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# pH DEPENDENT INSERTION OF A DIPHTHERIA TOXIN B FRAGMENT PEPTIDE INTO THE LIPID MEMBRANE : A CONFORMATIONAL ANALYSIS

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A peptide of diphtheria toxin B fragment (residues 147-266) has been shown to induce pore formation in lipid bilayer membranes at low pH. Such an effect was obtained at a much lower extent or not at all at pH=7. The region localized between residues 225 and 246 is highly hydrophobic (27.3% polarity) and characterized by a high concentration of proline residues. Since proline cis-trans isomerization is highly sensitive to the pH of the medium, we investigated the capability of the cis and trans isomers to penetrate into the lipid matrix. Obviously, the cis-trans isomerization of proline 242 and 245, assumed to be imposed by a low pH, uncovers the hydrophobic region and induces its insertion into a lipid layer of dipalmitoylphosphatidylcholine. The lipid matrix destabilization resulting from this process could promote the penetration into the lipid bilayer of an amphipatic structure (153-178) similar to the transverse lipid associating domains of membrane proteins. 

Diphtheria toxin (DT) is a bacterial toxin constituted by two fragments, A and B, linked by a disulfide bridge. The A fragment of DT (DTA) inhibits proteins synthesis in eukaryotic cells by ADP ribosylation of elongation factor 2 (1). DT B fragment (DTB) promotes the binding of toxin to specific cell surface receptors and the entry of DTA into the cytosol (2). Even if the molecular mechanism of this passage remains largely unknown, strong evidence now suggests that low pH is an essential requirement for the passage of the toxin into the cytoplasm (3,4). Using 1-anilino-naphtalene-8-sulfonic acid, we recently localized this pH sensitive region in CB1 (unpublished

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results), a cyanogen bromide peptide of DTB (5) . As DT, this peptide forms transmembrane channels in planar lipid bilayers at low pH (6), indicating that at least one region of CB1 is capable of inserting into the lipid bilayer. A fragment and the other cyanogen bromide peptides of B fragment did not modify the conductance of the lipid bilayer (6). From the structural analysis of the CB1 amino acid sequence(7), we identified two regions that could be involved in the insertion process:

(i) a segment localized between residues 153 and 178. Its helical net is characterized by an amphipatic structure with a large apolar face (8) similar to the transverse lipid associating domains of membrane proteins;

(ii) an hydrophobic segment of 22 residues (225 to 246) similar to membrane-penetrating segments (MPS), of which "signal" sequences are typical examples (9)(Ile-Lys-Ile-Thr-Ala-Glu-Asn-Thr-Pro-Leu-Pro-Ile-Ala-Gly-Val-Leu-Leu-Pro-Thr-Ile-Pro-Gly).

Using a conformational analysis procedure based on computer assisted modeling, we investigated the capability of the hydrophobic 22 residues segment of CB1 to insert into a lipid monolayer at neutral and acid pH. The conformational analysis procedure gives molecular information about the mode of assemblage of amphiphilic membrane components and about the interaction energy between them. It is has been applied mostly to amphiphilic drugs and lipids (10-14) but more recently to describe the orientation of Gramicidin A into a lysopalmitoyl-phosphatidylcholine monolayer (15).

## METHODS

The mean hydrophobic moment $\langle \mu H \rangle$ and mean hydrophobicity $\langle Hi \rangle$ were calculated according to the procedure of Eisenberg et al. (16,17). A segment of 7 amino acids was moved through the protein sequence and the mean hydrophobicity and mean hydrophobic moment per segment were calculated. These 2 parameters are plotted as a function of the mid-point of the amino acid

