

Mutation of the Catalytic Cysteine in *Anopheles gambiae* Transglutaminase 3 (AgTG3) Abolishes Plugin Crosslinking Activity without Disrupting Protein Folding Properties

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Summary

Malaria is a devastating parasitic infectious disease with an enormous impact on public health and economic growth, particularly in Sub-Saharan Africa. Besides advances in anti-malarial drugs and vaccine development, a successful malaria eradication program relies on controlling the mosquito vector. In this study, we pursue a novel approach for malaria vector control by inhibiting an enzyme important for ensuring reproductive success of the mosquito *Anopheles gambiae*. The enzyme, *A. gambiae* transglutaminase 3 (AgTG3), catalyzes the cross-linking of its native substrate Plugin, which is then transferred to a female mosquito in a coagulated mass known as the mating plug. Interfering with AgTG3-catalyzed mating plug formation prevents efficient sperm storage, with a direct consequence on fertility. This study demonstrates that the mutation of a highly conserved cysteine residue in AgTG3 (Cys323) abolishes its cross-linking activity without disrupting other properties of the enzyme such as protein folding and oligomeric assembly. These results suggest that Cys323 is an active site residue and support the design of specific inhibitors targeting this site as a promising strategy to reduce the malaria disease burden worldwide.

Introduction

Malaria is a devastating parasitic infectious disease with an enormous impact on public health and economic growth, particularly in developing countries (1). In Sub-Saharan Africa, *Anopheles gambiae* is the principal mosquito vector which harbors and spreads the disease-causing *Plasmodium* parasite. A successful malaria eradication program therefore requires controlling *A. gambiae*. Pyrethroid insecticides have been important chemical weapons in vector control; however, their increased use has created a selection pressure for mosquitoes to develop resistance against them (2-6). Novel methods for vector control are therefore urgently needed.

The sterile insect technique (SIT) has shown significant promise as a vector control strategy (7,8). SIT relies on the release of sterile, but sexually competitive, males into the wild to mate with females. *Anopheles* females have a high reproductive capacity that is ensured by a single mating event (9). Thus, interfering with the mating rituals and reproductive biology of male mosquitoes has the potential to reduce overall vector populations and therefore halt disease transmission. The application of SIT,

however, hinges upon a comprehensive understanding of the reproductive system of *A. gambiae*. During mating, the *A. gambiae* male transfers sperm along with additional protein secretions into the female in the form of a coagulated mass known as the mating plug (8,9). The mating plug effectively seals sperm inside the female reproductive chamber, or atrium, thereby increasing the chances of successful conception while simultaneously imposing monogamy. The plug is eventually broken down in the female reproductive tract, coinciding with major physiological and behavioral changes, including an aversion to further mating. Recent studies have identified *A. gambiae* seminal transglutaminase 3 (AgTG3) as a key determinant in mating plug formation (10,11). Since AgTG3 is specific to *A. gambiae*, it is a potential target for a species-specific malaria prophylactic.

Transglutaminases (TGs) are a family of enzymes widely distributed in living organisms which catalyze the irreversible cross-linking of proteins to create stable biological structures, including blood clots, skin, hair, and other tissues (11). The cross-linking reaction, which involves the formation of iso-peptide bonds between the carboxyl (-COOH) group of glutamine and the amine (-NH₂) moiety of a lysine substrate, relies on the nucleophilic activity of a cysteine thiol. The protein sequence of AgTG3 contains 11 cysteine residues. The sequence is highly conserved in the region around Cys323 (Figure 1A), by alignment of AgTG3 with the homologous TG sequences human factor XIII (FXIII), (12) human tissue transglutaminase 2 (hTG2), (13,14) human transglutaminase 3 (hTG3), (15) and red sea bream transglutaminase (PgTG) (16). This cysteine should cluster with a conserved aspartate and histidine to form a catalytic triad based upon crystal structures of other TGs (Figure 1B). This homology suggests that Cys323 is an active site residue in AgTG3.

