Membrane Protein Insertion Regulated by Bringing Electrostatic and Hydrophobic Interactions into Play

A CASE STUDY WITH THE TRANSLOCATION DOMAIN OF THE DIPHTHERIA TOXIN*

Received for publication, April 29, 2002, and in revised form, July 30, 2002
Published, JBC Papers in Press, August 21, 2002, DOI 10.1074/jbc.M204148200

Alexandre Chenal‡§, Philippe Savarin‡¶, Philippe Nizard‡§, Florent Guillaum‖, Daniel Gillet‡***, and Vincent Forge‡‡

From the ‡Département d’Ingenierie et d’Etudes des Protéines, CEA-Saclay, 91191 Gif sur Yvette cedex and the ¶Biophysique Moléculaire et Cellulaire, Unité Mixte de Recherche 5090, Département Réponse et Dynamique Cellulaires, Commissariat à l’Energie Atomique-Grenoble, 17 rue des Martyrs, 38054 Grenoble, cedex 9, France

The study of the membrane insertion of the translocation domain of diphtheria toxin deepens our insight into the interactions between proteins and membranes. During cell intoxication, this domain undergoes a change from a soluble and folded state at alkaline pH to a functional membrane-inserted state at acid pH. We found that hydrophobic and electrostatic interactions occur in a sequential manner between the domain and the membrane during the insertion. The first step involves hydrophobic interactions by the C-terminal region. This is because of the pH-induced formation of a molten globule specialized for binding to the membrane. Accumulation of this molten globule follows a precise molecular mechanism adapted to the toxin function. The second step, as the pH decreases, leads to the functional inserted state. It arises from the changes in the balance of electrostatic attractions and repulsions between the N-terminal part and the membrane. Our study shows how the structural changes and the interaction with membranes of the translocation domain are finely tuned by pH changes to take advantage of the cellular uptake system.

Folding and insertion of membrane proteins (1, 2), binding of hormones to membrane receptors (3), action of antibiotic peptides (4, 5), protein translocation, and internalization of toxins (6) are examples of phenomena that require the interactions of polypeptide chains with membranes. Because of the anisotropic nature of membranes, the initial steps of the association and the final structure and localization of polypeptide chains within membranes depend on a combination of hydrophobic and electrostatic interactions (7). Hydrophobic interactions are dominant for the insertion of transmembrane polypeptides. Electrostatic interactions are important for the binding of antibiotic peptides (5, 8), the association of proteins with the surface of the membrane (9–11), and as determinants of the topology of integral membrane proteins after biosynthesis (12). In most cases, electrostatic interactions are the result of the attraction between anionic phospholipid head groups and basic amino acid side chains (13, 14). However, there are exceptions where electrostatic repulsions are involved in the membrane association of peptides, particularly when their structure and localization within the membrane is regulated by the pH (15–19). The interplay of hydrophobicity and electrostatics and their distribution within the polypeptide sequence have only been studied in detail for small peptides (3–5, 7, 13–15, 17–20). In the case of proteins, the role of these effects on the association with and the insertion into membranes is still poorly understood (2, 21–24).

The study of the membrane insertion process of the translocation (T) domain of diphtheria toxin (25) can provide precious insight into the interactions between proteins and membranes and the refolding mechanisms of membrane proteins. During intoxication of cells (25), the toxin reaches the early endosomes through the clathrin-coated pathway (26). Because of the acid pH found in this compartment, the T domain changes from a soluble state with a stable tertiary structure to a functional membrane-inserted state (27–34) and helps the catalytic domain to reach the cytosol. In its soluble form (Fig. 1), the T domain (22 kDa) is structured in a bundle of nine α-helices organized in three layers (35, 36). A central hydrophobic helical hairpin made of helices TH8 and TH9 is hidden from the solvent by two amphiphilic layers made of the TH1-TH4 and TH5-TH7 groups of α-helices. Such topology is found for other membrane-penetrating proteins such as the pore-forming domain of colicins (37) and the pro- or anti-apoptotic proteins belonging to the Bcl-2 family (38). The N-terminal region (TH1-TH4) contains many negative and positive residues at neutral pH.

