Structure-Function Relationships in Diphtheria Toxin Channels: III. Residues which Affect the Cis pH Dependence of Channel Conductance

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Abstract. The conductance of channels formed by diphtheria toxin (DT) in lipid bilayer membranes depends strongly on pH. We have previously shown that a 61 amino acid region of the protein, denoted TH8–9, is sufficient to form channels having the same pH-dependent conductance properties as those of whole toxin channels. One residue in this region, Aspartate 352, is responsible for all the dependence of single channel conductance on trans pH, whereas another, Glutamate 349, has no effect. Here, we report that of the seven remaining charged residues in the TH8–9 region, mutations altering the charge on H322, H323, H372, and R377 have minimal effects on single channel conductance; mutations of Glutamates 326, 327, or 362, however, significantly affect single channel conductance as well as its dependence on cis pH. Moreover, Glutamate 362 is titratable from both the cis and trans sides of the membrane, suggesting that this residue lies within the channel; it is more accessible, however, to cis than to trans protons. These results are consistent with the membrane-spanning topology previously proposed for the TH8–9 region, and suggest a geometric model for the DT channel.

Key words: Diphtheria toxin — Site-directed mutagenesis — Planar lipid bilayers — Single channel conductance — Ion selectivity — pH dependence

Introduction

This is the third in a series of papers analyzing structure-function relationships in the ion-conducting channel formed by Diphtheria Toxin (DT). In the first paper (Silverman et al., 1994), we examined the channels formed by a series of natural and genetically engineered deletion mutants of DT and found that a short region, consisting of 61 amino acids in the B fragment, is sufficient to form a channel essentially identical to that formed by the intact toxin. This region, denoted TH8–9, corresponds to a pair of α-helices, TH8 and TH9, within the T (transmembrane) domain1 of the recently solved crystal structure of DT (Choe et al., 1992). In the solution form of DT, these helices form a “helical hairpin” connected by a short loop. Given the evidence that there are relatively minor changes in secondary structure as DT inserts into the membrane (Cabiaux et al., 1989), we suggested that the structure of this hairpin is conserved in the membrane.

In the second paper (Mindell et al., 1994), we compared channels formed by wild-type DT with those formed by point mutants in which the charges on one or both of the acidic amino acids in the loop connecting helices TH8 and TH9 were changed. Mutations of Glutamate 349 (to glutamine or lysine) had no effect on the single channel conductance or ion selectivity of the channel; in contrast, both of these properties were dramatically altered in channels with similar mutations of the other acidic residue, Aspartate 352. All of the effects of changing the charge at residue 352 appeared consistent with this group’s interacting electrostatically with channel-permeating ions. By titrating Aspartate 352 with trans pH, we demonstrated that this residue

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The T domain contains 9 α-helices, numbered TH1 through TH9; TH8 and TH9 are at the C-terminus of the T domain.
lies on or near the trans side of the membrane. In fact, under the conditions used in Mindell et al. (1994), Aspartate 352 accounts for all of the dependence on trans pH of the DT channel's conductance. The location of residue 352 led us to propose a membrane topology for the channel-forming region of DT (Fig. 1): the N-terminus of TH8 on the cis side; TH8 itself spanning the membrane; the loop connecting TH8 and TH9 (and containing D352) on the trans side; TH9 returning across the membrane; and the C-terminus of TH9 back on the cis side.

In this paper, we describe the results from mutating the remaining charged residues in the TH8–9 region. We find that titration of the three acidic amino acids (Glutamates 326, 327, and 362) seems to account for much of the substantial cis pH dependence of DT's single channel conductance. This finding is consistent with the proposed membrane topology of the TH8–9 region. In contrast, none of the positively charged groups in TH8–9 appears to play a major role in determining single channel conductance properties.

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**Materials and Methods**

Preparation of mutant toxins is described in detail elsewhere (J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, in preparation). Toxins were stable indefinitely at −20°C; working dilutions were stored at 4°C for no more than one week, with no change in activity.

Lipid bilayers were prepared as described in the previous paper (Mindell et al., 1994). Toxin was always added to the cis compartment. All solutions contained 1 M or 0.1 M KCl, 2 mM CaCl₂, 1 mM EDTA, and an appropriate pH buffer. For experiments at pH 7.0 cis/7.2 trans, or at pH 4.1 cis/7.2 trans, membranes were formed at pH 5.3 cis/7.2 trans (30 mM MES cis, 5 mM HEPES trans), toxin protein added, channel activity observed, and the cis pH brought to its final value by adding either 1 M HEPES pH 7.5 (to a final concentration of 100 mM, pH 7.0 cis) or 1 M glycerate pH 4.0 (to a final concentration of 75 mM, pH 4.1 cis). All voltages refer to that of the cis solution (to which protein was added); the potential of the trans solution is taken as zero.

**Results**

The DT channel's ion permeability is highly pH dependent; both its single channel conductance and its