Persister-promoting bacterial toxin TisB produces anion-selective pores in planar lipid bilayers

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Abstract

We studied membrane activity of the bacterial peptide TisB involved in persister cell formation. TisB and its analogs form multi-state ion-conductive pores in planar lipid bilayers with all states displaying similar anionic selectivity. TisB analogs differing by ±1 elementary charges show corresponding changes in selectivity. Probing TisB pores with poly-(ethylene glycol)s reveals only restricted partitioning even for the smallest polymers, suggesting that the pores are characterized by a relatively small diameter. These findings allow us to suggest that TisB forms clusters of narrow pores that are essential for its mechanism of action.

1. Introduction

Formation of dormant persister cells in a bacterial population is among the most important survival strategies employed by bacteria against antibiotic treatment and other harmful conditions [1]. Persister cells are not mutants but rather phenotypic variants of the wild type characterized by very slow or arrested growth, diminished protein synthesis, and reduced membrane potential [1]. Multidrug tolerant persisters are largely responsible for recalcitrance of chronic infections such as those caused by biofilms, to antibiotic treatment. Mechanisms of persister formation are highly redundant [1].

In Escherichia coli, toxin/antitoxin modules have been implicated in persister formation [2–5]. Of particular interest is the TisAB locus [5–8] which is controlled by the SOS response [6,9]. DNA damage induces the SOS response, which activates expression of DNA repair enzymes in the majority of cells. In a small fraction of cells, high levels of TisB lead to the formation of dormant persisters. Depending on conditions, either active repair or dormant state may lead to better survival. Interestingly, under conditions of DNA damage by fluoroquinolones, all regular cells that attempt to actively repair damage are killed, and only persisters produced through expression of TisB survive [5].

Based on its composition, TisB is a typical membrane-active antimicrobial peptide – it is small, 29 amino acids long, hydrophobic and positively charged (MSLVDIAILILKLIVAALQLLDAVLKYLK). Ectopic overexpression of TisB causes a drop in ATP and cell death [8]. Apparently, mild expression under conditions of SOS response leads to a drop in proton motive force, ATP level, and dormant state induction. Antibiotics kill by corrupting their targets, but in dormant cells, targets are inactive, leading to bacterial tolerance. Paradoxically, a typical antimicrobial peptide serves to protect cells from killing by other antibiotics.

Understanding persister formation by TisB is of considerable importance, but the mechanism of its action is unknown. In this study, we investigated the effect of TisB on ionic conductance of planar lipid membranes. We have demonstrated that TisB forms anion channels, providing a mechanistic understanding for the production of a dormant state leading to drug tolerance.

2. Materials and methods

Synthetic TisB, TisB K26A, and TisB D5A were kindly provided by Wayne Anderson, Northwestern University, Chicago, Illinois.

Experiments with planar lipid membranes were performed as described previously [10] using a two-(cis and trans) compartment Teflon chamber. Ag/AgCl electrodes with 2 M KCl/15% agarose bridges were used to apply voltages and to measure the...
transmembrane current. Potential was defined as positive when it is greater at the side of the TisB addition (cis side). Membranes were prepared from neutral diphtyanyol-phosphatidylcholine (DPhPC), negatively charged diphtyanyol-phosphatidylserine (DPhPS⁻), and positively charged didodecytrimethylammonium propane (DOTAP⁺) (Avanti Polar Lipids) using monolayer-apposition technique ([11] after [12]). Membrane-bathing solutions contained 0.1 M or 1.0 M KCl, 10 mM Na-phosphate, pH 7.4. TisB and its analogs were added to the cis compartment from ethanol stock solutions. Poly(ethylene glycol)s of various molecular weights (Sigma) were admixed to the final concentration of 30% (w/w) after the pore formation. The ionic selectivity of the pores formed by TisB and its analogs was determined by measuring the potential of zero current ($E_{rev}$) after establishing of a 10-fold gradient of KCl across the membrane (e.g., see [13]. The cation ($t_{cis}$) and anion ($t_{trans}$) transport numbers related to each other by $t_{cis} + t_{trans} = 1$, were calculated using,

$$E_{rev} = (1 - 2t_{cis}) \frac{k_B T}{e} \ln \frac{\alpha_{cis}}{\alpha_{trans}}$$

