}$ was incubated with 2 μg of commercial bovine Factor Xa (New England Biolab) in a total volume of 100 μl (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl2) at 37°C for 16 hr. Cleavage of the zymogen was examined by Western blot using anti-His antibody. To examine the amidase activity, 2 μl of the above reaction were transferred to a 96 well flat bottomed microplate (Corning) followed by the addition of 200 μl of 1000 μM Acetyl-IEAR-pNA (BioWorld) in buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM CaCl2). Absorbance changes at 405 nm were monitored immediately in a microplate reader (BioTek Instruments, Inc.) every 30 s for 20 min. One unit amidase activity was defined as ΔA405/min=0.001. Amidase activity of the serine proteinase was defined as the activity of enzyme minus the activity of Factor Xa alone.

Serpin inhibition assays - To examine the inhibitory effect of serpins on proteinase activity, 100 ng of activated CLIPB9$_{xa}$ in a volume of 2 μl was incubated with 4 μl of recombinant SRPN2 proteins at different molar ratios with the addition of 1 μl BSA (2 μg/μl) at 23°C for 20 min. The reaction was then subjected to an amidase activity assay as described above. Substitution of recombinant serpin with 4 μl buffer (20 mM Tris-HCl, pH 8.0,100 mM NaCl) was used to determine 100% enzyme activity. Residual amidase activity was plotted against the ratio of SRPN2 to activated CLIPB9$_{xa}$, and the stoichiometry of inhibition (SI) was determined as the x-intercept of the linear regression fit. All experiments were carried out with at least three independent replicates.

The second-order rate constant ($k_a$) of interactions between SRPN2-WT or SRPN2-S358E and CLIPB9$_{xa}$ was determined under pseudo-first order conditions as described previously (59). A fixed amount of CLIPB9$_{xa}$ (2.4 pmol) was mixed with different concentrations of recombinant SRPN2 in 1000 μM IEARpNa. The progress of product formation (P) at each concentration of SRPN2 was measured immediately as described above. For each combination of enzyme and inhibitor, a $k_{obs}$ was calculated by nonlinear regression using the following equation (P - amount product formation, V - initial velocity, t - time, and $k_{obs}$ - reaction rate.)

\[
P=\frac{V}{k_{obs}}\times(1-e^{-k_{obs}t})
\]

The $k_a$ was determined by plotting a series of $k_{obs}$ against the respective SRPN2 concentration and measuring the slope of the linear regression fit.
The association rate constant for the interaction between CLIPB9\textsubscript{Xa} and \textit{Ag}-SRPN2-S358W or \textit{Ag}-SRPN2-K198C/E359C was determined by a discontinuous second order rate constant inhibition assay as described previously with the following modifications (60). Briefly, \textit{Ag}-SRPN2-S358W or \textit{Ag}-SRPN2-K198C/E359C was added to activated CLIPB9\textsubscript{Xa} at a molar ratio 100:1 and incubated at RT for varying periods of time (t) including 0, 10, 20, 40, 60, 80, 100, and 120 min. The residual amidase activity was measured at each time point \((V_t)\) as described above. Initial enzyme activity \((V_0)\) was measured by replacing SRPN2 protein in the reaction with the same volume of buffer. The slope was calculated by plotting a series of \(\ln(V_t/V_0)\) against the respective incubation time. The \(k_a\) was calculated by dividing this negative slope by the concentration of the SRPN2 variants.

Detection of inhibitory complexes between SRPN2-CLIPB9\textsubscript{Xa} was performed as described previously (12). Activated CLIPB9\textsubscript{Xa} was mixed with purified SRPN2 at a molar ratio of 1:6, and incubated at RT for 10 min. The reaction mixtures were separated by 10% SDS-PAGE and stained with Coomassie blue.

