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Lipopolysaccharides promote binding and unfolding of the antibacterial colicin E3 rRNAse domain

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ABSTRACT

Nuclease colicins are antibacterial proteins produced by certain strains of *E. coli* to reduce competition from rival strains. These colicins are generally organized with an N-terminal transport (T)-domain, a central receptor binding (R)-domain, and a C-terminal cytotoxic nuclease domain. These colicins are always produced in complex with an inhibitory immunity protein, which dissociates prior entrance of the cytotoxic domain in the target cell. How exactly colicins traverse the cell envelope is not understood, yet this knowledge is important for the design of new antibacterial therapies. In this report, we find that the cytotoxic rRNAse domain of colicin E3, lacking both T- and R-domains, is sufficient to inhibit cell growth provided the immunity protein Im3 has been removed. Thus, while the T-domain is needed for dissociation of Im3, the rRNAse alone can associate to the cell surface without R-domain. Accordingly, we find a high affinity interaction (Kd ~1–2 μM) between the rRNAse domain and lipopolysaccharides (LPS). Furthermore, we show that binding of ColE3 to LPS destabilizes the secondary structure of the toxin, which is expectedly crucial for transport through the narrow pore of the porin OmpF. The effect of LPS on binding and unfolding of ColE3 may be indicative of a broader role of LPS for transport of colicins in general.

1. Introduction

Gram-negative bacteria have evolved a number of strategies to compete with other strains for scarce resources. The production of antimicrobial proteins termed colicins is one such strategy. These proteins or toxins use a variety of mechanisms to kill rivaling strains such as DNA or RNA degradation [1,2], inhibition of peptidoglycan synthesis [3], and formation of membrane pores [4]. To reach their intracellular targets, colicins must traverse the cell envelope of the competing strain.

Colicin E3 (ColE3; 59 kDa) is a ribosomal RNase (rRNase) that cleaves the 16S subunit [1]. Its overall architecture is similar to other nuclease colicins including an N-terminal transport domain (T-domain); a receptor-binding domain (R-domain), and a C-terminal rRNAse active domain (Fig. 1A). These colicins are expressed together as a complex along with inhibitory proteins that provide protection for the producing strain. The immunity protein for ColE3, Im3 (~10 kDa), is sandwiched between the T-domain and the nuclease domain (Fig. 1A; 5). The affinity between ColE3 and Im3 is extremely high; within the femtomolar range (~10 fM; 6). Such tight interaction is contributed in part by a charge difference between the highly basic ColE3 nuclease domain (pI ~9.92) and the highly acidic Im3 protein (pI ~3.96) (Fig. 1B).

Import of ColE3 into its target cell is a multistep process involving a number of membrane-bound and periplasmic proteins. It is initiated by the binding of the R-domain to the outer membrane receptor, BtuB [7,8]. This event triggers a partial unfolding of the R-domain and the dissociation of Im3 from the globular T-domain [6,9,10]. The T-domain is then threaded through the porin OmpF [11,12], allowing it to reach the periplasmic protein TolB [13]. The subsequent engagement of TolB with the inner membrane complex TolA-TolQ-TolR couples the proton-motive force with the import of the rRNAase domain of ColE3 in the periplasm [14,15,16,17]. The nuclease domain is eventually transported across the inner membrane by an unknown mechanism involving the protease FtsH [18,19,20].

The release of Im3 and subsequent unfolding of the ColE3 nuclease domain are two critical steps essential for the passage of the toxin through the narrow pore of OmpF. The current model asserts that association of the T-domain with the Tol system promotes the dissociation of Im3 and unfolding of the rRNAse domain to access OmpF [21]. However, thermodynamic studies indicate that Im3-free ColE3 rRNAse domain is still highly stable and resistant to unfolding [22]. An additional step may therefore be required to promote active unfolding of the colicin [23]. In this study, we report that truncated colicin comprising

