Probing the Structure of the Diphtheria Toxin Channel

Reactivity in Planar Lipid Bilayer Membranes of Cysteine-substituted Mutant Channels with Methanethiosulfonate Derivatives

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ABSTRACT Previous work has established that the 61 amino acid stretch from residue 322 to 382 in the T-domain of diphtheria toxin forms channels indistinguishable in ion-conducting properties from those formed by the entire T-domain. In the crystal structure of the toxin's water-soluble form, the bulk of this stretch is an α-helical hairpin, designated TH8-9. The present study was directed at determining which residues in TH8-9 line the ion-conducting pathway of the channel; i.e., its lumen or entrances. To this end, we singly mutated 49 of TH8-9's 51 residues (328–376) to cysteines, formed channels with the mutant T-domain proteins in planar lipid bilayers, and then determined whether they reacted with small, charged, lipid-insoluble, sulfhydryl-specific methanethiosulfonate (MTS) derivatives added to the bathing solutions. The indication of a reaction, and that the residue lined the ion-conducting pathway, was a sudden change in single-channel conductance and/or flickering behavior. The results of this study were surprising in two respects. First, of the 49 cysteine-substituted residues in TH8-9 tested, 23 reacted with MTS derivatives in a most unusual pattern consisting of two segments: one extending from 329 to 341 (11 of 13 reacted), and the other from 347 to 359 (12 of 13 reacted); none of the residues outside of these two segments appeared to react. Second, in every cysteine mutant channel manifesting an MTS effect, only one transition in single-channel conductance (or flickering behavior) occurred, not the several expected for a multimeric channel. Our results are not consistent with an α-helical or β-strand model for the channel, but instead suggest an open, flexible structure. Moreover, contrary to common sense, they indicate that the channel is not multimeric but is formed from only one TH8-9 unit of the T-domain.

KEY WORDS: helical hairpin • streptavidin • subunits • channel conductance • channel flickering

INTRODUCTION

Diphtheria toxin is a 535 amino acid polypeptide secreted by *Corynebacterium diphtheriae*; the polypeptide is coded for by the *tox* gene of the lysogenic bacteriophage, β, in the bacterial chromosome. Upon mild proteolysis, and then reduction of the disulfide bond that links cysteine 186 to 201, the polypeptide separates into two fragments. The NH_2-terminal A fragment (190, 192, or 193 amino acids, depending on where in the arginine-rich loop connecting the two fragments the protease has "nicked" the toxin) is an enzyme that catalyzes the ADP-ribosylation of elongation factor 2, thereby stopping protein synthesis and leading to cell death. For the A fragment to enter the cell and commit this dirty deed, the COOH-terminal B fragment (342, 343, or 345 amino acids) is required. This fragment binds the toxin to a membrane receptor and, after receptor-mediated endocytosis, translocates the A fragment from an acidic vesicle compartment into the cytosol, where elongation factor 2 resides (see Madshus and Stenmark [1992] for a review of diphtheria toxin and its action). The mechanism by which the B fragment translocates the A fragment across the vesicle membrane is not understood, but the machinery for this process is known to reside in the B fragment's NH_2-terminal 172 residues, known as the T-domain, which forms ion-conducting channels in both cell membranes and planar lipid bilayers at acidic pH (Sandvig and Olsnes, 1988; Kagan et al., 1981). The crystal structure of the water-soluble form of diphtheria toxin reveals that the NH_2-terminal T-domain of the B fragment consists of 10 α-helices (designated TH1–TH9 and TH5′), whereas the COOH-terminal R (receptor-binding) domain of the fragment is entirely β structure (Bennett et al., 1994).

Experiments with deletion mutants have established that the 61 amino acid stretch from residue 322 to 382

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in the T-domain[1] is all that is required to form channels in planar lipid bilayers that are indistinguishable in their conductance, ion selectivity, and the pH dependence of these quantities from those formed by the entire T-domain (Silverman et al., 1994*b*). It should be understood, however, that, although the channel formed by this minimal piece of the T-domain is the same as that formed by the whole domain, its ability to form channels is drastically reduced. Orders of magnitude higher concentrations of this piece are required to obtain comparable numbers of channels (Silverman et al., 1994*b*). Thus, other parts of the T-domain outside of this minimal piece are important in the insertion of the channel into the membrane and in its subsequent gating.

This 61 amino acid channel-forming stretch contains helices TH8 (22 amino acids), TH9 (21 amino acids), and the interhelical loop (8 amino acids) connecting them. In the crystal structure of the water-soluble form of the toxin, these three segments form an α-helical hairpin, designated TH8-9 (Choe et al., 1992). Because the helices are of sufficient length to span a lipid bilayer, it is reasonable to postulate that this α-helical hairpin structure, which exists in the water-soluble form of the toxin, is preserved in its membrane-associated form. Thus, one postulates that the channel is formed by a structure in which helices TH8 and TH9 lie within the bilayer, and the connecting loop resides in or near the *trans* solution; i.e., the side of the membrane opposite that to which the toxin was added (see Fig. 1). (Presumably, the channel would be a homooligomer of this structure, an issue we explore in DISCUSSION.) Consistent with this postulated structure is the finding, from studies on the effect of pH on the conductances of wild-type channels and those formed by site-directed mutants, that residue 352 of the interhelical loop lies close to the *trans* side of the membrane (Mindell et al., 1994*a*).

In this paper, we test the validity of this model of the channel by determining which residues in TH8, TH9, and their interhelical loop line the channel lumen or its entrances. To this end, we have mutated to a cysteine, one at a time, 49 of the 53 residues in TH8-9, formed channels in planar lipid bilayers with these mutated T-domains, and then determined whether the cysteines in these channels can react with sulfhydryl-specific methanethiosulfonate (MTS)[2] derivatives: $CH_3SO_2SCH_2CH_2NH_3^+$ (MTS-EA$^+$), $CH_3SO_2SCH_2CH_2N(CH_3)_3^+$ (MTS-ET$^+$), and $CH_3SO_2SCH_2CH_2SO_3^-$ (MTS-ES$^-$). The rationale for these experiments is that being small, charged, lipid-insoluble,[3] sulfhydryl-specific reagents, these MTS derivatives will have access to and react with only those residues (mutated to a cysteine) that are exposed to an aqueous milieu and can be reached by an aqueous pathway. The reaction of these reagents with a reduced cysteine converts the −SH group to $-SSCH_2CH_2R$, where R is either NH_3^+, $N(CH_3)_3^+$, or SO_3^-. The introduction of the $-SCH_2CH_2R$ moiety, either by virtue of its charge and/or its size, should have an effect on single-channel conductance, if the reacted cysteine-mutated residue lies in the ion-conducting pathway. In this way, those residues lining the channel lumen or lying near the channel entrances can be identified. (This approach using MTS derivatives to map the ion-conducting pathway of a channel was introduced by Akabas et al. [1992] for the nicotinic acetylcholine receptor channel and has since been used for the GABA receptor [Xu and Akabas, 1993], the CFTR channel [Cheung and Akabas, 1996], and in our earlier study of four residues in the interhelical loop of TH8-9 [Mindell et al., 1994*c*].) We report here a most unusual distribution for the residues accessible to the MTS derivatives, one that is totally inconsistent with the helical hairpin structure of the channel shown in Fig. 1. Our results also bring into question the multimeric nature of the channel.