segment along the sequence. In our calculations, we used the normalized hydrophobicity scale of Argos (18) as it is especially suitable for membrane-related proteins. Space filling structures of MPS were obtained from the prediction made by Falmagne et al.(7) using the Chou-Fasman (19) method and giving to the torsional angles $\phi$ and $\psi$ the values classically associated to each amino acid in $\alpha$ helices, $\beta$ sheets and $\beta$ - turn structures. To mimic the effect of the pH, we impose to the proline residues a "trans" conformation at pH 7.0 and a "cis" conformation at pH 4.0 (20,21). The cis and trans structures are defined by the orientation of the 2 $\alpha$ carbons with respect to the C-N bond. The procedure of conformational analysis used has been described previously (10-15). Each polypeptide segment is oriented at the air-water interface as defined earlier (15). The procedure used to surround one peptide with lipid molecules has been described elsewhere (15). We limited our analysis to a number of lipid molecules sufficient to surround one peptide molecule. Calculation were made on a CDC Cyber170 computer coupled to an Olivetti M24 with the PC-MGM (Molecular Graphics Manipulation) drawing program.

## RESULTS AND DISCUSSION

As it has already been shown by Collier et al.(22) using a segment of 7 amino acids, two domains in CB1 emerge clearly from the Kyte and Doolittle plot (23) of diphtheria toxin B fragment, the transverse lipid associating domain (TLAD; residues 153 to 178) and the membrane penetrating segment (MPS; residues 225 to 246). Since the other hydrophobic regions identified by this method were located in peptide regions that do not interact with lipid bilayers (6-8), we focused our analysis on these two hydrophobic domains of CB1. Moreover, no other clusters of prolines were associated with hydrophobic regions as defined by Kyte and Doolittle (23). If the mean hydrophobic moment $<\mu H>$ was plotted versus the mean hydrophobicity $<Hi>$ for all possible segments of DTB using a segment of 7 amino acids (fig.1), most of DTB residues were located in a region described by Eisenberg as characteristic of globular proteins (16). The MPS, on the contrary, is mainly located in a domain of the plot defined by a high hydrophobicity ($Hi \geq 0.5$) and a low hydrophobic moment ($0<\mu H<0.4$) associated with transmembrane structures (16), as illustrated

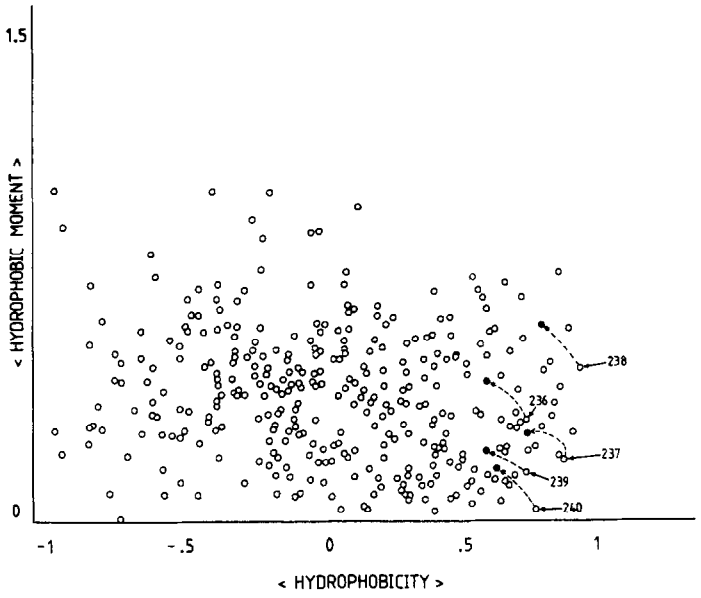


Figure 1.: Plot of the hydrophobic moment ( $< \mu_H >$ ) (vertical axis) against the hydrophobicity ($< H_i >$) (horizontal axis) for all seven residue-long segments of diphtheria toxin B fragment. Arrows indicate the position of MPS amino acids. The dotted line indicates the shift observed for the residues of MPS 228 .