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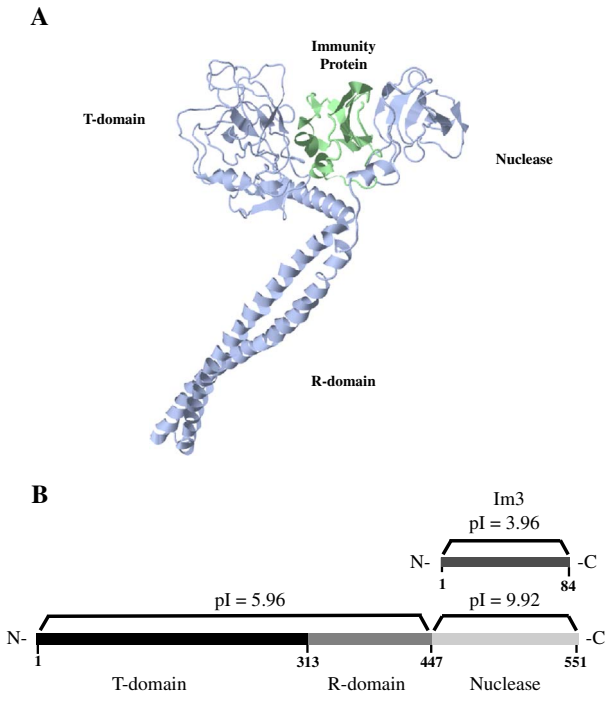

Fig. 1. Structure of the ColE3-Im3 complex. A) Crystal structure of the ColE3-Im3 heterodimer with colicin E3 shown in blue and Im3 shown in green (Soelaiman, 2001) (PDB: 1JCH). B) Location of ColE3 domains with T-domain in black (residues 1–313), R-domain in dark grey (residues 313–447), and nuclease domain in light grey (residues 447–551). Im3 is also depicted. The isoelectric point of each domain is indicated.

only the rRNAse domain is still cytotoxic. We show that the rRNAse domain has micromolar affinity for membrane lipopolysaccharide (LPS) and this interaction significantly affects the folding stability of the protein, which is critical for transport through OmpF. Membrane LPS is therefore an active contributor to the colicin transport process.

2. Materials and methods

2.1. Protein purification

The genes encoding for ColE3 and Im3 were cloned into plasmid pET28 between *Nde*I and *Xho*I, introducing a His_6-tag at the C-terminus of Im3. The plasmid was transformed into BL21 (DE3) and protein expression was induced with IPTG (1 mM) at OD_{600} ~0.6 in LB broth plus kanamycin (25 μg/ml). Cells were harvested after 3 h by centrifugation (3000 rpm for 10 min). Cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH = 7.9, 50 mM NaCl, 10% glycerol) and lysed through a Microfluidizer in the presence of PMSF (1 mM). Cell lysate was centrifuged (200,000 × *g* for 45 min) and supernatant was passed over a 5 ml IMAC-Co^{2+} column (GoldBio) equilibrated in buffer A. Proteins were eluted with a step gradient of buffer B (50 mM Tris-HCl, pH = 7.9, 300 mM NaCl, 10% glycerol, 600 mM imidazole). The gene encoding for ColE3-TR (residues 1 to 455) with a C-terminal His_6-tag was cloned into pET28. The protein was expressed and purified as described above. The ColE3-C96 nuclease domain (residues 455 to 551) with an N-terminal His_6-tag was cloned together with Im3 into pET28. The complex was purified as described above.

2.2. Removal of the immunity protein

ColE3 was separated from Im3 as previously described [24]. Briefly, the ColE3-Im3 complex was incubated with urea (6 M final; 1 h at room temperature) before injection onto a Superdex 200 HR 10/300 column equilibrated in buffer C (50 mM Tris-HCl, pH = 7.9, 50 mM NaCl, 6 M Urea). Eluted protein fractions were analyzed by 18% SDS-PAGE to confirm separation of ColE3 from Im3. ColE3 was refolded overnight at 4 °C by dialysis against buffer D (20 mM Tris-HCl, pH = 7.9, 50 mM NaCl). Purification of Im3-free ColE3-C96 was as described by Zakharov et al. [26]. Briefly, the complex bound onto a 5 ml IMAC-Co^{2+} column in buffer A was washed with 6 M guanidinium chloride (three column volumes) to dissociate Im3. The resin was then extensively washed with buffer A and ColE3-C96 was eluted with a step gradient of buffer B.