MATERIALS AND METHODS

The details of site-directed cysteine mutagenesis of the T-domain, which contains residues 202–378 and has no natural cysteines, and subsequent T-domain expression and purification are given by Zhan et al. (1995). Mutants expressed and purified this way, which is basically that described in the pET system manual from Novagen, Inc. (Madison, WI), contain a histidine tag at the NH_2 terminus. For some of the mutants tested, this was removed (as described in the Novagen, Inc. manual), leaving four additional residues at the NH_2 terminus: Gly-Ser-His-Met; for others, the histidine tag was not removed, leaving 21 additional residues at the NH_2 terminus: Met-Gly-Ser-Ser-$(His)_6$-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met. The number system used in this paper, however, is the same as that described for native diphtheria toxin (Greenfield et al., 1983); i.e., we do not count the additional 4 or 21 residues in our numbering. In a few instances, experiments were done on the same mutant both with and without the histidine tag attached. We saw no difference in single-channel conductance or reaction with MTS derivatives for channels formed by the same mutant with these different NH_2 termini.[4] The sites at which cys-

[1]The carboxy terminus of the T-domain is Pro 378. The construct in the work cited here terminated at Pro 382, thereby including four of the residues between the T- and the R-domain.

[2]*Abbreviations used in this paper:* DTT, dithiothreitol; MTS, methanethiosulfonate.

[3]The issue of the lipid insolubility of these reagents, particularly the neutral form of MTS-EA, is addressed later.

[4]There was, however, a major difference in the voltage gating behavior. The channels formed by all the mutant proteins retaining the histidine tag rapidly closed at small negative potentials (−10 to −60 mV), whereas those from which the histidine tag was removed closed very slowly at these voltages. This interesting phenomenon, which will be the subject of a later paper, is not relevant to the experiments described here.

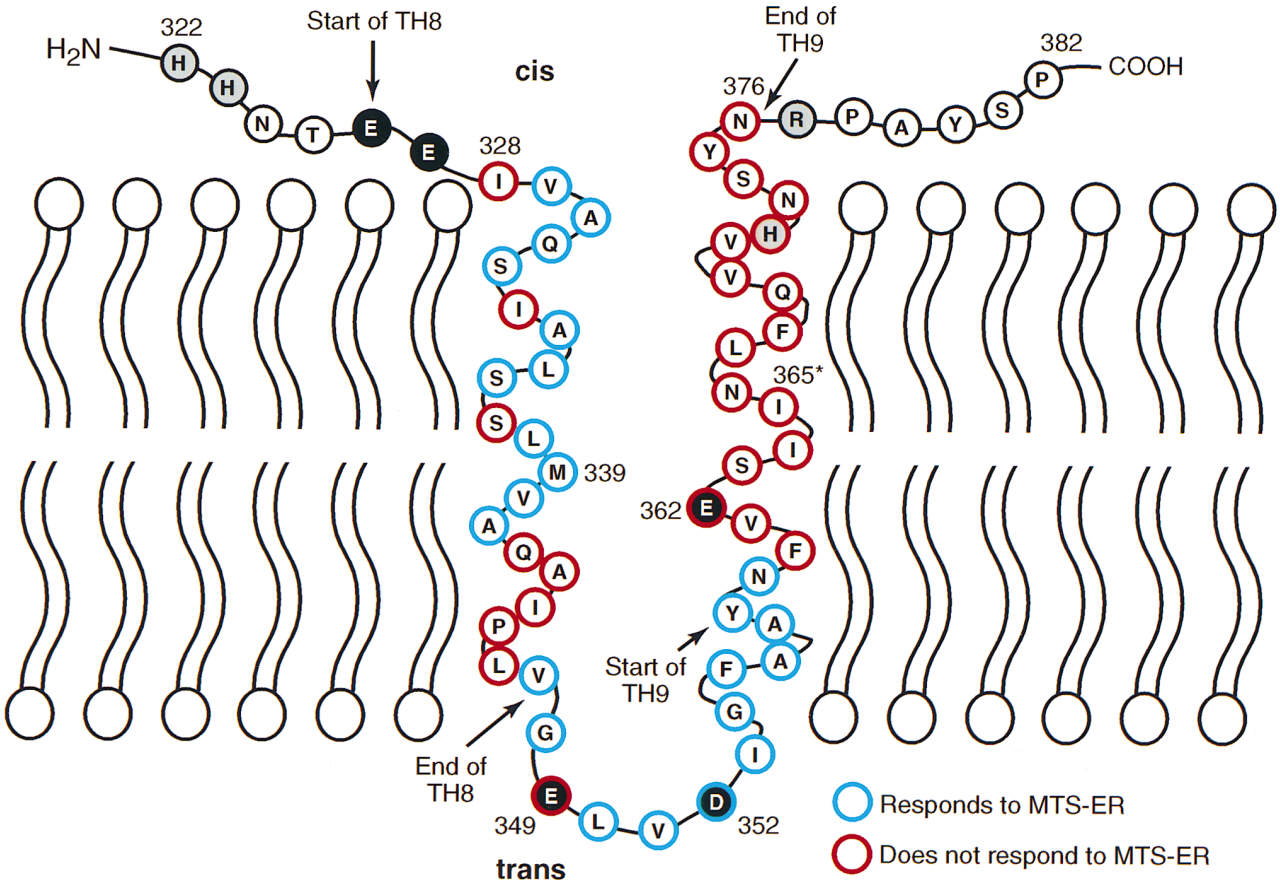

FIGURE 1. Residues in TH8-9 lining the channel. Previously proposed model of the TH8-9 hairpin membrane topology, with helices corresponding roughly to TH8 and TH9 spanning the bilayer and the interhelical loop on or near the *trans* side. The two ends of TH8 and TH9 are indicated. In the crystal structure of the aqueous form of DT, the region between TH9 and the interhelical loop is irregular and, therefore, the border between the end of the loop and the start of TH9 is ambiguous. Negatively charged amino acids are shaded black and positively charged ones are shaded gray. In this model, residues that line the channel lumen (or its entrances) were identified by substituting cysteines, one at a time, at positions 328–376, and their accessibility to MTS derivatives was determined. Cysteine-substituted residues that responded to an MTS derivative are colored blue, and those that did not respond are colored red. This pattern of accessibility to the MTS derivatives is not consistent with an expected periodicity of roughly three to four for an α-helix or a periodicity of two for a β-strand. *Residue I365C probably did not respond to the MTS derivatives, but the data for this mutant are slightly ambiguous. At all other positions, it is clear whether the mutant channels responded to the MTS derivatives.

teine mutagenesis was performed in this study were residues 328–376, inclusive.

Mutant proteins at concentrations of ~1 mg/ml were stored at −70 to −80°C in 20 mM TrisCl + 5 mM dithiothreitol (DTT), pH 8.0. For experiments with a given mutant protein, we took a thawed aliquot and added DTT to a concentration of 20 mM and incubated at room temperature for 10 min to insure reduction of the cysteine residue. Subsequent 10-fold serial dilutions were then made down to a concentration of ~1 μg/ml, and this last was stored on ice during the course of the experiments; at the end of the day, it was discarded. Less dilute solutions were stored at −20°C and could undergo numerous freeze-thaw cycles while still maintaining channel-forming activity and reactivity with the MTS derivatives. Generally, ~1 μl of the 1-μg/ml solution was added to one side of the bilayer (see below) to obtain single-channel activity.

