Characterization of Anopheles gambiae Transglutaminase 3 (AgTG3) and Its Native Substrate Plugin*

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Background: A. gambiae transglutaminase AgTG3 cross-links Plugin within seminal fluids.

Results: AgTG3 and Plugin were purified and their structure and activity characterized in vitro.

Conclusion: AgTG3 forms a dimer in solution, analogous to human FXIII. Plugin is nonglobular in solution. AgTG3 is Ca2+-dependent and prefers Plugin as a substrate.

Significance: Inhibition of AgTG3 cross-linking of Plugin is a possible method to chemosterilize male Anopheles mosquitoes.

Male Anopheles mosquitoes coagulate their seminal fluids via cross-linking of a substrate, called Plugin, by the seminal transglutaminase AgTG3. Formation of the “mating plug” by cross-linking Plugin is necessary for efficient sperm storage by females. AgTG3 has a similar degree of sequence identity (~30%) to both human Factor XIII (FXIII) and tissue transglutaminase 2 (hTG2). Here we report the solution structure and in vitro activity for the cross-linking reaction of AgTG3 and Plugin. AgTG3 is a dimer in solution and exhibits Ca2+-dependent nonproteolytic activation analogous to cytoplasmic FXIII. The C-terminal domain of Plugin is predominantly α-helical with extended tertiary structure and oligomerizes in solution. The specific activity of AgTG3 was measured as 4.25 × 10−2 units mg−1. AgTG3 is less active than hTG2 assayed using the general substrate TVQQEL but has 8–10× higher relative activity when Plugin is the substrate. Mass spectrometric analysis of cross-linked Plugin detects specific peptides including a predicted consensus motif for cross-linking by AgTG3. These results support the development of AgTG3 inhibitors as specific and effective chemosterilants for A. gambiae.

Transglutaminases (TGs)2 are ubiquitous enzymes that catalyze the deamidation and transamidation of glutamine and the cross-linking of proteins by formation of ε-(γ-glutamyl)-lysine isopeptide bonds (1). Mammals possess multiple TGs that are involved in processes such as blood clotting by Factor XIII (FXIII), regulating cellular responses to stress by tissue transglutaminase (TG2), formation of the epithelial barrier (TG3), and coagulation of seminal plasma (TG4) (2). Similar roles for TGs have been identified in insects yet with a reduced gene set. The single TG gene present in Drosophila is involved in cuticle morphogenesis (3) and coagulation of hemolymph in response to septic injury (4). This TG is conserved in mosquitoes (Culicidae) including the malaria vector Anopheles gambiae (AGAP009100, or AgTG1). A second TG is specific to Culicidae (A. gambiae AGAP009098, or AgTG2), whereas a third TG is specific to Anopheles: A. gambiae AGAP009099, hereafter termed AgTG3.

Although AgTG1 and AgTG2 have yet to be functionally characterized, AgTG3 has recently been found to play a role in male Anopheles fertility (5). In many animals the coagulation of male seminal fluids results in the formation of a “copulatory plug” or “mating plug” that completely occludes the female reproductive tract. A recent study of A. gambiae seminal fluid proteins and male accessory glands showed that the plug is formed by cross-linking of a substrate protein called Plugin (AGAP009368) by AgTG3 (5). Furthermore, dsRNAi-mediated knockdown of AgTG3 inhibited proper sperm storage by females, indicating that correct formation of the mating plug is required for reproductive success of Anopheles males. Within Culicidae, formation of a mating plug is specific to Anopheles (6). TG-mediated cross-linking of seminal fluid proteins occurs in rodents (7), but other invertebrates use different mechanisms, such as secretion of O-glycosylated mucins in Caenorhabditis elegans (8) and homopolymerization of PEBII in Dro sophila melanogaster (9).

