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GhoT of the GhoT/GhoS toxin/antitoxin system damages lipid membranes by forming transient pores

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ABSTRACT

GhoT is a bacterial toxin of the type V toxin/antitoxin system that allows *Escherichia coli* to reduce its metabolism in response to oxidative and bile stress. GhoT functions by increasing membrane permeability and reducing both ATP levels and the proton motive force. However, how GhoT damages the inner membrane has not been elucidated. Here we investigated how GhoT damages membranes by studying its interaction with lipid bilayers and determined that GhoT does not cause macroscopic disruption of the lipid bilayer to increase membrane permeability to the dye carboxyfluorescein. Using circular dichroism, we found that GhoT forms an alpha helical structure in lipid bilayers that agrees with the structure predicted by the I-TASSER protein structure prediction program. The structure generated using I-TASSER was used to conduct coarse-grained molecular dynamics simulations, which indicate that GhoT damages the cell membrane, as a multimer, by forming transient transmembrane pores.

1. Introduction

Toxin/antitoxin (TA) systems in bacteria reduce the metabolism of cells to allow them to weather nutritional, oxidative, and antibiotic stress [1]. TA systems consist of two major parts, a toxin protein that disrupts essential cellular processes and an antitoxin (protein or RNA) that inhibits the activity of the toxin [2]. Without stress, toxins are inactivated, but during stress, antitoxins are degraded and the toxin reduces metabolism [2].

TA systems are classified into seven different types based on how the antitoxin neutralizes the toxin. In type I systems (e.g., Hok/Sok), the antitoxin RNA interferes with the translation of the toxin [3]. In type II systems (e.g., CcdB/CcdA), the antitoxin protein binds to toxin and inhibits its activity [4]. In type III systems (e.g., ToxN/ToxI), the antitoxin RNA binds the toxin to inhibit it [5]. In type IV systems (e.g., CbtA/CbeA), the antitoxin protein inhibits the toxin by binding its target [6]. In type V systems (e.g., GhoT/GhoS), the antitoxin is a specific RNase that cleaves the toxin's mRNA [7]. In type VI systems (e.g., SocB/SocA), the antitoxin protein promotes the degradation of the toxin [8]. In type VII systems (e.g., Hha/TomB), the antitoxin is an enzyme that oxidizes specifically the toxin to inactivate it [9].

The cellular targets of toxin proteins of TA systems vary and include (i) replication by inhibiting DNA gyrase (CcdB, ParE) [10,11], (ii) translation by cleaving many mRNA (SymE, MazF, HicA, RelE, MqsR) [7,10–13], (iii) translation by phosphorylating EF-Tu (HipA) [14], (iv) cellular structure by inhibiting FtsZ (YeeV, CptA) [6,15] and (v) adenosine triphosphate (ATP) synthesis by cell membrane damage (Hok, TisB) [16,17].

Previously, we found the first type V TA system, GhoT/GhoS, in which ribonuclease GhoS (antitoxin) cleaves the mRNA of GhoT toxin to inhibit its translation [18]. The GhoT/GhoS TA system is also controlled by the MqsR-MqsA TA system (type II) in which toxin MqsR degrades specifically the GhoS antitoxin mRNA during oxidative stress and bile stress (the GhoT mRNA lacks MqsR cleavage sites) [19,20]. Critically, the formation of persister cells by MqsR depends partially on toxin GhoT [18]. Production of toxin GhoT damages the cell membrane and reduces ATP levels, the proton motive force (PMF), and cell growth [12]. GhoT consists of 57 amino acids, 68% of which are hydrophobic. GhoT is predicted to contain an amphiphilic a-helix, and localization of the non-toxic GhoT variant (GhoT F38R) indicates GhoT is membrane protein [12].

In this study, the mechanism of GhoT toxicity was determined using lipid bilayers to mimic the *E. coli* inner membrane [21]. Circular dichroism (CD) showed that GhoT changes its conformation in the presence of lipids to form a structure that agrees with protein modeling results [12]; hence, we confirm that GhoT is a membrane protein with alpha-helical structure. Furthermore, GhoT does not

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cause macroscopic disruption of liposomes. Simulations of GhoT in the lipid bilayer indicate that GhoT damages the cell membrane by forming transient pores through the membrane. This mechanism is different from that of many toxins, such as TisB of the TisB/IstR TA system [22], that disrupt the membrane by forming permanent pores. However, other peptides (especially antimicrobial peptides) cause leakage by transiently forming pore-like aggregates that disrupt the sharp hydrophobic/hydrophilic interface within the cell membrane [23,24], similarly to what we observe with GhoT.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli BW25113 Δ*ghoS* with plasmids pCA24N [25], pCA24N-GhoT [25], and pCA24N-GhoT F38R [12] were utilized; chloramphenicol (34 μg/mL) was used to retain the plasmids. To induce protein expression, isopropyl β-*D*-1-thiogalactopyranoside (0.1 mM) was added after 1 h. Strains were grown in LB medium [26] at 37 °C with shaking at 250 rpm. To test for membrane damage, GhoT and GhoT F38R (0.25 μg/mL) were electroporated into BW25113 (200 Ω, 1.25 kV) [26].

2.2. Peptide synthesis

GhoT and GhoT F38R peptides were synthesized by Lifetein LLC (New Jersey, USA). The purities the syntheses were over 90% as confirmed by HPLC, and the identities of the proteins were confirmed by mass spectrum (MS). The peptides were dissolved in DMSO or methanol as indicated.