**RESULTS**

**Structure of SRPN2-S358E** – The previously determined crystal structure of \textit{An. gambiae} SRPN2 wild type (SRPN2-WT) revealed a characteristic serpin fold consisting of three \(\beta\)-sheets (A, B, C) flanked by nine \(\alpha\)-helices (A through I) (31). The SRPN2-WT RCL hinge region (residues L356-A360) was inserted into \(\beta\)-sheet A between strands \(\beta\)A3 and \(\beta\)A5. P14 residue S358 is located at the apex of the loop involved in the hinge insertion. The corresponding P14 residue in ATIII (S380) was also located in this position, and its mutation to a glutamate (ATIII-S380E) resulted in an expulsion of the hinge region. Importantly, ATIII-S380E inhibitory activity against factor Xa is increased nearly 200-fold, to a level comparable to wild-type ATIII upon H5 activation (32,44). To investigate the consequences of the hinge region insertion on SRPN2 structure and activity, we mutated S358 to a glutamate (SRPN2-S358E) in an attempt to constitutively expel the residue from \(\beta\)-sheet A in the native serpin conformation.

The conformation of the hinge region in SRPN2-S358E was characterized from the crystal structure, determined to a resolution of 2.0Å (Table 1, Fig. 1A) and contained three molecules in the asymmetric unit (Fig. 1B). The three subunits are nearly identical except for the number of residues that could be traced in the RCL hinge region (Fig. 1C). Although most of the RCL was disordered in each subunit, the RCL in chain A could be traced to residue A360 (L356-A360 were inserted in SRPN2-WT) (Fig. 1D). Therefore the A-chain was used for all further analysis of the SRPN2-S358E structure.

The overall structure of SRPN2-S358E is very similar to the SRPN2-WT structure. Superposition of the two structures using secondary structure matching (61) yielded a root mean square deviation (rmsd) of 0.76Å between C\(\alpha\) atoms of the 353 residues aligned (Fig. 2A). As predicted, the most notable difference between the wild type and SRPN2-S358E structures is the RCL hinge region (Fig. 2B). The SRPN2-S358E hinge region is indeed expelled from \(\beta\)-sheet A, translocated approximately 12Å from the inserted position in SRPN2-WT. E358 forms hydrogen bonds with K47 in helix A of the A-chain of a symmetry-related molecule, R252 located between \(\beta\)B2 and \(\beta\)B3, and K114 located between helix D and \(\beta\)A1 (Fig. 2C). Overall, these structural data confirm that SRPN2-S358E assumes the native serpin fold and contains the predicted expulsion of the hinge region.

**Structure of SRPN2-S358W** – The fact that the SRPN2-S358E structure revealed an expelled RCL hinge region prompted us to develop a mutant in which the hinge region insertion could be further stabilized compared to the wild-type protein. Therefore, we generated a SRPN2-S358W mutant, in which W358 could potentially interact with the hydrophobic residues between strands \(\beta\)A3 and \(\beta\)A5 and thus be buried within the hydrophobic interior of the serpin.

The crystal structure of SRPN2-S358W was determined to a resolution of 1.9Å (Table 1) and is isomorphous to SRPN2-WT with a single molecule in the asymmetric unit. The rmsd between C\(\alpha\) atoms in SRPN2-S358W and SRPN2-WT was 0.33Å for 358 residues aligned (Fig. 3A). As predicted, the RCL hinge region in SRPN2-S358W adopts a similar conformation compared to SRPN2-WT, buried within the interior of \(\beta\)-sheet A (Fig 3B). The SRPN2-S358W RCL could be modeled to
residue E374 and W358 side chain density was clearly defined (Fig. 3C). W358 is located between βA3 and βA5 and is embedded within a hydrophobic pocket composed of I351 and I353 on βA5, F197 on βA3, F400 on βB5, I390 on βB4, and Y251 located between βB2 and βB3 (Fig. 3D). Residues F197, F400, and Y251 are highly invariant and conserved in approximately 75% of serpins (62). Subtle conformational changes are observed in multiple residues within this hydrophobic pocket to accommodate the tryptophan side chain. The structure of SRPN2-S358W suggests that W358 may stabilize the hinge region in an inserted conformation.