(1)

where $\alpha_{cis}$ and $\alpha_{trans}$ are the KCl activities in the cis and trans sides of the membrane-bathing solutions, respectively; $k_B$, $T$, and $e$ have their usual meaning of the Boltzmann constant, absolute temperature, and electron charge.

Transmembrane currents were resolved with an Axopatch 200B amplifier and recorded and analyzed using PClamp 9.2 (Molecular Devices) and Origin 8.1 (OriginLab) software. All measurements were performed at room temperature (23 ± 1.5 °C).

MG1655 pZS’34tisB cells were grown to mid-exponential phase in 0.1 M HEPES-buffered Mueller-Hinton broth and 1 mM IPTG was added to induce expression of tisB. Membrane potential was assessed using the protocol of the Invivopt Baclight membrane potential kit. Briefly, cells expressing tisB were diluted 100-fold into pre-warmed PBS, incubated with cyanine dye DiOC(2)3 for 30 min and analyzed in a BD FACSaria. As depolarized control, 5 μM protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to one sample for 10 min. Membrane potential was estimated by dividing population mean red fluorescence values by population mean green fluorescence values.

3. Results and discussion

In the present study we applied TisB to only one side of the planar lipid membranes thus mimicking the natural asymmetry, where TisB interacts with the cytoplasmic side of the bacterial inner membrane. We found, that application of pulses of relatively high transmembrane voltage (2–5 s: ±250 mV) promotes formation of ion-permeable structures. In total, we observed more than 50 incorporations with TisB and ~20 with its analogs. In most cases, upon incorporation, the TisB produces a stable ion-permeable state, with the conductance ranging from 0.5 to 3 nS in 1 M KCl solution.

Transmembrane voltage affects the TisB-induced conductance. Fig. 1A shows typical behavior of the TisB-induced conductance as the transmembrane voltage was incrementally increased from 20 to 100 mV (the initial conductive state formation was induced by a 250 mV pulse of 5 s duration). Here, in the range of 20–60 mV the TisB-induced conductance of 1.3 nS is ohmic and insensitive to the voltage polarity (negative voltage applications are not shown) or the duration of the applied voltage. Increasing the voltage above 60 mV (in other cases above 40 mV) produces instability in conductance, causing current flickering between various levels. This instability and intensive current flickering remained even after returning to the voltages below 60 mV (not shown).

Current–voltage dependences in Fig. 1B characterize several realizations of the TisB-induced conductance (regardless of their effective lifetimes), examples of which are given in panel A. The figure allows us to recognize the existence of at least three conductive states. The initial 1.3 nS conductive state (solid squares), observed in 0–60 mV voltage range, was the most stable one; voltage increase above 60 mV produced higher conductive states (diamonds and open squares). The observed states are likely realized within the same TisB aggregate because of the clear evidence of the cooperativity of the “pore gating”: indeed, all the conductive states exhibited occasional one-step transitions to zero transmembrane current and back. The lasting application of the increased voltages (>60 mV) induced instabilities in the aggregate; subsequent returns to the voltages below 60 mV did not restore the initial stable conductive state and produced various smaller

![Fig. 1. TisB-induced pores in planar lipid bilayer from diphtyanyol-phosphatidylcholine, and their reaction on the transmembrane voltage. Panel A demonstrates a typical reaction of the initial TisB-induced conductance on the increase of the positive transmembrane voltage; reaction on the negative applied voltage was qualitatively the same (the trace not shown). An increase of voltage induces formation of multiple conductive levels. Panel B gives current–voltage characteristics of the TisB-induced pores; the data obtained from the current tracks in panel A: solid squares denote the most stable observed conductive level; open diamonds and open triangles correspond to newly-formed conductive levels stimulated by voltage increase. Panel C summarizes the statistics of initial insertion conductances. Because of the broad range of conductances, the distribution is given in logarithmic scale.](image-url)
(triangles) and larger conductances. Longer (minutes) application of voltages above 60 mV produced gradual increase of the membrane conductance, probably reflecting the incorporation of new TisB pores.