We have found that first hydrophobic, and then electrostatic, interactions are involved during the insertion of the T domain in a membrane. On the basis of our results we propose a model that describes how these two kinds of interactions are controlled by pH and involve distinct regions of the domain. In a first step, hydrophobic interactions by the C-terminal TH8-TH9 helices are allowed by the pH-induced partial unfolding of the soluble form. In a second step, further acidification changes

* This work was supported in part by the Commissariat à l’Energie Atomique, the CNRS, the Ligue Nationale Contre le Cancer, and Association pour la Recherche Contre le Cancer Grant 5876. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by the Ministère de l’Education Nationale, de la Recherche et de la Technologie.
¶ Supported by the Commissariat à l’Energie Atomique.
†† To whom correspondence may be addressed. Tel.: 33-1-69-08-76-46; Fax: 33-1-69-08-94-30; E-mail: daniel.gillet@cea.fr.
‡‡ To whom correspondence may be addressed. Tel.: 33-4-38-75-94-05; Fax: 33-4-38-75-94-87; E-mail: forge@dsvuad.cea.fr.

1 The abbreviations used are: T, translocation; EPA, egg phosphatidic acid; EPC, egg phosphatidyl choline; FRET, fluorescence resonance energy transfer; λmax, maximum emission wavelength; L/P, lipid/protein; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; NB-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt; Dansyl-DHPE, N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt.
the balance of electrostatic attractions and repulsions between the N-terminal part of the domain and the membrane surface, leading to increased penetration into the membrane. Our study shows how the structural changes and the interaction with membranes of the T domain are finely tuned by pH changes to take advantage of the cellular uptake system.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—The recombinant T domain has been described previously (39, 40). Cys-201 (native diphtheria toxin numbering) has been mutated to Ser. The protein was further purified on a 1-ml HiTrap™ Q-Sepharose™ HP; Amersham Biosciences).

**Lipid Vesicles**—Anionic lipid vesicles were prepared in 10 mM sodium citrate buffer (pH 7.8) from egg phosphatidyl choline (EPC) and egg phosphatidic acid (EPA) (Avanti Polar Lipids, Alabaster, AL) at a 9:1 molar ratio by reverse phase evaporation (41, 42) to obtain large unilamellar vesicles (LUV) or by sonication to obtain small unilamellar vesicles (SUV).

**Experimental Buffers**—The protein was kept in 20 mM phosphate buffer and was diluted in a range of 5 mM citrate buffers of various pH before spectroscopic measurements. The pH of the diluted protein was checked afterward. All experiments were done at 22 °C.

**CD Spectroscopy**—CD experiments were performed on a CD6 spectrodichrograph (Jobin-Yvon Instruments, Longjumeau, France) as described previously (40). The scans were recorded using a bandwidth of 2 nm and an integration time of 1 s as a scan rate of 0.5 nm s⁻¹. The spectra were corrected for the blank, and a smoothing algorithm was then used with the minimum filter in the CD6 software (CDMax, filter 5).

**Fluorescence Spectroscopy**—Fluorescence measurements were performed with an FP-750 spectrophotometer (Jasco, Tokyo, Japan) in a thermostated cell holder, using a 1-cm path length quartz cell as described previously (40). A bandwidth of 5 nm was used for both excitation and emission beams. The excitation wavelength was fixed at 295 nm where the contribution of Tyr is negligible. The emission spectra were recorded from 300 to 500 nm at a scan rate of 60 nm/min⁻¹. Maximal emission wavelength (λ_max) represents the average of five values obtained from emission spectra that were corrected for blank measurements.

**Binding Curves**—The various pH dependence monitored in this study were fitted with the following binding curve equation: 

\[ S = S_i + (S_f - S_i)\frac{1}{1 + (K/H)^n}, \]

where \( S \) is the signal used to monitor the reaction, \( S_i \) and \( S_f \) are the initial and final values, respectively, \( H \) is the proton concentration, \( H = 10^{-pH} \), \( K \) is the dissociation constant, and \( n \) is the Hill coefficient. When intrinsic fluorescence was used, we chose to show \( \lambda_{\text{max}} \) as a function of pH, because it provides direct information about the Trp environment. Unlike the fluorescence intensity at a fixed wavelength, \( \lambda_{\text{max}} \) is not an extensive variable. To be exact one should not use \( \lambda_{\text{max}} \) to be fitted by a binding curve. In the case of intrinsic fluorescence of proteins, however, it can provide reliable thermodynamic parameters of a reaction (43). This is confirmed by our study where the pH dependence of \( \lambda_{\text{max}} \) was systematically compared with a different kind of data (44).