2.3. Spot test and survival assays

Strains of *E. coli* DH5α (pBad22 or Im3-producing ColE3-Im3-pET28) and AW740 (ΔompF zcb::Tn10 ΔompC) (pHX405) were grown in LB at 37 °C to OD_{600} ~0.8. Aliquots (100 μl) were spread onto LB agar plates supplemented with 100 μg/ml ampicillin. Serial dilutions of ColE3 (150 μM) and ColE3-C96 (150 μM) purified with or without Im3, and dialyzed into 20 mM Tris-HCl, pH = 8.0, 50 mM NaCl were spotted onto the plates (6.5 μl aliquots). Plates were placed at 37 °C and inspected for zones of inhibition after overnight incubation.

During the survival assay the above strains were grown to OD_{600} = 1.0 at 37 °C. 100 μl of culture was then added to 100 μl of LB containing either 1 μM colicin E3, with or without Im3 or 50 μM ColE3-C96, with or without Im3. After 20 min of incubation at 37 °C 1.8 ml of LB was added to each culture, incubated for two additional hours at 37 °C, and absorbance was then measured. Percent survival was determined from the absorbance ratio of control and treated cells.

2.4. Purification of lipopolysaccharides

LPS was extracted from *E. coli* DH5α using the hot phenol-water technique [25,26]. Briefly, cells were incubated with proteinase K (100 μg/ml) for 1 h at 65 °C, then overnight at 37 °C in the presence of DNase (20 μg/ml), RNase (40 μg/ml), $MgSO_4$ (0.02%) and chloroform (4 μl/ml). Cells were subsequently treated for 15 min at 65 °C with shaking with an equivalent volume of 90% phenol. After centrifugation (10 min at 8500 × *g*), the supernatant was diluted with H_2O and supplemented with sodium acetate (0.5 M final) and 10 volumes ethanol (95%). The solution was stored overnight at − 20 °C. The LPS was pelleted by centrifugation (2000 × *g* for 10 min) and resuspended in 1 ml H_2O. The LPS preparation was analyzed by SDS-PAGE and silver staining to assess purity and concentration in comparison to commercial LPS (Sigma-Aldrich, product # L2630). Concentration of LPS was determined using ImageJ to compare density of a known molecular standard and assuming a molecular mass of 2000 Da per LPS molecule.

2.5. Isothermal titration calorimetry

Prior to analysis, ColE3 (20 μM), ColE3-C96 (50 μM) and LPS (0.5 mM) were dialyzed overnight against buffer D (20 mM Tris-HCl, pH = 7.9, 50 mM NaCl) using 12–14 kDa and 3.5 kDa cutoff membranes, respectively (SpectraPor). ITC measurements were conducted at 25 °C using a MicroCal ITC-200 apparatus (GE Healthcare). Titrations of LPS (~0.5 mM) into the cell were initiated with a single 0.5 μl injection followed by 19 injections of 1.8 μl each. Data were corrected for heats of dilution, which was determined by injecting LPS (~0.5 mM) into buffer. Binding curves, stoichiometry (N), dissociation constant (K_d), and enthalpy changes (ΔH) were analyzed using the one-site binding model on Origin 7 software (MicroCal Inc.).

2.6. Fluorescence measurements

ColE3-C96 (3 μM), with or without Im3, and LPS (90 μM) were incubated together overnight at 4 °C then heated at the indicated temperature (20–80 °C) for 5 min before fluorescence measurements. Measurements were taken on a Cary Eclipse fluorometer (Agilent Technologies) in 10 mM potassium phosphate buffer (pH = 7.4). Spectra were recorded between 300 nm and 400 nm with an excitation wavelength (λ_{ex}) of 280 nm. Measurements were repeated three times and data were corrected from blank spectrum (buffer or LPS-buffer only).