**Structure of SRPN2-K198C/E359C** - Despite the structural data from SRPN2-S358W, it is possible that the region could become intermittently expelled in a dynamic equilibrium as observed previously in ATIII-S380W (40). Therefore, we also created a SRPN2-K198C/E359C mutant in which a disulfide bond is introduced between the hinge region residue E359 and the adjacent K198C residue located on βA3. Hypothetically, a disulfide bond at this position would strengthen the interaction and maintain the inserted hinge loop region with limited possibility for its expulsion. The introduction of a disulfide bond at this position was previously used for similar purposes in study of ATIII (42).

The crystal structure of SRPN2-K198C/E359C was determined to a resolution of 2.9Å (Table 1), containing twelve molecules in the asymmetric unit. The overall conformation of SRPN2-K198C/E359C was very similar to SRPN2-WT with an rmsd of 0.63Å between Cα atoms for 351 residues aligned (Fig. 4A). As predicted, the RCL hinge region in the SRPN2-K198C/E359C structure is inserted into β-sheet A (Fig. 4B). The hinge region of the RCL in the SRPN2-K198C/E359C structure could be modeled up to residue A360 and shows clear density corresponding to the disulfide bond introduced between C359 and C198 (Fig. 4C). We further confirmed the presence of the disulfide bond by analyzing SRPN2-WT, SRPN2-K198C, SRPN2-E359C, and SRPN2-K198C/E359C on SDS-PAGE under reducing and nonreducing conditions. All proteins ran at the same size under both conditions except for SRPN2-K198C/E359C, which ran faster under nonreducing compared to reducing conditions, indicating the presence of a disulfide bond (Fig. 4D). Therefore, SRPN2-K198C/E359C exists in a native conformation and contains a disulfide bond that stabilizes the partial RCL hinge insertion.

**Differences in Thermostability and Structure Amongst the SRPN2 Variants** – The SRPN2 variant structures clearly reveal either expulsion or stabilization of the RCL hinge region. To investigate the thermodynamic effects of these mutations we used differential scanning calorimetry (DSC) to determine mid-point transition temperature, Tm of each variant (Fig. 5A). The thermal unfolding transition was absent during repeated scans (data not shown) due to irreversible aggregation of the samples at high temperature. The Tm of SRPN2-WT was 55.9˚C, and a similar Tm of 54.6˚C was determined for SRPN2-S358W. The expulsion of the hinge region in SRPN2-S358E resulted in a decrease in Tm to 52.9˚C. While E358 formed new hydrogen bonds with K114 and R252, the network of stabilizing bonds found in the hinge of SRPN2-WT were lost, reducing overall thermodynamic stability. As a consequence, hinge expulsion led to sixteen residues in the RCL being left unresolved (residues 361-376), twice as many as those unresolved in the SRPN2-WT structure (residues 367-374). Lastly, we determined that SRPN2-K198C/E359C had a significant increase in thermostability, with Tm of 60.7˚C. This Tm increase reflects the enhanced protein stability provided by the disulfide bond. Together, these results indicated that differences in the RCL conformation and stabilizing intramolecular interactions between the SRPN2 variants are reflected in their respective thermostabilities.

The structural differences found in the hinge region of the SRPN2 variants also affect the respective conformations of β-sheet A. During the formation of the inhibitory complex, the RCL must insert between βA3 and βA5 as an additional β-strand. Therefore, the distance between the β-strands must increase in order to accommodate insertion of the RCL. In SRPN2-WT, βA3 and β5 are separated by a distance of approximately 8.6Å at the location of the partial hinge insertion (Fig. 5B). Similar distances are found for SRPN2-S358W (9.0Å) (Fig. 5C) and SRPN2-
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K198C/E359C (8.7 Å) (Fig. 5D). However, with the hinge expelled in SRPN2-S358E this distance reduced to 6.3 Å (Fig. 5E).