**RESULTS**

**Mechanisms of the pH-induced Conformational Change of the T Domain**—The far-UV CD spectrum of the T domain (Fig. 2A) indicates a substantial α-helical content, consistent with the structure deduced from x-ray crystallography (35, 36). The amount of secondary structure does not depend on pH (Fig. 2A).

The near-UV CD spectrum of T at neutral pH, i.e. in the S-state, displays a large signal at 292 nm, which vanishes at acid pH (Fig. 2B). This signal can be attributed to Trps stabilized in a rigid chiral environment, and it reveals their embedding in tertiary structure at neutral pH (44). Therefore, CD spectra show that at acid pH, i.e. in the A-state, the tertiary structure is lost whereas the secondary structure remains native-like. 1-Aminonaphthalene-8-sulfonic acid (ANS) binding experiments (data not shown) also indicate that hydrophobic surfaces become exposed to solvent, in agreement with earlier work (28, 45). Moreover, the one-dimensional NMR spectrum of T at acidic pH shows a drastic diminution of the chemical shift dispersion, together with broader peaks, as compared with the spectrum in the native state (data not shown). Such an NMR spectrum indicates that important conformational exchanges occur in the A-state on the millisecond time scale (46–48). Overall, the structural characteristics of the A-state are those commonly accepted for the molten globule state, which is observed in the folding reaction of many proteins (49).

The conformational change induced by acid pH is fully reversible, because the peak at 292 nm detected by CD in the near-UV at neutral pH and lost at acid pH is recovered after neutralization of the acid sample (Fig. 2B). It is not possible to monitor the tertiary structure of T as a function of pH by near-UV CD because of aggregation of T around pH 5.5 at the protein concentrations needed for CD experiments (40). How-
Membrane Insertion of the Diphtheria Toxin T Domain

5.5, and a Hill coefficient, \(n\), observed rate constant (\(k_{\text{obs}}\)) strongly increases from 0.7 s\(^{-1}\) at pH 4.85 to 44.4 s\(^{-1}\) at pH 2.8 (Fig. 4) and seems to reach a plateau for pH values lower than 3 (Fig. 4, inset). It is difficult to obtain experimental \(k_{\text{obs}}\) values for pH values lower than 2.8, because T becomes unstable. When the experiment is conducted in the reverse direction, i.e. starting from T prepared at pH 4.6 and then mixed with a buffer to reach a final pH of 7.8, the reaction is slower with \(k_{\text{obs}} = 0.14\) s\(^{-1}\). The pH dependence of the rate constant indicates that a binding of protons is associated with the rate-limiting step of the conformational change (50–53). A simple way to describe the pH dependence of \(k_{\text{obs}}\) is to consider that the proton binding is a fast event that precedes the conformational change. In that case \(k_{\text{obs}}\) is controlled by this event and depends on the proportion of the protonated species. The final conditions used for the kinetic measurements are highly favorable to the A-state. Indeed, in these experiments the final pH is lower than 4.8, and according to the equilibrium experiments (Fig. 3), a large majority of the protein is in the A-state under these conditions. Therefore, as an approximation, we can consider the reaction as a one-way reaction. Then, the pH dependence of \(k_{\text{obs}}\) can be written in terms of binding constants as follows: \(k_{\text{obs}} = k_0/1 + 10^{-n_H(pK_{\text{K}} - \text{pH})}\), where \(k_0\) is the rate of the conformational change of the protonated S-state, \(pK_{\text{K}}\) is the dissociation constant of the proton binding to the S-state, and \(n_H\) reflects the cooperativity of the binding. The pH dependence of \(k_{\text{obs}}\) can be described with the following parameters: \(pK_{\text{K}} = 3.6, n_H = 1.4\), and \(k_0 = 48\) s\(^{-1}\) (± 1 s\(^{-1}\)) (Fig. 4). The \(pK\) and the cooperativity of the proton binding associated with the rate-limiting step of the conformational change are lower than those obtained at equilibrium, \(pK_{\text{Eq}} = 5.5\), and \(n_H = 2.8\). Thus, an additional binding of protons must occur after the rate-limiting step monitored in the kinetics. This additional binding must be of high affinity to increase the apparent \(pK\) and \(n_H\) of the transition at equilibrium in contrast to those of the binding.