2.3. Determination of secondary structures of GhoT via CD spectrum

Egg L-α-phosphatidylcholine (PC, Avanti, #840051) and brain L-α-phosphatidylserine (PS, Avanti, #840032) lipids were dissolved in chloroform (10 mg/mL), and GhoT was dissolved in methanol (1 mg/mL). To make 1 mL of liposomes (1 mg/mL), 179 μL of egg PC and 21 μL of brain PS were mixed (1:1) with 100 μL of GhoT in a round tube at a final molar ratio of peptide:lipid = 1:85. The chloroform and methanol were removed by a rotary evaporator (180 rpm, 420 mbar) at 42 °C. Lipid and peptide films were incubated in the vacuum chamber for an additional 3 h to ensure evaporation of chloroform and methanol. 1 mL of 100 mM sodium phosphate buffer (pH 7.4) was added to the lipid-peptide films and incubated overnight at 4 °C to dissolve the films in buffer via a stirrer. The mixture of lipid and peptides dissolved in phosphate buffer was extruded through track-etched polycarbonate membrane filters (Whatman, Maidstone, UK) with diameters through 400 nm (5 times), 200 nm (5 times), 100 nm (10 times) to prepare the liposomes containing GhoT at a size of ~100 nm diameter. The quality of the liposomes was determined by dynamic light scattering (Zetasizer Nano ZS, Malvern). The CD spectrum was analyzed by Jasco J-1500 CD Spectrometer (Easton, MD, USA).

2.4. Liposomal content release assay

Egg PC (Avanti, 840051) and brain PS (Avanti, 840032) stocks were dissolved in chloroform (10 mg/mL) and used to prepare the PC-PS liposomes. *E. coli* total lipid extracts (Avanti, 100500) were dissolved in chloroform (10 mg/mL) and used for the *E. coli* liposomes. A 50 mM 5(6)-carboxyfluorescein (CF) (Sigma-Aldrich, 21877) solution was prepared in 50 mM HEPES buffer (pH 7.4). CF solution (2 mL, 50 mM) was added to the lipid film and incubated overnight at 4 °C to dissolve the lipid film in solution by stirring. The lipid dissolved in 50 mM carboxyfluorescein solution was extruded through a 100 nm polycarbonate filter (Whatman, Maidstone, UK) 10 times to generate a consistent 100 nm diameter liposomes. The size of all the prepared liposomes were confirmed by dynamic light scattering (Zetasizer Nano ZS, Malvern). Liposomes (50 μL) were mixed with 40 μL of 50 mM HEPES buffer to make a final concentration of 0.5 mg/mL and pre-incubated in the fluorometer (TECAN, Infinite M200 pro) for 10 min. Various concentrations of native GhoT and GhoT F38R (final conc. 0.25, 0.125, 0.05 mg/mL) dissolved in DMSO were added to the liposomes. The fluorescence signal was measured with excitation at 490 nm and emission at 520 nm (gain 42) at 25 °C. Triton X-100 (1%) was added to determine the value for 100% release of carboxyfluorescein. The percentage of CF release was calculated as $\frac{(CF_t - CF_0)}{(CF_{tx-100} - CF_0)} \times 100$.

2.5. Simulations

From the known amino acid sequences of the native and F38R variant GhoT peptides, we used the I-TASSER structure prediction program [27,28] to determine the most probable structure for each peptide. We then converted the all-atom structures obtained from I-TASSER to MARTINI coarse-grained structures [29–32] using the martinize.py script [30,31]. We then combined these structures with lipids, either anionic 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) or zwitterionic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and water, also represented using the MARTINI coarse-grained model. Initial configuration construction and simulations were both conducted using the Gromacs program for biological simulations [33]. Images of our simulations were made using Visual Molecular Dynamics software [34]. For the remaining simulation details, please see the supplementary methods.

3. Results

3.1. Conformational changes of GhoT in a lipid bilayer

Since GhoT is very toxic, it has never been visualized by SDS-PAGE or Western blotting [12]; hence, in order to study it, we synthesized GhoT chemically. GhoT is poorly soluble so it was dissolved in methanol.

The secondary structure of GhoT in the presence or absence of a lipid bilayer was determined based on its CD spectrum. The *E. coli* inner membrane is a highly impermeable bilayer. Since the *E. coli* inner membrane consists of up to 25% of negatively charged lipids (phosphatidylglycerol (PG) and cardiolipin) [35], we used PC-PS liposomes (9:1 ratio) which contains 10% of negatively charged lipids (PS). As expected as a membrane protein, GhoT displayed a conformational secondary structure in the presence of the lipid bilayer while no secondary structure was determined in the absence of lipid (Fig. 1). The CD spectrum had a negative peak around 222 nm and 208 nm and a positive peak around 195 nm. This result confirms that GhoT forms an α-helix when it is contacted with lipids as previously predicted [12].

3.2. GhoT promotes release of liposomal contents

In the previous research, membrane damage, depletion of intracellular ATP, and disruption of the proton motive force by GhoT was demonstrated *in vivo* [12]. To show directly that GhoT damages membranes, a liposomal contents release assay was performed. CF is a self-quenching fluorescein that fluoresces only at low concentrations [36]; hence, damage of membranes is monitored by the % CF released from liposomes. Since fluorescence generated by CF started to increase at 25 mM (Fig. S1), small unilamellar liposomes

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