**Stoichiometry of Inhibition of SRPN2 variants**

- Having established the structure and thermostability of the SRPN2 variants, we determined their ability to form an inhibitory complex with the activated cognate proteinase CLIPB9xₐ and the stoichiometry of inhibition (SI) of this interaction (Table 2, Fig. 6). SRPN2-WT forms an SDS-stable complex with CLIPB9xₐ with a stoichiometry of inhibition (SI) of 1.7 ± 0.1. SRPN2-S358E was also able to inhibit CLIPB9xₐ and form an SDS-stable complex with the proteinase with a slightly increased SI of 2.5 ± 0.7, indicating that the expelled hinge mutant is capable of forming an inhibitory complex at a level comparable to SRPN2-WT. Conversely, SRPN2-S358W did not form an inhibitory complex with CLIPB9, reflected in its significantly increased SI of 50 ± 12 (Table 2, Fig. 6). However, CLIPB9xₐ efficiently degraded SRPN2-S358W, leaving a cleavage product at a size consistent with cleavage at the RCL P1-P1’. Therefore, although SRPN2-S358W cannot inhibit CLIPB9xₐ it is efficiently cleaved at the RCL. Surprisingly, SRPN2-K198C/E359C was able to form an inhibitory complex with CLIPB9xₐ although the SI was greatly increased to 20 ± 5. SRPN2-K198C/E359C also contained a band in SDS-PAGE consistent with cleavage at the RCL.

**Rate of CLIPB9xₐ inhibition by SRPN2 variants**

- Having established that the SRPN2 variants, with the exception of SRPN2-S358W, each form an inhibitory complex with CLIPB9, we determined the second order rate constants (kₛ) of the SRPN2 variants’ inhibition against CLIPB9xₐ (Table 2). SRPN2-WT was able to inhibit CLIPB9xₐ with a kₛ of (1.5 ± 0.2) x 10³ M⁻¹s⁻¹. Combining this reaction rate with the SI (SI x kₛ) provides a measure of total flux down both the substrate and inhibitory pathways of SRPN2-WT with CLIPB9xₐ of (2.6 ± 0.4) x 10³ M⁻¹s⁻¹. This level of activity is relatively low compared to ATIII, which has inhibitory activity against factor Xa in the 1.0 x 10⁷ M⁻¹s⁻¹ range when fully activated (40). To determine whether the expelled hinge region increases the inhibitory activity of SRPN2, we measured the kₛ of SRPN2-S358E against CLIPB9xₐ. The kₛ of SRPN2-S358E was (2.8 ± 0.3) x 10³ M⁻¹s⁻¹, a 1.9-fold increase compared to the kₛ of SRPN2-WT. Combing the kₛ and SI of SRPN2-S358E (7.1 ± 0.3) x 10³ M⁻¹s⁻¹ indicates that the overall reaction kinetics of SRPN2-S358E are increased nearly 3-fold compared to SRPN2-WT.

We also investigated the second order rate constants of the hinge-inserted SRPN2 variants SRPN2-S358W and SRPN2-K198C/E359C (Table 2). Based on its high SI and inability to form an SDS-stable inhibitory complex, it is not surprising that SRPN2-S358W had a kₛ of (6.1 ± 0.2) x 10¹ M⁻¹s⁻¹. These results confirm that SRPN2-S358W has effectively no inhibitory activity against CLIPB9xₐ. We determined that the kₛ of SRPN2-K198C/E359C was (2.3 ± 0.4) x 10² M⁻¹s⁻¹, which is a 6.7-fold decrease compared to SRPN2-WT. However, the SI of SRPN2-K198C/E359C was significantly increased indicating that the disulfide bond may hinder this variant’s ability to form an inhibitory complex independent of the effect of the inserted hinge region we are investigating. Combining the SI and kₛ for SRPN2-K198C/E359C of (4.6 ± 0.4) x 10³ M⁻¹s⁻¹ indicates that this hinge-inserted variant has a similar flux down inhibitor and substrate pathways when interacting with CLIPB9xₐ comparable to SRPN2-WT and SRPN2-S358E.

**DISCUSSION**

SRPN2 is a critical negative regulator of the melanization pathway in *An. gambiae*. The phenotype of SRPN2 depletion from adult female mosquitoes shows accelerated mortality rates and decreased feeding propensity at the time when malaria-infected mosquitoes are able to transmit the parasite to the next human host (11, 12; Michel, unpublished). SRPN2 may therefore be a promising target for small molecule inhibitors to be utilized as LLA insecticides for vector control.