![Figure 2](image1.png)

**Fig. 2.** Effect of pH on the CD spectra of the T domain. Shown are far-UV (A; 5 μM) and near-UV (B; 30 μM) CD spectra of the T domain subjected successively to neutral (pH 7; black), acid (pH 4; red), and neutralized (pH 7; blue) pH.

![Figure 3](image2.png)

**Fig. 3.** Fluorescence changes of the T domain as a function of pH. λ\(_{\text{max}}\) of Trp fluorescence of the T domain (1 μM) as a function of pH (open circles) and after neutralization (open squares) are shown. The continuous line is the best fit of a binding curve to the data.

![Figure 4](image3.png)

**Fig. 4.** Observed rate constants (\(k_{\text{obs}}\)) for fluorescence changes induced by various pH jumps (open squares). The continuous line is the best fit to the data using the equation given in the text. Inset, as shown in logarithmic scale, the observed rate constants reach a plateau.
Membrane Insertion of the Diphtheria Toxin T Domain

that control the rate of the conformational change. According to the two different \( n_H \) values obtained from kinetic and steady-state experiments, the binding of at least two protons is associated with the rate-limiting step of the conformational change (\( n_H = 1.4 \)), whereas at least three protons are involved in the whole conformational change (\( n_H = 2.8 \)). For the sake of simplicity in the model proposed below to interpret our results, we show the binding of only two and one-third protons for each step, respectively. Scheme 1 is the minimal scheme to describe both the steady-state and kinetic experiments.

![Scheme 1](image)

The sequential binding of two protons to the S-state accounts for the cooperative binding that controls the rate of the conformational change (S- to A-state). \( K_{1a} \) and \( K_{1b} \) can be calculated from the parameters of the pH dependence of \( K_{3a} \), as follows: \( K_{1a} = K_K/(4/n_H - 2) \) and \( K_{1b} = K_K \times (4/n_H - 2) \). The values we obtain with \( pK_{1a} = 3.6 \) and \( n_H = 1.4 \) are \( pK_{1b} = 3.55 \) and \( pK_{1b} = 3.7 \). These two initial protonations induce a conformational change that, in turn, exposes to the solvent at least one additional amino acid, now open to protonation. The binding of the third proton determines the dissociation constant and the \( n_H \) observed in the steady-state experiment. S, SH, and SH₂ (the S-state with various amount of bound protons in Scheme 1) have the same fluorescence characteristics, i.e. \( \lambda_{max} = 336 \) nm. SH₂ spontaneously changes its conformation in AH₂ (the A-state with two bound protons), and this rate-limiting equilibrium \( (K_2 = \text{SH}₂/\text{AH}₂ = K_{\text{alkaline}}/K_{\text{acid}}) \) favors the AH₂ species, which could be stabilized by a further protonation in AH₃. Within AH₂ and AH₃, the Trps are more exposed to solvent, i.e. \( \lambda_{max} = 342 \) nm. In the rate-limiting equilibrium, \( K_{\text{acid}} \) is determined from the pH dependence of \( K_{\text{alkaline}}, K_{\text{acid}} = k_{\text{0}} = 48 \) s⁻¹. The rate of the conformational change induced by a pH jump from 4.6 to 7.5, which is highly favorable to the S-state, can be used as an approximation for \( k_{\text{alkaline}}/K_{\text{alkaline}} \sim 0.14 \) s⁻¹. Then, \( K_{3} \) can be estimated, \( pK_{3} \approx 2.5 \), and \( K_{3} \) can be calculated from these values and the parameters of the steady-state experiments (\( pK_{3} = 5.5 \)).