Planar lipid bilayer membranes made of asolectin (lecithin type IIS; Sigma Chemical Co., St. Louis, MO) from which neutral lipids were removed (Kagawa and Racker, 1971) were formed at room temperature, using a modification of the folded film method (Montal, 1974), across a hole (90–100-μm diameter) in a polystyrene cup (Wonderlin et al., 1990) as previously described (Silverman et al., 1994*b*). The solutions both inside the cup (0.5 ml) and outside (1 ml) contained 1 M KCl, 2 mM $CaCl_2$, and 1 mM EDTA; in addition, the *cis* solution (the solution to which the mutant protein was added) contained 30 mM MES, pH 5.3, and the *trans* solution contained 50 mM HEPES, pH 7.2. For experiments in which the pH of the *cis* solution was subsequently raised to 7.0 by the addition of concentrated HEPES solution, it contained only 5–10 mM MES. The solutions on both sides of the membrane could be stirred by miniature magnetic stir bars.

After the membrane formed, ~1 ng of the mutant protein du jour was added to the *cis* solution, a known voltage (generally +60 mV) was applied across the membrane, and the resulting current responses were monitored on a chart recorder (frequency response = 100 Hz) as previously described (Silverman et al., 1994*b*). In addition, the current responses were filtered at 10 kHz by an EPC-7 patch clamp amplifier (List Medical Systems, Darmstadt, Germany) and recorded on digital tape at a 44-kHz

TABLE I

Cysteine-mutant Channels that Reacted with MTS Derivatives

Mutant	pH Conditions (*cis/trans*)	Reagents *cis*	Reagents *trans*	Percent change in conductance	Δ Flickering	No. reactions observed
V329C	5.2/7.2	EA	EA			0*
	7.2/7.2		EA	+50	+++++	2
	7.2/7.2	EA		+50	+++++	3–4
	7.2/7.2	EA	EA	+50	+++++	1
A330C	5.2/7.2		EA	−10	NC	2–3
	7.2/7.2		EA	−27	NC	7
Q331C	5.2/7.2		EA	−73	NC	1
	7.2/7.2		EA	−81	NC	3
S332C	5.2/7.2		EA			0*
	7.2/7.2		EA	−23	NC	2
	7.2/7.2	EA		−23	NC	2
A334C	5.2/7.2		EA	−44	NC	1
	7.2/7.2	EA		−60	NC	3
L335C	7.2/7.2	EA		−25	NC	2
	7.2/7.2	EA	EA	−25	NC	3
S336C	5.2/7.2		EA			0*
	7.2/7.2	EA		+18	+++++	5–6
	7.2/7.2	EA	EA	+18	+++++	1
L338C	5.2/7.2		EA	−29	NC	3
M339C	5.2/7.2	EA	EA			0*
	7.2/7.2		EA	+80	+++++	1
	7.2/7.2	EA		+100	+++++	3
	7.2/7.2	EA	EA	+100	+++++	1
V340C	5.2/7.2		EA	+20	+	8
	5.2/7.2		ES	+15	+	16
A341C	5.2/7.2		EA	−13	−	32
V347C	5.3/7.2		EA	−60	+	7
	5.3/7.2	EA		−60	+	3
	5.3/7.2		ET	−67	++	43
	5.3/7.2	ET				0*
	5.3/7.2		ES	−45	+	4
G348C	5.3/7.2		EA	−50	NC	10
	5.3/7.2	EA		−50	NC	6
	5.3/7.2		ET	−50	+	45
	5.3/7.2	ET		−50	+	10
L350C	5.3/7.2		EA	NC	++	22
	5.3/7.2		ET	NC	+++	48
	5.3/7.2		ES	+10	++	10
V351C	5.3/7.2		EA	−48	+	39
	5.3/7.2		ET	−70	+	47
	5.3/8.0		ET	−73	+	21
	5.3/8.8	ET		−73	+	3
	7.7/8.0		ET	−73	+	1
	7/7.2		ET	−73	+	2
	7/7.2	ET		−73	+	12–15
	5.3/7.2		ES	+24	+	23
	5.3/7.2		MMTS	+15	NC	6
D352C	5.3/7.2		EA	−18	NC	Several
	5.3/7.2		ET	−27	NC	Several
	5.3/7.2		ES	+57	NC	Several
I353C	5.3/7.2		EA	−30	NC	6
	5.3/7.2	EA		−30	NC	4

TABLE I
Continued

Mutant	pH Conditions (*cis*/*trans*)	Reagents *cis*	Reagents *trans*	Percent change in conductance	Δ Flickering	No. reactions observed
G354C	5.3/7.2		EA	−20	NC	43
	5.3/7.2	EA		−20	NC	5
	5.3/7.2		ET	−15	+	18
F355C	5.3/7.2		EA	−70	NC	31
	5.3/7.2	EA		−70	NC	5
A356C	5.3/7.2		EA	−46	NC	15
A357C	5.3/7.2		EA	NC	+++	Few
Y358C	5.3/7.2		EA	−57	NC	7
N359C	5.3/7.2		EA	−64	NC	8
	5.3/7.2	EA		−64	NC	7
	5.3/7.2		ET	−75	NC	16
	5.3/7.2	ET		−75	NC	2
	5.3/7.2		MMTS	−35	+	2

The membrane potential was held at +60 mV, except for some reactions of G348C with *trans* ET, which were at −60 mV. In rows where an MTS derivative is listed under both *cis* and *trans* reagents, it means the MTS derivative was added to both sides of the same membrane. EA = MTS-EA$^+$; ET = MTS-ET$^+$; ES = MTS-ES$^-$; MMTS = methylmethanethiosulfonate; and NC = no change. *Observed several channels for many minutes without getting any reaction.

sampling rate through an A/D converter (Instrutech Corp., Great Neck, NY). Tape recorder output was filtered at 1 kHz (with an 8-pole Bessel filter; Frequency Devices Inc., Haverhill, MA) and sampled at 5 kHz on playback to a PC data acquisition system. Voltages are those of the *cis* compartment with respect to that of the *trans*, which is taken as zero. After one or a few channels appeared, one of the MTS derivatives was stirred into the *cis* or *trans* compartment to a concentration of several hundred micromolar or higher (see RESULTS). In those experiments in which the pH of the *cis* solution was raised to pH 7.0, this was done after several channels had appeared; the voltage was maintained throughout at +60 mV and not switched to negative voltages, as once channels were turned off with the *cis* solution at pH 7.0, they did not reappear. Even with the voltage held at +60 mV, the channels spontaneously disappeared over a period of seconds to a few minutes, so that by the time the MTS derivative had been stirred into the appropriate compartment, there were usually only one or two channels still present. MTS-EA$^+$, MTS-ET$^+$, and MTS-ES$^-$ were generous gifts from Dr. David Stauffer (Columbia University, New York), and methylmethanethiosulfonate (MMTS) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

Accessibility of a Given Cysteine-mutated Residue to MTS Derivatives

We singly mutated 49 of the 53 residues of TH8-9 (residues 328–376) to cysteines and determined whether a residue's location in the channel structure is such that it can react with an MTS derivative, and hence is accessible to that reagent. The results of this investigation are summarized in Fig. 1 and Table I, and are the heart of this paper. Before we present an exegesis of that figure, with various details and caveats, let us explain the criteria used to establish whether a given cysteine had, or had not, reacted with an MTS derivative.

Each of the 49 cysteine-substituted residues was initially tested with MTS-EA$^+$ added to the *trans* side under pH conditions of *cis* pH 5.3/*trans* pH 7.2.[5] With the

[5]The one exception was residue 363, which was tested with MTS-ES$^-$ added to the *trans* side and MTS-ET$^+$ added to the *cis* side, under the pH conditions *cis* pH 7.0/*trans* pH 7.2.