The structure of SRPN2-WT revealed a partial insertion of the RCL hinge region into beta sheet A. A very similar partial RCL insertion was linked previously to allostERIC activation of ATIII. The ATIII data revealed a model whereby heparin-induced conformational changes resulted in expulsion of the hinge region increasing accessibility of the RCL P1-P1' and exosites, thereby boosting inhibition of target proteinases.
Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2

Due to the relative rarity of the inserted hinge region among serpins, we hypothesized that it likewise functions as a regulatory mechanism in SRPN2. To test this hypothesis, we developed SRPN2 mutants with constitutively expelled (SRPN2-S358E) or stabilized (SRPN2-S358W, SRPN2-K198C/E359C) hinge regions, analyzed the structure and thermostability of the variants, and determined the effects of the mutations on inhibition of CLIPB9xa. Our data reveal that a hinge-expelled SRPN2 variant had a limited increase in inhibitory activity against CLIPB9xa and that stabilization of the inserted hinge region did not affect RCL accessibility. Together, these results strongly suggest that SRPN2 does not follow the ATIII model of allosteric activation by H5.

The hinge-expelled SRPN2-S358E mutant did indeed gain an approximately 3-fold increase in CLIPB9xa inhibition, indicating that serpin activity does generally benefit from increased RCL extension and flexibility. However, this increase is much less than the 190-fold increase in the equivalent ATIII-S380E mutant against factor Xa (44). An explanation for the limited advantage in SRPN2 is the possibility that hinge expulsion is achieved by exosite interactions between SRPN2 and CLIPB9 prior to cleavage of the scissile bond. SRPN2-S358E would therefore circumvent this step, gaining 3-fold efficiency. Such exosite interaction would increase the specificity of SRPN2 for CLIPB9, which may be crucial in the physiological context of an open circulatory system. Further structural and mutagenesis studies and investigations into the SRPN2-CLIPB9 interaction will be necessary to test this hypothesis.

An equally parsimonious explanation for the minimal activation of SRPN2-S358E is that accessibility of the P1-P1’ bond is not strongly hindered by the potential hinge insertion. Therefore, CLIPB9 inhibition simply does not require extension of the RCL. We found that CLIPB9xa cleaves the RCL of both SRPN2-S358W and SRPN2-K198C/E359C, despite their stabilization of the hinge insertion. SRPN2-S358W is unable to form an inhibitory complex with CLIPB9xa, likely due to the bulky P14 residue inhibiting the complete insertion into β-sheet A, which has been reported for other such mutants (63). However, SRPN2-K198C/E359C can form an inhibitory complex with CLIPB9xa and its increased SI is likely due to effects of the disulfide bond on complete insertion. Furthermore, when SI is taken into consideration, SRPN2-K198C/E359C has an efficiency of interaction with CLIPB9xa comparable to SRPN2-WT. Therefore, these variants indicate that the partial hinge insertion does not hinder the ability for CLIPB9 to target and cleave the SRPN2 RCL. These data can be interpreted in the context of a recently proposed expansion of the allosteric ATIII activation model (64). This model envisions that lowered activity of hinge-inserted ATIII in absence of H5 is largely due to repulsive exosite interactions with factors IXa and Xa. These proposed negative exosite interactions are diminished physiologically by conformational changes induced by H5, and presumably are overcome by mutational expulsion of the hinge region as seen in ATIII-S380E. Following this model, efficient cleavage of SRPN2-S358W and SRPN2-K198C/E359C can be explained by the lack of such repulsive exosites in the interaction of SRPN2 with CLIPB9.

