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Crucial Role of H322 in the Folding of Diphtheria Toxin T-Domain into the Open-Channel State

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Abstract

The translocation (T) domain plays a key role in the entry of diphtheria toxin into the cell. Upon endosomal acidification, the T-domain undergoes a series of conformational changes that lead to its membrane insertion and formation of a channel. Recently, we have reported that the triple replacement of the C-terminal histidines H322, H323 and H372 with glutamines prevents the formation of open channels in planar lipid bilayers. Here, we report that this effect is primarily due to the mutation of H322. We further examine the relationship between the loss of functionality and membrane folding in a series of mutants with C-terminal histidine substitutions using spectroscopic assays. The membrane insertion pathway for the mutants differs from that of the wild type as revealed by membrane-induced red-shift of tryptophan fluorescence at pH 6.0–6.5. T-domain mutants with replacements at H323 and H372, but not at H322, regain wild type-like spectroscopic signature upon further acidification. Circular dichroism measurements confirm that affected mutants misfold during insertion into vesicles. Conductance measurements reveal that substituting H322 dramatically reduces the numbers of properly folded channels in a planar bilayer, but the properties of the active channels appear to be unaltered. We propose that H322 plays an important role in the formation of open channels and is involved in guiding the proper insertion of the N-terminal region of the T-domain into the membrane.

Diphtheria toxin enters the cell via an endosomal pathway, where the translocation (T) domain plays a critical role in the cell infection. Upon endosomal internalization, the T-domain undergoes a series of conformational changes in response to the acidification of the endosome, which lead to the insertion of the T-domain into the membrane and the translocation of the catalytic domain into the cytosol (1). The molecular mechanism of this insertion/translocation pathway, however, is not well understood.

The crystallographic structure of the soluble form of T-domain at neutral pH (2) reveals the presence of two central hydrophobic helices, TH8-9, (Fig. 1A, brown helices) surrounded by seven amphipathic helices (Fig. 1A, gray helices). Although no high resolution structure of the T-domain in its membrane-inserted state is available, multiple studies suggest that helices TH8-9 adopt a transmembrane topology (3–7), with a possibility of other regions of the sequence deeply embedding into the lipid bilayer to form various intermediates (6–9), as well as the functionally relevant Open-Channel State (OCS) (5).

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The insertion/translocation pathway is modulated by staggered pH-dependent transitions (10), with several studies implicating the protonation of the six histidines of the T-domain in various stages of the process (11, 12). For example, protonation of H257 has been linked to the early stages of the conformational switching, involving the destabilization of the folded structure of the T-domain in solution and the formation of the membrane-competent state (13). In contrast, the C-terminal histidines, H322, H323 and H372, located at the top of the insertion unit TH8-9 (color coded in Fig. 1A), affect the later stages of the folding in the membrane (14). Recently, we have reported that a triple substitution of these residues does not affect the insertion of the TH8-9 domain, yet affects the physiological activity of the T-domain (14). That study raised two important questions: 1) why does the replacement of C-terminal histidines result in a loss of activity despite the apparently normal interactions with the lipid bilayer, and 2) are any of the three histidines more important than the other, or will single and double replacements show a cumulative effect? In this study, we addressed these questions by generating a series of mutants of the C-terminal histidines and testing their ability to effectively fold into an OCS. Spectroscopic and conductivity measurements indicate a strong correlation between the inability to form the OCS and the misfolding of the T-domain in the membrane and reveal a critical role of H322 in the folding process.

MATERIALS AND METHODS

Materials

POPC, POPG, POPS and POPE were purchased from Avanti Polar Lipids (Alabaster, AL). Diphtheria toxin T-domain (amino acids 202-378) was cloned into NdeI-EcoRI-treated pET15b vector containing an N-terminal 6xHis-tag and a thrombin cleavage site. Both the mutants and the WT protein were expressed and purified as described in (15).