Formation of A. gambiae mating plug is interesting because it represents not only the independent evolution of a seminal TG in an insect but also a target for control of Anopheles, which are vectors for malaria, the world’s most devastating parasitic disease. Targeting fertility of insect populations, the sterile insect technique, is a classic method of pest control first used to con-
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AgTG3 is homologous at \(~30\%\) sequence identity to all TGs of known structure: FXIII, hTG2, hTG3, and \textit{Pagrus major} TG (17–21). FXIII is a dimer in solution whereas other TGs are monomeric. All TGs are activated by calcium (Ca\(^{2+}\)), but only one structure, hTG2, bound by a peptidomimetic inhibitor, exhibits the presumed active conformation (21); other structures adopt a conformation in which the catalytic site is occluded by the \(\beta\)-barrel domains even in the presence of Ca\(^{2+}\). Other mechanisms of regulation exist for TGs besides Ca\(^{2+}\). Serum FXIII requires proteolysis by thrombin for activation, although cytoplasmic FXIII can be activated without proteolysis (22). The activation of hTG2 is inhibited by the binding of GTP (23). Hence, some initial questions regarding the mechanism of AgTG3-mediated cross-linking of Plugin are the oligomeric states of both enzyme and substrate in solution, the specific activity of AgTG3, and the requirements for its activity.

Here we report the expression, purification, and biophysical characterization of AgTG3 and its substrate Plugin. The \textit{in vitro} reconstitution of Plugin cross-linking by AgTG3 was performed, and specific cross-linking sites within Plugin were identified. We demonstrate that AgTG3 can exist as both a monomer and dimer whereas Plugin has an extended or partially unfolded structure and tendency to oligomerize in solution. We measured the specific activity of purified AgTG3 by a fluorescent assay both in solution and in a high throughput plate-based format and confirm that the activity of AgTG3 is dependent on Ca\(^{2+}\) but not GTP. Our results provide the basis for further molecular characterization of the AgTG3-Plugin mechanism of seminal fluid coagulation and screening for specific AgTG3 inhibitors.

EXPERIMENTAL PROCEDURES

Cloning—Full-length clones of Plugin (AGAP009368) and AgTG3 (AGAP009099) were amplified from male accessory gland cDNAs prepared from 4-days old virgin \textit{A. gambiae} males and cloned into a pBluescript plasmid. Amplification was performed using the following primers: Plugin_FWD, 5’-GAATTCCATATGAGCTTGGTAGCTCTGC-3’ and Plugin_REV, 5’-GAATTCTTCACTGCAGGAAAGCATTCC-3’; AgTG3 9099_FWD, 5’-GGCCAGCCATATGCTTCACTCAGGAAAGCATTCC-3’; and AgTG3 9099_REV, 5’-GGCCAGCCATATGCTTCACTCAGGAAAGCATTCC-3’ (EcoRI and Ndel restriction sites underlined). AgTG3 was subcloned into pET28a with a C-terminal Hisk tag. A C-terminal fragment of Plugin encoding amino acid residues 345–557 (Plugin-C) was amplified by PCR with primers 5’-GCTTCACTCAGGAAAGCATTCCAGGAAAGCATTCC-3’ and 5’-GCTTCACTCAGGAAAGCATTCCAGGAAAGCATTCC-3’ (NcoI/XhoI restriction sites underlined). The PCR product was digested with NcoI/XhoI and ligated into the pHis-parallel vector (24), comprising an N-terminal His6 tag and tobacco etch virus cleavage site. All constructs were verified by DNA sequencing.

Purification of AgTG3 and Plugin—Recombinant protein was expressed in \textit{Escherichia coli} using LB medium, Rosetta\textsuperscript{TM} (Novagen) for AgTG3, and BL21(DE3) for Plugin-C. Cells were induced at \(A_{600} \sim 0.5\) with 0.5 mm isopropyl-\(\beta\)-D-galactopyranoside and grown overnight at 18\(^{\circ}\)C. All purification steps were performed at 4\(^{\circ}\)C.
For AgTG3 purification, cells were lysed by sonication in 20 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% (w/v) glycerol. The lysate was clarified by centrifugation at 40,000 × g at 4 °C for 30 min, loaded onto a HisTrap HP column (GE Healthcare), and eluted with a 0–1 M imidazole gradient. AgTG3 was further purified by Superdex 200 (GE Healthcare) size exclusion chromatography (SEC) in 25 mM HEPES, pH 7.5, 0.1 M NaCl, 10% (w/v) glycerol, 2 mM TCEP.