Interactions of the T Domain with Membranes—The two Trps of the T domain are located in the TH1 and TH5 helices. When Trp fluorescence is used to monitor the pH-induced conformational changes of the T domain in the presence of anionic SUV made of a mixture of neutral and negative phospholipids (EPC and EPA 9/1, respectively), two steps are detected (Fig. 5A). The first step occurs between pH 7 and pH 6. The shift of \( \lambda_{max} \) from 336 to 344 nm indicates that the Trps become more accessible to the solvent. During the second step, between pH 6 and pH 4, the \( \lambda_{max} \) moves from 344 nm at pH 6 to 333 nm at pH 4. This shows that the Trps penetrate a hydrophobic environment. A similar pattern for the pH dependence of \( \lambda_{max} \) is obtained when the experiment is performed with LUV instead of SUV (data not shown). After the first step, the secondary structure of the T domain is preserved. Indeed, the far-UV CD spectra recorded in the presence of anionic SUV at pH 6 and pH 7 are identical (Fig. 6A). During the second step, however, a small increase of the helicity (Fig. 6B) and the burying of the Trps in a hydrophobic environment (Fig. 5A) are concomitant.

The two steps can be monitored separately either by measuring the partition between the membrane-bound domain and the free domain or by measuring the FRET between dansyl (a fluorescent probe linked to phospholipid head groups) and Trps (Fig. 5B). Partition measurements indicate that the domain binds to the membrane during the first step (between pH 7 and pH 6). The establishment of the FRET between the dansyl and Trps occurs in the second step. No conclusion can be drawn from the FRET data about the location of the Trps within the membrane. The distance between Trps and dansyl molecules is not the only factor that determines the level of FRET. It also depends on the \( \lambda_{max} \) and the intensity of the Trp fluorescence, two factors which vary. The second step is also related to a permeabilization of the membrane (between pH 6 and pH 4). Indeed, a fluorescent probe, trapped previously in the liposomes, is released and quenched for pH values lower than 6 (Fig. 5C). The permeabilization appears in the same range of pH when SUV or LUV are used.

Both transitions are determined by the binding of protons. The best fit of a binding curve to the partition data gives \( pK = 6.6 \) and \( n_H = 3.3 \) (Fig. 5B). The high cooperativity of the first
transition suggests that the formation of the A-state, described previously in the absence of membrane, is a prerequisite for binding to the membrane. The pH dependence of the first step is shifted toward more alkaline pH values in the presence of membranes as compared with the pH dependence of the formation of the A-state in the absence of membrane. This could be explained by the interaction with the membrane, which stabilizes the partially folded state. The best fit to the FRET data, which is related to the second step of the interaction, gives $pK_a = 5.7$ and $n_{H} = 1.3$. To estimate $\lambda_{\text{max}}$ after the first step, the pH dependence of $\lambda_{\text{max}}$ is fitted for each of the two parts of the binding curve in Fig. 5A. The parameters of proton binding for the first step are $pK = 6.4$ and $n_{H} = 2.7$, and those for the second step are $pK = 5.75$ and $n_{H} = 1.1$. These values of the binding parameters are similar to those obtained from the partition and FRET data. This fit provides an estimation of $\lambda_{\text{max}}$ after the first transition of 350 nm. It shows that, after the first step, the environment of the Trps is more polar than in the A-state in solution ($\lambda_{\text{max}} = 342$ nm).