Conductance assays in planar bilayers

The current response was measured under voltage-clamp conditions in planar phospholipid bilayers separating two compartments: the cis compartment with acidic buffer into which the proteins were injected from a stock solution and the trans compartment with neutral buffer, as previously described (16). Asolectin planar bilayers were formed and voltage-clamp recordings were performed as previously described (17). The voltage is defined as the potential of the cis compartment, relative to that of the opposite trans compartment. Both aqueous solutions contained 1 M KCl, 2 mM CaCl₂, and 1 mM EDTA; in addition, the cis compartment contained 20 mM malic acid, pH 5.3, and the trans compartment contained 20 mM HEPES, pH 7.2.

LUV preparation

Large unilamellar vesicles (LUV) of 0.1 μ m diameter were prepared by extrusion as previously described (18, 19). The vesicles were composed of molar mixtures of POPG:POPC 3:1, POPG:POPC 1:3, POPG:POPE 3:1, and POPS:POPC 3:1.

Tryptophan fluorescence measurements

Fluorescence was measured using a SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating. The measurements were made at 25°C in 2x10 mm cuvettes oriented perpendicular to the excitation beam. Tryptophan residues were excited at 280 nm and the emission spectra were recorded between 290–500 nm using excitation and emission spectral slits of 2 and 4 nm, respectively. Normally, we added purified WT or mutant T-domain from a concentrated stock to 50 mM of sodium phosphate buffer at pH 8.0. We then mixed the sample with LUV to reach a final concentration of T-domain and LUV of 1 μ M and 1 mM, respectively, and

the rapid acidification was achieved by addition of small amounts of concentrated acetic buffer. We recorded at least 5 scans after an incubation time of 30 min to assure the equilibration of the sample. All spectra were corrected for background and fitted to a log-normal distribution as described in (20) to determine the position of maximum of emission. Normally, positions of maximum for 3 to 6 samples were averaged.

CD measurements and analysis of thermal unfolding

CD measurements were performed using an upgraded Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo). Normally, 100 scans were recorded between 190 and 260 nm with a 1 nm step at 25°C, using a 1 mm optical path cuvette. The sample contained 4 μ M of T-domain, either WT or mutant, and 1 mM of LUV in 50 mM phosphate buffer at pH 8.0. The rapid acidification was reached as described in the fluorescence experiments. All spectra were corrected for background; however, only the data collected at wavelengths longer than 200 nm, where effects of scattering are negligible (21), were presented. The thermal unfolding of T-domain WT and mutant in solution at pH 8.0 and pH 6.0 was measured at 222 nm with a 1 degree/minute scan rate. The analysis of thermal stability, yielding transition temperature T_m and transition enthalpy ΔH° , was performed as described before (14).

RESULTS AND DISCUSSION

In our previous study we demonstrated that a triple replacement of all C-terminal histidines in the T-domain affects the formation of the OCS by uncoupling the insertion of the TH8-9 helical hairpin (highlighted in brown in Fig. 1A) from the translocation of the N-terminus across the lipid bilayer (14). Here we examine the role of individual histidines in the formation of the OCS by replacing them one at a time with either lysine or glutamine. First, we tested their activity by measuring the conductance in a planar lipid bilayer as described previously (14, 16, 17). As shown in Fig. 2, the addition of any of the single histidine mutants to the low-pH cis compartment (first arrow) generally caused a lesser increase in the conductance than that of the WT T-domain (second arrow). The mutation of H322 resulted in the most dramatic effect, as the activities of both H322Q and H322K were almost undetectable (Fig. 2A–B). Indeed, the relative activities of these mutants were two orders of magnitude lower than that of the WT T-domain (Table 1). The effect of substituting H323 and H372 (Fig. 2C–F) was less pronounced, resulting in relative activities within a factor of four of that of the WT T-domain (Table 1). A closer look at the current-voltage dependence, even of the least active mutant H322Q (see Fig S1), reveals the characteristic blocking of the channels by the His-tag when a negative potential was applied (5). Thus, the loss of activity is likely to be associated with reduction in the number of channels rather than with changes in the properties of the individual channel. These results suggest that each of the three C-terminal histidines affects the formation of the OCS, but the role of H322 appears to be critical.

