Locating a residue in the diphtheria toxin channel

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ABSTRACT We are studying structure-function relationships in the Diphtheria Toxin (DT) channel using a combination of site-directed mutagenesis and electrophysiology in artificial lipid bilayers. We made site-directed mutations of charged residues in the toxin's channel-forming B fragment, and after expressing the mutant proteins in *Escherichia coli,* we analyzed the single channels they formed in lipid bilayers. Changing aspartate 352, which is located in a short hydrophilic loop separating two hydrophobic stretches, to asparagine or lysine dramatically reduces the single-channel conductance of the pore at pH 5.3 *cis,* 7.2 *trans* (5.3/7.2). Lowering the pH on both sides of the membrane essentially eliminates the difference between wild-type and D352N; this finding is consistent with the idea that an aspartate with a (protonated) neutral side-chain and the always neutral asparagine have similar electrostatic influences on permeant ions. Using a high concentration of permeant buffer to clamp the pH of the *cis* compartment and the pore, and varying the pH on the *trans* side, we have located D352 at or near the *trans* compartment. We further find that D352N channels, in contrast to wild-type, display conductances independent of *trans* pH. This observation allows us to determine the titration curve of aspartate 352 in the wild-type toxin, establishing its pK_a at ~5.5.

RESULTS AND DISCUSSION

DT is a 62 kD single-chain protein that can be split by site specific proteolysis and thiol reduction into an A fragment (~21 kD) and a B fragment (~40 kD). DT forms channels in lipid bilayer membranes (1, 2); this

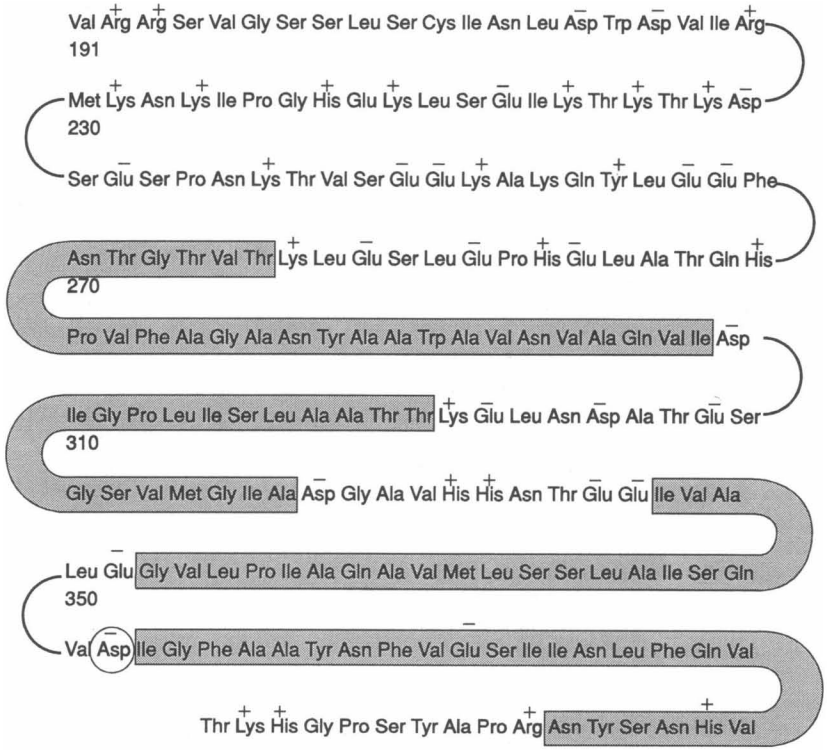

FIGURE 1 The amino acid sequence of B_{45}, the channel-forming region of DT. Here hydrophobic regions are grey, charged amino acids are noted with +'s and −'s, and aspartate 352, the focus of this paper, is circled. The numbers in this figure refer to locations in the whole toxin; the A fragment accounts for residues 1–190.

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channel-forming activity is localized within B_{45} (Fig. 1), the NH_2-terminal domain of the B-fragment (1). Several properties of the DT channel are pH dependent, including single-channel conductance, selectivity, and specific activity of channel formation (1, 3). In these studies we use the pH dependence of single-channel conductance to probe the effects of our mutations.

In Fig. 2, we see that mutating aspartate 352 to asparagine (D352N) significantly reduces the sizes of single-channel transitions at pH 5.3/7.2 (*cis*/*trans*) in 1M KCl. Fig. 3 presents these data as a conductance vs voltage plot with the addition of data for the mutant D352K, where this same aspartate is mutated to the positively charged lysine. Note that changing the negatively charged aspartate to the neutral asparagine reduces the single-channel conductance nearly two-fold: from ~47 pS in the wild-type (WT) channel to 27 pS in D352N (all conductances evaluated at −100 mV). Further changing the charge at this residue to positive (D352K) decreases the conductance another factor of two to 14 pS. These changes are all consistent with the charge at residue 352 having an electrostatic effect on ions permeating the channel, because under these pH conditions DT channels are largely cation selective (40 mV reversal potential for WT channels in a 10:1 KCl gradient). In the remainder of this paper we focus only on the comparison between WT and the D352N mutant.

