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Balance of Electrostatic and Hydrophobic Interactions in the Lysis of Model Membranes by *E. coli* α-Haemolysin

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Abstract. The relative weight of electrostatic interactions and hydrophobic forces in the process of membrane disruption caused by *E. coli* α-haemolysin (HlyA) has been studied with a purified protein preparation and a model system consisting of large unilamellar vesicles loaded with water-soluble fluorescent probes. Vesicles were prepared in buffers of different ionic strengths, or pHs, and the net surface charge of the bilayers was also modified by addition of negatively (e.g., phosphatidylinositol) or positively (e.g., stearylamine) charged lipids. The results can be interpreted in terms of a multiple equilibrium in which α-haemolysin may exist: aggregated HlyA ⇄ monomeric HlyA ⇄ membrane-bound HlyA. In these equilibria both electrostatic and hydrophobic forces are significant. Electrostatic forces become substantial under certain circumstances, e.g., membrane binding when bilayer and protein have opposite electric charges. Protein adsorption to the bilayer is more sensitive to electrostatic forces than membrane disruption itself. In the latter case, the irreversible nature of protein insertion may overcome electrostatic repulsions. Also of interest is the complex effect of pH on the degree of aggregation of an amphipathic toxin like α-haemolysin, since pH changes are not only influencing the net protein charge but may also be inducing protein conformational transitions shown by changes in the protein intrinsic fluorescence and in its susceptibility to protease digestion, that appear to regulate the presence of hydrophobic patches at the surface of the molecule, thus modifying the ability of the toxin to either aggregate or become inserted in membranes.

Key words: Bacterial toxins — Model membranes — α-haemolysin — Electrostatic forces — Hydrophobic forces — Membrane protein insertion

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Introduction

Protein toxins acting at the level of cell membranes offer a good example of water-soluble peptides that can, at some stage, become irreversibly inserted in membranes. This change from soluble to integral membrane proteins is a complex process in which various stages can be distinguished: (i) a conformational transition that brings the protein in solution to a ''competent state'', (ii) adsorption of the activated protein onto membrane surface, and (iii) insertion into the bilayer (Merrill, Cohen & Crammer, 1990; van der Goot et al., 1991; Helenius, 1992; Gonzalez-Mañas, Lakey & Pattus, 1992; Muga et al., 1993; Shin et al., 1993; Bañuelos & Muga, 1996). These stages may not necessarily occur in the said order. Individual toxins may act in very different ways, e.g., the competent state may require acidic pH (colicin A, Parker et al., 1990) or neutral pH (perfringolysin, Nakamura et al., 1995), colicin A adsorbs only on negatively charged surfaces (Lakey et al., 1991; van der Goot et al., 1991; Gonzalez Mañas et al., 1992) while perfringolysin or colicin E1 require electrically neutral bilayers (Shin et al., 1993; Nakamura et al., 1995). The last stage, insertion, is the one on which less is known. For some toxins (aerolysin, van der Goot et al., 1993; *S. aureus* α-toxin, Gouaux et al., 1994) an oligomerization process appears to be involved.

E. coli extracellular toxin α-haemolysin (HlyA), a 107 kDa amphipathic protein, constitutes a good example of a large protein that can be transferred from the aqueous medium to the hydrophobic bilayer matrix of cell membranes (Cavalieri, Bohach & Snyder, 1984; Coote, 1992). Previous studies from this laboratory have

shown that HlyA has an isoelectric point at pH = 4.1 (Ostolaza et al., 1991) and that it binds irreversibly to liposomal membranes, thereby producing vesicle leakage (Ostolaza et al., 1993). HlyA binding to membranes does not by itself lead to vesicle leakage, and the toxin must bind calcium ions prior to its attachment to the membrane for lysis to occur (Ostolaza et al., 1995; Ostolaza & Goñi, 1995).