The refolding/insertion pathway of the T-domain is initiated by the acid-induced destabilization of the folded membrane-incompetent state and the formation of the membrane-competent state, a partially refolded intermediate capable of interaction with membranes (10). While the triple replacement of C-terminal histidines does not appear to affect the thermodynamic stability of the membrane-incompetent state (14), the effect on the stability of the membrane-competent state has not been explored. Here, we examine the stability of the WT and mutant proteins by measuring temperature-dependent changes in ellipticity associated with the helical structure using CD spectroscopy. The data collected at pH 8 and 6 (Fig. 3), corresponding to membrane-incompetent and membrane-competent states, respectively, indicate a very similar behavior for all proteins. Mild acidification results in similar reduction in transition temperature and enthalpy (Table S1). No

reproducible data, however, could be collected at lower pH as the protein aggregation in the denatured state renders thermodynamic analysis impossible. Nevertheless, it is clear that the reason for the loss of functional activity due to histidine replacements is not associated with changes in stability in solution, but is rather related to altered interactions with the membranes at some point along the insertion pathway.

Previously, we demonstrated that triple substitutions of C-terminal histidines do not prevent efficient insertion of the TH8-9 helical hairpin (14) (brown helices in Fig. 1A), suggesting that they affect proper insertion of the rest of the structure (grey helices in Fig. 1A). Here we follow up on this result using intrinsic fluorescence from W206 and W281, located in the N-terminal helices TH1 and TH5, respectively (shown in yellow in Fig. 1A). Representative examples of fluorescence spectra of the WT and H322Q mutant of the T-domain are shown as open symbols in Fig. 4. Although the spectra in the membrane-incompetent state are superimposable (panel A), there is a pronounced difference when WT and mutant interact with the membrane (panel B). In order to accurately measure spectral shifts, fluorescence spectra are fitted to a log-normal distribution (fits are shown as solid lines), as described in Methods. This procedure allows for accurate and reliable measurements of subtle changes in tryptophan spectral position (20).

Complementing our thermal stability CD results, the spectral position of the Trp fluorescence maximum in solution is not affected by the mutations at any pH value tested (e.g., compare H322Q and H322K with WT in Fig. 5A). On the other hand, the mutants show a distinct red-shift of emission in the presence of membranes, normally associated with increased exposure of fluorophores to the aqueous phase (Fig. 5B–D). These changes are detected at all lipid compositions that we tested, and appear to be exactly the same for 3:1 mixtures of anionic:neutral lipids, regardless of the substitution of PC with PE, or PG with PS (Fig. 5C). At lower anionic content, the same effect is observed, but the curves are shifted to more acidic pH (Fig. 5D). These changes are indicative of a membrane-induced misfolding of intermediate states on the path toward the OCS.

As illustrated in Fig 6A, this misfolding-associated red shift in Trp fluorescence occurs at pH 6.0–6.5 in all mutants, including those with substitutions in H323 and H372. The latter mutants regain the WT-like spectroscopic signature at pH 5.3 (blue and green symbols) and are relatively active in electrophysiological assay (Table 1). Mutants with replacements of H322 (red, orange and magenta symbols), however, do not show a WT-like spectral shift at lower pH and are inactive. It is possible that fluorescence spectra for these mutants will be blue shifted under more acidic conditions; however, this will not be physiologically relevant.

To corroborate that the inactive mutants adopted a misfolded conformation within the bilayer, we recorded the CD spectra of the WT T-domain, and the active and inactive mutants in the presence of LUV (POPG:POPC 3:1) at pH 5.3 (Fig. 6B). The CD spectra of the membrane-inserted state of the WT T-domain (black spectrum) and the active mutant H372Q (green spectrum) are identical and exhibit the characteristics of an α -helical structure, with two minima at 222 and 208 nm. In contrast, inactive mutants H322Q (red spectrum), H322,323Q (orange spectrum) and H322,323,372Q (magenta spectrum) showed CD spectra with loss of ellipticity and characteristic changes in shape, associated with the misfolding and aggregation of membrane proteins (22, 23), indicating that they do not fold into a WT-like structure. Previously we demonstrated that non-inserted intermediates of another protein that undergoes pH-dependent membrane insertion, annexin B12, are prone to aggregation on the interfaces (24, 25). The relatively active mutant, H323Q (blue spectrum), does not quite follow this pattern; it has a similar CD spectrum to that of the inactive mutant H322Q. Fluorescence data indicate that H323Q is strongly misfolded at the intermediate pH of 6.0, yet inserts like the WT at pH 5.3 (Fig. 6A). The likely reason why it cannot regain a