For Plugin-C purification, cells were lysed by sonication in 20 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole, and the lysate was clarified by centrifugation. The supernatant was loaded onto a HisTrap HP column (GE Healthcare), equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM TCEP. The supernatant was then further purified by Superdex 200 (GE Healthcare) size exclusion chromatography (SEC) in 25 mM HEPES, pH 7.5, 0.1 M NaCl.

Circular Dichroism Spectroscopy (CD)—Both AgTG3 and Plugin-C were concentrated to A280 = 0.5 in 10 mM KH2PO4, pH 7.0, 1 mM DTT. Spectra were obtained on a Jasco J-810 (average of three scans, 1-nm step) at 25 °C. Data were deconvoluted using K2D2 (25).

Size Exclusion Chromatography with Multiangle Laser Light Scattering (SEC-MALLS)—Plugin-C (0.3 mg/ml) was loaded onto a Superdex 200 (10/300) column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM TCEP. Peaks were detected with an in-line UV 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 differential refractive index (dn/dc). The molecular mass was determined from the Debye plot of light scattering intensity versus scattering angle (Astra software; Wyatt Technology Corp.).

Analytical Ultracentrifugation (AUC)—AgTG3 and Plugin-C (0.5 mg/ml) were analyzed by sedimentation velocity in a Beckman XL-1 centrifuge at 20 °C and 42,000 × g (AgTG3), 55,000 × g (Plugin). Data were analyzed with SEDFIT (26) with parameters λ = 0.7337 cm³ g⁻¹, a = 1.0096 cm⁻³, η = 0.01 P for AgTG3, and λ = 0.7369 cm³ g⁻¹, a = 1.0050 cm⁻³, η = 0.01 P for Plugin-C.

Small Angle X-ray Scattering (SAXS)—Ca2⁺-AgTG3 was purified by SEC in 25 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM CaCl2, 1 mM glycerol, and prepared at 1.0–5.0 mg/ml. Plugin-C was prepared in TBS at 0.5 mg/ml. SAXS data were collected at NSLS beamline X9 (Brookhaven National Laboratory). A 50-μl sample was loaded by autosampling into a capillary cuvette (1 mm) at 60 μl min⁻¹, 2-s exposures. Data were analyzed using in-house (pyXS) and ATSAS (27) software.

FITC Incorporation Assay—Plugin-C cross-linking was performed in 25 mM HEPES, pH 7.5, 10 mM CaCl2, 10 mM DTT, 0.5 mM FITC-cadaverine (FITC-CAD) at room temperature. After 2 h, the reaction was quenched by heat denaturation of proteins in the presence of Laemmli buffer and subjected to sodium...
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was scanned using Alpha Imager 2200 software.

**Specific Activity Assays**—All assays are the average of three measurements. For the solution assay, Plugin-C was reductively methylated with dimethylamine borane using standard procedures (28). Assay conditions were 1.25 ml of 150 mM NaCl, 55 mM HEPES, pH 7.5, 10 mM DTT, 100 μM FITC-CAD, 1 mg/ml Plugin-C, 9.6 μg/ml AgTG3, and 5 mM CaCl2. Solutions were prewarmed to 30 °C before addition of CaCl2 (6.25 μl of 1 M stock) to initiate reactions. At the indicated time points, 200-μl aliquots were removed, mixed with 22 μl of 0.5 M iodoacetamide, and incubated for 30 min at room temperature. Aliquots were diluted to 0.5 ml with PBS and loaded onto a PD-10 desalting column (GE Healthcare) followed by 2 ml of PBS, eluted with 3.5 ml with PBS. Fluorescence (485/515) was measured on a JY Horiba Fluorolog-3 and fluorescence quantified relative to a FITC-CAD standard curve.

ELISAs for specific activity relative to guinea pig TG2 were performed with a standard kit (Sigma CS1070). For Plugin-plated-based assay, Plugin-C (25 μg/well) was incubated in black 96-well Ni-coated plates (Pierce 15342) for 2 h at room temperature or overnight at 4 °C. Plates were washed three times with 200 μl of TBS and loaded with 50 μl of AgTG3 (diluted to given concentration with water), then 50 μl of 2× assay buffer (2× TBS, 20 mM CaCl2, 100 μM FITC-CAD, 2 mM DTT). Wells were incubated for 60 min at room temperature, washed three times with 200 μl of TBS, and filled to 100-μl final volume for fluorescence measurement in a microplate reader (Biotek Synergy 2). Inhibition was performed by 30-min preincubation with 50 mM iodoacetamide (Sigma A3221).