If the difference in conductance between the WT (Asp 352) and the mutant (Asn 352) is simply due to an electrostatic effect, then we expect that at low pH, where the aspartate in the WT is neutral, the two channels should have similar conductances. This is indeed our observation: at symmetric pH 4.0, both WT and D352N channels have essentially the same conductance (Fig. 4). We used these pH-dependent conductance differences to establish that residue 352 is on the *trans* side of the membrane. Employing a high concentration (55 mM) of the channel-permeant buffer glycerate (pK_a = 3.5) on the *cis* side, and a low concentration of relatively impermeant buffer (5 mM Hepes, EDTA, or Bis-Tris-

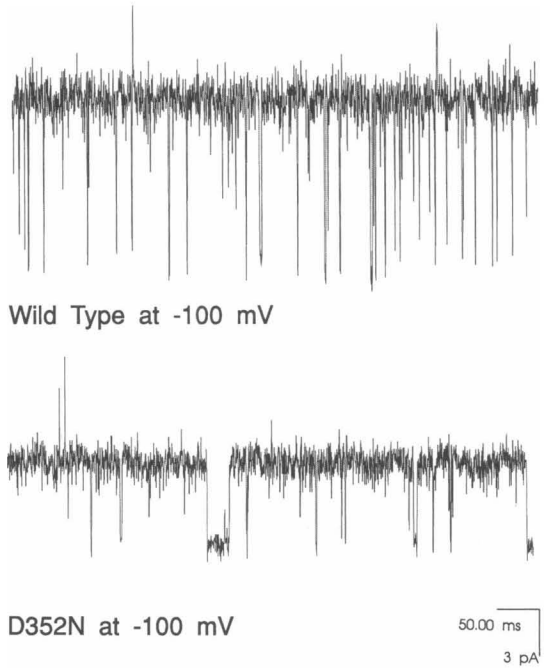

FIGURE 2 Single-channel records from wild-type (WT) diphtheria toxin and the mutant D352N. Solutions for these experiments are: 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, and an appropriate pH buffer (here 30 mM MES pH 5.3 *cis*, 5 mM HEPES pH 7.2 *trans*). Membranes were formed from asolectin, and whole, nicked DT was added from a crude periplasmic extract of *E. coli* to a final concentration of 3–30 ng/ml. Currents were amplified using a patch clamp amplifier, filtered at 1 kHz, digitized at 3 kHz, and analyzed using pClamp software. For these experiments, "wild-type" actually refers to the nontoxic parent mutant DT-E148S, from which all other mutants in this study are derived. Channels formed by DT-E148S behave identically to those formed by truly wild-type DT.

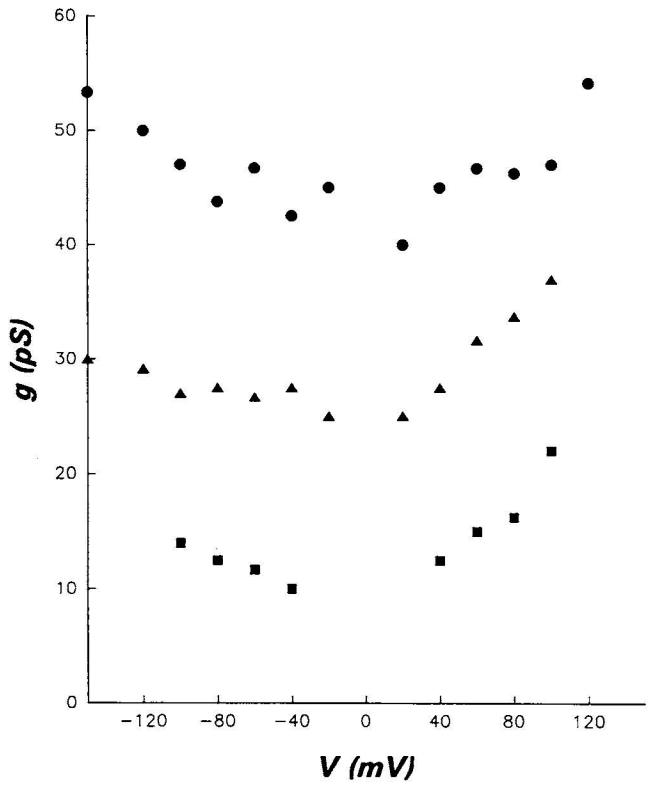

FIGURE 3 Single-channel conductance-voltage relations for DT and mutants at position 352. The data from the experiments described in Fig. 1 are plotted: *circles* represent WT, *triangles* represent D352N, and *squares* D352K. Conductance is calculated as the single-channel current divided by the command voltage.