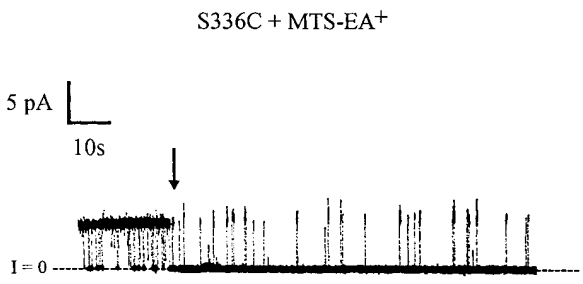

FIGURE 2. Reaction of a cysteine-mutant channel with an MTS derivative: change in flickering pattern. The membrane initially separated solutions of *cis* pH 5.3/*trans* pH 7.2. Before the start of the record, S336C was added to the *cis* compartment to a concentration of 0.2 ng/ml, and the voltage across the membrane was held at +60 mV. After a channel opened, MTS-EA$^+$ was added to both the *cis* and *trans* solutions to concentrations of 504 and 864 μM, respectively. When no reaction had occurred after several minutes, the pH of the *cis* solution was raised to 7.3 with HEPES buffer. The current record shows a channel with a normal flickering pattern under these pH conditions; that is, the channel spends most of its time in the open state, with brief transitions to the flicker-closed state. The arrow marks the instant that the channel's flickering pattern suddenly changes, presumably because of its reaction with MTS-EA$^+$. The channel now spends most of its time in the closed state, with brief transitions to the open state. The record is filtered at 100 Hz by the chart recorder.

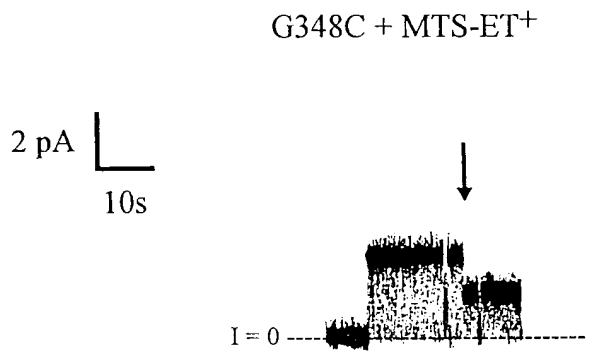

FIGURE 3. Reaction of a cysteine-mutant channel with an MTS derivative: change in single-channel conductance. The membrane separated solutions of *cis* pH 5.3/*trans* pH 7.2. Before the start of the record, G348C was added to the *cis* solution to a concentration of 40 ng/ml, and the voltage across the membrane was held at +60 mV. After single-channel activity appeared, the channels were turned off by a negative voltage. MTS-ET$^+$ was then added to the *trans* solution to a concentration of 183 μM, and the voltage across the membrane was then again held at +60 mV. The current record shows a channel opening under these conditions at its normal conductance, and after ~17 s (*arrow*), its conductance suddenly drops, presumably because of its reaction with MTS-ET$^+$. (For extended periods of time after a conductance change such as the one shown here, we have never seen a second conductance change in any of the mutant channels.) The record is filtered at 100 Hz by the chart recorder.

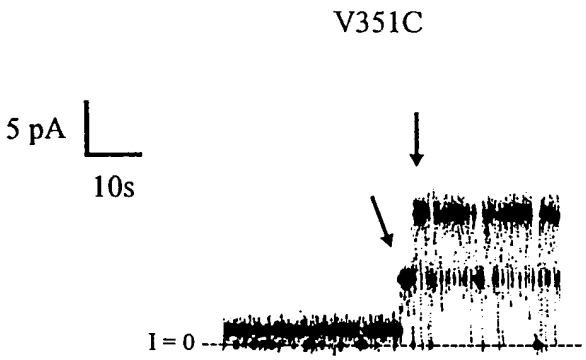

FIGURE 4. Reversal by DTT of the MTS-ET$^+$ reaction with a V351C channel. The membrane initially separated solutions of *cis* pH 5.3/*trans* pH 8.0. Before the start of the record, V351C was added to the *cis* compartment to a concentration of 25 ng/ml, and the voltage across the membrane was held at +60 mV. After a channel opened, MTS-ET was added to the *trans* solution to a concentration of 90 μM, and subsequently the channel reacted with it, causing its conductance to drop by about a factor of three. The pH of the *cis* solution was then raised to 7.7 with HEPES buffer and DTT was added to the *trans* solution to a concentration of 20 mM. The current record shows the low conductance, MTS-ET$^+$-reacted channel. The first arrow marks the sudden increase of the channel conductance to its normal value, presumably because of its reaction with DTT. The second arrow marks the appearance of another channel (which had never reacted with MTS-ET$^+$) with normal conductance. The record is filtered at 100 Hz by the chart recorder.

exceptions of residues 337 and 352, which we comment upon later, the single-channel conductance of the cysteine-mutated channel under these conditions was within 20% of that of the wild-type channel (≈40 pS). A subsequent step change in channel conductance, and/or a sudden change in flickering pattern, established that MTS-EA$^+$ had reacted with the cysteine at that site and therefore was entered as a MTS-accessible residue in Fig. 1. Examples of these changes in channel behavior subsequent to reacting with MTS-EA$^+$ and MTS-ET$^+$ are shown in Figs. 2 and 3, respectively (see Mindell et al. [1994*c*] for further examples). The change in single-channel behavior resulting from the reaction of the cysteine at residue 351 with MTS-ET$^+$ was reversed by DTT (Fig. 4) (this was the only site at which we attempted to reverse the reaction). The magnitude of the change in single-channel conductance produced by a given MTS derivative for a given mutant was very reproducible, and in those cases in which reactions were obtained from both the *trans* and *cis* application of the MTS derivative, the changes in single-channel conductance were identical.

It turned out that all but four of the MTS-accessible residues (329, 332, 336, and 339) were covered by the above criterion. That is, if no effect was obtained with *trans* MTS-EA$^+$ under the conditions *cis* pH 5.3/*trans* pH 7.2, then no effect was obtained under any of the other conditions we tried, and the residues were entered as MTS-inaccessible in Fig. 1.[6] These conditions included, for most of the mutant channels failing the initial test, 5–10× higher concentrations of *trans* MTS-EA$^+$, *cis* MTS-EA$^+$ under the same pH conditions, and *cis* and *trans* MTS-EA$^+$ under pH conditions *cis* pH 7.0/*trans* pH 7.2 (the rationale for raising the *cis* pH is that the MTS reaction is with the ionized sulfhydryl group, and we were therefore eliminating the possibility that the failure to obtain a reaction was because the pH at the site being examined was ~5.3). In addition, some of these unreactive mutant channels were also tested with *cis* or *trans* MTS-ET$^+$ and MTS-ES$^-$ under the pH conditions *cis* pH 5.3/*trans* pH 7.2, and *cis* pH 7.0/*trans* pH 7.2.

Character of the Single Channel Response to the Reaction with MTS Derivatives

There were two invariant features of the response of each of the 23 different cysteine mutant channels for which there was an MTS effect. First, the MTS reaction resulted in only one transition in single-channel con-

[6]It should be understood that calling these residues "MTS inaccessible" is an overinterpretation of the data. Such residues could have reacted with the reagent, but the addition of the $-SCH_2CH_2R$ group to the cysteine might not have resulted in a change of single-channel conductance or flickering. We feel that for most of the residues, this is an unlikely possibility (see DISCUSSION).

ductance (and/or flickering). Thus, in Fig. 3 for example, there is only a single step decrease in conductance when MTS-ET$^+$ reacted with the channel, not the n steps that one might expect for a multimeric channel, where n is the number of monomers forming the channel (and hence is the number of cysteine residues in the channel). Second, when MTS-ET$^+$ or MTS-ES$^-$ was in the *trans* solution, the channel always initially opened normally (i.e., with normal conductance and flickering) before it was converted to its modified state. That is, it never opened ab initio already chemically modified by the MTS derivative.[7] (Once modified, the channel could close and then reopen in its modified form.) This implies that in the channels' closed state, which existed either before they ever opened or after they were closed by a large negative voltage, the MTS derivatives did not have access from the *trans* solution to any of the cysteine residues with which they were capable of reacting; i.e., these residues were shielded from the *trans* solution when the channel was in its closed state, but became exposed to that solution when the channel opened (the closed state we have been discussing is distinct from the one associated with the rapid open–closed flickering that we shall address shortly). In contrast, when an MTS derivative was in the *cis* solution (see below), the reaction could occur not only when the channel was already open (as was the case with an MTS derivative in the *trans* solution), but also before its opening, as evidenced by the channel opening ab initio already chemically modified by the reagent.

