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Location of tryptophan residues in free and membrane bound *Escherichia coli* α-hemolysin and their role on the lytic membrane properties

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Abstract

α-Hemolysin (HlyA) is an extracellular protein toxin secreted by *Escherichia coli* that acts at the level of plasma cell membranes of target eukaryotic cells. Previous studies showed that toxin binding to the bilayers occurs in at least two ways, a reversible adsorption and an irreversible insertion. Studies of HlyA insertion into bilayers formed from phosphatidylcholine show that insertion is accompanied by an increase in the protein intrinsic fluorescence. In order to better define structural parameters of the membrane-bound form, the location of tryptophan residues was studied by means of quenchers of their intrinsic fluorescence located at 7, 12 and 16 positions of the acyl chain of phosphatidylcholine. The quenching was progressively weaker suggesting an interfacial location of the Trp. In parallel, HlyA was subjected to oxidation with *N*-bromosuccinimide to study the role of Trp residues exposed to aqueous media in its structure–function relationship. In the folded toxin molecule, a single residue was susceptible to oxidation with NBS, whereas incubation with LUV of the toxin prior modification prevents its oxidation, suggesting that Trp residue(s) are directly involved in toxin binding and insertion. Finally, the modification of residues exposed to solvent resulted in a complete impairment of the lytic activity. It was concluded that the modification-sensitive Trp residues are essential for the structure and function of native HlyA. These results are consistent with the model proposed by Soloaga et al. (Mol. Microbiol. 31 (1999) 1013–1024) according to which HlyA is bound to a single monolayer through a number of amphipathic instead of inserted transmembrane helices.

Keywords: Chemical modification; α-Hemolysin; Lytic toxin; Protein–membrane interaction; Tryptophan fluorescence

1. Introduction

Escherichia coli α-hemolysin (HlyA) is a cytolytic exotoxin secreted by pathogenic strains of this bacterium, involved in human extraintestinal diseases, like urinary tract infections, peritonitis, meningitis and septicemia [1]. It is a member of a wider family of toxin named RTX elaborated by Gram-negative organisms which share several functional and genetical features [2–3].

In the case of HlyA, (MW 107 kDa), it is a single polypeptide chain whose complex molecular organization was identified by different mutational and

Abbreviations: ANTS, 1-anilino-naphthalene-1,3,5-trisulfonate; DPX, *p*-xylenebis pyridinium; HlyA, α-hemolysin; LUV, large unilamellar vesicle; NBS, *N*-bromosuccinimide; POPC, 1-palmitoyl-2-oleyl phosphatidylcholine; Trp, tryptophan

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structural prediction studies. It comprises: (a) amphiphilic α-helix at the N-terminal end [4]; (b) a region rich in amphipathic stretches organized as α-helices [5]; (c) a Ca^{2+}-binding domain containing 11–17 copies of a glycine-rich repeated nonapeptide [6]; and (d) a C-terminal export sequence [7]. The toxin is synthesized as an inactive precursor activated via a post-translational acylation at two internal lysine residues [8].

Unlike other membrane active proteins, this toxin has been shown to exert its activity directly through the lipid bilayer of the cellular membrane rather than interact with specific protein target [9] and the membrane damage is the direct result of polypeptide activity. For this reason, this toxin constitutes a good example of a large protein that can be transferred from the aqueous medium to the hydrophobic bilayer matrix of cell membranes. The importance of the physical state of the lipid bilayer in the insertion of HlyA has been described in detail [10]; however, hydrophobic forces are not the only ones involved in HlyA interaction; polar and electrostatic forces may play a role in this process [11]. The binding of HlyA to membranes does not by itself lead to leakage, and the toxin must bind calcium prior to its attachment to the membrane for lysis occurrence. Calcium binding induces a competent state in the protein, enabling its insertion into the membrane [12,13].

A large variety of physical techniques has been used to characterize lipid–HlyA interactions, including indirect evidence for irreversible binding from erythrocyte lysis, intrinsic fluorescence studies and differential scanning calorimetry. Those experiments support the notion that *E. coli* HlyA binds and inserts into phospholipid bilayers, becoming an intrinsic, but non-transmembrane protein [5]. However, the mechanism of cell lysis by HlyA is not yet known. There are two different hypotheses about this, one is about a toxin, such as the pore-forming protein and the other is the protein with a 'detergent-like' effect.