**Mass Spectrometry**—Cross-linked Plugin-C was separated on SDS-PAGE, excised, and subjected to in-gel tryptic digest. Peptides were separated on a Waters nanoACQUITY (75 μm × 250 mm eluted at 300 nl/min) and analyzed on a LTQ Orbitrap
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RESULTS

AgTG3 was expressed and purified to homogeneity in E. coli (Fig. 2A). Purification and SDS-PAGE were equivalent in reducing and nonreducing conditions; hence, AgTG3 contains no disulfide bonds. The AgTG3 CD spectrum (Fig. 2B) corresponds to 40–50% β-sheet and 5–15% α-helix. In the presence of 10 mM CaCl₂ AgTG3 elutes from Superdex 200 column with an apparent molecular mass of ~160 kDa suggesting a dimeric species (Fig. 2C). In the presence of 1 mM EDTA AgTG3 appears more heterogeneous, with the appearance of a shoulder peak at the apparent molecular mass of a monomer. AUC sedimentation velocity analysis of Ca²⁺-AgTG3 (Fig. 2D) gives a molecular mass estimate of ~170 kDa (s₂₀,₆₅ = 7.0, f₀/₀ = 1.4), consistent with the estimate from SEC.

We used SAXS to further analyze the structure of Ca²⁺-AgTG3 in solution. In 1 M glycerol Ca²⁺-AgTG3 gave consistent scattering curves in the concentration range 1.0–5.0 mg/ml (Fig. 2E). Guinier analysis (Fig. 2E, inset) at 1 mg/ml determined a radius of gyration R_G = 42.7 Å. Scattering at

FIGURE 6. Identification of Plugin cross-linking sites. A, CID-MS/MS analyses and corresponding fragment ion series for three specific peaks identified by mass spectrometric analysis of cross-linked Plugin-C tryptic digest. B, location of cross-linking sites within Plugin-C sequence. C, alignment of α-helices in Plugin C-terminal domain. The positions of known cross-linked Gln residues are highlighted in green, conserved or highly similar residues are shown in bold.

mass spectrometer. Results were analyzed using MassMatrix (29). Identities of crosslinked peptides were confirmed by Collision-induced Dissociation tandem mass spectrometry (CID-MS/MS).
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zero angle (I_o) was directly proportional to concentration, although a systematic increase in R_g suggests some nonideality of the solute. Calibration with a 1.0 mg/ml solution of BSA gave an estimated molecular mass of 173 kDa, consistent with that of a dimer. The radial distribution function p(r) calculated by GNOM (30) requires D_{max} = 160 Å (Fig. 2F), almost 4 \times R_g, implying that the Ca^{2+}-AgTG3 dimer is a prolate ellipsoid. Ab initio shape determination was performed using 20 independent models calculated by DAMMIF (31) with P2 symmetry and normalized spatial discrepancy (32) NSD = 0.67 ± 0.18.

The most probable volume for the Ca^{2+}-AgTG3 dimer (Fig. 2G) suggests a structure similar to that of the Ca^{2+}-bound FXIII dimer (18), yet with an open conformation for each AgTG3 monomer, analogous to that observed for inhibitor-bound hTG2 (21). A homology model for Ca^{2+}-AgTG3 based on hTG2 (Protein Data Bank ID 2Q3Z) was superimposed on the FXIII dimer (Protein Data Bank ID 1GGU) and aligned with the most probable volume; imposition of P2 symmetry gives a good fit to the model.