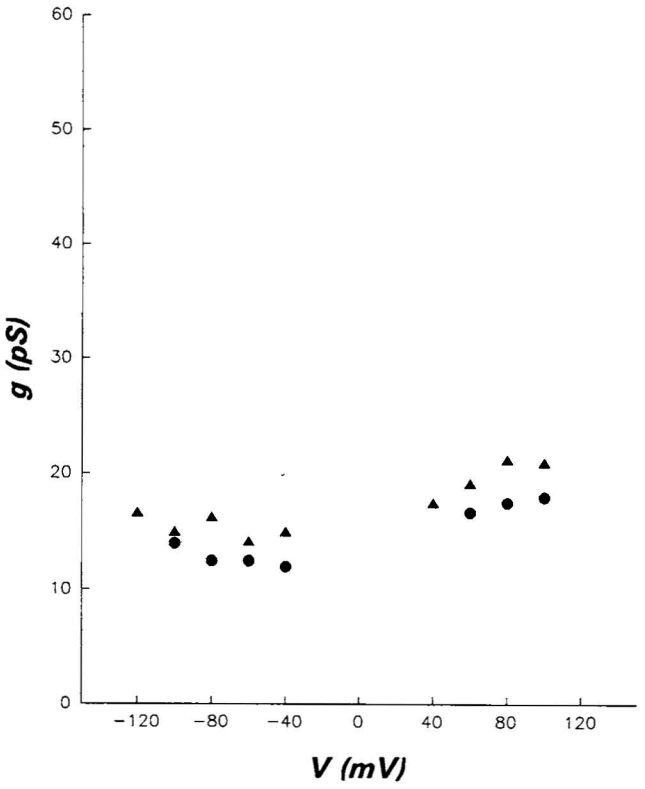

FIGURE 4 g-V plot for WT and D352N channels at symmetric pH 4.0. Solutions on both sides: 1M KCl, 2 mM $CaCl_2$, 1 mM EDTA, and 10 mM citrate, pH 4.0. Other aspects of experiments identical to those in Fig. 1. *Circles* represent WT; *triangles* D352N.

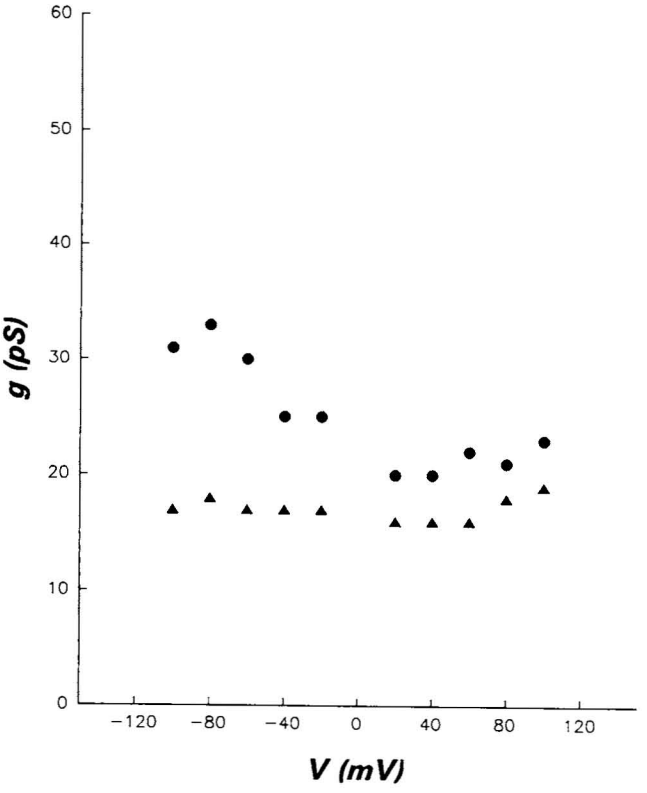

FIGURE 5 *g-V* plot for WT and D352N channels under luminal pH clamp: pH 4.0 *cis*, 7.2 *trans*. 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA on both sides; 55 mM glycerate *cis*, pH 4.0, 5 mM HEPES *trans*, pH 7.2. Because glycerate is a channel-permeant molecule, the high *cis* concentration of this buffer should also clamp the channel lumen at pH 4.0. Again: *circles* = WT, *triangles* = D352N.