The polypeptide region interacting with the membrane should be thoroughly known in order to understand the mechanism of membrane damage. Additional details about protein–lipid interactions can be approached by spectroscopic studies of the intrinsic toxin fluorescence. HlyA has four Trp residues which can serve as intrinsic fluorophores. They are located at positions 431, 480, 579 and 914.

In the present study, the changes in Trp fluorescence emission and its quenching by doxyl labels incorporated into membranes were used to identify Trp residues inserted into the lipid bilayer. In parallel, HlyA was oxidized with *N*-bromosuccinimide to estimate the Trp residues exposed to solvent, and the significance of Trp residues for the toxin structure and function.

2. Materials and methods

2.1. Materials

Plasmid-encoded α-hemolysin (HlyA) was purified from the culture filtrates of an over-producing strain of *E. coli*, according to the method of Ostolaza et al. [14]. The toxin purified from a plasmid determinant shows high specific activity indicating that the protein had been acylated correctly [14–16]. The protein was dialyzed against 150 mM NaCl, 20 mM Tris HCl, pH 7.0 (buffer TC) before its use. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 7-, 12- and 16-doxyl POPC were supplied by Avanti Polar Lipids (Alabaster, AL). 1,3,5-Trisulfonate-8-anilinonaphthalene (ANTS) and *p*-xylenebis pyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR).

Large unilamellar vesicles (LUVs) were prepared by extrusion and sized using 0.1 μm-pore size Nucleopore membranes as described by Mayer et al. [17].

2.2. Measurement of intrinsic fluorescence of HlyA

Fluorescence spectra were recorded on a SLM 4800 Aminco spectrofluorometer with a temperature-controlled sample holder at 25°C. The excitation wavelength was 295 nm in order to minimize tyrosine emission [18]. Slit width was 8 nm for both, excitation and emission. Protein (30 μg) was added to 3-ml cuvettes containing buffer TC under continuous stirring. NBS or LUV was added to reach a final ratio NBS/Trp 2:1 (mol:mol) or 1000:1 lipid:protein (mol:mol). After equilibrating for 10 min, the emission spectra were recorded in the wavelength range

from 300 to 400 nm. Fluorescence measurements were corrected for any dilution factor.

2.3. Quenching of intrinsic fluorescence by doxylphosphatidylcholines

Large unilamellar vesicles were prepared as described above with palmitoyl-oleoylphosphatidylcholine without or with phosphatidylcholines containing doxyl groups at various positions along the acyl chain (7, 12 and 16). The vesicle suspension (0.1 mM lipid) was incubated with 10 μg/ml of protein as indicated above and the intrinsic tryptophanyl emission spectra of HlyA were recorded. A correction factor was also applied for the attenuation of excitation light intensity by doxyl-PC concentration [19].

2.4. Chemical modification of HlyA with N-bromosuccinimide

A freshly prepared solution of NBS was added to HlyA in 20 mM Tris-HCl buffer, pH 6.5, to achieve the NBS/Trp molar ratio ranging from 0.25:1 to 2:1. Aliquots of the modified toxin were removed and tested for residual lytic activities.

2.5. Assessment of vesicle leakage

Vesicle-content leakage was measured by ANTS/DPX methods according to Ellens et al. [20]. Liposomes containing 12.5 mM ANTS, 45 mM DPX, 50 mM Tris-HCl (pH 7.0) were separated from unencapsulated material by column chromatography on Sephadex G75 using 50 mM Tris-HCl, 85 mM NaCl (pH 7.0) as the elution buffer. The same buffer was used in the leakage assays.

Fluorescence assays were performed in a 4800 SLM Aminco spectrofluorometer with continuous stirring at room temperature (25°C). For the ANTS/DPX system, the excitation wavelength was at 355 nm and emission at 530 nm. A lipid suspension was diluted in buffer to give a final lipid concentration of 10^{-4} M; the fluorescence level of this preparation did not vary with time and was set as 0% leakage. Leakage experiments were started by adding an appropriate amount of native toxin equivalent to obtain 400 HU ($\approx$1 μg) at 10 μM lipid concentration, and measured 5 min after toxin addition. One HU was defined as the dilution factor required for a given HlyA preparation to produce 50% lysis of a standard red blood cell suspension. One hundred percent release was measured after addition of Triton X-100 to 1 mM final concentration.