Full-length Plugin is susceptible to proteolysis and aggregates in solution. Plugin-C (residues 345–557) was purified to homogeneity (Fig. 3A). The CD spectrum (Fig. 3B) has minima at 208 nm and 222 nm consistent with predominant α-helical secondary structure. At ≈0.5 mg/ml Plugin-C exhibits an apparent molecular mass of 90 kDa on SEC, but static light scattering (Fig. 3C) is consistent with a monomeric molecular mass of ~30 kDa, suggesting that the protein has a nonglobular conformation in solution. AUC sedimentation velocity analysis (Fig. 3D) also yielded a molecular mass estimate of ~30 kDa for Plugin-C (s_{20,w} = 2.1 S, f/s_{0} = 1.4).

We analyzed Plugin-C (0.5 mg/ml) by SAXS to confirm its extended structure in solution. The scattering curve (Fig. 3E) resembles that of an unfolded or heterogeneous sample, precluding calculation of a p(r) function. Guinier analysis (Fig. 3F) yields an estimated molecular mass of ~30 kDa based on I_o (calibrated with BSA) but an unusually large radius of gyration (R_g = 43 Å). In such cases the Kratky plot provides a qualitative measure of flexibility in a scattering particle (33, 34). Both absolute (Fig. 3G) and dimensionless (Fig. 3H) Kratky plots for Plugin-C suggest a partly unfolded tertiary structure. At concentrations >0.5 mg/ml Plugin-C forms oligomers or soluble aggregates that interfere with particle analysis.

Cross-linking of Plugin by AgTG3 was previously demonstrated using endogenous extracts of male accessory glands, monodansylcadaverine, Ca^{2+}, and GTP (5). We therefore reconstituted AgTG3 cross-linking of Plugin-C in vitro. Combining AgTG3 and Plugin-C in the presence of Ca^{2+} produced a ladder of cross-linked Plugin-C bands of increasing molecular mass (Fig. 4A). The presence of FITC-CAD led to fluorescent labeling of both the monomer and cross-linked bands (Fig. 4B), confirming that cross-linking was the result of transamidation. The requirement for Ca^{2+} was confirmed by the inhibitory effect of the chelating agent EGTA. Whereas Ca^{2+} binding sites are conserved between AgTG3 and other TGs, the GTP binding site found in tissue TG (TG2) is not: only two binding site residues are conservative substitutions (S171A, F174G, R476E, R478K, S482E, R580K). Consistent with this observation, no effect on AgTG3 cross-linking of Plugin-C was detected in the presence of GTP.

Next, we used the conjugation of FITC-CAD to Plugin-C to determine the specific activity of transamidation for AgTG3. Reductive methylation of Plugin-C prevents the transamidation of lysine residues. Fluorescence was measured after incubation for 0–60 min at 30 °C and buffer exchange (PD-10 column) to remove unbound FITC-CAD (Fig. 5A). In a 1.25-ml total volume, 1 mg/ml (40 μM) Plugin-C, 100 μM FITC-CAD, 1.2 μg of AgTG3 cross-linked FITC-CAD to Plugin-C at 0.408 μM min^{-1}, yielding a specific activity of 4.25 × 10^{-2} unit mg^{-1} or 3.5 unit μmol^{-1} AgTG3 (1 unit = 1 μmol min^{-1} FITC-CAD).

The specific activity of AgTG3 determined above is 100× lower than is typically observed for standard TGs such as guinea pig TG2 (35). To confirm this result we performed a standard ELISA for transamidation of a biotinylated peptide (BntTVQHEL-OH) to polylysine-coated plates, with guinea pig TG2 (1.5 unit mg^{-1}) as a standard (Fig. 5B). The relative specific activity of AgTG3 was 0.04 ± 0.01 unit mg^{-1}, significantly less than is measured for human Factor XII or TG2 (1 unit = 1 μmol min^{-1} hydroxamate from Na₂-Z-Gln-Gly, NH₄OH, pH 6.0 at 37 °C).

To test whether the specific activity of AgTG3 may be substrate-dependent we repeated our FITC-CAD assay in a plate-based format. His₆-tagged Plugin-C was adsorbed to Ni-coated plates and incubated with FITC-CAD and either guinea pig TG2 or AgTG3 for 60 min at room temperature (Fig. 5C). The specific activity of AgTG3 was 0.3–0.4 unit mg^{-1} relative to guinea pig TG2, an 8–10-fold increase compared with the previous assay. Iodoacetamide irreversibly inhibits transglutaminases by acetylating the active site cysteine. AgTG3 (10 μg) was efficiently inhibited by 30-min preincubation with iodoacetamide in the plate-based Plugin/FITC-CAD assay (Fig. 5D).