Very little is known at present of the mechanism of HlyA insertion into cell membranes. By analogy with other toxins, it can be reasonably assumed that HlyA in solution undergoes some kind of conformational change that enables it to interact with the membrane. The importance of the physical state of the lipid bilayer in the insertion of haemolysin has been described in detail recently (Bakás et al., 1996) thus showing the fundamental role of hydrophobic interactions. In addition, considering its large size, its overall negative charge at physiological pH, and the fact that hydrophobic regions appear to be concentrated in a domain near the N-terminal end of the protein (Ludwig et al., 1991), it is to be expected that hydrophobic forces are not the only ones involved in HlyA-membrane interaction, and that polar and electrostatic forces may play a role in this process. As in our previous studies, liposomes (large unilamellar vesicles) constitute a convenient and simple experimental system to test this hypothesis, notwithstanding the fact that cell membranes, with their multiple charged groups on their surfaces, will offer many more possibilities in this respect. According to our experimental results, HlyA exists at neutral pH in solution in a conformational state that we have denoted as a ''competent state'' for binding. At pH <5 the protein exists in a noncompetent conformation, however under particular circumstances certain bilayer properties may also induce the active state.

Materials and Methods

Materials

Egg phosphatidylcholine (PC) and phosphatidylinositol (PI) were grade I from Lipid Products (South Nutfield, England). Stearylamine (SA) was from Sigma (St. Louis, MO). 8-Amino-naphthalene-1,2,3-trisulphonic acid (ANTS) and *p*-xylenebis-pyridinium bromide (DPX) were from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Plasmid-encoded α-haemolysin (HlyA) was purified from the culture filtrates of an overproducing strain of *E. coli*, according to Ostolaza et al. (1991); prior to its use, the protein was dialyzed against 150 mM NaCl, 6 M urea, 20 mM Tris/HCl, pH 7.0. The protein could be stored for months in this buffer, at −20°C, without significant loss of activity. In the assays, the protein was diluted so that final urea concentration did not exceed 0.25 M, a concentration that does not modify the toxin-induced haemolysis (Ostolaza et al., 1993). For liposome preparation, lipids were mixed in organic solvent and the mixture freeze-dried. The lipids were then resuspended in 12.5 mM ANTS, 45 mM DPX, 50 mM Tris/HCl, pH 7.0, unless otherwise stated. Large unilamellar vesicles (LUV) were prepared by extrusion and sized using 0.1-μm pore-size Nuclepore membranes as described by Mayer et al. (1986).

Assessment of Vesicle Leakage

Release of vesicle contents was measured by the ANTS/DPX method, according to Ellens, Bentz & Szoka, (1986). Additional details have been given elsewhere (Ostolaza et al., 1993).

Buffers for Varying Ionic Strength and pH Conditions

In experiments designed to test the influence of ionic strength, liposomes were prepared in one of these buffers (in mM): (1a) 12.5 ANTS, 45 DPX, 50 Tris/HCl, pH 7.0, (2a) 12.5 ANTS, 45 DPX, 420 NaCl, 50 Tris/HCl, pH 7.0, (3a) 12.5 ANTS, 45 DPX, 905 NaCl, 50 Tris/Hcl, pH 7.0. Nonencapsulated probes were removed by eluting the liposomes in a gel-filtration column with the following buffers, respectively (in mM): (1b) 85 NaCl, 50 Tris/Hcl, pH 7.0, (2b) 500 NaCl, 50 Tris/HCl, pH 7.0, (3b) 50 Tris/HCl, pH 7.0, and 1 M NaCl. Liposomes were assayed for leakage in the following buffers, respectively (in mM): (1*c*) 65 NaCl, 10 $CaCl_2$, 50 Tris/HCl, pH 7.0, (2*c*) 485 NaCl, 10 $CaCl_2$, 50 Tris/HCl, pH 7.0, (3c) 985 NaCl, 10 $CaCL_2$, 50 Tris/HCl, pH 7.0. The osmolality of the buffer families (1), (2) and (3), measured with an Osmomat osmometer (Gonotec, Berlin) was respectively of about 0.2, 1 and 2 osm. The protein was added in small volumes, that did not contribute significantly to the overall ionic strength or osmotic pressure of the assay medium. For experiments under varying pH conditions, the corresponding buffers contained 50 mM Tris/acetic acid, adjusted to the desired pH with concentrated NaOH or HCl. Liposomes were prepared in (mM): 12.5 ANTS, 45 DPX and 50 Tris/acetic acid, eluted in 80 NaCl, 50 Tris/acetic acid, and assayed in 65 NaCl, 10 $CaCl_2$, 50 Tris/acetic acid. The osmolality of all these solutions was adjusted to ≈200 mosm with concentrated NaCl. A relatively high (10 mM) Ca^{2+} concentration is used, i.e., about two orders of magnitude above the calcium dissociation constant (Ostolaza et al., 1995) in order to compensate for the expected decreased affinity for Ca^{2+} at high ionic strengths or low pH.