3. Results

HlyA was subjected to oxidation with *N*-bromosuccinimide in order to determine the accessibility of the Trp residues to this reagent. Our experiments were performed under neutral conditions to increase the specificity for Trp residues. A side reaction of NBS with tyrosine was reported by Oshini [21]; however, we did not find an increased absorbance at 280 nm due to the unspecific tyrosine modification. Under these conditions, the reaction of NBS with Trp in the HlyA molecules was almost accomplished within 10 min of incubation (data not shown).

Fig. 1 shows HlyA titration with NBS in the presence or absence of POPC–LUVs. For HlyA in buffer,

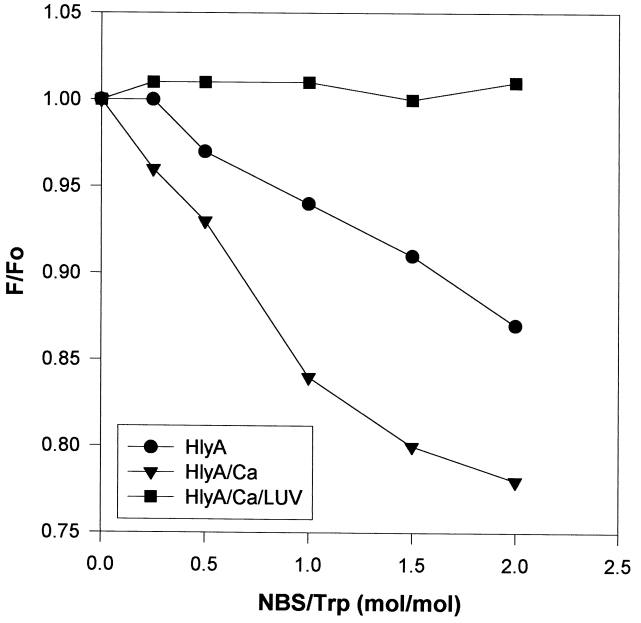

Fig. 1. Modification of HlyA with NBS. Relative intrinsic fluorescence of HlyA (10 μg/ml) was measured after NBS addition. ▼, HlyA in buffer 20 mM Tris-HCl, pH 7.0, 20 mM $CaCl_2$; ●, HlyA in buffer 20 mM Tris-HCl, pH 7.0; ■, HlyA was preincubated with 0.1 mM LUV (POPC). F_o and F represent the intrinsic fluorescence of native and oxidized toxin, respectively.

a fluorescence decrease was observed. This effect is enhanced by the presence of Ca^{2+} because this cation induces protein conformational changes that imply modifications in the tertiary structures with the exposition of superficial hydrophobic areas [13]. In this case, 25% of Trp fluorescence was quenched corresponding to one out of four Trp residues present in HlyA. This result indicates that other Trp residues are either non-reactive or more probably buried and therefore, not accessible to NBS. Yet, when the protein was incubated with POPC–LUV all residues are protected from NBS modification as shown in the same figure. Then, some Trp residues might be accessible for NBS oxidation, whereas the other Trp residues are mostly buried in both free and membrane-bound toxin.

HlyA fluorescence may be heterogeneous due to a distinct solvent exposure of each particular indolyl residue. For proteins containing a single Trp, the wavelength of maximal fluorescence is thus diagnostic of the environment, but the complexity of those systems containing multiple intrinsic Trp residues does not allow an accurate photophysical interpretation. However, fluorescence spectroscopy offers a very powerful tool for searching changes in protein structures.

In order to get further information on the Trp location in the membrane-bound form, NBS was added either before or after the binding of HlyA to lipid vesicle, and then the emission spectrum was obtained. As shown in Fig. 2A, the fluorescence emission spectrum of native toxin was significantly increased when POPC–LUVs were added. As expected, no change was observed when NBS was added to the membrane-bound form. The Trp fluorescence of oxidized toxin exhibited an interesting behavior upon membrane binding as shown in Fig. 2B. When the protein was previously oxidized, its fluorescence emission spectrum was increased by adding LUV (POPC). These results indicate that even when Trp residues were mostly buried, the protein conformational changes induced on membrane-binding exposed some buried Trp to membrane.