When neutral membranes made of zwitterionic phospholipids (EPC only) are used, binding to the membrane and partial unfolding are detected, but the second step associated with the burying of the Trps in a hydrophobic environment (Fig. 5A) is not. The best fit to a binding curve gives $pK = 7.0$ and $n_{H} = 1.9$. The $\lambda_{\text{max}}$ of the Trp fluorescence after binding is around 342 nm, similar to that found for the A-state. Therefore, after the initial binding, the environment of the Trps is less polar with neutral membranes as compared with anionic ones ($\lambda_{\text{max}} = 350$ nm). In the presence of neutral membranes, the second step does not occur (Fig. 5A). In this case, a weak permeabilization of the membrane can be detected only for small values of $L/P$ ($L/P = 75$) but not when values of $L/P$ are similar to those leading to permeabilization with anionic membranes, i.e. $L/P = 3000$ (Fig. 5C). These results show that electrostatic interactions between the T domain and acid phospholipids are essential for the second step, i.e. the membrane permeabilization. As a confirmation, the addition of 0.1 m NaCl inhibits partially the second transition observed in the presence of anionic membranes, whereas the first step remains unchanged (Fig. 7). One can notice that in this experiment the pH dependence is slightly shifted by comparison with the previous one (Fig. 5A). This is probably because of the use of a different batch of phospholipids for this last experiment. All of the previous experiments were made with the same batch of phospholipids but various preparations of T domain.

**DISCUSSION**

The formation at acid pH of the A-state of the T domain that is able to interact with membranes depends on the cooperative binding of at least three protons. The binding of at least two protons to the S-state is a prerequisite to the conformational change leading to the A-state. The $pK$s of these two protons suggest that either glutamates or asparagines are involved. In solution, the binding of at least a third proton (probably on a histidine) stabilizes the A-state. Then, two steps can be distinguished in the interaction between the T domain and a membrane, first a binding step and then a structural reorganization associated with a permeabilization of the membrane.

The high cooperativity of the pH dependence of the first step of the interaction suggests that the binding of the T domain to the membrane is controlled by the formation of the A-state. In this state, the hydrophobic regions of the protein are more accessible to the solvent. Most likely, the hydrophobic TH8 and TH9 $\alpha$-helix hairpin becomes available for insertion in the membrane, as it was shown to penetrate the bilayer (27, 29, 32). Once bound to the membrane, the structure of the domain depends on the charge of the membrane surface. After the first step of the interaction (around pH 6) (Fig. 5A), Trps are in a more polar environment when the membrane is negatively charged than when the membrane is neutral. In addition, on neutral membranes the interaction cannot proceed to the second step observed at pH < 6 on negative membranes. Both Trps being located in the N-terminal part of the domain (on TH1 and TH5), this difference is probably because of electrostatic interactions between anionic phospholipids and charged residues also located in this region. The $\alpha$-helices of T are preserved (Fig. 6). This suggests that the $\alpha$-helices of the N-terminal part of the T domain are lying on the membrane surface. Furthermore, in the absence of interaction with other
α-helices, it is likely that the TH8 and TH9 hydrophobic α-helix hairpin is inserted in the hydrophobic core of the membrane (27, 29, 32). Likewise, in the presence of neutral membranes, without the possibility of electrostatic interactions between the membrane surface and the N-terminal part of the domain, TH8 and TH9 are likely to be responsible for the binding through hydrophobic interactions. In this case the conformational change due to the interaction with the membrane occurs for higher pH values than previously, and the apparent cooperativity of the binding is lower (Fig. 5A). Probably, states other than AH₂ are also able to bind to neutral membranes. This suggests that, in the case of anionic membranes, electrostatic repulsions between anionic phospholipids and some of the acid residues that are protonated during the transition from the S- to the A-state, are involved in the pH regulation of the binding to the membrane. Acid residues located in the loop linking TH8 to TH9 are good candidates for this regulation. There is much evidence indicating that residues Glu-349 and Asp-352 are responsible for the pH dependence of the insertion of TH8 and TH9 in the membrane (55–57). Nevertheless, it has been reported that other residues may also contribute to the pH-dependent membrane insertion of TH8–TH9 (34).