Measuring the Size of HlyA Aggregates

The average diameter of HlyA aggregates in buffer was estimated by quasi-elastic light-scattering using a Malvern Zeta-sizer instrument operating in the multimodal mode.

Measurements of Protein-Liposome Binding

The amount of liposome-bound toxin was measured by the flotation method of Ostolaza and Goñi (1995). Liposomes (1 mM) are incubated with the appropriate amounts of HlyA to give 2,000–20,000 lipid:protein molar ratios for 30 min at room temperature, in buffers prepared with D_2O instead of H_2O. The mixture is then centrifuged in a 120.2 Beckman rotor (500,000 × *g*, 20°C, 120 min). Under these conditions liposome-bound protein remains with the upper fraction of the buffer, while free protein aggregates and sediments.

Measurements of Intrinsic Fluorescence of HlyA

Fluorescence spectra were recorded at 25°C on a Perkin Elmer MPF-66 spectrofluorimeter. The excitation wavelength was 295 nm in order to

minimize Tyr emission. Slitwidths were 5 nm for both excitation and emission (Lakowicz, 1983; Valpuesta, Goñi & Macarulla, 1987). Protein (10 μg) was added to 1-ml cuvettes containing the appropriate solutions at different pH or NaCl concentration, under continuous stirring. After equilibrating for 10 min emission spectra were recorded.

Sensitivity of HlyA to Proteolytic Digestion

Haemolysin (30 μg) was incubated in 300 μl buffer at different pH for 30 min, after which time 6 μg papain (E.C. 3.4.22.2) (Sigma) in 20 mM Tris-HCl, was added to the solutions. The mixtures were further incubated for 10 min at room temperature, then 0.1 mM phenylmethanesulphonyl fluoride was added to stop proteolysis.

Gel Electrophoresis

The fragments of proteolytic digestion were resolved on a Biorad minislab gel SDS-PAGE apparatus. The solution resulting from papain digestion was mixed with the sample diluter (2% SDS, 10% glycerol (w/v), 0.01% bromophenol blue, 10 mM Tris-HCl, pH 6.8). The samples were electrophoresed on 10% acrylamide gels in the presence of SDS according to Laemmli (1970). Protein bands were visualized by Coomassie Blue staining.

Results

Effect of Ionic Strength

As a first approach in evaluating the contribution of electrostatic and hydrophobic forces in the overall process of HlyA-bilayer interaction, the effect of increasing ionic strength on the protein in solution as well as on its interaction with the lipid membrane was studied.

Vesicle leakage induced by α-haemolysin was tested on LUV composed of PC and loaded with ANTS/DPX as described under Methods. LUV were prepared and tested in buffers of osmolarities 0.2, 1.0 and 2.0 respectively, differing only in the concentration of NaCl, i.e., in ionic strength (*see* Materials and Methods for buffer composition). The time course of vesicle leakage is shown in Fig. 1 for all three LUV preparations. It can be seen that increasing the solution ionic strength decreases the extent of leakage at a given time (15 min) and increases the half-time of leakage; both data suggest that increasing the ionic strength makes more difficult the formation of HlyA lytic complexes (whatever their structure) in the liposomal membranes. It should be noted that before HlyA action, the fluorescence of LUV dispersions was higher the higher the ionic strength while, after detergent addition, the fluorescence intensity was about the same in all cases.

The extent (after 15 min) and half-time of leakage experiments shown in Fig. 1 are given numerically in Table 1, together with two relevant parameters, namely the average diameter of HlyA aggregates in buffer (in the absence of vesicles) and the protein/lipid molar ratios in the vesicles after incubation with a constant amount of protein, measured by the flotation method. Increasing ionic strength increases the aggregate size (Table 1), this may be due to a decreased repulsive surface potential, in turn leading to aggregation by dispersion forces, and/or to aggregation driven mainly by hydrophobic forces, as suggested by Soloaga et al. (1996). Correspondingly, as the tendency of the protein to aggregate increases, the amount of α-haemolysin bound to the vesicles decreases (Table 1), indicating a multiple equilibrium: aggregate ⇄ monomer ⇄ vesicle-bound HlyA. [The free monomer intermediate has been suggested (Bhakdi et al., 1986; Ostolaza et al., 1991, 1993), but, to the authors' knowledge, its existence has not been demonstrated].