proper fold under the conditions of the CD experiment is that the latter requires much higher concentrations than those used in fluorescence or conductance measurements. Indeed, when the concentration of this mutant in the tryptophan fluorescence assay was increased, the spectrum no longer returned to the WT position (data not shown).

SUMMARY

We illustrate our findings in the scheme (Fig. 1B) summarizing membrane insertion of the WT T-domain (upper panel) and the mutants carrying substitutions of the C-terminal histidines (lower panel). Upon initial formation of the membrane-competent state and binding to the membrane, the process continues through the insertion of TH8-9 into the bilayer and the subsequent refolding of the rest of the protein until reaching the open-channel state (10). In the case of the WT protein, we propose that the C-terminal histidines are involved in guiding the conformation of the N-terminal region through productive folding intermediate states towards the OCS. There is no high resolution structure of the OCS available (or that of any membrane-associated intermediate); however, the electrophysiological data are consistent with helices TH8, TH9 and TH5 adopting a transmembrane conformation (5). When C-terminal histidines are replaced, the protein still undergoes a proper pH-dependent destabilization in solution (Fig. 3), binds to membranes (Supplement Fig. S2) and inserts a TH8-9 helical hairpin (14) similar to that of the WT (Fig. 1B, lower panel). Histidine replacement, however, leads to the formation of a non-productive intermediate which is characterized by greater exposure of W206 and W281 to the aqueous phase at pH values of ~6–6.5, manifesting itself in red-shifted fluorescence spectra (Figs. 5 and 6). The replacement of H322 appears to be particularly damaging, as the corresponding mutants tend to misfold on the membrane, dramatically reducing the number of properly folded and functional channels (Figs. 2 and S1). Interestingly, the replacement of H322 with the charged lysine or neutral glutamine has a similar effect on the folding pathway (Fig. 5B). [This is different from replacements of another critical residue, H257, involved in destabilization of the folded structure in solution (13). While replacement of H257 with a charged residue promoted the unfolding, replacement with a neutral residue slowed it down.] Thus, we propose that the C-terminal histidines play a crucial role in guiding the folding of the N-terminal region into the OCS, where the residue H322 would have particular importance that likely goes beyond its mere protonation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

T-domain	diphtheria toxin T-domain
WT	wild type (T-domain)
Trp	tryptophan
CD	circular dichroism
LUV	large unilamellar vesicles
POPC	palmitoylcholinephosphatidylcholine

POPG	palmitoyloleoylphosphatidylglycerol
POPE	palmitoyloleoylphosphatidylethanolamine
POPS	palmitoyloleoylphosphatidylserine
OCS	open-channel state

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Figure 1.

A) Crystal structure of the soluble form of diphtheria toxin T-domain at neutral pH (2). The C-terminal histidines H322, H323 and H372 are color coded. The insertion unit comprised of a helical hairpin TH8-9 is highlighted in brown. Tryptophan residues W206 and W281 are shown in yellow, and the rest of the protein is shown in grey. B) Model of the role of the C-terminal histidines in the refolding process of the T-domain within the bilayer. Top (WT T-domain): Upon initial destabilization of the WT T-domain and its association with the lipid bilayer, the N-terminal region of the protein adopts a conformation that leads to the insertion of the TH8-9 unit into the bilayer. The N-terminal region refolds to form the OCS. Bottom (mutants with C-terminal histidine replacements): Membrane interaction of these mutants results in a different conformation from that of the WT, specifically in the more exposed N-terminal part as revealed by a red-shifted fluorescence. While the insertion of TH8-9 is not compromised (14), replacement of C-terminal histidines, especially that of H322, affects efficient folding of the T-domain into the OCS (see text for details).