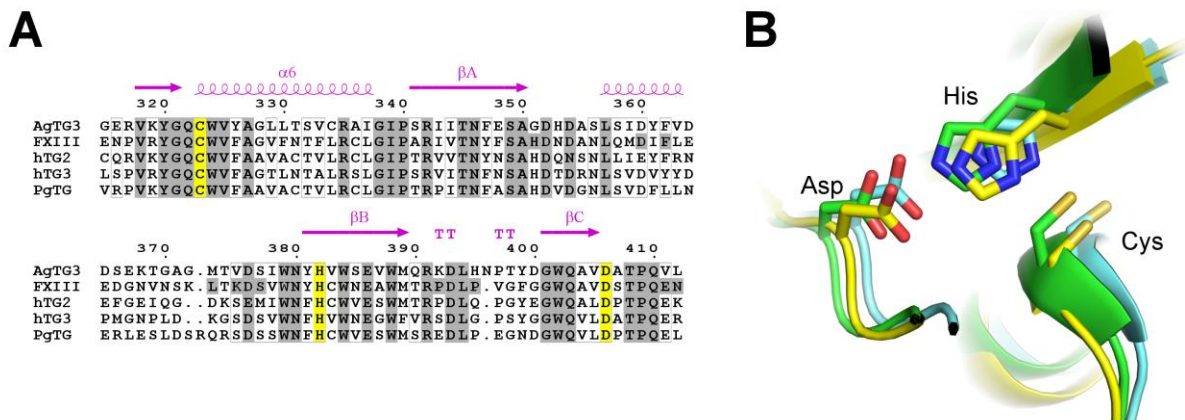


Figure 1: Identification of Cys323 as a putative catalytic residue. (A) Multiple sequence alignment of AgTG3 with transglutaminases of known structure: human factor XIII (FXIII), human tissue transglutaminase 2 (hTG2), human transglutaminase 3 (hTG3), and *P. major* transglutaminase. Conserved residues are shaded in grey, whereas catalytic residues are shaded in yellow. (B) The active

site catalytic triad based on superposition of the crystal structures of human transglutaminases FXIII (yellow), hTG2 (cyan), and hTG3 (green).

In this study, we demonstrate that the C323A mutation in AgTG3 is sufficient to disrupt cross-linking activity and prevent formation of the mating plug. Additional biophysical measurements demonstrate that the mechanism of inhibition is due to removal of the catalytic nucleophile and does not affect the general behavior of AgTG3 in solution. Our findings identify Cys323 in AgTG3 as a catalytic residue and highlight the potential of AgTG3 active-site inhibitors as chemosterilants for *A. gambiae*.

Results

C323A mutant AgTG3 can be recombinantly expressed in *E. coli* and purified to homogeneity

The first step in evaluating Cys323 in AgTG3 as a potential antimalarial drug target required the expression and purification of the C323A mutant protein (Figure 2). DNA encoding the C323A mutant AgTG3 was generated by site-directed mutagenesis PCR using the template pET28a-AgTG3 vector encoding wild-type (WT) AgTG3 with a C-terminal six-histidine tag. The resultant mutant plasmid was transformed into the *E. coli* Rosetta strain (Novagen) for large-scale expression. The C323A protein was purified to homogeneity using nickel affinity and size exclusion chromatography. The overall yield for the C323A mutant was significantly lower compared to WT AgTG3, suggesting that this residue is important for protein stability or function and that the C323A mutation is not favored for natural selection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified WT and C323A mutant AgTG3 revealed a protein band between 80 – 90 kDa (Figure 2C), consistent with theoretical molecular weight values. To assess purity and determine exact molecular mass values, both WT and C323A mutant AgTG3 were subjected to liquid-chromatography electrospray ionization mass spectrometry (LC-ESI-MS) to give molecular masses of 84,175 Da and 84,143 Da, respectively (Figure 2D). The 32 Da difference in mass corresponds to the loss of a sulfur atom from the cysteine to alanine mutation.