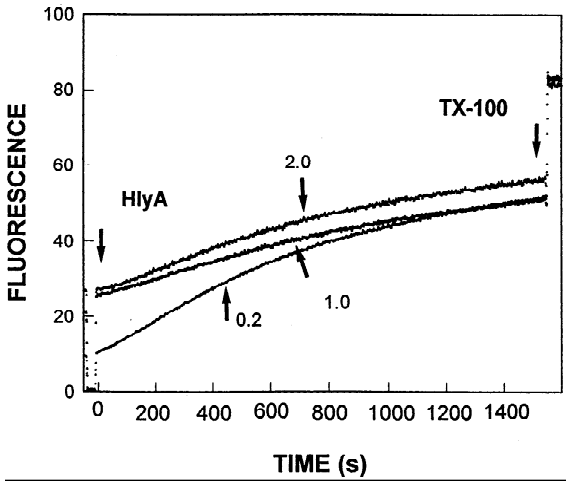

Fig. 1. The effect of ionic strength of the medium on HlyA-induced liposomal leakage. Large unilamellar vesicles consisting of PC (0.1 mM) were prepared in buffers of varying ionic strength in the presence of ANTS/DPX and treated with HlyA (2000 U/ml) at time 0. 100% leakage is obtained by addition of detergent. Plots are autoscaled to a common 100% value.

Table 1. Effect of the ionic strength of the aqueous medium on HlyA self-aggregation, binding to vesicles and HlyA-dependent vesicle leakage

Osmolality[a]	Aggregate size (nm)[b]	Bound protein[c]	Leakage[d] (%)	$t_{1/2}$ (sec)
0.2	573	1.87	59	446
1.0	610	0.73	44	576
2.0	626	0.23	34	624

[a] Changes in osmolarity are achieved through changes in NaCl concentration. See Materials and Methods for details on buffer composition.
[b] Average size of protein aggregates in the corresponding buffer, in the absence of vesicles.
[c] Vesicle-bound protein, expressed as protein:lipid molar ratio × 10^4.
[d] Percent leakage after 15 min. Hemolytic activity: 1600 U/ml. Lipid concentration: 0.1 mM.

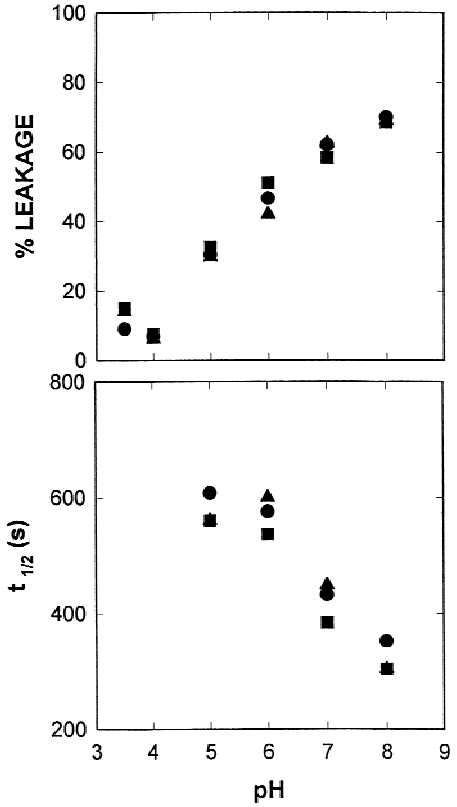

Fig. 2. The effect of pH on HlyA-induced liposomal leakage. (*A*) Extent of leakage, measured as % leakage after 15 min. (*B*) Half-times for leakage. Data points corresponding to three independent experiments are shown. Lipid was 0.1 mM and hemolytic activity, 2050 U/ml.

An important observation is that a decrease of about one order of magnitude, due to the change from 0.2 to 2.0 osm in ionic strength, in the vesicle protein:lipid ratio leads to a decrease of less than 50% in the extent of leakage, and to a similar increase in $t_{1/2}$ (Table 1). This lack of correlation between changes in toxin binding and changes in liposomal leakage has been repeatedly made in experiments described in this and previous papers (Ostolaza & Goñi, 1995), and will be discussed below.

