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Towards understanding the Tat translocation mechanism through structural and biophysical studies of the amphipathic region of TatA from *Escherichia coli*

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ABSTRACT

The twin-arginine translocase (Tat) system is used by many bacteria and plants to move folded proteins across the cytoplasmic or thylakoid membrane. In most bacteria, the TatA protein is believed to form a defined pore in the membrane through homo-oligomerization with other TatA protomers. The predicted secondary structure of TatA includes a transmembrane helix, an amphipathic helix, and an unstructured C-terminal region. Here biophysical and structural investigations were performed on a synthetic peptide representing the amphipathic region of TatA (residues 22 to 44, abbreviated TatAH2). The C-terminal region of TatA (residues 44–89) was previously shown to be accessible from both the cytoplasmic and periplasmic sides of the membrane only when the membrane potential was intact, suggesting dependence of its topology on an energized membrane (Chan et al. 2007 *Biochemistry* 46: 7396–404). Such observation suggests that the TatAH2 region would have unique lipid interactions that may be related to the function of TatA during translocation and thus warranted further investigations. NMR and CD spectroscopy of TatAH2 show that it adopts a predominantly helical structure in a membrane environment while remaining unstructured in aqueous solution. Differential scanning calorimetry studies also reveal that TatAH2 interacts with DPPG lipids but not with DPPC, suggesting that negatively charged phospholipid head groups contribute to the membrane interactions with TatA.

1. Introduction

The twin-arginine translocase (Tat) system is used by many bacteria to move proteins across the cytoplasmic membrane. Tat substrates are pre-folded in the cytoplasm and contain an S/TRRxFLK twin-arginine motif in their N-terminal leader sequence. The Tat translocon consists of the TatA, TatB, and TatC subunits. The current model identifies TatA as the pore subunit and the TatBC complex as the substrate recognition and delivery vehicle (reviewed in Natale et al. [1]). Two other subunits, TatD and TatE also exist but their roles are not understood and the translocon seems to function properly in their absence.

TatA is the smallest of the three functionally important Tat subunits with a length of only 89 amino acids (in *Escherichia coli*) and the protein is approximately 9.8 kDa. It is suggested to homo-oligomerize with other TatA protomers to form a pore or channel in the cytoplasmic membrane to allow passage of large, folded polypeptide substrates. The protein is predicted to form two α-helices at the N-terminal half followed by an unstructured C-terminal half, which was confirmed by circular dichroism (CD) spectroscopy [2]. Truncation studies show that TatA can be shortened to 49 residues, by removing the C-terminal region, and still participate in translocation [3]. Further investigations reveal that a DDE acidic motif at residues 45 to 47 is essential for translocation [4]. Mutagenesis studies have also identified many important residues in both helices [3,5,6], further implicating these two helices in translocation and formation of the channel. The first helix is highly hydrophobic and is presumed to be a transmembrane helix whereas the second helix is amphipathic and is believed to lie parallel to the cytoplasmic membrane. Protease and chemical accessibility experiments showed that the entire C-terminal half immediately following the second helix of TatA was accessible from both the cytoplasm and the periplasm, suggesting that this protein has dual topologies in the membrane [7,8]. Therefore, it was suggested that TatA can be found in a single transmembrane structure with helix-2 lying parallel to the membrane or a double transmembrane mode with both helices inserted into the membrane (Fig. 1). Interestingly, it appears that TatA can be trapped in the single transmembrane topology when the membrane

Abbreviations: Tat, twin-arginine translocase; TatAH2, TatA helix-2 peptide (residues 22–44 of full-length sequence); NMR, nuclear magnetic resonance; CD, circular dichroism; DSC, differential scanning calorimetry; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPC, dodecylphosphocholine; eggPC, egg derived phosphatidylcholine; eggPG, egg derived phosphatidylglycerol; eggPE, egg derived phosphatidylethanolamine; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles

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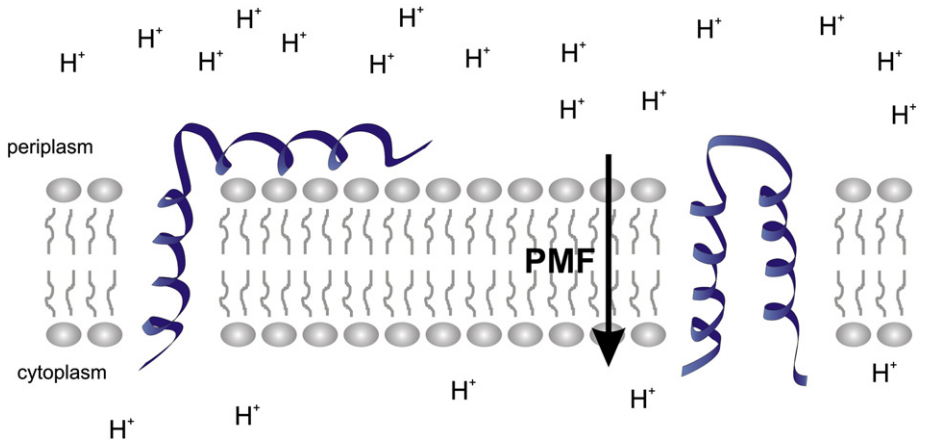