Consistent with the idea that RCL accessibility is not a limiting factor of SRPN2 activity, the structural data suggest that the hinge-inserted conformation of SRPN2 provides increased efficiency for complete hinge insertion after P1-P1’ cleavage, thus facilitating inhibitory complex formation. The SRPN2-WT hinge insertion naturally results in a local increase in the distance between βA3 and βA5 that decreases once the hinge is expelled in SRPN2-S358E. Previous studies on ATIII indicated the requirement for coordination of βA strand separation and RCL insertion (44). However, the drastically increased level of RCL cleavage upon hinge expulsion in ATIII would overcome negative effects due to increased βA strand interaction. However, because the SRPN2 RCL is cleaved at similar rates in inserted and expelled forms, the local separation of βA strands may provide an energetic advantage towards rapid insertion. The SRPN2-S358E inhibitory data supports this, with a 1.9-fold increase in SRPN2-S358E $k_a$ (perhaps due to increased RCL accessibility) and a 1.5-fold decrease in SI (due to decreased insertion). The partially inserted hinge region in SRPN2-WT therefore gains an energetic head start towards complete insertion that is not negated by diminished RCL accessibility.
While the data did not reveal that the inserted hinge in SRPN2 functions as a regulatory mechanism against CLIPB9 inhibition, it is nevertheless conceivable that hinge expulsion is required for inhibition of a yet to be identified proteinase target. While this possibility is actively pursued, the low level of CLIPB9 inhibition in vitro remains intriguing and suggests that a different unidentified mode of SRPN2 regulation may exist in vivo. Activation of ATIII against thrombin requires a bridging mechanism mediated by full-length heparin that binds both proteins, in contrast with the allosteric activation mediated by H5 that governs inhibition of factors IXa and Xa (reviewed in (41)). This divergence has been attributed to the poorer suitability of ATIII as a thrombin substrate (44) and the inability of thrombin to utilize exosites critical for contact between ATIII and factors IXa and Xa (41). Molecules that facilitate initial interaction between thrombin and ATIII are therefore far more likely to increase ATIII activity against thrombin than those that extend the RCL (44). The ATIII-S380E mutation only increases the $k_a$ against thrombin 1.9-fold (44), which correlates intriguingly well with our SRPN2-CLIPB9Xa data. It is tempting to speculate that a bridging mechanism rather than allosteric regulation modulates SRPN2 activity. There are a number of inactive proteinases in the CLIPA family that coordinate protein-protein interactions in different pathways within insect immune systems (65-69). The specific molecular roles of CLIPAs within melanization in mosquitoes await elucidation, and some may indeed regulate SRPN2 activity and thus PO activation in An. gambiae.
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FOOTNOTES
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¶These authors contributed equally to this work.

FIGURE LEGENDS

FIGURE 1. Structure of SRPN2-S358E. A. Crystal structure of SRPN2-S358E determined to a resolution of 2.0Å. β-sheet A (red), β-sheet B (blue), β-sheet C (yellow), Reactive Center Loop (RCL, cyan), serpin helices (A-I) and βA strands (1-5) are identified. B. Non-crystallographic symmetry (NCS) trimer of SRPN2-S358E viewed along the 3-fold NCS axis showing chain-A (orange), chain-B (cyan), and chain-C (pink). C. Structural alignment of the RCL, βA3, and βA5 from SRPN2-S358E chain-A (orange), chain-B (cyan), and chain-C (pink). D. Fo-Fc omit electron density map (3σ) of the SRPN2-S358E RCL hinge region (I353-A360).

FIGURE 2. Structural comparison of SRPN2-S358E and SRPN2-WT. A. Structural alignment of SRPN2-WT (PDB code: 3PZF, blue) and chain-A of SRPN2-S358E (orange). B. Close-up of the alignment of the RCL hinge region from SRPN2-WT (blue) and SRPN2-S358E (orange) from the inset in A with S358 from WT and E358 from S358E side chains shown. C. In the SRPN2-S358E mutant, hydrogen bonds are formed between E358 and K114 and R252 within the protein, and with K47 in chain-A of a symmetry related molecule (gray).

FIGURE 3. Structure of SRPN2-S358W and comparison with SRPN2-WT. A. Crystal structure of SRPN2-S358W determined to a resolution of 1.9Å (green) aligned with SRPN2-WT (PDB code: 3PZF, blue). B. Close-up of the alignment of the RCL hinge region from SRPN2-WT (blue) and SRPN2-S358W (green) from the inset in B with S358 from WT and W358 from S358W side chains shown. C. Fo-Fc omit electron density map (3σ) of the SRPN2-S358W RCL hinge region (I353-E374). D. In the SRPN2-S358W mutant, W358 (green) inserts into a hydrophobic pocket within the serpin. Conformation shift of residues in SRPN2-S358W (green) surrounding W358 compared to their position in SRPN2-WT (blue).