In our description up to this point of the MTS derivatives reacting with a channel after it had opened, we have neglected the rapid open–closed flickering that occurs, characterized by the channel's spending most of its time (95%) in the open state (examples of this flickering are seen in the records in Figs. 2 and 3). When the MTS reactions were viewed at higher time resolutions than could be obtained from the chart recordings, it became apparent that at all of the sites examined in this way, there was a marked preference for the reaction to occur in the channel's flickered closed state, rather than in the flickered open state. An example of this is shown in Fig. 5, and a summary of the results obtained at high time resolution is presented in Table II. A striking example is residue 351. Of the 23 reactions resolvable at high time resolution, every one of them occurred in the flickered closed state. Even for those sites that showed more reactions occurring in the flickered open state than in the flickered closed state

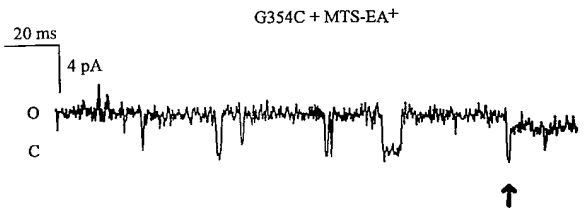

FIGURE 5. Resolution of whether a channel's reaction with an MTS derivative occurred in its open state or in its flickered closed state. This current record is a playback at much higher time resolution of a channel's reaction with an MTS derivative. In this instance, it is the reaction of a G354C channel with *trans* MTS-EA$^+$ (169 μM). The record is filtered at 1 kHz and sampled at 5 kHz. Note that the channel spends most of its time in the open state (*O*), with brief excursions to the flickered closed state (*C*). The arrow marks the time of the channel's reaction with MTS-EA$^+$, resulting in a smaller channel conductance. Note that in this instance, the transition to this smaller conductance occurs not from the open state, but after a brief closure. Solutions are *cis* pH 5.3/*trans* pH 7.2; the applied voltage is +60 mV.

(e.g., residue 352), when one corrects for the fact that the channel spends only ~5% of its time in the flickered closed state, it is apparent that this is the preferred state for the occurrence of the reaction.

Accessibility of Sites to MTS Derivatives from the cis and trans Solutions

For several of the sites that reacted with MTS derivatives from the *trans* solution, their accessibility to MTS derivatives from the *cis* solution was also tested; the results of these experiments are summarized in Table III. Of the sites that reacted with *trans* MTS derivatives, every one that we tested with *cis* MTS derivatives reacted with at least one of them. Because MTS-EA in its uncharged form could conceivably have traversed the lipid bilayer from the *cis* to the *trans* solution, and then have gained access to the site from there (Holmgren et al., 1996), or even have partitioned into the bilayer and gained access to the site in that way, there is an ambiguity in those experiments as to whether the site was truly accessible to MTS derivatives from the *cis* solution. However, for three of the four mutant channels tested with *cis* MTS-ET$^+$, where the issue of the reagent's inability to cross the bilayer or significantly partition into it is not in question, the mutant channels reacted with it, thereby demonstrating that these sites were accessible to MTS derivatives from both the *trans* and the *cis* solutions (Table III).

Way in which the Introduction of the $-SCH_2CH_2R$ Group at a Site Affected Single-channel Behavior

In general, the reactions with the MTS derivatives resulted in a sudden change in single-channel conduc-

[7]This was generally true also for MTS-EA$^+$. The few possible exceptions that we observed can be attributed to the membrane permeability of its neutral form (Holmgren et al., 1996), which, having traversed the membrane to the *cis* side, could now react with cysteine-modified mutant either in solution or at the membrane surface.

TABLE II

Resolvable Reactions in the Flicker-open and -closed States of Cysteine-mutant Channels with MTS Derivatives

Mutant	Reagent (*cis*)	Reagent (*trans*)	No. of reactions analyzed	No. reacted from open state	No. reacted from closed state
V347C		EA	3		1 (?)
		ET	43	2 (?)	1; 2 (?)
G348C		EA	10	8	1; 1 (?)
		ET	14	6	3; 1 (?)
	EA		6	1; 1 (?)	2; 1 (?)
V351C		EA	5		4
		ET	39		7
		ES	21		7
	ET*		14		5; 2 (?)
D352C		EA	Several	1	
		ES	Several	6; 1 (?)	2
I353C		EA	6		3; 1 (?)
	EA		4		2
G354C		EA	40		9
	EA		5		1
F355C		EA	29	8; 1(?)	6; (2?)
	EA		5	1; 2 (?)	
A356C		EA	15	1	4; 3 (?)
Y358C		EA	7	1	1
N359C		ET	15	2	3
		EA	8	3	5
	EA		4	4	

The membrane potential was held at +60 mV. The pH conditions were *cis* 5.3/*trans* 7.2, except for the reactions of V351C with *cis* ET, which were *cis* pH 7.2/*trans* pH 7.2. A question mark in parenthesis next to an entry in a "No. reacted from . . ." columns indicates that, in addition to the number of resolvable reactions, this many more reactions probably reacted from the indicated flicker open or closed state also, but it is not entirely clear. Note that only a small fraction of the number of reactions analyzed was resolvable. The reactions of some mutants with MTS derivatives were not analyzed at high time resolution to determine whether they reacted from the flicker-closed or -open state. Some mutants that were analyzed, but could not be resolved, are not included in the table. EA = MTS-EA$^+$; ET = MTS-ET$^+$; and ES = MTS-ES$^-$. *pH Conditions *cis* 7.2/*trans* 7.2.

tance. Sometimes this was accompanied by a change in the channel flickering pattern, and at two sites (350 and 357) this was the only change. The sign of the conductance change was generally that, for the two positive reagents, MTS-EA$^+$ and MTS-ET$^+$, the single-channel conductance dropped; in fact, only at four sites (residues 329, 336, 339, and 340) out of the 23 that reacted with the positive reagents did the single-channel conductance increase. Conversely, for the negative MTS-ES$^-$ reagent, the single-channel conductance increased, with the exception of residue 347. At those sites at which both MTS-EA$^+$ and MTS-ET$^+$ reacted, the drop in conductance produced by MTS-ET$^+$ was larger than that produced by MTS-EA$^+$, with the lone exception of site 354. The data from all of these experiments are summarized in Table I. We shall comment in greater detail on these findings in DISCUSSION, but it is superficially clear that the sign of the effect is strongly dependent on the sign of the charge introduced at the site, which is consistent with an electrostatic effect on the principal current carrier (K$^+$) in these experiments. The generally larger effect produced by MTS-ET$^+$ compared with that produced by MTS-EA$^+$ indicates that a steric effect is also operating.