Fig. 1C shows the results of statistical analysis of the conductances of the initial stable states performed over 58 TisB incorporation events. Because of the wide spread of these conductances we also included a logarithmic scale histogram (insert) that shows a maximum around 1.4 nS.

Experiments with DPhPS−1 and DOTAP+1 (data not shown) to check for the effect of the charge of lipid heads did not reveal any measurable effect on channel conductance thus suggesting that the channel structure does not directly involve lipid molecules the way the syringomycin E channel structure does [14,15].

The transitions between the multi-level conductive states within the TisB-induced pores may be a result of the changes in pore diameter, analogously to the case for a benchmark channel-former alamethicin [16]. In the case of alamethicin, the lower conductive states demonstrate pronounced cationic selectivity, compared with the generally less selective higher conductance states, in agreement with the barrel-stave model of alamethicin channel [17,18]. Alternatively, multi-level conductance may result from additional pores joining the conductive aggregate and gating with various degrees of cooperativity [14,15].

To address this question, we compared the ionic selectivity of the TisB-induced pores at the level of initial conductive aggregates with that of the highly conductive states (a representative experiment is shown on Fig. 2). Incorporating a stable TisB conductive aggregate in 0.1 M KCl, we added concentrated salt solution to impose a 1.0 vs 0.1 M KCl cis/trans gradient across the membrane. As shown in Fig. 2A, at these conditions the zero applied voltage corresponds to a finite negative current through the TisB-modified membrane. We had to apply a potential of about 40 mV to reach the point of current reversal. Such a reversal potential, \( E_{\text{rev}} \), yields about 90% pore anionic selectivity as calculated using the Eq. 1. The anionic selectivity is not surprising considering the net positive charge of the TisB molecule (three lysine residues vs. two aspartic acids). Following the measurement on an initial conductive aggregate, we added extra TisB into the chamber and increased voltage to 150 mV for 5 min, inducing a pronounced increase in conductance (incorporations are not shown). However, the reversal potential for the 30-fold more conductive system was \(-38 \pm 3 mV\) (Fig. 2B), which corresponds to only slightly less selective TisB aggregate (87% anionic selectivity) than the initial conductive state. These observations suggest that the higher conductance states are characterized by similar electrostatics as the lower-conductive pores, implying that the effective radii of the differently-conductive TisB states are similar.

We also studied the role of the charge balance in the structure of TisB, measuring the selectivity of the pores induced by two differently charged TisB analogs: neutral TisB K26A with alanine replacing lysine in position 26 and doubly positively charged TisB D5A with alanine in the place of aspartic acid in position 5. The two analogs manifest essentially the same pore-forming activity in planar lipid bilayers as TisB. Typical traces first reveal stable conductance upon pore formation at low voltages, and then show multi-state higher conductive pores at elevated voltages (Fig. 3).

At the same time, the ionic selectivity of the three studied peptides correlated well with the peptide net charge. TisB K26A showed a decrease in anionic selectivity, producing almost non-selective pores, while TisB D5A was slightly more anion-selective than TisB. The results of these experiments are summarized in Table 1. We have also employed two modes of the salt gradient application, with the higher salt concentration from either cis or trans side of the membrane, while the pore-forming peptide was added from the cis side. This protocol of gradient application is aimed at