FIGURE 4. Structure of SRPN2-K198C/E359C and comparison with SRPN2-WT. A. Crystal structure of SRPN2-K198C/E359C determined to a resolution of 2.85Å (purple) aligned with SRPN2-WT (PDB code: 3PZF, blue). B. Close-up of the alignment of the RCL hinge region from SRPN2-WT (blue) and SRPN2-K198C/E359C (pink) from the inset in B with S358 shown. C. Fo-Fc omit electron density map (3σ) of the SRPN2-K198C/E359C RCL hinge region (I353-A360) contoured at 1.5σ. D. Coomassie blue stained SDS-PAGE gel showing SRPN2 cysteine mutants in the presence of 0% and 10% dithiothreitol.
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(DTT). The higher weight molecular band in 0% DTT corresponding to E359C is believed to be due to an intermolecular disulfide bond.

**FIGURE 5.** Thermostability of SRPN2 variants and structural changes in β-sheet A. A. Differential scanning calorimetry (DSC) measurements of SRPN2-WT (blue), SRPN2-S358W (green), SRPN2-K198C/E359C (purple), and SRPN2-S358E (yellow) indicating the relative mid-point unfolding temperature ($T_m$, dotted lines) as measured by maximum heat capacity (Cp). B. SRPN2-WT structure with the RCL hinge region and βA3/βA5 (blue) shown including the distance measured between βA3 and βA5. The identical orientation and measurement is shown for C. SRPN2-S358W (green), D. SRPN2-K198C/E359C (pink), and E. SRPN2-S358E (orange).

**FIGURE 6.** Inhibitory complex formation between CLIPB9 and SRPN2 variants. SDS-PAGE gel with Coomassie staining after incubation of recombinant SRPN2 variants and CLIPB9Xa. CLIPB9Xa must be activated by factor Xa for full catalytic activity. Notable bands are described on the right; fl – full length, cl – cleaved, cat – catalytic domain.
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TABLE 1. Crystallographic data for SRPN2 mutant structures.

| Data Collection | SRPN2 S358E | SRPN2 S358W | SRPN2 K198C/E359C |
|-----------------|-------------|-------------|-------------------|
| **Unit-cell parameters**<br>\(a=171.33, b=42.26\) | \(a=96.95\) | \(a=97.93, b=164.39\) |
| \(c=185.62, \beta=116.9\) | \(c=78.57\) | \(c=186.18, \beta=90.02\) |
| **Space group**<br>\(P2_1\) | \(P6_3\) | \(P2_1\) |
| **Resolution (Å)**<br>93.5-2.00<br>\(2.04-2.00\) | 48.47-1.90<br>\(1.94-1.90\) | 48.94-2.90<br>\(2.95-2.90\) |
| **Wavelength (Å)**<br>1.0000 | 1.0000 | 1.0000 |
| **Temperature (K)**<br>100 | 100 | 100 |
| **Observed reflections**<br>268,964 | 309,621 | 441,482 |
| **Unique reflections**<br>80,845 | 33,155 | 129,666 |
| **\(<I/\sigma(I)>\)**<br>13.8 (2.1) | 18.4 (2.7) | 7.9 (2.0) |
| **Completeness (%)**<br>99.7 (99.7) | 100 (100) | 99.5 (99.8) |
| **Multiplicity**<br>3.3 (3.5) | 9.3 (8.9) | 3.4 (3.3) |
| **\(R_{merge}\) (%)**<br>4.6 (46.9) | 7.9 (79.8) | 13.5 (63.5) |
| **\(R_{meas}\) (%)**<br>6.5 (67.4) | 8.4 (84.7) | 16.1 (76.3) |
| **\(R_{pim}\) (%)**<br>3.5 (35.8) | 2.7 (28.2) | 8.6 (41.8) |
| **CC\(_{1/2}\) (%)**<br>0.999 (0.849) | 0.999 (0.881) | 0.990 (0.752) |
| **Refinement**<br>No. of molecules/ASU<br>3 | 1 | 12 |
| **Resolution (Å)**<br>38.18-2.00 | 39.29-1.90 | 47.82-2.90 |
| **Reflections**<br>76,752 / 4,050 | 31,401 / 1,671 | 123,334 / 6,285 |
| **\(R_{factor} / R_{free}\) (%)**<br>19.0 / 23.8 | 17.2 / 21.0 | 19.4 / 25.5 |
| **No. of atoms**<br>8,324 / 249 | 2,872 / 107 | 33,765 / 0 |
| **Model Quality**<br>R.m.s deviations<br>Bond lengths (Å)<br>0.009 | 0.010 | 0.006 |
| Bond angles (°)<br>1.150 | 0.951 | 1.080 |
| **Average B factor (Å\(^2\))**<br>All Atoms<br>41.1 | 40.4 | 43.4 |
| Protein<br>41.4 | 40.6 | 43.4 |
| Water<br>38.7 | 36.4 | - |
| **Coordinate error (Å)**<br>0.22 | 0.19 | 0.24 |
| **Ramachandran Plot**<br>Favored (%)<br>96.4 | 98.0 | 95.4 |
| Allowed (%)<br>3.1 | 2.0 | 4.3 |