by the seven transmembrane helices of bacteriorhodopsin which mainly fall in the region defined by values of $< Hi >$ above 0.5 and of $< \mu_H >$ ranging from 0.0 to 0.4 (16). Figure 2 shows the conformational states of the MPS at pH 4.0 and pH 7.0 and illustrates the strong pH dependency of its conformation. We limited our analysis to the 230-247 segment which was identified as hydrophobic in the Kyte and Doolittle plot. At pH 7.0 the peptide adopts an almost completely extended conformation whereas at pH 4.0 it takes a much more folded conformation. The cis-trans isomerization of Pro242 and Pro245 is mainly responsible for the observed conformational change indicating that conformation is highly sensitive to the transconformation process of prolines associated with $\beta$-sheet and $\beta$-turn structures (7). At the opposite, no significant structural modifications were induced by the cis-trans isomerization of

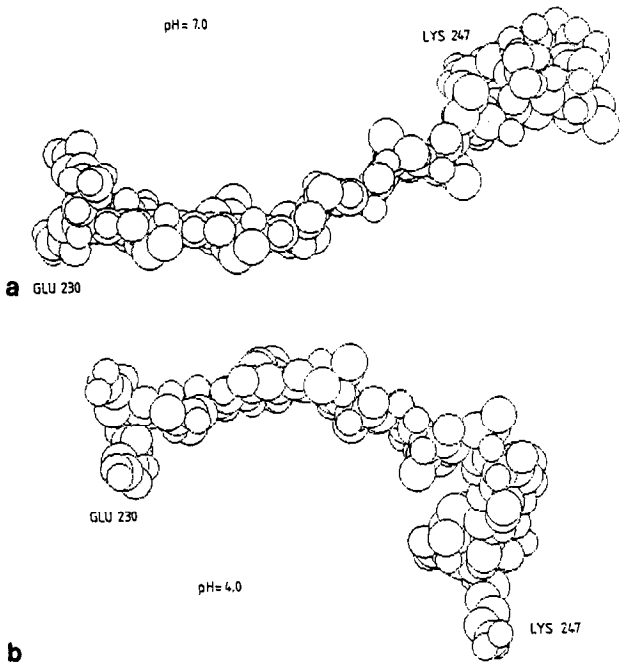


Figure 2.: Space filling model of the membrane penetrating segment (230-247) conformation at a) pH=7.0 and b) pH=4.0.

Pro233 and Pro235 associated with a random coil structure. This raises the question of the relative capability of these 2 conformers to insert into the lipid layer. We used an another approach based on the evaluation of the distance ( Δ ) between the hydrophobic and hydrophilic centers which has been demonstrated to determine the mode of insertion of an amphiphilic agent into a lipid matrix (13,14,15). Previous conformational studies of more than 80 molecules, including phospholipids (10,12), ionophores (11,24), antibiotics and other various drugs (13-15) confirm that the Δ values must be higher than 2 Å in order to allow the insertion of the molecule into the lipid layer. The calculated Δ values at pH 4.0 (3.4Å) and pH 7.0 (1.92Å) indicate that only the acid form of MPS will penetrate into the lipid matrix; the neutral form has no tendency to interact with the lipid layer. In order to describe

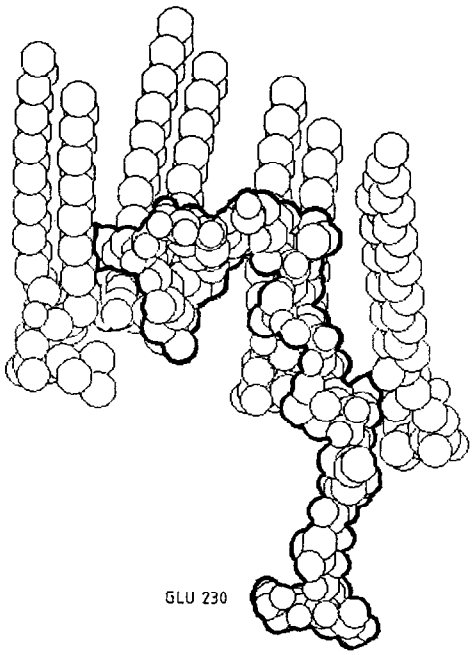


Figure 3.: Computer visualization of the mode of insertion of the membrane penetrating segment (230-247) into the dipalmitoylphosphatidylcholine matrix (DPPC). Lipids placed in front of the polypeptide were not represented. Heavy lines indicate the external surface of the peptide pH=4.0.