Relative Distances of Sites from the cis and trans Sides

Although we did not make a detailed study, an idea of the relative distance of some sites from the *cis* and *trans* sides can be gleaned from the data in Table III. One indication of the relative distance of a site from the *cis* and *trans* sides can be obtained from a comparison of the rates of the reaction at that site with *cis* and *trans* application of MTS derivatives. Because of the permeability of the membrane to the neutral form of MTS-EA$^+$ (Holmgren et al., 1996), the unstirred layers on the two sides of the membrane, and the pH gradient in most experiments, it is not safe to use the MTS-EA$^+$ data to draw inferences. The three entries in Table III with MTS-ET$^+$, however, are not confounded by these factors, and are revealing. It is clear that the substituted cysteine at residue 348 reacts much more rapidly with MTS-ET$^+$ from the *trans* side than from the *cis* side, thereby placing that site close to the *trans* side. Simi-

TABLE III

Accessibility of Cysteine-mutant Channels to MTS Derivatives from both the cis and trans Solutions

Mutant	pH Conditions (*cis*/*trans*)	Reagent *cis*	Reagent *trans*	No. reactions observed	Waiting time for reaction
		μM	μM		*s*
V329C	7.2/7.2		EA (460)	2	10–25
	7.2/7.2	EA (300–380)		3–4	<3
S332C	7.2/7.2		EA (810)	2	15, 17
	7.2/7.2	EA (135)		2	<5
A334C	5.2/7.2		EA (510)	1	25
	7.2/7.2	EA (170)		3	<4
M339C	7.2/7.2		EA (680)	1	~60
	7.2/7.2	EA (475)		3	<4
V347C	5.3/7.2		EA (140–240)	7	1–3
	5.3/7.2	EA (560)		3	~60
	5.3/7.2		ET (150)	43	~1
	5.3/7.2	ET (150)		0*	
G348C	5.3/7.2		EA (180)	10	~1
	5.3/7.2	EA (120)		6	10–25
	5.3/7.2		ET (126–180)	45	~7
	5.3/7.2	ET (250–300)		10	1–60
V351C	5.3/7.2		ET (90–2170)	47	1–20
	5.3/8.0		ET (90–270)	21	1–3
	5.3/8.8	ET (350)		3	5–10
	7/7.2		ET (150)	2	5–10
	7/7.2	ET (180–270)		12–15	10–120
I353C	5.3/7.2		EA (170)	6	≤0.5
	5.3/7.2	EA (170)		4	120–360
G354C	5.3/7.2		EA (60–170)	43	1–10
	5.3/7.2	EA (170)		5	120–180
F355C	5.3/7.2		EA (7.0–100)	31	3–60
	5.3/7.2	EA (70–200)		5	2–60
N359C	5.3/7.2		ET (175)	16	2–60
	5.3/7.2	ET (180)		2	—
	5.3/7.2		EA (170)	8	0.5–10
	5.3/7.2	EA (170–250)		7	5–25

The membrane potiential was held at +60 mV, except for some reactions of G348C with *trans* ET, which were at −60 mV. Mutants not included in the table were not tested with MTS derivatives from both sides of the membrane. EA = MTS-EA$^+$; ET = MTS-ET$^+$. *Observed several channels for many minutes without getting any reaction.

larly, the fact that with the *cis* solution at pH 5.3, *cis* MTS-ET$^+$ reacted with the substituted cysteine at residue 351 only when the pH of the *trans* side was raised to 8.8, indicates that this site also lies close to the *trans* side. The results for residue 359 are not as clear cut, but suggest that the site is not as close to the *trans* side as sites 348 and 351.

The voltage dependence of the MTS-ET$^+$ reaction with the cysteine at residue 348 is also consistent with that site being close to the *trans* side. With MTS-ET$^+$ (183 μM) on the *trans* side, the average time for the reaction to occur at +60 mV (5.8 s, 12 events) was essentially the same as that at −60 mV (7.7 s, 16 events). In contrast, with MTS-ET$^+$ (306 μM) on the *cis* side, the times for the reaction to occur at +60 mV ranged from 1 to 20 s (four events), whereas no reaction occurred at −60 mV even after 30–120 s (11 channels).

Another indication of the location of several sites is obtained from the data in Table IV. We note that the cysteine-substituted residues from 329 to 336 reacted slowly or not at all when the pH of the *cis* solution was 5.2 and that of the *trans* solution was 7.2, but subsequently reacted when the pH of the *cis* solution was raised to 7.2. Since the substrate for the MTS derivatives is the S$^-$ form of the cysteine's sulfhydryl group, the implication of these results is that the reason that the reaction did not occur (or occurred slowly) at these sites when the pH of the *cis* solution was 5.2 and that of the *trans* solution was 7.2 was that the local pH was low. That is, residues 329–336 lie closer to the *cis* side than

TABLE IV

Waiting Times for Reactions of Cysteine-substituted Residues 329–336 with MTS-EA$^+$

Mutant	pH Conditions (*cis/trans*)	EA concentration *cis*	EA concentration *trans*	No. reactions observed	Waiting time for reaction
		μM	μM		*s*
V329C	5.2/7.2	EA (233)		0*	
	5.2/7.2		EA (465)	0*	
	7.2/7.2		EA (460)	2	10–25
	7.2/7.2	EA (380)	EA (920)	1	<3
	7.2/7.2	EA (300–380)		3–4	<3
A330C	5.2/7.2		EA (600–680)	2–3	>30
	7.2/7.2		EA (460–760)	7	5–40
Q331C	5.2/7.2		EA (678)	1	140
	7.2/7.2		EA (508)	3	12, 30, 60
S332C	5.2/7.2		EA (400–810)	0*	
	7.2/7.2		EA (810)	2	15, 17
	7.2/7.2	EA (135)		2	<5
I333C	5.2/7.2	EA (373–466)	EA (373–746)	0*	
A334C	5.2/7.2		EA (508)	1	25
	7.2/7.2	EA (170)		3	<4
L335C	7.2/7.2	EA (339)		2	1 and 90
	7.2/7.2	EA (424)	EA (847)	3	1–10
S336C	5.2/7.2	EA (504)	EA (864)	0*	
	7.2/7.2	EA (216–504)		5–6	5–25
	7.2/7.2	EA (504)	EA (864)	1	~30

The membrane potential was held at +60 mV. In rows where EA is listed under both the *cis* and *trans* column, it means EA was added to both sides of the same membrane. EA = MTS-EA$^+$. *Observed several channels for many minutes without getting any reaction.

to the *trans* side and therefore "feel" the *cis* pH more than the *trans* pH.

DISCUSSION

Previous work has established that the 61 amino acid stretch from residue 322 to 382 of the T-domain of diphtheria toxin forms channels in planar phospholipid bilayer membranes that are indistinguishable in their ion-conducting and permeability properties from those formed by the entire T-domain (Silverman et al., 1994*b*). In the crystal structure of the water-soluble form of the toxin, the bulk of this stretch (from residue 326 to 378) consists of an α-helical hairpin, designated TH8-9 (Choe et al., 1992). Since the two helices of the hairpin, TH8 and TH9, are long enough to cross a lipid bilayer, it was postulated that the channel is formed by a structure (presumably a homooligomer) in which TH8 and TH9 span the bilayer as α-helices, and the connecting loop resides in or near the *trans* solution. In the present study, we used the substituted-cysteine-accessibility method (Akabas et al., 1992) to determine which residues in TH8-9 line the ion-conducting pathway of the channel; i.e., line the channel lumen or its entrances. The method consists of mutating one at a time the residues of interest (in our case, residues 328–376) to cysteines, and then determining whether they are accessible to react with small, charged, hydrophilic, sulfhydryl-specific MTS derivatives. We anticipated that our results would be consistent with the helical hairpin model of the channel, with perhaps some caveats. Instead, we have uncovered a pattern of residue accessibility to the MTS derivatives that is completely at variance with this model. Moreover, the channel's response to the reaction of its accessible residues with MTS derivatives is such as to strongly suggest that the channel is not a homooligomer of TH8-9, but instead contains only one TH8-9 unit.