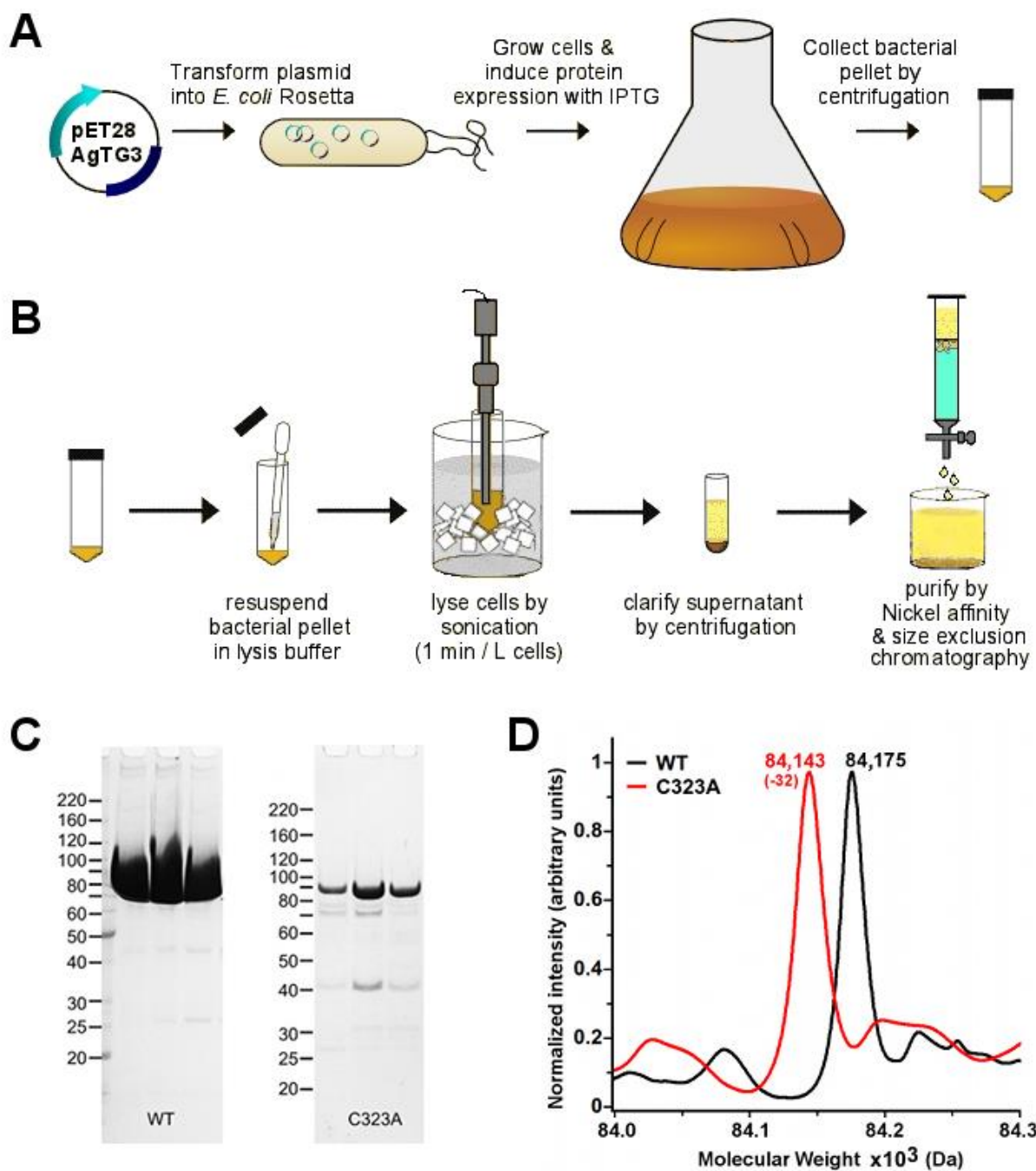


Figure 2: Expression and purification of WT and C323A AgTG3. (A-B) Schematic diagrams for the expression and purification of AgTG3 from *E. coli* Rosetta cells. (C) SDS-PAGE gels of WT and C323A mutant AgTG3 purified to >95% purity based on Coomassie staining. (D) Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) verifies the purity and exact molecular masses of WT and C323A mutant AgTG3. The loss of 32 Da in the C323A correlates with the loss of a sulfur atom, as expected.

The C323A mutation is sufficient to abolish AgTG3 enzyme activity

To evaluate the catalytic activity of the C323A mutant, we reconstituted AgTG3 cross-linking of Plugin-C in vitro using two orthogonal, yet complementary, techniques: (a) SDS-PAGE analysis of Plugin cross-linking to monodansyl-cadaverine (MDC), a fluorescent amine substrate mimic, under UV illumination and (b) plate-based fluorescence detection of Plugin cross-linked to fluorescein isothiocyanate-cadaverine (FITC-CAD). The gel-based MDC incorporation assay was a qualitative measure of Plugin cross-linking, whereas the plate-based FITC-CAD fluorescence assay was a quantitative assessment of catalytic activity.