The fluorescence emission maximum of the membrane-bound toxin was red shifted to 345 nm, suggesting an interfacial location into the membrane and the other maximum appears to be defined. It is blue

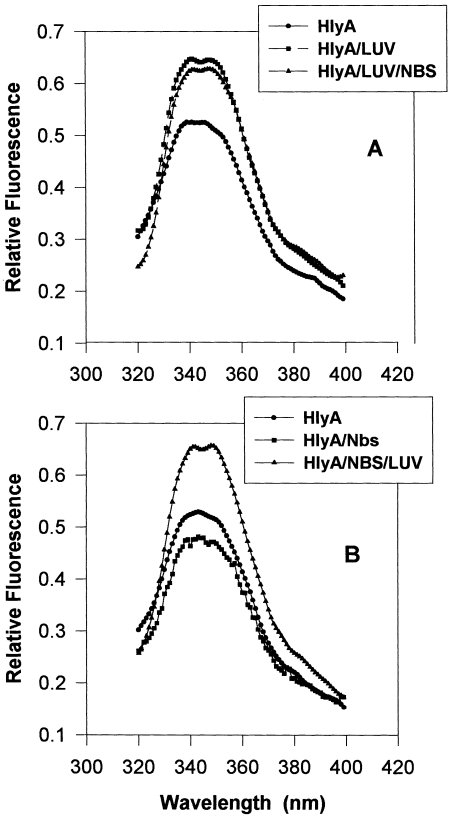

Fig. 2. Fluorescence spectroscopy of native and NBS modified HlyA (10 μg/ml) in 20 mM Tris-HCl, pH 7.0, in the presence of 20 mM $CaCl_2$ and 0.1 mM LUV (POPC). Fluorescence emission spectra of HlyA in 20 mM Tris-HCl in the presence of 20 mM $CaCl_2$ and LUV (POPC). NBS was added either after (A) or before (B) incubation with LUV.

shifted corresponds to a non-polar environment location, most probably the interior of the protein.

It is tempting to speculate that the red shift and the increase in intensity are related to conversion to a less tightly folded form when HlyA binds to phospholipid, whereas the increase in the intensity may indicate the disappearance of tertiary interactions that quench the fluorescence in the native state

In parallel, we have studied the perturbation produced by HlyA on pure phosphatidylcholine bilayers in the form of large unilamellar vesicles as a function of oxidation degree, under conditions in which the toxin induces vesicle leakage. HlyA-induced release of aqueous contents from LUV is due to the bilayer disruption in a 'detergent-like' effect of the toxin. This effect was characterized in a series of experiments in which lipid and protein concentration was

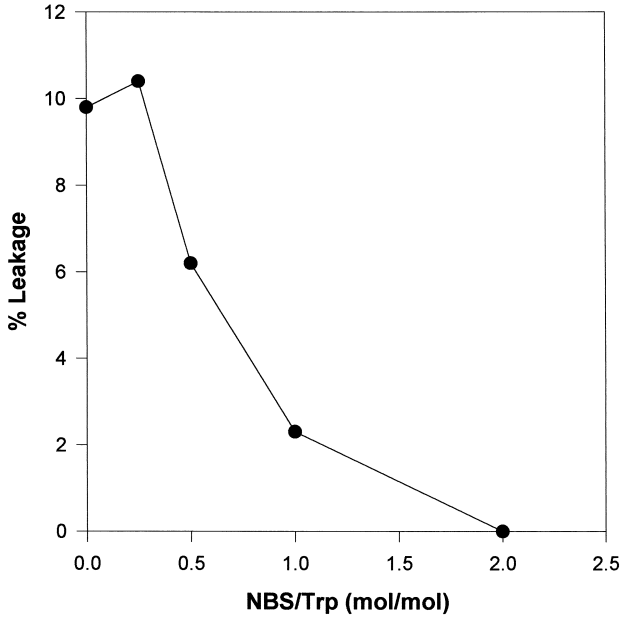

Fig. 3. Leakage of ANTS-DPX from LUV (POPC) as a function of toxin modification by NBS. One hundred percent release was measured after addition of Triton X-100 to 1 mM final concentration. Percent release was measured 5 min after addition of 400 HU (≈1 μg) HlyA. Lipid concentration was 10 μM. One HU being defined as the dilution factor required for a given HlyA preparation to produce 50% lysis of a standard red blood cell suspension.