Fig. 1. Dual topology models of TatA. Previous protease and chemical accessibility experiments demonstrate that the C-terminus of TatA is accessible from both the cytoplasm and periplasm whereas the N-terminus was fixed in the cytoplasm, suggesting that the C-terminal half of TatA has two topological states in the membrane [7,8]. Both single (left) and double (right) transmembrane topologies were only detected when the proton motive force (PMF) was intact, whereas only the single transmembrane topology was detected when the PMF was destroyed by a membrane uncoupler [8]. The C-terminal unstructured region following the two helices is not shown for simplicity.

potential is destroyed by a chemical uncoupler [8]. Recent NMR structural analysis of intact $TatA_d$ from *Bacillus subtilis* shows that it has an 'L'-shaped structure of a short first helix with a turn to a longer second helix [9,10], which supports the single transmembrane topology model described in Fig. 1.

Here structural and biophysical investigations were carried out with a synthetic peptide encompassing the second helix of *E. coli* TatA (TatAH2) consisting of residues 22 to 44. NMR and CD spectroscopy show that the helical structure of this amphipathic helix in TatA is dependent on the presence of a stable membrane mimetic environment. Its similarities to helical cationic antimicrobial peptides led us to evaluate its interaction with DPPG and DPPC phospholipids using differential scanning calorimetry, and the results demonstrate that TatAH2 associates with negatively charged lipids while having little effect on zwitterionic bilayers.

2. Materials and methods

2.1. Materials

The TatAH2 peptide was commercially synthesized to 95% purity (Invitrogen Custom Services). All phospholipids and dodecylphosphocholine (DPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) as stock solutions dissolved in chloroform. D_2O was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada), deuterated sodium dodecyl sulfate (SDS) and 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Spectroscopic grade SDS and all other reagents were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The lipid concentrations in the small unilamellar vesicle (SUV) samples were determined by measuring the phosphate concentration according to the assay described by Ames [11].

2.2. Circular dichroism spectroscopy

CD spectra were recorded with a J810 spectropolarimeter (Jasco) from 260 to 190 nm with a 1 nm bandwith, 0.5 nm pitch at 50 nm/min in a 1 mm quartz cuvette (Hellma). Experiments were done with a final peptide concentration of 40 μM in 25 mM phosphate buffer (pH 7.4), 50% v/v trifluoroethanol, SUVs composed of *E. coli* polar lipids, and 30 mM SDS or DPC micelles. The structure of TatAH2 was also monitored by titrating eggPC or eggPG SUVs into a solution of peptide. Briefly, a 200 μl solution of 50 μM peptide was prepared in buffer and spectra were acquired as described above. An aliquot of 10 μl eggPC or eggPG SUVs (lipid concentration 2.25 mM) was added to the cuvette and another spectrum was collected. A total of ten 10 μl aliquots were added to the peptide sample with a spectrum acquired after each addition.

2.3. Differential scanning calorimetry

TatAH2 was dissolved in 3:1 methanol:chloroform and mixed with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DPPG) at a 1:100 peptide-to-lipid ratio. The organic solvent was evaporated in a stream of N_2 gas and placed under vacuum for ~2 h to generate lipid films. Pure lipid films were prepared in a similar fashion to a final concentration of 0.5 mg/ml. The films were resuspended in buffer (20 mM phosphate, 130 mM NaCl, pH 7.4) at 60 °C and vortexed vigorously. The lipid suspension was incubated at 60 °C for an additional 5 min and vortexed again. Data was collected on a Microcal VP-DSC instrument and the resulting thermograms were analyzed in Origin (version 7). A total of four scans were acquired between 20 and 60 °C using a scan rate of 10 °C/h. The calorimeter cells were passively cooled between heating scans. The thermogram from the fourth scan was used in the final analysis.

2.4. Calcein leakage

Calcein leakage experiments were performed according to the protocol described by Matsuzaki et al. [12]. Briefly, lipid films composed of 1:1 mixtures of eggPE:eggPG lipids were prepared from stock solutions dissolved in chloroform. The organic solvent was evaporated in a stream of N_2 gas followed by exposure to vacuum for 2 h. The lipid films were then resuspended in buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 70 mM calcein. This lipid suspension was subjected to 5 cycles of liquid nitrogen freezing and thawing under warm water, followed by 15 passes through two 0.1 μM polycarbonate filters using a mini-extruder apparatus (Avanti Polar Lipids). The calcein encapsulated LUVs were separated from free calcein using a Sephadex G50 gel filtration column. The percentage of calcein released from the calcein LUVs was followed using a Varian Cary Eclipse Fluorimeter (Varian Inc. Palo Alto, CA). Calcein containing LUVs were added to a final lipid concentration of 10 μM and the fluorescence emission intensity at 520 nm (excitation 490 nm) was monitored. Once the fluorescence reading stabilized, peptide was added to a final concentration of 1 μM. To establish 100% leakage, 20 μl