Monomeric Plugin-C appeared as a single band by SDS-PAGE (Figure 3A, lane 3), with an apparent molecular weight of approximately 36 kDa. Combining WT AgTG3 and Plugin-C in the presence of calcium (Figure 3A, lane 5) produced a ladder of bands of increasing molecular weight, indicating intermolecular crosslinks among Plugin monomers into higher-order oligomers. Crosslinking was absent in the sample containing C323A and calcium, demonstrating that the C323A mutant is inactive and suggesting that Cys323 is indeed the catalytic cysteine residue. Interestingly, a faint band corresponding to monomeric Plugin was present in this sample, suggesting that the calcium-treated C323A mutant AgTG3, although catalytically inactive, retains the ability to bind its amine substrate. Preincubation of both WT and C323A AgTG3 with iodoacetamide (IA), which alkylates free cysteine residues, revealed only a single band corresponding to free MDC, confirming the requirement of a catalytically-active cysteine residue for AgTG3 activity.

The FITC-CAD plate-based assay produced similar results and enabled a statistical analysis of the data (Figure 3B). Only the sample containing WT AgTG3 and Plugin-C with calcium demonstrated a statistically significant ($p < 0.001$) increase in fluorescence intensity. Samples containing the C323A mutant or IA showed no statistically significant crosslinking relative to the buffer-only control.

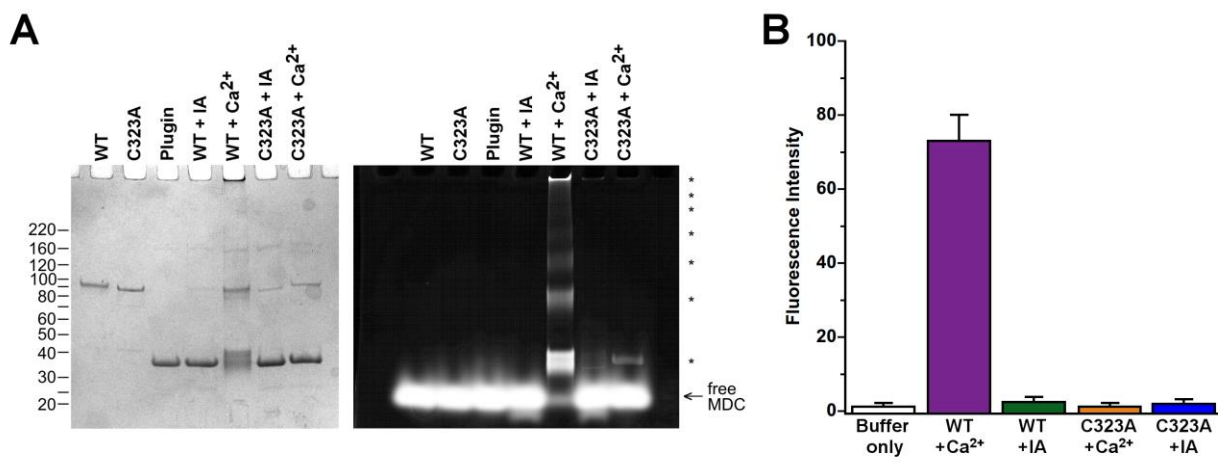


Figure 3: Activity assays for WT and C323A mutant AgTG3. (A) MDC-incorporation assay. Cross-linked Plugin forms high molecular weight bands (lane 5) as shown by Coomassie (left) and chemiluminescence (right). WT AgTG3 activity is abolished in the presence of iodoacetamide (IA) (lane 4). C323A mutant AgTG3 does not demonstrate cross-linking activity, even in the absence of iodoacetamide (lanes 6-7). (B) FITC fluorescence for conjugation of FITC-CAD to Plugin-coated plates.

The C323A mutation does not affect properties of AgTG3 in solution

We have demonstrated that the C323A mutation is sufficient to abolish Plugin crosslinking activity. However, it remains unclear whether the lack of enzyme activity is due to specific removal of the catalytic nucleophile or whether it is a consequence of mutant protein misfolding or aggregation. To address the basis for inactivity of the C323A mutant, we used size-exclusion chromatography (SEC) and multi-angle laser light scattering (MALLS) to determine whether the C323A mutant has similar bulk properties in solution compared to WT AgTG3.

Since calcium is required for AgTG3 activity, the solution characteristics of both WT and C323A mutant AgTG3 were evaluated in the presence and absence of calcium. EGTA, a chelating agent that binds specifically to calcium ions, was used to simulate a calcium-free environment. To allow for a direct comparison of the results, either calcium or EGTA was pre-incubated with WT and C323A mutant AgTG3 at a fixed protein concentration. Samples were injected individually and successively onto a Superdex 200 (10/300) size-exclusion column in line with light scattering and refractive index detectors to measure the hydrodynamic properties of each protein in solution.