varied [9], demonstrating its dependence on the protein concentration and finally both the extent and the initial rates of leakage reached a plateau. Fig. 3 shows the leakage of ANTS-DPX encapsulated in PC vesicles as a function of the NBS/Trp ratio. These results show that the chemical modification of Trp residues exposed to solvent abolish lytic toxin effect. These results correlated well with the ones present in Fig. 1 in which titrating out one Trp, titrates out lytic activity at the same time.

As mentioned earlier, the membrane-bound HlyA has a conformation different from the one in the soluble form. We were interested in establishing the location of the non-solvent exposed Trp. For this reason, we used PC analogs containing a fatty acyl chain with the doxyl label at different positions along the chain to see if the Trp residues in question were interacting with the lipids, and to estimate the depth of Trp penetration into the membrane. Fig. 4 shows that 7-doxyl-PC strongly quenched fluorescence of Trp. The effect was less pronounced for the 12-label and least for the 16-doxyl PC. These results indicate that no Trp penetrates beyond an interfacial region on the membrane. The relatively high wavelength of maximal emission intensity (345 nm) when HlyA is bound to membrane, also supports this conclusion. The lack of a blue shift in HlyA Trp fluorescence emission is consistent with an interfacial location when it binds to LUV despite its accessibility to doxyl-POPC, which is not without precedence for other proteins [23].

In order to determine the Trp number inserted in the membrane, we added HlyA to vesicles prepared from POPC and 7-doxyl derivate at variable concentration. In the event of all Trp in a protein being accessible to quencher, the fluorescence quenching can be described by the Stern–Volmer equation. However, if all Trp in a protein are not accessible to quencher, i.e. buried in the interior protein, the Stern–Volmer plot deviates from linearity and the quenching process can be described by a modified equation of Lehrer [22]

$$I_o/(I_o - I) = 1/K_{eff}\, f_a\, Q + 1/f_a$$

where $(I_o - I)$ refers to a change in fluorescence inten-

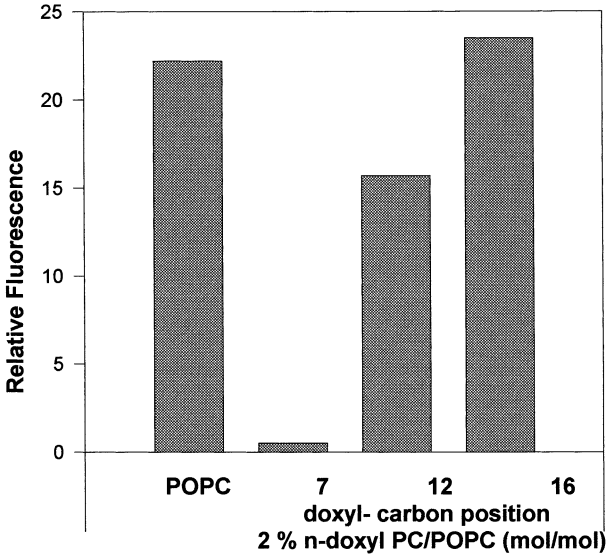

Fig. 4. Quenching of HlyA intrinsic fluorescence by doxyl label phospholipids. Increase in HlyA fluorescence relative to buffer in the presence of 0.1 mM LUV (POPC) or LUV (POPC) containing doxyl labels at the 7-, 12- and 16-position of PC-acyl chain. 2% *n*-doxyl-PC respect to total lipids were used in all cases. HlyA concentration was 10 μg/ml.

sity by quenching addition, I_o is the intensity in the absence of quencher, Q is the concentration of the quencher, f_a is the accessible Trp fraction and K_{eff} the effective quenching constant. Analysis of data by Lehrer equation is shown in Fig. 5, indicating that 1/4 of Trp is accessible to quencher and so involved in lipid–protein interaction.