To identify specific AgTG3 cross-linking sites within Plugin-C the high molecular mass band observed on SDS-PAGE (Fig. 4) was excised and subjected to in-gel tryptic digestion and tandem mass spectrometry. Mass spectrometry data were input into the MassMatrix (29) search engine, which evaluates the monoisotopic masses of peptides, looking for a mass shift of ~17 Da due to release of ammonia during isopeptide formation. Three specific peaks: m = 2947.5 ([M + H]^{+}, m/z = 590.5), m = 2977.4 ([M + H]^{+}, m/z = 596.5), and m = 3429.7 ([M + H]^{+}, m/z = 572.6) were detected. The first peak corresponds to Plugin peptides 432–445 (m = 1729.9) and 500–509 (m = 1233.7) minus 17 Da resulting in a monoisotopic mass m = 2946.6. The calculated mass is within error of m = 2947.5 (±1 atomic mass unit, accuracy ±0.02%). The identity of cross-linked species was confirmed by y and b ion fragments (Fig. 6A). On the basis of the fragments observed and the sequences of two tryptic peptides, we deduce that Lys-433 in peptide 432–445 and either Gln-504 or Gln-506 in peptide 500–509 are linked by an e-(γ-glutamyl)lysine isopeptide bond. By similar calculations, we found that the second peak (m = 2977.4) corresponds to Plugin peptides 432–445 and 408–417 with the
isopeptide bond formed between Lys-433 and either Gln-411 or
Gln-412. The third peak (m = 3429.7) corresponds to Plugin
peptides 409–431 and either 442–445 or 496–499 with the
isopeptide bond formed between Lys-417 and either Gln-442 or
Gln-496.

Interestingly, whereas Plugin-C contains 10 lysine residues,
two of the three cross-linked peptides detected involve the
same lysine residue Lys-433 cross-linked to Gln residues Gln-
411 and Gln-506, in the preceding and subsequent α-helices,
respectively (Fig. 6B). The third cross-link is between Lys-417
and the sequence QIGR, which occurs twice in Plugin-C within
the consensus repeat near the N terminus of each predicted
α-helix. The Gln residues involved in cross-linking are present
in a majority of the six α-helices within the C-terminal domain
of Plugin (Fig. 6C).

**DISCUSSION**

We have characterized *A. gambiae* seminal transglutaminase
AgTG3 and the cross-linking of its native substrate Plugin in vitro.
Intriguingly, we find that AgTG3 forms a dimer in solution,
similar to the mammalian blood-clotting enzyme FXIII,
yet distinct from other transglutaminases of known structure.
AgTG3 has similar (∼30%) sequence identity to all of these
enzymes. This raises the question: what features of the TG
sequence are responsible for dimerization of AgTG3/FXIII versus
tissue-type transglutaminases?

This question can be addressed by mapping the dimer inter-
facing of the Ca2⁺-bound FXIII reported by PISA (36) to the
alignment of AgTG3 with TGs of known structure (Fig. 7). For
the majority of this interface AgTG3 is no more similar to FXIII
than other TGs with two exceptions. First, tissue-type TGs have
an extended loop following the second α-helix of the core
domain (hTG2 200–206), absent from AgTG3 and FXIII,
which would interrupt the FXIII dimer interface. Second,
AgTG3/FXIII share a charged residue in the loop prior to the
catalytic aspartate within the core domain, the βCD loop, at the
center of the dimer interface. Specifically FXIII Arg-408
(AgTG3 Lys-417) is replaced by hTG2 Cys-370, which forms a
vicinal disulfide with Cys-371 in the active conformation of
hTG2 and is implicated in oxidative inhibition of tissue TGs
(37).