Effect of pH

PC vesicles loaded with ANTS/DPX were prepared in a series of Tris/acetic acid buffers, pH 3–8, at constant ionic strength. The fluorescence properties of ANTS-DPX allow performing measurements of leakage throughout this pH range (Ellens et al., 1985). Leakage induced by α-haemolysin was tested in each case, the results are summarized in Fig. 2. The extent of leakage is maximum at the highest pH, and reaches a minimum at pH 4, i.e., near the isoelectric point of the protein (pH ≈ 4.1) (Fig. 2*A*). The half-times for leakage vary inversely, with the longest at pH values near the isoelectric point

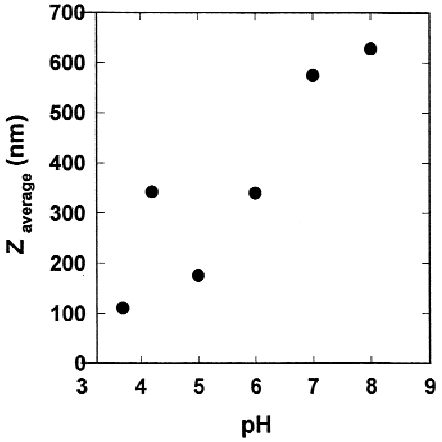

Fig. 3. The effect of pH on HlyA self-aggregation properties. Average sizes of HlyA aggregates in the absence of vesicles are plotted as a function of pH.

(Fig. 2*B*). These results would be interpreted most easily considering that at the isoelectric point the tendency of the protein to form aggregates was maximal, thus the concentration of the membrane-active species (monomer) would be lowest and leakage minimal.

In practice, the situation is more complicated, as shown in Fig. 3, where the average diameter of the HlyA aggregates in buffer, in the absence of vesicles, has been plotted as a function of pH. At the isoelectric point (pH ≈ 4.1) there is a local maximum in particle diameter, but even larger aggregates are detected at pH 7 and 8. This may be explained by assuming that pH has, among others, the effect of changing the protein conformation and, with it, the amphipathic character of α-haemolysin. Neutral and alkaline pHs would make the protein more amphiphilic, thus favoring its aggregation. Furthermore, aggregation at neutral pH must be essentially different from that caused by high ionic strength or by isoelectric pH conditions, since in the former case high aggregation is accompanied by high lytic activity (Fig. 2) and the opposite occurs under the latter conditions. One possible reason for that difference is that the monomeric species giving rise to the aggregates are different in one and the other case. This is supported by data on the intrinsic fluorescence of the protein under varying ionic strength and pH conditions. Changes in ionic strength do not modify the fluorescence emission intensity ratio F330/F350, nor the maximum wavelength of fluorescent emission λmax, both known to detect changes in the microenvironmental polarity of the Trp residues (Wharton et al., 1988; Ostolaza & Goñi, 1995) (Fig. 4*A*). However a remarkable pH effect on both parameters is detected (Fig. 4*B*), that might be indicating a conformational change, or a polarity change in the vicinity of the tryptophanyl fluorophores, any of which could in turn influence protein aggregation. Fluorescence studies