1Values in parenthesis are for the highest resolution shell.
2\(R_{merge} = \sum_{hkl} (I(hkl) - \langle I(hkl)\rangle) / \sum_{hkl} I(hkl)\), where \(I(hkl)\) is the intensity measured for the \(ih\)th reflection and \(\langle I(hkl)\rangle\) is the average intensity of all reflections with indices \(hkl\).
3\(R_{meas} = \sum_{hkl} |F_{obs}(hkl)| / \sum_{hkl} |F_{calc}(hkl)|\); \(R_{free}\) is calculated in an identical manner using 5% of randomly selected reflections that were not included in the refinement.
4\(R_{pim}\) is the correlation coefficient of the mean intensities between two random half-sets of data (73,74).
### TABLE 2. *In vitro* inhibition of CLIPB9 by SRPN2 and mutants.

| Protein         | SI ± SD | $k_o$ ± SD (M$^{-1}$s$^{-1}$) | $k_o$×SI ± SD (M$^{-1}$s$^{-1}$) |
|-----------------|---------|-------------------------------|----------------------------------|
| SRPN2-WT        | 1.7 ± 0.1 | (1.5 ± 0.2) x 10$^3$           | (2.6 ± 0.2) x 10$^3$            |
| SRPN2-S358E     | 2.5 ± 0.7 | (2.8 ± 0.3) x 10$^3$           | (7.1 ± 0.3) x 10$^3$            |
| SRPN2-S358W     | 50 ± 12  | (6.1 ± 0.2) x 10$^1$           | (3.1 ± 0.1) x 10$^3$            |
| SRPN2-K198C/E359C | 20 ± 5  | (2.3 ± 0.4) x 10$^2$           | (4.6 ± 0.4) x 10$^3$            |
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**FIGURE 1**

(A) A helical representation of the Serpin-2 protein structure with labeled helices.

(B) A cartoon representation of the protein structure with chains labeled.

(C) A more detailed view of the hinge loop region.

(D) A close-up view of the amino acids A360 and E358 in the hinge loop.
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FIGURE 2
FIGURE 3

Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2
Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2

**FIGURE 4**

A. WT K198C/E359C

B. S358

C. C359, A360, C198

D. DTT treatment 0% and 15%
Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2

**FIGURE 5**
FIGURE 6

Mutagenesis of the hinge loop in *Anopheles gambiae* Serpin-2
Structural and Inhibitory Effects of Hinge Loop Mutagenesis in Serpin-2 from the Malaria Vector *Anopheles gambiae*

Xin Zhang, David A. Meekins, Chunju An, Michal Zolkiewski, Kevin P. Battaile, Michael R. Kanost, Scott Lovell and Kristin Michel

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