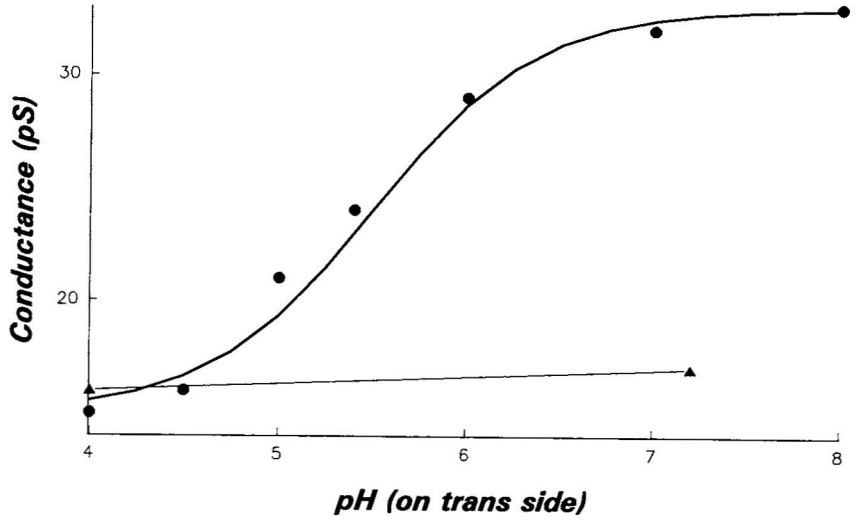

FIGURE 6 The titration curve of aspartate 352. Experiments are similar to those in the previous figure, at a series of *trans* pH's. Because the WT *g-V* curve rectifies, conductances at −100 mV, where WT and D352N channels differ most, are illustrated. The heavy line is a standard titration curve with $pK_a = 5.5$. Solutions are identical to those in Fig. 5, except that the *trans* buffer is 5 mM succinate, MES, or Tris, as appropriate for the desired pH. *Circles* = WT, *triangles* = D352N.

Propane) on the *trans* side, we clamped the *cis* compartment and channel lumen at pH 4.0, leaving only the *trans* compartment at pH 7.2. Under these conditions, we expect that if residue 352 is in the lumen or on the *cis* side, the WT and mutant channels should have identical conductances, because they would experience a pH of 4.0 where they are both neutral. If, on the other hand, residue 352 is on the *trans* side of the membrane, then the WT should be negatively charged and the mutant neutral, and we expect that the their channels would have different conductances. Fig. 5 demonstrates that under these conditions the conductances of the two channels differ; therefore residue 352 is on or near the *trans* side of the membrane. Another feature of the plots in Fig. 5 is their shape: while the g-V curve for D352N is nearly flat over all voltages, that for the WT channel rectifies significantly, with higher conductances at *cis* negative voltages. We do not discuss this behavior here, but our conclusions are independent of its nature.

Comparison of the g-V plots of D352N in Figs. 4 and 5 reveals that the conductance of the mutant is roughly identical in the two experiments. Because these experiments are done at significantly different *trans* pH's, with all other conditions identical, we conclude that the conductance of the D352N protein is independent of *trans* pH. Given this observation, all of the *trans* pH dependence of the WT channel must be due to residue 352. Therefore, by measuring the conductance of the WT channel at a series of *trans* pH's, we can find the titration curve for aspartate 352. These data are illustrated in Fig. 6; here, each point in the WT curve is measured with the *cis* compartment and channel lumen clamped at pH 4.0 as described above, and the single-channel conductance is measured (at −100 mV for consistency) at a series of *trans* pH's with appropriate buffers. The data are well fit by a theoretical titration curve with a pK_a of 5.5 (*heavy line*). Note that under the same conditions the conductance of the D352N channel is independent of *trans* pH.

In summary, we have located Asp 352 of DT on the *trans* side of the membrane and determined its pK_a. Referring to the sequence of the channel-forming region of the protein (Fig. 1), we find that this residue is part of a very short (four amino acids) hydrophilic loop connecting two long hydrophobic stretches (residues 328–348 and 353–376). We thus predict that these two hydrophobic stretches together form a membrane-spanning hairpin loop with residues 349–352 protruding on the *trans* side of the membrane, and residues 327 and 377 on the *cis* side. When plotted on a helical wheel, the stretch containing amino acids 353–376, which is adjacent to Asp 352, displays a marked amphiphilic character. Combined with our results that Asp 352 is near enough to influence channel conduction, and those of Deelers et al. (4), which show that a proteolytic fragment containing this region forms channels in lipid bilayers, this amphiphilicity makes this helix a good candidate for at least part of the pore lining. We are currently constructing mutations to test this hypothesis.

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