Figure 2.

Functional assay of H322, H323 and H372 mutants measured in planar bilayers through electrophysiology as previously described (16). Briefly, the transmembrane current (upper traces) was measured at ± 30 mV (lower traces) in the presence of 150 pM of the mutant T-domain in the cis compartment at pH 5.3 (first arrow) and followed by the addition of 150 pM of the WT T-domain (second arrow). The replacement of H322 by either glutamine or lysine (panels A–B) resulted in a dramatic loss of the ability to permeabilize the planar bilayer, whereas the substitution of H323 (panels C–D) and H372 (panels E–F) did not greatly affect the activity of the T-domain.

Figure 3.

Thermal stability of H322Q, H323Q and H372Q mutants at pH 8 and 6 measured by CD spectroscopy. The loss of secondary structure was followed at 222 nm upon increase in the temperature at pH 8 (solid symbols) or pH 6 (open symbols). Lines represent the fitting curves obtained as described in (14) and the values for enthalpies and transition temperatures are summarized in Supplemental Table S1. None of the replacements (color coded as in Fig 1) caused noticeable change in stability when compared to the WT protein (black) at either pH, confirming that the replacement of C-terminal histidines does not alter the acid destabilization process in solution.

Figure 4.

Tryptophan spectra of WT and H322Q mutants in the presence of LUV at pH 8 (A) and pH 5.3 (B). All spectra (open symbols) were corrected for background, normalized to maximal intensity for visual comparison of spectral changes and fitted to a log-normal function (lines) according Ladokhin et al. (20) to estimate the position of maximum.

Figure 5.

Membrane-induced misfolding of the T-domain mutants with replaced H322 detected by Trp fluorescence. A and B) Position of maxima of intrinsic fluorescence emission as a function of pH for WT T-domain and H322Q and H322K mutants in the absence of LUV (A), or in the presence of 75POPG/25POPC LUV (B). The presence of membranes induces the red-shift of the tryptophan emission spectrum at pH 6–6.5 for the mutants, whereas the further acidification induces the blue-shift of the spectra for the WT and mutant T-domain. The final state adopted by the mutants is different from that adopted by the WT T-domain. C and D) Position of maxima of intrinsic fluorescence emission as a function of pH for WT T-domain and the mutant H322Q in LUV composed of 75POPG/25POPC, 75POPG/25POPE and 75POPS/25POPC (C), and 25POPG/75POPC (D). The effect of replacing H322 on the emission spectra is maintained regardless of the composition of the LUV. Error bars represent the standard deviation of at least three independent measurements.

Figure 6.

Folding of the T-domain C-terminal histidine mutants followed by Trp fluorescence (A) and CD (B). A) Wavelength of maximum tryptophan emission represented as a function of the pH for active and inactive mutants in the presence of LUV. All of the mutants show red-shifted spectra at pH 6–6.5. At pH 5.3, the spectra are blue-shifted for the active mutants (black box), but not in the case of the inactive mutants (red box). (The spectra of the double and triple mutants are even red-shifted relative to the pH 8 spectra). The error bars represent the standard deviation of three independent measurements. B) CD spectra of WT T-domain for representative active and inactive mutants at pH 5.3 in the presence of LUV. The inactive mutants show atypical CD spectral shape and a decrease in ellipticity.

TABLE 1

Relative activity of the C-terminal histidine mutants of the diphtheria toxin T-domain. The values are calculated relative to the total increase of conductance of the WT. For the single replacements, the values correspond to the average and standard deviation of 3 independent assays.

T-DOMAIN MUTANT	RELATIVE ACTIVITY
WT	1
H322K	0.028 ± 0.004
H322Q	0.011 ± 0.005
H323K	0.38 ± 0.06
H323Q	0.26 ± 0.04
H372K	0.83 ± 0.09
H372Q	0.41 ± 0.04
H322,323Q	0.007
H322,323,372Q	0.002