Pattern of the Residues Accessible to the MTS Derivatives

We consider here the distribution of the cysteine-substituted residues that reacted with the MTS derivatives. We remind the reader that the criterion for a reaction is a change in single-channel conductance and/or single-channel flickering pattern. A positive result is an unambiguous indication that the MTS derivatives had access to the site and reacted there, and therefore the residue at the site is exposed to an aqueous milieu (presumably the ion-conducting pathway of the channel). A negative result is an indication that the residue is not exposed to an aqueous milieu (i.e., it does not face the channel lumen or its entrances), but it is not conclusive. The MTS derivative might have reacted at the site

without producing a change in channel conductance or flickering behavior, or it might have access to the site but is sterically prevented from reacting with the −SH of the cysteine. We shall elaborate on these points later, but to facilitate the discussion and avoid awkward circumlocutions, we shall refer to those sites at which MTS derivatives produced no change in single-channel properties as "unreactive" or "inaccessible". We also note that with the exception of residue 337, mutation of a residue to a cysteine resulted in channels having essentially the same conductance as that of wild type, or, in the case of 352, a smaller conductance consistent with the removal of a negative charge. Thus, we can reasonably assume that the structure of the mutant channels is fundamentally the same as that of wild-type channels.

Of the 49 sites in TH8-9 that we tested, 23 of them were accessible to MTS derivatives. The pattern of the MTS-accessible residues is displayed in Fig. 1 and consists of two segments: one extending from residue 347 to 359, and the other from 329 to 341. Of the 13 residues in the former, MTS-EA$^+$ produced an effect on every cysteine-substituted mutant channel except E349C; of the 13 residues in the latter, MTS-EA$^+$ produced an effect on every mutant channel except I333C and S337C. The failure of any of the MTS derivatives to affect the conductance of the E349C channel was anticipated, since previous site-directed mutagenesis experiments revealed that the single-channel conductance of E349Q and E349K was the same as that of the wild-type channel (Mindell et al., 1994*a*). The absence of an effect of MTS-EA$^+$ on the S337C channel is difficult to interpret, since the conductance of that channel was less than half that of the wild-type channel, suggesting that the mutation disrupted channel structure. None of the other 23 cysteine-substituted residues outside of these two segments appeared to react with the MTS derivatives. The two MTS-accessible segments are separated by a stretch of five unreactive residues.

What is one to make of this unusual pattern of MTS-accessible sites? For sure, it is not easily reconciled with an α-helical or β secondary structure! This type of pattern is not unprecedented in the channel literature. A segment of three consecutive aqueous exposed residues was found, using MTS derivatives, for the CFTR channel (Cheung and Akabas, 1996), and a stretch of four consecutive residues was found, using Ag^+ as a probe for reactivity with cysteine mutants, for the P-region of the Shaker potassium channel (Lü and Miller, 1995). But the extent of the two apparently structureless segments in the diphtheria toxin channel is remarkable. The implication of this pattern of MTS-accessible sites is that these two segments are not rigid, but instead are sufficiently flexible and mobile that most of their residues are at some time exposed to an aqueous environment; i.e., the channel lumen or its entrances.

Relative Distance of MTS-accessible Sites from the cis and trans Sides of the Membrane

Based on the relative rates of the reaction of the substituted cysteine at residue 348 with *cis* and *trans* application of MTS-ET$^+$, we conclude that this site is close to the *trans* side of the membrane. This result is consistent with the titration data of Mindell et al. (1994*a*) that placed residue 352 near the *trans* side. On the other hand, since cysteine-substituted residues from 329 to 336 reacted slowly or not at all with MTS derivatives when the pH of the *cis* solution was 5.2 and that of the *trans* solution was 7.2, but did react readily when the pH of the *cis* solution was subsequently raised to 7.2, we conclude that this region is closer to the *cis* than the *trans* side. Thus, any model for the diphtheria toxin channel must place the 329–336 region closer to the *cis* than to the *trans* side, and the 348–352 region close to the *trans* side.

Helix TH9

The helical wheel representation of TH9 reveals an amphipathic helix, and it would be reasonable to suppose that its hydrophilic face lined the channel lumen. Indeed, Oh et al. (1996) concluded from spin label studies that TH9 is inserted into the bilayer of phospholipid vesicles as an α-helix and that polar groups of this helix line the channel lumen. The residues particularly cited as lining the lumen were A356, N359, E362, N366, Q369, and H372. Our MTS results are markedly different from this. The only sites in TH9 that were clearly accessible to MTS derivatives were the four at the NH_2-terminal end of the helix, residues 356–359 (see Fig. 1). These residues make the first turn in TH9, and in the crystal structure of the water-soluble form of diphtheria toxin, this segment is irregular (Bennett and Eisenberg, 1994). Thus, their MTS accessibility, although somewhat at variance with the spin label results, which place 356 and 359, but not 357 and 358, in an aqueous environment, is not inconsistent with the α-helix seen for TH9 in the crystal structure. What is most inconsistent with that structure is our failure to identify any other residues in TH9 that are accessible to the MTS derivatives.

As we pointed out earlier, our failure to see a change in single-channel conductance or flickering in the presence of the MTS derivatives does not necessarily mean that the cysteine-mutated site in the channel does not line the channel lumen. We know, for example, from site-directed mutagenesis experiments, that when the negatively charged glutamate at 362 was mutated to a neutral glutamine or alanine (Mindell et al., 1994*b*), the

single-channel conductance was smaller than that of the wild-type channel (under certain pH conditions), consistent with the negatively charged glutamate lining the channel lumen. Of even more relevance is that when it was mutated to the positively charged arginine (Silverman et al., 1994*a*), its conductance was even smaller. Therefore, had either MTS-EA$^+$ or MTS-ET$^+$ reacted with the −SH at this site, we certainly should have seen a drop in single-channel conductance. We must therefore suppose that for local steric reasons, the MTS derivatives could not react with the −SH of the cysteine at 362. (We were unable to observe a reaction with MTS-EA$^+$ in the *cis* and *trans* solutions even at symmetric pH 8.0 conditions.) Note that one cannot argue that the MTS reagents were blocked from reaching residue 362, since residue 359 was accessible to MTS-EA$^+$ and MTS-ET$^+$ from both the *cis* and the *trans* sides (see Table III).

Given this failure of the MTS derivatives to give a positive result at a site (362) at which a priori they were expected to do so, one could take the position that TH9 is indeed inserted into the membrane as an α-helix, and that for local steric reasons the MTS reagents fail to react at its sites lining the channel lumen. Or, alternatively, the lumen might be so wide beyond residue 362 that, even if MTS-EA$^+$ reacted at sites there, the introduction of positively charged groups would not significantly affect single-channel conductance. Consistent with this possibility is that the mutant H372R channel had the same conductance as that of the wild-type channel, even when both the *cis* and *trans* solutions were at pH 7.2 (Mindell et al., 1994*b*); i.e., at a pH at which the histidine should be mainly neutral, its replacement by the positively charged arginine did not affect single-channel conductance. In summary, our results do not preclude that TH9 is inserted into the membrane as an α-helix with most of its polar residues lining the channel lumen, but they offer no support for that hypothesis.

