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Stabilization of the Tertiary Structure of the Cholera Toxin A1 Subunit Inhibits Toxin Dislocation and Cellular Intoxication

Shane Massey[1], Tuhina Banerjee[1], Abhay H. Pande[1], Michael Taylor[1], Suren A. Tatulian[2] and Ken Teter[1]*

[1]*Burnett School of Biomedical Science, College of Medicine, University of Central Florida, Orlando, FL 32826, USA*

[2]*Department of Physics, University of Central Florida, Orlando, FL 32816, USA*

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Cholera toxin (CT) moves from the cell surface to the endoplasmic reticulum (ER) by retrograde vesicular transport. The catalytic subunit of CT (CTA1) then crosses the ER membrane and enters the cytosol in a process that involves the quality control mechanism of ER-associated degradation. The molecular details of this dislocation event have not been fully characterized. Here, we report that thermal instability in the CTA1 subunit—specifically, the loss of CTA1 tertiary structure at 37 °C—triggers toxin dislocation. Biophysical studies found that glycerol preferentially stabilized the tertiary structure of CTA1 without having any noticeable effect on the thermal stability of its secondary structure. The thermal disordering of CTA1 tertiary structure normally preceded the perturbation of its secondary structure, but in the presence of 10% glycerol the temperature-induced loss of CTA1 tertiary structure occurred at higher temperatures in tandem with the loss of CTA1 secondary structure. The glycerol-induced stabilization of CTA1 tertiary structure blocked CTA1 dislocation from the ER and instead promoted CTA1 secretion into the extracellular medium. This, in turn, inhibited CT intoxication. Glycerol treatment also inhibited the in vitro degradation of CTA1 by the core 20S proteasome. Collectively, these findings indicate that toxin thermal instability plays a key role in the intoxication process. They also suggest the stabilization of CTA1 tertiary structure is a potential goal for novel antitoxin therapeutic agents.

Keywords: cholera toxin; circular dichroism; endoplasmic reticulum-associated degradation; surface plasmon resonance; toxin translocation

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Introduction

Cholera toxin (CT) is an AB_5 protein toxin that ADP-ribosylates and activates the stimulatory α subunit of the heterotrimeric G protein ($G_{s\alpha}$).[1,2] The catalytic A moiety of CT is synthesized as a CTA protein of 27 -kDa molecular mass. Nicking of CTA by the *Vibrio cholerae* hemagglutinin protease or other proteases generates a disulfide-linked CTA1/CTA2 heterodimer. Enzymatic activity is a property of the 22 -kDa CTA1 subunit, while the 5 -kDa CTA2 subunit interacts noncovalently with the B pentamer and thereby tethers CTA1 to the CTB domain. The cell-binding B moiety of CT is assembled from 12 -kDa monomers as a homopentameric ring-like structure that adheres to G_{M1} gangliosides on the eukaryotic plasma membrane.

A substantial portion of surface-bound CT is delivered to the lysosomes and degraded, but the functional pool of toxin is instead transported to the endoplasmic reticulum (ER) through a series of vesicular trafficking events.[3–6] The resident redox state of the ER reduces the CTA1/CTA2 disulfide

*Corresponding author. E-mail address: kteter@mail.ucf.edu.

Present address: A. H. Pande, Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sec-67, S.A.S. Nagar (Mohali)-160062, India.

Abbreviations used: BfA, brefeldin A; CT, cholera toxin; ER, endoplasmic reticulum; ERAD, ER-associated degradation; EDTA, ethylenediaminetetraacetic acid; β-ME, β-mercaptoethanol; GSH, glutathione; PBS, phosphate-buffered saline; PBST, PBS containing 1% Triton X-100; PDI, protein disulfide isomerase; SPR, surface plasmon resonance.

bond, which then permits chaperone-assisted dissociation of CTA1 from CTA2/CTB_5.[7–9] The isolated CTA1 subunit subsequently crosses the ER membrane and enters the cytosol where it interacts with $G_{s\alpha}$. Activated $G_{s\alpha}$ stimulates adenylate cyclase function and the production of cAMP. This leads to the opening of chloride channels on the apical face of intoxicated intestinal epithelial cells; the osmotic movement of water that follows chloride efflux into the gut generates the profuse watery diarrhea of cholera.[1,2]

To move from the ER to the cytosol, CTA1 uses the ER-associated degradation (ERAD) dislocation mechanism.[9–13] ERAD recognizes misfolded or misassembled proteins in the ER and exports them to the cytosol for degradation by the 26S proteasome.[14] CTA1 export probably occurs through Sec61 and/or Derlin-1-protein-conducting channels in the ER membrane.[15–17] Although CTA1 is processed as an ERAD substrate, it avoids the standard ERAD route of ubiquitin-dependent proteasomal degradation because its arginine-over-lysine bias limits the number of potential sites for ubiquitin conjugation.[18] Other AB toxins such as Shiga toxin and ricin also move from the cell surface to the ER and exploit ERAD for entry into the cytosol.[19,20]