An important assumption in the analysis above is that
both the monomeric structure and dimeric interface of
AgTG3 are equivalent to those of FXIII. Further structural
studies of AgTG3 are necessary to test this assumption.
Hence, a caveat to our analysis is that AgTG3 diverges sig-
nificantly from all mammalian TGs, including numerous
amino acid substitutions and unique extensions, notably in
two loops within the β-sandwich domain. A high resolution
structure of AgTG3 would be required to address the molec-
ular basis of AgTG3 dimerization.

Whereas AgTG3 adopts a folded tertiary structure, Plugin-C
has an extended or partially unfolded tertiary structure despite
clear α-helical secondary structure and a propensity for oligo-
erization or aggregation in solution. Oligomerization of TG
substrates is found for both FXIII and seminal TGs. Fibrin αC
domains preassemble into polymers which are then reinforced
by cross-linking with FXIIIs (38, 39), and seminal vesicle sec-
retion proteins in rodents form disulfide-bridged high molecular
mass complexes prior to cross-linking by the seminal transglu-
taminase TG4 (40). We suggest that oligomerization of Plugin is
mediated by coiled-coil interactions between the repetitive
helical regions of the C-terminal domain that are subsequently
reinforced by AgTG3 cross-linking. The N-terminal domain of
Plugin has a high density of putative cross-linking sites and is
expected to form a densely cross-linked polymer, so association
of the C-terminal domain may have a significant effect on the
density and mechanical properties of the *Anopheles* mating
plug.

The specific activity of AgTG3 is 8–10 times lower than that of
other well characterized TGs. Although this may arise from
the use of *E. coli* to express AgTG3, we note that both FXIII and
hTG2 have been expressed in an active form from this host (37,
41). From studies of other TGs we propose two explanations.
First, AgTG3 includes an N-terminal extension of ∼20 amino
acids that may bind across the dimer interface, as is observed in
FXIII. Activation of serum FXIII includes cleavage of this
sequence by thrombin: if a similar mechanism held for AgTG3
the purified enzyme may exhibit autoinhibition resulting in
suboptimal activity in vitro. Second, hTG2 has been shown to
exhibit redox regulation of activity (37). AgTG3 contains a
number of cysteine residues that are not conserved with mam-
malian TGs; despite the lack of evident disulfides in the purified
protein these cysteines may be involved in oxidative inhibition
of AgTG3.

AgTG3 has higher relative activity toward Plugin-C/FITC-
CAD versus Btn-TVQVQL-OH and polylysine-coated plates.
Hence, AgTG3 may have some preference for specific sequence
motifs in Plugin. Among the cross-linking sites identified is the
motif QIGR present in the majority of α-helices in the C-
terminal domain (Fig. 6C). Its similarity to the motif present in
the N-terminal domain of Plugin suggests that QI(G/P) is a pre-
ferred motif for cross-linking by AgTG3.

AgTG3 activity toward Plugin-C/FITC-CAD, however, may
be due to a preference for (i) polypeptide or α-helical versus
hexapeptide substrates, or (ii) soluble polyanine versus
surface-bound lysine. Further experiments are necessary to
discriminate between these hypotheses. Nevertheless, the current
signal-to-noise achieved with the nonspecific inhibitor iodac-
etamide is sufficient for in vitro high throughput screening of
prospective AgTG3 inhibitors (Z’ = 0.84).

The mechanism for formation of the copulatory plug in *A.
gambiae* bears intriguing similarities to the mammalian

![Figure 7: Sequence alignment of AgTG3(22–748) with transglutaminases of known structure: FXIII, hTG2, hTG3, and *P. major* TG. Secondary structure elements colored and labeled by domain. Identical residues are shaded gray, similar residues are boxed in gray. Catalytic residues are shaded yellow, Ca²⁺-binding residues (based upon hTG3) are shaded pink. FXIII residues at the dimer interface are shaded green. Numbering corresponds to AgTG3 full-length sequence. Figure was generated with ESPRIPPT (42).](image-url)
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blood-clotting cascade: a dimeric TG activated by calcium to act upon a specific substrate capable of preassembly of a oligomeric/polymeric state. Given their diverse functions, these similarities appear to be an instance of convergent evolution. Future investigation of AgTG3 will provide insight into the evolution of TGs and advance our basic understanding of fertility as well as the development of potential chemosterilants for male Anopheles.

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