in molecular terms the MPS-lipids interaction at pH 4.0, the isolated peptide was oriented at the air-water interface and surrounded with lipids, according to the procedure previously described. DL- $\alpha$ -dipalmitoylphosphatidylcholine (DPPC) was chosen since its structure has been extensively investigated in previous studies (10). As shown in fig.3, MPS at pH 4.0, is capable of strongly destabilizing the lipid layer by breaking the parallelism between lipid acyl chains. The large calculated peptide-lipid interaction (-19 Kcal/mole) as compared to the lipid-lipid interaction (-13 Kcal/mole) explains the observed destabilization. CRM 228, a product of multiple missense mutations in the diphtheria toxin gene, has a partially defective B fragment. One mutation leads to the replacement in B228 of Gly238 by Ser, right in the middle of

the hydrophobic core of the MPS. As a consequence, this segment is shifted toward less hydrophobic regions of the Eisenberg plot (fig.1); it has a decreased capability of insertion into the lipid layer which leads to a strongly decreased effect on the lipid matrix organization (data not shown). These results suggest that, at low pH of the endosome, the isomerization of the MPS proline residues might induce the interaction of diphtheria toxin B fragment with the endosomal membrane by the consecutive destabilization of the lipid matrix organization resulting from the insertion of the MPS in the lipid bilayer. The lipid destabilization could initiate the insertion of other hydrophobic regions of B fragment (17), namely the transverse lipid-associating domain located at the N-terminus of CB1 (residues 153 to 178) (fig.4). The conformational analysis procedure clearly demonstrated the capability of this segment to insert into a DPPC lipid layer; the computed energy of interaction between the transverse lipid-associating domain and DPPC (-33.6 Kcal/mole) indicates the stability of this

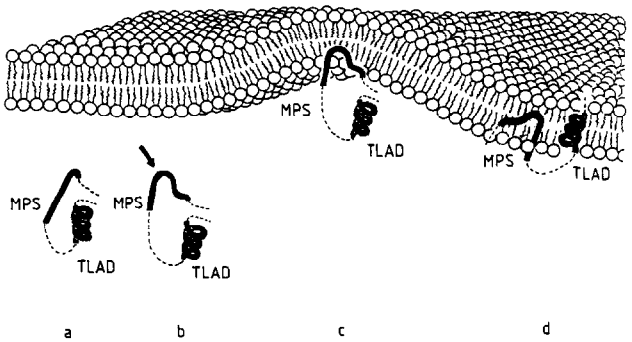


Figure 4.: Hypothetical representation of the sequence of events leading to the insertion of the membrane penetrating segment (MPS) and of the transverse lipid associating domain (TLAD) into the lipid bilayer.
a) pH=7.0.
b) pH=4.0. The isomerization of the proline residues (Pro233 and Pro235) uncovers an hydrophobic segment (arrow).
c) Insertion of the membrane penetrating segment (MPS) into the bilayer induces a lipid desorganization.
d) The lipid destabilization makes possible the insertion of the transverse lipid associating domain (153-178; TLAD).

interaction. The destabilization of the lipid layer organization could ultimately facilitate the export of diphtheria toxin A fragment from the endosome into the cytoplasm. The present work suggests also that a pH induced cis-trans proline isomerization could be the driving force leading to peptide insertion into a lipid matrix. Such a modulation of protein conformation by the distribution of proline cis-trans isomers has been held responsible for the interaction of immunoglobulins and lipid bilayers at low pH (26).

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