In addition, Fig. 6 shows that the maximum fluorescence emission of the unquenched fraction apparently inaccessible for 7-doxyl PC was blue shifted to 338 nm, suggesting a location in a non-polar environment, most probably the interior of the protein.

The data presented here indicate that no Trp penetrates beyond an interfacial site on the membrane.

Quenching results evidence HlyA-membrane interaction and thus they explain that the fluorescence increase observed by HlyA–LUV (PC) interaction is not the consequence from the loss of Trp–Trp energy transfer.

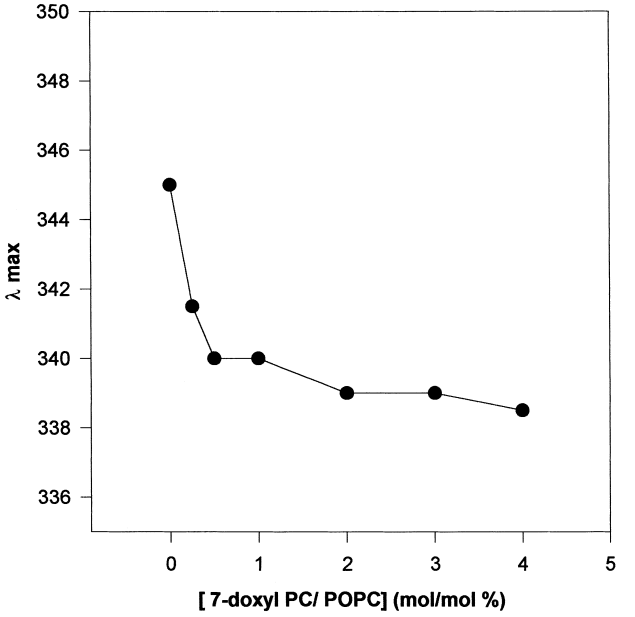

Fig. 6. Wavelength emission maximum of HlyA membrane bound as a function of %7-doxyl labels incorporated in LUV (POPC). The lipid:protein ratio was in all cases 1000:1 (mol/mol).

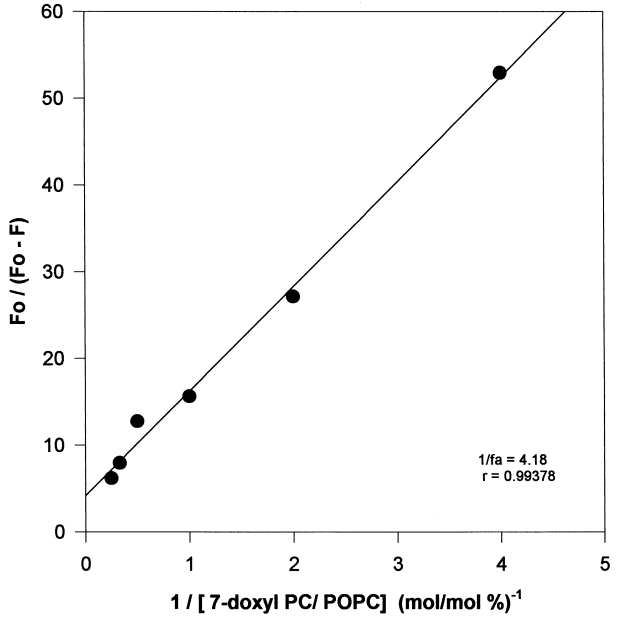

Fig. 5. Lehrer plot for the quenching of membrane bound HlyA by 7-doxyl-PC. Variable concentrations of 7-doxyl-PC were incorporated in 0.1 mM LUV (POPC). HlyA concentration was 10 μg/ml. The fluorescence quenching data were analyzed according to modified Stern–Volmer (Lehrer): $F_o/(F_o-F) = 1/(Q\, f_a\, K_q)+1/f_a$ where f_a is the fraction number of accessible Trp and K_q their collisional constant. The wavelengths of excitation and emission were 295 and 340 nm, respectively.

4. Discussion

The association of water-soluble proteins with membranes is a complex process that has been postulated to occur through distinguishable steps which include: (1) adoption of a competent conformational state in solution by the protein; (2) adsorption of this active state to the membrane surface; and (3) either partial or complete insertion into membranes.