In the absence of calcium, both WT and C323A mutant AgTG3 proteins eluted from the column as single, monodisperse peaks, indicating that both proteins are homogeneous and well folded. The elution volume, centered at ~15 ml (Figure 4), corresponds to a 100 kDa apparent molecular weight, ~20% greater than the actual molecular weight of AgTG3, indicating that both proteins are non-globular in shape. When calcium was added to the solution, the elution volume shifted to ~14 ml, indicating a protein with an increased hydrodynamic radius. To confirm this result, the average molecular weight of the protein in solution was calculated from the light scattering intensity. The peak at 15 ml corresponded to an average molecular weight of 93-95 kDa, consistent with a monomer, whereas the peak at 14 ml corresponded to an average molecular weight of 150-164 kDa, consistent with a dimer (Figure 4). Minor differences in the shape of the peak shoulders in the calcium-containing samples are likely the result of small changes in the ratio of dimer to monomer. These results demonstrate that the C323A mutant, while catalytically inactive, has similar physical properties to the wild type, including dimerization in the presence of calcium.

In samples containing EGTA, a significant peak was observed at an elution volume of ~8 ml in the light scattering profile but not the absorbance profile. The intensity of light scattered by an object is directly related to its molecular weight (larger molecules scatter more light), so the peak at ~8 ml corresponds to large protein aggregates. The fact that this peak is disproportionately greater in the presence of EGTA suggests that monomeric AgTG3 is less stable than dimeric AgTG3.