The need for electrostatic interactions suggests that the highly charged N terminus of the T domain, TH1-TH4, plays a key role in the second step of the interaction, which is related to the permeabilization of the membrane. The need for electrostatic interactions and the pH dependence of the second step are difficult to explain if we assume that the N-terminal part of the domain adopts a stable transmembrane conformation. Likely, TH1-TH4 remains lying on the membrane surface with basic residues in interaction with anionic phospholipids. The behavior of the T domain during the membrane permeabilization shows some similarities with that of antibiotic (4, 58) and toxin peptides (59). They are made of an amphiphilic helix that binds to anionic phospholipids. These peptides form transient pores made of a peptide-lipid supramolecular complex, and upon membrane permeabilization a fraction of the peptide is translocated (20, 58). Electrostatic interactions between anionic phospholipids and basic residues are of prime importance in this phenomenon (20). One can imagine such behavior for the N-terminal part of the T domain. It would explain the membrane permeabilization and the central role played by electrostatic interactions in this activity. Moreover, structural studies have shown that the TH1-TH4 region is translocated in relation to channel formation (33), as described for peptides (20, 58). The weak permeabilization of neutral membranes can be attributed to residual binding of the N-terminal part to the membrane surface. A similar behavior has been described for peptides forming transient pores (59). The membrane permeabilization becomes weaker, and the lipid/protein ratio needed decreases when the amount of acid phospholipids in the membrane decreases (59), in a similar way as we observed for the T domain in the presence of neutral membranes.

The pH dependence of the interaction of the N-terminal part of the domain with the membrane can be illustrated with the α-helix TH1 (Fig. 8). Its structure is quite peculiar with a hydrophobic face separated from a negatively charged one by two positively charged bands (27). After binding to the membrane around pH 6, it is likely that both basic and acid residues carry electric charges. Therefore, the electrostatic interactions are a combination of attractions and repulsions between residues and anionic phospholipid head groups. As a consequence, the N-terminal part of the domain lies on the membrane surface within the interface area (Fig. 8). At this stage of the membrane interaction, the repulsive component is strong enough to keep the hydrophobic face of TH1, where one of the Trp is located, and the hydrophobic face of TH5, which contains the second Trp, away from the hydrophobic core of the membrane. Within the pH range of the second transition, the acid phospholipids (EPA) are still negatively charged (pKₐ = 2.9), and the basic residues remain positively charged, whereas the acid residues may lose their negative charge by protonation of their side chain. Considering that the local pH within the anionic membrane interface can be more acid than the bulk pH by up to two orders of magnitude (60, 61), the apparent pK = 5.7 for the second step is in agreement with the protonation of acid residues (pKₐ ~ 3.9–4.2). After the electrostatic repulsion has vanished, the interaction between the N-terminal part and the membrane depends on the electrostatic attraction and hydrophobic interaction. As a consequence, the localization of the N-terminal part within the membrane interface changes. The hydrophobic regions can contact the hydrophobic core of the membrane. The basic side chains can interact with anionic lipids. In the case of TH1, the positively charged bands running along this helix should be in tight interaction with anionic phospholipid head groups. At the same time, the hy-
Membrane Insertion of the Diphtheria Toxin T Domain

drophobic face, where one of the Trps is located, should be in contact with the hydrophobic phase of the membrane (Fig. 8), as shown by the blue shift of $\lambda_{\text{max}}$ (Fig. 5A). Similar changes in the position of a helix at the membrane interface, with changes in the balance between electrostatic and hydrophobic interactions, have already been described for peptides (14, 17). At this stage, the N-terminal part could be translocated to equilibrate its distribution on both sides of the membrane. On the basis of the observations made on antibiotic and toxin peptides (20, 58), we propose that membrane permeabilization results from this translocation. This can be related to the function of the T domain within the diphtheria toxin, which is to translocate the catalytic domain, bound to the N terminus of TH1 by a disulphide bridge (25). The translocation of the TH1-TH4 part is probably necessary for the translocation of the catalytic domain (62).

The translocation of the catalytic domain is sensitive to a transmembrane pH gradient between the endosome and the cytosol (63, 64). One can predict the effect of such transmembrane pH gradient with a simple model, which takes into account a pH-dependent translocation of the TH1-TH4 part of the T domain. Scheme 2 describes this model.