![Fig. 2. Current–voltage relationships for a small initial TisB-induced conductance (panel A) and a highly-conductive multi-pore system (panel B) in membranes separating 1 M (cis) and 0.1 M (trans) KCl solutions (10 mM Na-phosphate buffer, pH 7.4). The insets show direct measurements of the reversal potential. For both systems the reversal potentials are close in value, being \((40 \pm 1.5)\) mV for the initial small conductance (A) and \((38 \pm 1.5)\) mV for the highly-conductive system (B). Both systems are characterized by \(-0.8\) anionic selectivity.](image-url)
Each data point is an average of 3–4 independent experiments ± S.E. The polymer probing experiments allow us to suggest that the TisB pores are characterized by an unexpectedly narrow inner radius. Because the conductance never reaches the equipartitioning value designated by the lower horizontal line in Fig. 4 even in the presence of the smallest PEGs (this conductance reduction would correspond to the solution conductivity reduction due to the presence of 30% w/w PEG [23]), it is problematic to apply the usual quantitative analysis of the partitioning curves [20]. However, the dependence of TisB conductance on PEG molecular weight shows some characteristic features of polymer partitioning in other channels. If, based on this, we assume that the molecular weight of \( w_{TisB} \) is the cut-off size for PEG partitioning in TisB pores, it should be compared with the cut-off PEG size for alpha-Hemolysin, \( w_{\alpha-HL} \approx 2200 \) Da [22]. Then an estimate for the TisB pore radius is [20] \( r_{TisB} = r_{\alpha-HL}(w_{TisB}/w_{\alpha-HL})^{0.6} \), where \( r_{\alpha-HL} \) is the effective radius of the alpha-Hemolysin pore. Substituting the cut-off weights given above into this formula, we obtain \( r_{TisB} \approx 0.3r_{\alpha-HL} \). Thus, according to this crude estimate, the radius of the TisB pore is only 30% that of the alpha-Hemolysin pore, meaning that its aperture, which is most relevant for conductance evaluation, is more than ten times smaller. Taking into account that the TisB average initial conductance is very close to that of the alpha-Hemolysin pore, we suggest that TisB induces honeycomb-like cluster pores that synchronously switch between open and closed states. The virtual independence of pore selectivity on its conductance (Fig. 2) suggests similar structural organization of all its states, including those of much higher conductance than the initial one. Taking into account PEG partitioning that indicates very narrow pore radius, we can picture the structure of the TisB conductive aggregate as a cluster of narrow parallel channels akin to a honeycomb. It is our interpretation that even the initial conductive state is represented by a cluster. Therefore, the voltage-induced increase in conductance in Fig. 1 A may be due to the increase of the initial cluster size by 30 to 150%. Similar type of pore cluster organization was earlier proposed for channel-forming peptides including latrotoxin and syringomycin E [24,25]. Unfortunately, in the case of TisB we could not deduce the unitary conductance of a single channel in its cluster – the cooperativity was always maintained to some level, even for the smallest initial pore formation was found to be very sensitive to changes in solution osmolarity, with higher osmolarity stimulating pore formation. To deal with the osmotic stress induced by PEG addition into the membrane-bathing solution, we first acquired the stable TisB pores (as the one shown on Fig. 1A at 20–60 mV range) by maintaining the low voltage conditions and then added aliquots of PEG solution under stirring, continuously monitoring the pore current. To deal with the osmotic stress induced by PEG addition to the initial current.

Fig. 4 summarizes the experimental data on pore probing with the differently-sized PEGs. To our surprise, the relatively high-conductive TisB pores were found to allow very little partitioning even for the smallest probing PEGs, suggesting that the pores are rather narrow. Only mono- and di-ethylene glycols affect the pore current substantially, while PEG 600 is nearly fully excluded. Though the polymer partitioning experiments were performed only on the conductance states of 0.5–3 nS, their conclusions seem to extend to the whole range of the TisB-induced ionic conductance. For a comparison we have added the PEG-exclusion profile for the alpha-Hemolysin channel [22], which is characterized by a similar conductance (~1 nS) at the same experimental conditions. Even at lower PEG concentrations, the alpha-Hemolysin shows pronounced polymer partitioning up to PEG 1000.