In the case of HlyA, the protein in solution adopts a competent state for insertion in the presence of calcium and at pH > 5. Under these conditions, the protein has a large exposed hydrophobic surface and the bilayer provides room for accommodating the toxin [11,13]. Our previous studies on the insertion of α-hemolysin in lipid bilayers show that this toxin may bind the membranes in at least two ways: a reversible adsorption and an irreversible insertion. Adsorption appears to be largely independent of bilayer fluidity or composition, whereas the insertion is highly dependent on the bilayer physical properties. The lipid bilayer properties that facilitate the irreversible insertion of HlyA are fluid state over gel state and the disordered liquid over ordered liquid or gel

states. The hypothesis that HlyA becomes irreversibly bound to lipid bilayers when they are in fluid state is also supported by experiments in which toxin binding is assessed by an increase in the intrinsic Trp fluorescence of the protein [10].

The binding of amphipathic proteins to lipid bilayers is commonly followed through in the protein intrinsic fluorescence. This is the case of hemolysin. An essentially similar effect is observed with prohemolysin [16]. This may be compatible with an amphipathic tertiary structure present in both HlyA and proHlyA. However, previous data [16] indicate that despite the amphipathic stretches known to be essential for the lytic activity, proHlyA is unable to impair bilayer permeability. No hemolytic activity was detected for proHlyA even at protein concentration two orders of magnitude above the mature toxin. Trp residues susceptible to be oxidized and thus localized at hydrophilic environment are probably involved in the lytic toxin activity. Previous results from Goñi [12] show that hemolysin binding to membranes does not always lead to the lytic activity. In fact, the protein must bind Ca^{+2} prior to its binding in order to exert a lytic activity. We showed that Ca^{2+} produces specific changes in the emission spectra of HlyA [13]. Unlike the mature protein, the intrinsic fluorescence of proHlyA is not modified in the presence of Ca^{2+}. Probably, the Trp residue responsible to those changes is Trp-579, located near the toxin acylation site and thus it is related to lytic activity. This interpretation correlated well with data presented in Fig. 3.

Considering our result about the ability of 7-doxyl PC incorporated into lipid vesicles to quench intrinsic fluorescence of a Trp residue fraction, it is tempting to conclude on the basis of percentage quenching data, that one of the four Trp residues is membrane embedded and the other three are buried inside in the membrane-bound HlyA because the NBS oxidation data indicate that membrane-bound HlyA has no NBS-accessible Trp residues. This residue is not exposed initially to solvent, and as seen in Fig. 2B, a fluorescence increase is produced even when the toxin had been previously oxidized with NBS indicating that only after conformational changes, this residue is accessible to the bilayer. The protein adsorption on the membrane surface could provide the energy necessary for protein conformational changes which would trigger its partial membrane insertion.

The present results support the notion that *E. coli* α-hemolysin, which behaves as a soluble protein when secreted by bacterium into the extracellular medium, binds and inserts into cell membranes becoming an intrinsic protein of the host membrane. A preliminary model for the conformation of HlyA inserted in a bilayer was published by Soloaga et al. [5]. Its main feature is the protein binding to a single monolayer through a number of amphipathic helices according to the structure prediction studies. The amphipathic α-helices are often an encountered secondary structural motif in biologically active peptides and proteins, and it was the first described as a unique structure–function motif involved in lipid interaction by Segrest in 1974 [24]. The functional properties suggested for amphipathic helices include lipid association, membrane perturbation in the form of lysis or fusion, transmembrane signal transduction, and so on.

An amphipathic helix is defined as the one with opposed polar and non-polar faces oriented along its long axis. For this reason, they are ideal for anchoring a protein to a single monolayer. There are several lytic peptides with helical structure that have been proposed in late years to lie oriented parallel to the surface membrane. This is the case of magainins 1 and 2, as well as the model peptide 18L [25–27]. In addition, the amphipathic helices are also involved in both intra- and intermolecular protein–protein interactions in a number of proteins [28]

In HlyA, a conclusion could be drawn that only the Trp located in the amphipathic domain at position 431, is involved in toxin–lipid interaction.