$K_H$ is the dissociation constant of the protonation of acid side chains. For the sake of simplicity we assume that $K_H$ is the same on both sides of the membrane, $K_{Hc} = K_{Ht} = K_H$. $T_c \times H_c / T_c H_c$ and $T_t$ are the proton concentrations on the cis- and trans-sides, respectively. $K_{MB}$ is the equilibrium constant for the distribution of the N terminus on the cis ($T_c$) and trans ($T_t$) sides of the membrane. We assume that, when protonated, the N terminus is equally distributed between the two faces of the membrane, $K_{MB} = T_t / T_c$. Inside the endosome, when the catalytic domain is translocated, the pH is $\sim 5.5–6$ (65). With this value for the pH on the cis-side we can calculate the proportion of N terminus on the cytosolic side as a function of the pH on that side of the membrane, i.e. the trans-side (Fig. 9). Thus, in the absence of a pH gradient, the TH1-TH4 part is equally distributed on both sides of the membrane. However, in the presence of a pH gradient, the TH1-TH4 part remains trapped on the alkaline side of the membrane, because acid side chains are negatively charged. When the pH on the trans-side is equal to the pH generally found in the cytosol (pH 7.2), a large majority of the N terminus of the T domain would be on the trans-side (Fig. 9). Thus, the yield of translocation of the catalytic domain, together with the N terminus part of the T domain, is enhanced with a pH gradient. The predictions based on our model are in agreement with measurements made on purified endosomes (64). With a cis pH of 5.5, the largest effect of a transmembrane pH gradient on the translocation yield is observed when the trans pH is between 6 and 7.

Our study provides a new insight into the mechanism by which the pH, the composition of the membrane, and a transmembrane pH gradient can regulate the activity of the diphtheria toxin T domain. This allows the toxin to take advantage of the cellular uptake system with high efficiency and specificity. The set of experiments and data presented herein will be used as a basis for further characterization of the interplay between the various interactions involved in protein-membrane association and particularly to unveil how the primary sequence codes for the interaction of proteins with membranes.

Acknowledgments—We thank A. Urvoas for help with the spectrophotometer and E. Mintz and Y. Gaudin for careful reading of the manuscript.
Membrane Insertion of the Diphtheria Toxin T Domain

48. Forge, V., Wijesinha, R. T., Baibach, J., Brew, K., Robinson, C. V., Redfield, C., and Dobson, C. M. (1999) *J. Mol. Biol.* **288**, 673–688
49. Arai, M., and Kuwajima, K. (2000) *Adv. Protein Chem.* **53**, 209–282
50. Kuwajima, K., Mitani, M., and Sugai, S. (1989) *J. Mol. Biol.* **206**, 547–561
51. Jamin, M., Geierstanger, B., and Baldwin, R. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6127–6131
52. Guillain, F., Gingold, M. P., Buschlen, S., and Champeil, P. (1980) *J. Biol. Chem.* **255**, 2072–2076
53. Forge, V., Mintz, E., and Guillain, F. (1993) *J. Biol. Chem.* **268**, 10961–10968
54. Falnes, P. O., Madshus, I. H., Sandvig, K., and Olsnes, S. (1992) *J. Biol. Chem.* **267**, 12284–12290
55. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1996) *Biochemistry* **35**, 11361–11368
56. Matsuzaki, K., Yoneyama, S., Murase, O., and Miyajima, K. (1996) *Biochemistry* **35**, 8450–8456
57. Oh, K. J., Senzel, L., Collier, R. J., and Finkelstein, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8467–8470
58. Beaumelle, B., Bensammar, L., and Bienvenue, A. (1992) *J. Biol. Chem.* **267**, 11525–11531
59. Clague, M. J. (1998) *Biochem. J.* **336**, 271–282
60. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J. Mol. Graph.* **14**, 51–55
Membrane Protein Insertion Regulated by Bringing Electrostatic and Hydrophobic Interactions into Play: A CASE STUDY WITH THE TRANSLOCATION DOMAIN OF THE DIPHTHERIA TOXIN
Alexandre Chenal, Philippe Savarin, Philippe Nizard, Florent Guillain, Daniel Gillet and Vincent Forge

J. Biol. Chem. 2002, 277:43425-43432.
doi: 10.1074/jbc.M204148200 originally published online August 21, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204148200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 66 references, 19 of which can be accessed free at http://www.jbc.org/content/277/45/43425.full.html#ref-list-1