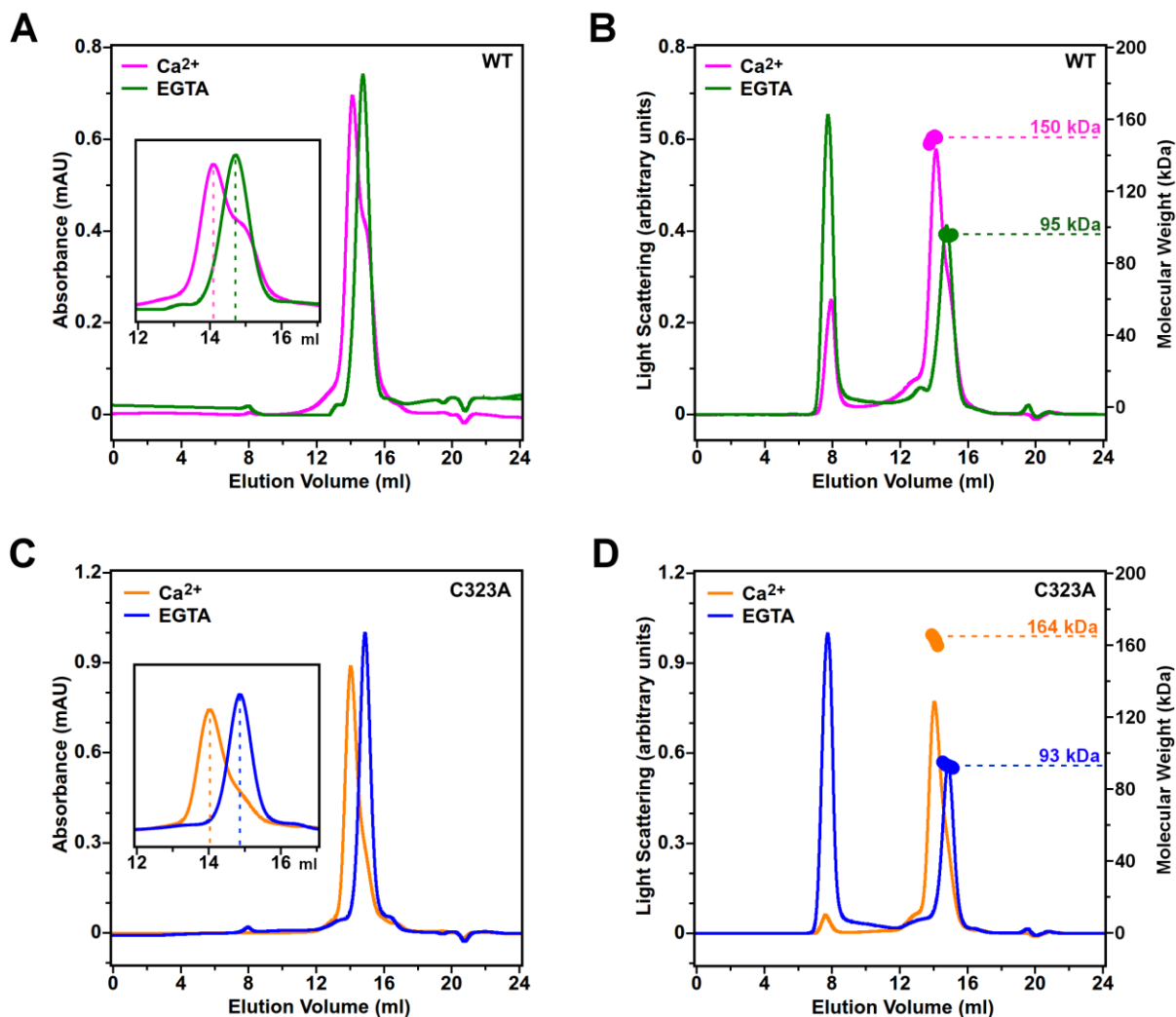


Figure 4: Biophysical parameters of WT and C323A AgTG3 from SEC-MALLS experiments. (A-B) WT and (C-D) C323A AgTG3 elute from a Superdex 200 (10/300) size-exclusion column as monomers in the absence of calcium (WT, green line; C323A, blue line). For both proteins, the elution volume shifts to that of a dimer in the presence of calcium (WT, purple line; C323A, orange line). Light scattering data confirm a shift in the oligomeric state of AgTG3 from monomers to dimers upon calcium activation.

Discussion

Malaria remains an important parasitic disease with a devastating impact on global health. Although significant gains have been made in controlling malaria in developing countries through advances in diagnostics, antimalarial drugs and insecticides, current tools and treatments are insufficient for global eradication of this disease. The *A. gambiae* seminal transglutaminase AgTG3 is important for male fertility and thus represents a novel target for the development of new strategies to control this vector (10,11).

This study identifies Cys323 as a catalytically important residue in AgTG3 and presents a framework for the design of novel inhibitors targeting this enzyme. We demonstrate that a single-point mutation, C323A, is sufficient to abolish AgTG3-mediated Plugin crosslinking activity without affecting substrate binding activity. Because the location of Cys323 is far from the proposed dimerization interface, its mutation should not have a significant impact on the structural integrity of the dimer and overall hydrodynamic properties of the enzyme. Indeed, the C323A mutation did not affect these biophysical properties of AgTG3 in solution, and we conclude that the observed lack of enzyme activity in the C323A mutant is due to direct interference with the catalytic mechanism, rather than structural changes such as protein misfolding or aggregation.

Our results support previous data that calcium is a required cofactor for AgTG3 activation and that its presence leads to dimerization of the enzyme. Whether dimerization is necessary for the AgTG3 catalytic mechanism or merely a coincidental feature remains unknown. Indeed, several homologous human transglutaminases, hTG2 and hTG3, have demonstrated a calcium requirement but do not dimerize in solution (14,15). Exploring the catalytic properties of dimerization-defective mutants could lead to the identification of novel molecular targets. Experiments to generate specific mutations at the dimerization interface and predicted calcium binding sites, without affecting the catalytic residues, are currently underway.

Although we were able to recombinantly express and purify the C323A mutant in bacteria, the protein expression level of the mutant was significantly lower than WT AgTG3. Discrepancies in expression levels could be a consequence of using *E. coli* and may not be observed using an alternative expression system, such as insect cells, which would more closely mimic the natural environment in which the proteins are produced and would also permit post-translational modifications (17). Moreover, although the recombinantly produced WT and mutant AgTG3 proteins exhibit similar bulk properties in solution, suggesting that the mutant is not structurally impaired compared to the WT protein, a more rigorous structural study is necessary to support this conclusion.

Overall, this study provides a framework for the development of inhibitors for AgTG3-mediated formation of the mating plug. The C323A mutant AgTG3 was deficient in Plugin cross-linking activity, due to specific elimination of the catalytic nucleophile. Compounds specifically designed to covalently modify the active site Cys323 residue are potential safe and effective chemosterilants for male *A. gambiae* mosquitoes and represent a novel strategy towards global eradication of malaria.

Materials and Methods

Bench work was performed using published procedures and under direct supervision.