On the other hand, Trp was found to be important for the activity of some polypeptides isolated from snake venom [29], and in the honey bee melittin [30]. In the case of melittin, a peptide with detergent-like activity similar to HlyA, Trp was proved spectrofluorometrically to participate in the interaction of the toxin molecule with membrane lipids. Considering our results about NBS oxidation, Trp residues exposed to the solvent in the soluble form of HlyA are not involved in the hydrophobic interaction with membrane lipids; however, this modified Trp residue is essential for the native conformation of HlyA as well as for the lytic activity.

References

[1] S. Cavalieri, G. Boehch, I. Snyder, Microbiol. Rev. 48 (1994) 326–343.
[2] V. Koronakis, M. Cross, B. Senior, E. Koronakis, C. Hughes, J. Bacteriol. 169 (1987) 1509–1515.
[3] J. Devenish, S. Rosendal, R. Johnson, S. Hubler, Infect. Immunol. 57 (1989) 3210–3213.
[4] K. Erb, M. Vogel, W. Wagner, W. Goebel, Mol. Gen. Genet. 208 (1987) 88–93.
[5] A. Soloaga, P. Veiga, L. García Segura, H. Ostolaza, R. Brasseur, F. Goñi, Mol. Microbiol. 31 (1999) 1013–1024.
[6] H. Ostolaza, A. Soloaga, F. Goñi, Eur. J. Biochem. 228 (1995) 39–44.
[7] C. Chevaux, I. Holland, J. Bacteriol. 178 (1996) 1232–1236.
[8] P. Standley, V. Koronakis, K. Hardie, C. Hughes, Mol. Microbiol. 20 (1996) 813–822.
[9] H. Ostolaza, B. Bartolome, I. Ortiz de Zarate, F. De la Cruz, F. Goñi, Biochim. Biophys. Acta 141 (1991) 81–88.
[10] L. Bakas, H. Ostolaza, F. Goñi, Biophys. J. 71 (1996) 1869–1876.
[11] H. Ostolaza, L. Bakas, G. Goñi, J. Membr. Biol. 158 (1997) 137–145.
[12] H. Ostolaza, F. Goñi, FEBS Lett. 371 (1995) 303–306.
[13] L. Bakas, M. Veiga, A. Soloaga, H. Ostolaza, F. Goñi, Biochim. Biophys. Acta 1368 (1998) 225–234.
[14] H. Ostolaza, B. Bartolome, J. Serra, F. de la Cruz, FEBS Lett. 289 (1991) 195–198.
[15] M.I. Gonzalez Carrero, J.C. Zabala, F. de la Cruz, J.M. Ortiz, Mol. Gen. Genet. 199 (1985) 106–110.
[16] A. Soloaga, H. Ostolaza, F. Goñi, Eur. J. Biochem. 238 (1996) 418–422.
[17] L. Mayer, M. Hope, P. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[18] J. Valpuesta, F. Goñi, J. Macarulla, Arch. Biochem. Biophys. 257 (1987) 285–292.
[19] J. Lackowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, NY, 1984, pp. 357–359.
[20] H. Ellens, J. Bentz, F. Szoka, Biochemistry 25 (1986) 285–294.
[21] M. Ohnishi, T. Kawagishi, T. Abe, K. Hiromi, J. Biochem. 87 (1980) 273–279.
[22] S. Leher, Biochemistry 10 (1971) 3254–3263.
[23] P. Meers, Biochemistry 29 (1990) 3325–3330.
[24] J. Segrest, R. Jackson, J. Morisett, A. Gotto, FEBS Lett. 38 (1974) 247–253.
[25] K. Matsuzaki, O. Murase, H. Tokuda, S. Funakoshi, N. Fuji, K. Miyajima, Biochemistry 33 (1994) 3342–3349.
[26] B. Bechinger, J. Membr. Biol. 156 (1887) 197–211.
[27] I. Polozov, A. Polozava, E. Tytler, G. Anantharamaiah, J. Segrest, G. Woley, R. Epand, Biochemistry 36 (1997) 9237–9245.
[28] C. Cohen, D. Parry, Trends Biochem. Sci. 11 (1986) 245–248.
[29] S. Nishida, N. Tamiya, Toxicon 29 (1991) 429–439.
[30] J. Dufourcq, J. Faucon, Biochim. Biophys. Acta 467 (1977) 1–11.