Molecularity of the Channel

Before one can think constructively about channel architecture, it is necessary to establish the channel's subunit composition. Although the present investigation was not directed at that issue, our results have a strong bearing on its resolution and lead to a surprising conclusion.

The most salient finding reported in this paper is that for every one of the cysteine mutant channels for which there was an MTS effect, there was only one transition in single-channel conductance (or flickering behavior). There may be Byzantine scenarios that can be devised in which, for example, several reactions must occur at a site before the channel can undergo a conductance or flickering change, or only the first of many subsequent reactions affects channel behavior, but it strains credulity to believe that this is the case for 23 different sites. The most plausible and obvious interpretation of this finding is that only one reaction with an MTS derivative occurs at a site, and the simplest explanation for this is that this is because there is only one cysteine at the site. In other words, the diphtheria toxin channel is not multimeric, but is formed from only one molecule. Given that previous work has shown that only the TH8-9 region of the toxin is required to make a channel (Silverman et al., 1994*b*), this conclusion is so contrary to one's usual ideas about channel structures that alternative explanations for the occurrence of only one reaction at a site must be carefully considered before adopting it.

The only reasonable explanation that we can think of for getting only one reaction per site in a multimeric channel is that the reaction with an MTS derivative of one of the cysteines at a site prevents the reaction of other cysteines at that site. How might this come about? One possibility is that the reaction of one cysteine at the site leads to a conformational change in the channel such that the other cysteines are no longer accessible to the reagent. This is very unlikely for at least two reasons. First, for most of the mutant channels that reacted, the response to the MTS derivatives is simply explained by an electrostatic and/or steric effect on the conducting ions (primarily K^+) by the new moiety attached to the cysteine sulfur, without invoking any change in channel structure. (For the few mutants, particularly M339C, in which a significant change in flickering behavior occurred, a case can be made for a conformational change.) Second, and more importantly, it is not very probable that the reaction at every one of 23 different sites within a channel would result in a conformational change that made the other cysteines at the site inaccessible to the MTS derivatives.

A more reasonable possibility is that the new moiety, attached to the cysteine sulfur after its reaction with these charged MTS derivatives, blocks electrostatically or sterically the access of that MTS derivative to other cysteines at the site. We think that this explanation is also unlikely. With regard to electrostatic block by the attached moiety, we have shown for the V351C and D352C channels that once they had reacted with MTS-EA$^+$ or MTS-ET$^+$, they could not subsequently react with the oppositely charged MTS-ES$^-$ and, conversely, reaction first with MTS-ES$^-$ prevented a subsequent reaction with the positively charged reagents (Mindell et al., 1994*c*). With regard to steric block, we note that the size of the group at the site after cysteine's reaction with MTS-EA$^+$ is not much larger than the original leucines, isoleucines, or tyrosines originally present at some of the sites before their mutation to cysteines. Moreover, at those sites that we reacted with MMTS ($CH_3SO_2SCH_3$), which attaches only a small and neu-

tral $-SCH_3$ group to the cysteine sulfur, we still saw only one reaction (see Table I). It therefore is difficult to invoke steric blockage by one reacted cysteine as the reason that other presumed cysteines at that site cannot react.[8]

There is other evidence suggesting that the diphtheria toxin channel is not a homooligomer. The side chain at the 352 site after the reaction of a cysteine with MTS-EA$^+$ (CH_2-S-S-CH_2-CH_2-NH_3^+) is similar to that of lysine (CH_2-CH_2-CH_2-CH_2-NH_3^+), and the conductance of the D352C channel after reacting with MTS-EA$^+$ is almost identical to that of the D352K channel (Mindell et al., 1994*a*). The identity of the conductances of these two types of channels does not make sense for a multimeric channel, where the former has one positively charged residue at a site and the latter has n similar positively charged residues there, where n is the number of subunits forming the channel. Similarly, the conductance of the D352C channel is smaller than that of the wild-type channel, which is consistent with the replacement of a negative charge by a neutral one. The subsequent reaction with MTS-ES$^-$ restores channel conductance to that of wild type. Again, it is surprising, if this is a multimeric channel, that the conductance of a mutant having only one negative charge at 352 should be the same as that of the wild-type channel having n negative charges at that site. Finally, in unpublished experiments designed for getting at the molecularity of the channel, we mixed in a test tube different proportions of wild-type and D352K toxin before adding the mixture to the bilayer chamber. In those experiments, we saw channels of either wild-type or D352K conductance (and in one experiment we saw both sized channels), but none of an intermediate conductance expected for a heteromultimer.

All of the results discussed in this section argue against a multimeric channel. The only observation suggesting that the channel is formed from more than one toxin molecule is that the rate of channel formation increases with about the second power of the toxin concentration, rather than linearly with its concentration (Kagan et al., 1981). It is, however, quite possible that a cooperative process facilitates insertion of the toxin in the membrane, thereby giving a nonlinear dependence of rate of channel formation on concentration, whereas the channel that actually forms is a monomer. Thus, contrary to common sense, we are forced to the reluctant conclusion that only one TH8-9 unit of the T-domain interacts with a phospholipid bilayer to form a channel. What the structure of this channel is, and what is the contribution of lipid to this structure, is unknown.

Further Problem to Envisioning the Channel Structure

As if it were not difficult enough to construct a model for the diphtheria toxin channel with phospholipid and just one TH8-9 segment, there is one other fact that is difficult to reconcile with our data. As we pointed out earlier, a number of independent observations lead us to conclude that residues 350 and 351 lie close to the *trans* side. We would therefore expect that if the cysteine-substituted mutants at these sites were biotinylated, the resulting channels would be somehow affected by streptavidin added to the *trans* compartment (analogous to the type of effects seen with biotinylated channels formed by colicin Ia) (Qiu et al., 1996). In fact, however, we saw no effect of *trans* streptavidin on either single-channel behavior or macroscopic voltage gating. This by itself is not too disturbing, since one could argue that the streptavidin is sterically prevented from binding to the biotin. One could also invoke this argument to explain our failure to see any effect of *cis* streptavidin on channel gating or activity. What is most disturbing and incomprehensible, however, is that even when we bound streptavidin to these biotinylated cysteine mutants in a test tube (and confirmed on denaturing and nondenaturing gels that indeed the streptavidin was bound to the cysteine mutant), we observed no effect on channel activity or gating. How a channel could still form with streptavidin attached to residues that are supposed to lie on the *trans* side of the membrane is a mystery. To suggest that the streptavidin somehow rapidly detaches from the biotinylated residue during channel formation is an act of desperation, given the tight binding of streptavidin to biotin.

Reactions of MTS Derivatives with the Flickered Closed State

We have noted (see RESULTS) that there is apparently a marked preference for the MTS derivatives to react with the cysteine-substituted residues when the channel is in its flickered closed state, where it spends only ~5% of its time, rather than when it is in its open state. This may be just one other of the inexplicable characteristics of the channel, but there is a possible benign explanation for the observation. Namely, that MTS derivatives actually react with the channel in the open state, but the released product of that reaction, $CH_3SO_2^-$, temporarily blocks the channel. Thus, the initial consequence of the reaction is to block current flow through the channel, before the channel is seen in its reacted state, and we misinterpret this block by $CH_3SO_2^-$ as a normal flickered closed state.

[8]In the presumed heptameric channel formed by the PA_{63} component of anthrax toxin, several (not one!) step changes of conductance were produced by MTS-ET$^+$ on the N306C mutant channel (unpublished data). Although not directly relevant to the present study of the diphtheria toxin channel, this does show that the substituted-cysteine-accessibility method employed here is capable of displaying multiple reactions in a multimeric channel.

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