Cloning

The plasmid encoding the C323A mutant AgTG3 was generated by site-directed mutagenesis using the Quik-change polymerase chain reaction (PCR) protocol on the wild-type pET28a-AgTG3 template using the primers 5'-GGGTCAAGTACGGCCAGGCCTGGGTGTATGCCGGGCTG-3' and 5'-CAGCCCCGGCATACACCCAGGCCTGGCC GTACTTGACCCTC-3'. The resultant PCR product was treated with DpnI for 1 h at 37 °C to digest the wild-type parental DNA template. The remaining mutant plasmid was then transformed by heat-shock treatment into competent DH5α *E. coli* cells and purified using a QIAgen Miniprep kit. DNA sequencing was performed to confirm the C323A mutation.

Purification of WT and C323A mutant AgTG3

WT and C323A mutant AgTG3 were expressed in competent Rosetta *E. coli* cells. Cells were induced at OD₆₀₀ ~0.5 with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and grown for 48 h at 18 °C. The cells were pelleted by centrifugation at 5,400 rpm for 15 min, and the supernatant was discarded. Each cell pellet was resuspended in 20 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% (w/v) glycerol and subsequently lysed by sonication. The lysate was centrifuged at 40,000 rpm at 4 °C for 30 minutes. The supernatant was loaded onto a nickel affinity HisTrap HP column (GE Healthcare), and eluted with a

0-600 mM imidazole gradient. Peak fractions were analyzed by SDS-PAGE to verify the presence of WT and C323A mutant AgTG3 proteins. The fractions were collected and concentrated for successive injections onto a HiLoad 16/60 Superdex 200 size exclusion column in 25 mM HEPES, pH 7.5, 0.1 M NaCl, 10% (w/v) glycerol, 2 mM TCEP. SDS-PAGE was used to assess the presence and purity of both proteins. Yields were 20-30 mg per liter of cell culture for WT AgTG3 and 2-3 mg per liter for the C323A mutant.

SDS-PAGE MDC Incorporation Assay

Plugin-C cross-linking was performed in 25 mM HEPES, pH 7.5, 10 mM CaCl₂, 10 mM DTT, 0.5 mM monodansyl cadaverine (MDC) at 37 °C in the presence of WT and C323A mutant proteins with and without pretreatment with 10 mM CaCl₂ or 5 mM iodoacetamide. After 30 min, the reaction was quenched by heat denaturation of proteins at 95 °C in the presence of Laemmli buffer and subjected to SDS- PAGE. The gel was scanned using Alpha Imager 2200 software to determine the extent of Plugin cross-linking to MDC.

Plate-based FITC-CAD Incorporation Assay

100 µl of Plugin-C solution (200 µl of Plugin in 5 ml of water) was placed in a 96-well nickel-coated plate (Pierce Cat# 15342) for 2 h at room temperature. The plates were washed three times with 200 µl of reaction buffer consisting of 25 mM Tris-HCl and 100 mM NaCl. 50 µl of protein solution containing 10 µg of protein and 5 µl of FITC-CAD were added to each plate with each of the conditions tested in the MDC incorporation assay. The wells were incubated for 60 min at room temperature. The wells were then washed three times with 200 µl of reaction buffer to remove excess FITC-CAD and then filled with 100 µl of reaction buffer for fluorescence measurements in a microplate reader (Biotek Synergy 2).

Size Exclusion Chromatography with Multiangle Laser Light Scattering (SEC-MALLS)

WT and C323A mutant AgTG3 were pre-incubated with 2 mM TCEP or 10 mM CaCl₂ for 1 h at

4 °C prior to the experiment. Proteins in the presence of TCEP were loaded successively onto a Superdex 200 (10/300) column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.4 mM TCEP. To assess hydrodynamic properties of the proteins in the calcium-activated form, the column was re-equilibrated overnight in 20 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM CaCl₂. Peaks were detected with an in-line detector (Jasco UV975) at 280 nm, a light scattering detector (DAWN EOS; Wyatt Technology Corp) at 690 nm, and a refractive index detector (Optilab; Wyatt Technology Corp). Protein concentration was based on the differential refractive index (dn/dc). The molecular weights (MW) were determined from the Debye plot of light scattering intensity versus scattering angles (ASTRA software; Wyatt Technology Corp).

Acknowledgements

The authors wish to thank Dr. Binh Le and Jennifer Nguyen for scientific guidance, technical assistance, and manuscript revisions. We also appreciate Marni Williams and Stephanie Reikine for their critical reading of the manuscript.