### Table 1

<table>
<thead>
<tr>
<th>1.0 M KCl cis</th>
<th>Reversal potential, mV</th>
<th>Cation transport number</th>
<th>Anion transport number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TisB</td>
<td>40.2 ± 2.2</td>
<td>0.1 ± 0.015</td>
<td>0.9 ± 0.015</td>
</tr>
<tr>
<td>TisB D5A</td>
<td>47.0 ± 1.5</td>
<td>0.06 ± 0.010</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>TisB K26A</td>
<td>10.5 ± 2.0</td>
<td>0.38 ± 0.025</td>
<td>0.62 ± 0.025</td>
</tr>
<tr>
<td>0.1 M KCl cis</td>
<td>3.8 ± 1.5</td>
<td>0.11 ± 0.015</td>
<td>0.89 ± 0.015</td>
</tr>
<tr>
<td>TisB</td>
<td>-38.0 ± 1.5</td>
<td>0.085 ± 0.015</td>
<td>0.91 ± 0.015</td>
</tr>
<tr>
<td>TisB D5A</td>
<td>-43.0 ± 0.5</td>
<td>0.39 ± 0.025</td>
<td>0.61 ± 0.025</td>
</tr>
<tr>
<td>TisB K26A</td>
<td>-11.5 ± 2.0</td>
<td>0.39 ± 0.025</td>
<td>0.61 ± 0.025</td>
</tr>
</tbody>
</table>

Each data point is an average of 3–4 independent experiments ± S.E.
conductive states. We will further probe the cluster organization of TisB pores with structural methods in our future studies.

Since TisB is considered to act on the inner membrane of bacteria, and its production was accompanied by a decrease in intracellular ATP concentration [8], we expect that the experimentally found ability of TisB to form pores in membranes may directly contribute to the dissipation of a proton motive force [5] in cell culture. We used the cyanine dye DiOC(2)3, which accumulates in the membrane in the presence of the proton motive force, shifting its emission spectrum from green towards red. Dividing red fluorescence by green fluorescence values gives a good approximation of the presence of the proton motive force independent of the cell size. The effect of TisB overproduction on *E. coli* MG1655 cells is illustrated in Fig. 5. After 30 min of induction, the proton motive force decreased and within 90 min reached a similar level to that of the control cell population, depolarized with a protonophore CCCP (Fig. 5D).

Thus, TisB production indeed leads to membrane depolarization, suggesting that the ability to form pores in lipid bilayers may be related to TisB protective function in vivo. The anionic selectivity of TisB pores suggests that these pores do not work as protonophores but could rather make the membrane permeable for hydroxyl anions, which, in turn, dissipate the proton-motive force. The narrow size of TisB pores renders them impermeable for water-soluble intracellular components crucial for cell survival.

4. Conclusions

We have shown that the linear peptide TisB forms anion-selective pores in planar lipid bilayers with a characteristic conductance on the order of 1 nS in 1 M KCl. Polymer-exclusion experiments suggest that the aperture of the TisB pore is very narrow. The relatively high conductance of the initial state and the finding that anionic selectivity does not change with the conductance increase strongly suggest cluster organization for the TisB conductive aggregate. The anionic selectivity of TisB pores is an expected consequence of the net-positive charge of the molecule. Neutralization of one negative charge in TisB D5A leads to a slight increase in anionic selectivity, while neutralization of a positive charge in TisB K26A results in a decreased anionic selectivity. In a bacterial cell culture, the TisB production reduced the proton-motive force, likely related to TisB pore formation. Overall, our findings offer an insight into the mechanism of biological activity of this toxin which induces specific membrane permeability that leads to cellular energy level decrease and promotes formation of dormant drug-tolerant persisters, contributing to biofilm survival.

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