Two major predictions have been derived from the ERAD model of CTA1 dislocation: (i) the C-terminal hydrophobic region of CTA1 (residues 162–192; the A13 subdomain) triggers ERAD-mediated toxin entry into the cytosol, and (ii) the translocated pool of CTA1 is stable in the eukaryotic cytosol.[6,21] It is hypothesized that components of the ERAD machinery interact with the CTA13 subdomain and subsequently unfold the toxin for passage into the cytosol.[9] CTA1 is then thought to spontaneously refold in the cytosol, producing a stable conformation that is resistant to proteasomal degradation.[18]

We have shown that the $CTA1_3$ subdomain is not required for toxin entry into the cytosol and that the translocated pool of CTA1 is not stable in the cytosol.[10,22] Both observations may be linked to the unstable, heat-labile nature of the CTA1 subunit.[23,24] CTA1 is held in a stable conformation when associated with other components of the holotoxin,[24–26] but it can unfold spontaneously after dissociation from CTA2/CTB_5.[24] This unfolding event would activate the ERAD system and thereby promote CTA1 export to the cytosol. Following dislocation into the cytosol, CTA1 could retain significant enzymatic activity because of its association with host proteins such as the ADP-ribosylation factors that serve as cofactors to enhance CTA1 activity.[23,24,27] However, the structural state of the isolated CTA1 subunit leaves it susceptible to ubiquitin-independent degradation by the core 20S proteasome.[24] With this model of toxin–ERAD interactions, an inherent physical property of the CTA1 subunit (i.e., thermal instability) is linked to both toxin dislocation into the cytosol and toxin degradation in the cytosol. Our model suggests CTA1 is processed as a typical misfolded/unfolded ERAD substrate, whereas a prevailing model of toxin dislocation treats CTA1 as a stable protein that requires chaperone-assisted unfolding in order to move from the ER to the cytosol.[6,9,16,18,28]

According to our model, structural stabilization of the CTA1 subunit will inhibit CTA1 dislocation and thereby prevent CT intoxication. The thermal stabilization of CTA1 should also block its degradation by the 20S proteasome, which only acts on unfolded substrates.[29] To test these predictions, we examined the impact of glycerol on CTA1 structure, CTA1 dislocation/degradation, and CT intoxication. Glycerol is a chemical chaperone that stabilizes protein structures and is commonly used to disrupt ERAD–substrate interactions.[30–34] Glycerol has also been shown, by an unknown mechanism, to protect cultured cells against intoxication with either ricin or Shiga toxin 2.[35,36] In this work, we show that glycerol prevents the temperature-induced loss of CTA1 tertiary structure, which in turn prevents CTA1 dislocation into the cytosol and productive intoxication. Glycerol also inhibited the in vitro degradation of CTA1 by the 20S proteasome. These observations provide mechanistic insight into the molecular events underlying CTA1–ERAD interactions and suggest a new therapeutic approach for antitoxin countermeasures.

Results

Effect of glycerol on CTA1 protease sensitivity

As a first step toward evaluating the stabilizing effect of glycerol on CTA1 structure, we employed a protease sensitivity assay (Fig. 1). A final concentration of 10% glycerol was used in order to maintain consistency with other reports that have used this concentration of glycerol to disrupt ERAD–substrate and/or host–toxin interactions.[31–33,35,36] Protease sensitivity assays are used to probe the folding state of a protein, as proteins often become more susceptible to proteolysis upon (partial) unfolding.[9,24,37] Samples of the reduced CTA1/CTA2 heterodimer were incubated in the absence or presence of 10% glycerol for 45 min at 4, 25, 33, 37, or 41 °C. All samples were then placed on ice and exposed to thermolysin, a metalloendoprotease that cleaves the peptide bonds in proteins at the surface-exposed hydrophobic residues. Ethylenediaminetetraacetic acid (EDTA) and sample buffer were added after 45 min to halt the digests, and the samples were subsequently resolved by SDS-PAGE with Coomassie staining. Since all protease treatments were performed at 4 °C, differential degradation of the toxin samples could only result from temperature-induced changes to the structure of CTA1.

Previous work has shown that our reducing condition of 10 mM β-mercaptoethanol (β-ME) is sufficient for complete separation of CTA1 from CTA2;[24] this result was also confirmed for CTA1/