References

1. Hemingway J, Craig A. "New ways to control malaria." *Science* 303 (2004): 1984. doi:10.1126/science.1096548
2. Wondji CS, Coleman M, Kleinschmidt I, Mzilahowa T, Irving H, Ndula M, Rehman A, Morgan J, Barnes KG, Hemingway J. "Impact of pyrethroid resistance on operational malaria control in Malawi." *Proc Natl Acad Sci USA* 109.47 (2012): 19063-70. doi:10.1073/pnas.1217229109
3. Asidi A, N'Guessan R, Akogbeto M, Curtis C, Rowland M. "Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes, Benin." *Emerg Infect Dis* 18 (2012): 7. doi:10.3201/eid1807.120218
4. Haji KA, Khatib BO, Smith S, Ali AS, Devine GJ, Coetzee M, Majambere S. "Challenges for malaria elimination in Zanzibar: pyrethroid resistance in malaria vectors and poor performance of long-lasting insecticide nets." *Parasit Vectors* 6 (2013):82. doi:10.1186/1756-3305-6-82
5. Briët OJT, Penny MA, Hardy D, Awolola TS, Bortel WV, Corbel V, Dabiré RK, Etang J, Koudou BG, Tungu PK, Chitnis N. "Effects of pyrethroid resistance on the cost effectiveness of a mass distribution of long-lasting insecticidal nets: a modelling study." *Malaria J* 12 (2013): 77. doi:10.1186/1475-2875-12-77
6. Butler D. "Mosquitoes score in chemical war." *Nature* 475 (2011):19. doi:10.1038/475019a
7. Dame DA, Curtis CF, Benedict MQ, Robinson AS, Knols BGJ. "Historical applications of induced sterilisation in field populations of mosquitoes." *Malaria J* 8 (2009): S2. doi:10.1186/1475-2875-8-S2-S2
8. Howell PI, Knols BGJ. "Male mating biology" *Malaria J* 8 (2009): S8. doi:10.1186/1475-2875-8-S2-S8
9. Baldini F, Gabrieli P, Rogers DW, Catteruccia F. "Function and composition of male accessory gland secretions in *Anopheles gambiae*: a comparison with other insect vectors of infectious diseases." *Pathog Glob Health*, 106.2 (2012): 82-93. doi:10.1179/2047773212Y.0000000016

10. Rogers DW, Baldini F, Battaglia F, Panico M, Dell A, et al. "Transglutaminase-Mediated Semen Coagulation Controls Sperm Storage in the Malaria Mosquito." *PLoS Biol*, 7.12 (2009): e1000272. doi:10.1371/journal.pbio.1000272

11. Le BV, Nguyen JB, Logarajah S, Wang B, Marcus J, Williams HP, Catteruccia F, Baxter RHG. "Characterization of *Anopheles gambiae* transglutaminase 3 (AgTG3) and its native substrate Plugin." *J Biol Chem* 288.7(2013):4844-53. doi:10.1074/jbc.M112.435347

12. Yee VC, Pedersen LC, Le Trong I, Bishop PD, Stenkamp RE, and Teller DC. "Three-dimensional structure of a transglutaminase: human blood coagulation factor XIII." *Proc Natl Acad Sci USA* 91.15 (1994): 7296-7300.

13. Liu S, Cerione RA, Clardy J. "Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity." *Proc Natl Acad Sci USA* 99.5 (2001): 2743–2747. doi:10.1073/pnas.042454899

14. Pinkas DM, Strop P, Brunger AT, Khosla C. "Transglutaminase 2 undergoes a large conformational change upon activation." *PLoS Biol*. 5.12 (2007): e327. doi:10.1371/journal.pbio.0050327

15. Ahvazi B, Kim HC, Kee SH, Nemes Z, Steinert PM. "Three-dimensional structure of the human transglutaminase 3 enzyme: binding of calcium ions changes structure for activation." *EMBO J* 21.9 (2002): 2055–2067. doi:10.1093/emboj/21.9.2055

16. Noguchi K, Ishikawa K, Yokoyama K, Ohtsuka T, Nio N, Suzuki E. "Crystal structure of red sea bream transglutaminase." *J Biol Chem* 276 (2001): 12055-9. doi:10.1074/jbc.M009862200

17. Unger T, Peleg Y. "Recombinant Protein Expression in the Baculovirus-Infected Insect Cell System" *Methods Mol Biol* 800 (2012): 187-199. doi:10.1007/978-1-61779-349-3_13