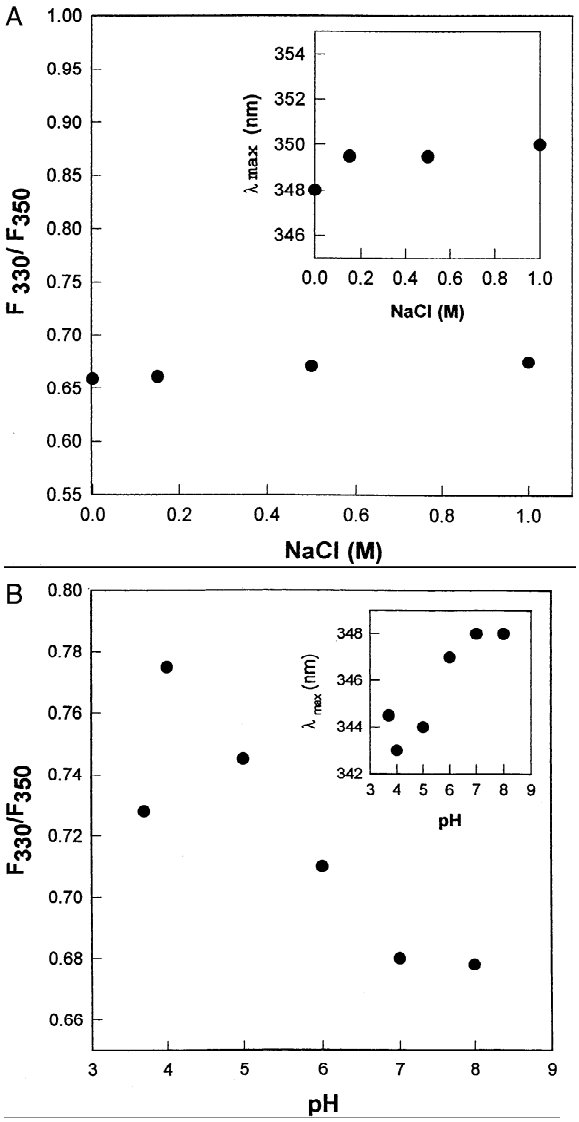

Fig. 4. Dependence of spectral parameter (F330/F350) of HlyA intrinsic fluorescence with (*A*) Ionic strength and (*B*) pH. Insert: ionic strength and pH dependence of maximum emission wavelength. Protein concentration 10 μg/ml.

show that at pH 7–8 the protein displays a low F330/F350 ratio and, at the same time, HlyA exhibits high bilayer binding and high membrane lytic activity (Table 2). We propose that, under these conditions, the protein is in an optimum conformation for interacting with membranes, that we define as ''competent state.''

Proteolysis is an alternative method to investigate pH-induced conformational changes. Papain proteolytic activity is known to be relatively unaffected by pH in the 3–7 range. Figure 5 shows increased HlyA digestion by papain at pH 7.0 relative to pH 3.7. Papain detects an additional site or sites that become exposed at high pHs. Very little digestion is found below pH 5.

Table 2. Effect of pH on HlyA self-aggregation and binding to vesicles and HlyA-dependent vesicle leakage

pH	Aggregate size (nm)[a]	Bound protein[b]	Leakage[c] (%)	$t_{1/2}$ (sec)
7.0	573	1.87	59	446
5.0	176	1.02	42	576
3.7	110	0.74	31	n.d.[d]

[a] Average size of protein aggregates in the corresponding buffer, in the absence of vesicles.
[b] Vesicle-bound protein, expressed as protein:lipid molar ratio × 10^4.
[c] Percent leakage after 15 min. Hemolytic activity 1600 U/ml. Lipid concentration: 0.1 mM.
[d] Not determined. Half-time too long and leakage too small for accurate estimation.

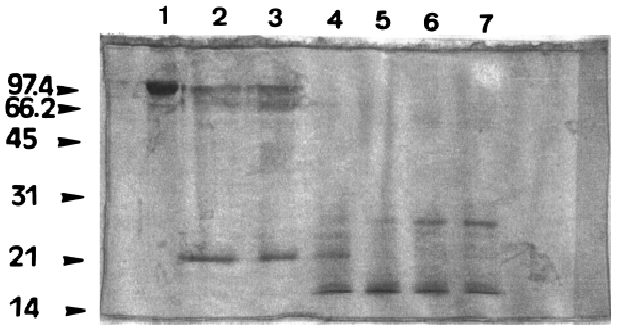

Fig. 5. Effect of pH upon proteolysis of HlyA by papain. lane 1: HlyA control, lanes 2–7: HlyA digested by papain at pH 3.7, 4, 5, 6, 7 and 8 respectively.

The relationship between bilayer disruption, protein aggregation and protein binding to bilayers at various pHs can be seen in detail in Table 2. Again the extent of leakage decreases with the protein/lipid ratio in the vesicles, as seen for the effect of ionic strength (Table 1). The paradoxal increase in HlyA aggregate size and simultaneous increase in vesicle binding at pH 7 is explained by the hypothesis of the competent state as discussed above.

Effect of Bilayer Surface Charge

Phosphatidylcholine is a zwitterionic phospholipid, thus bilayers containing only this phospholipid do not have a net electric charge. The effect of bilayer surface charge on HlyA insertion and subsequent leakage was tested by preparing mixed bilayers of phosphatidylcholine and phosphatidylinositol (PI) (net negative charge) or stearylamine (SA) (net positive charge). The results are summarized in Fig. 6. At pH 7.0, i.e., when HlyA has a net negative charge, and at the low protein concentration, a given amount of toxin produces decreasing amounts of leakage on PC:SA (9:1), pure PC and mixed PC:PI (1:1)

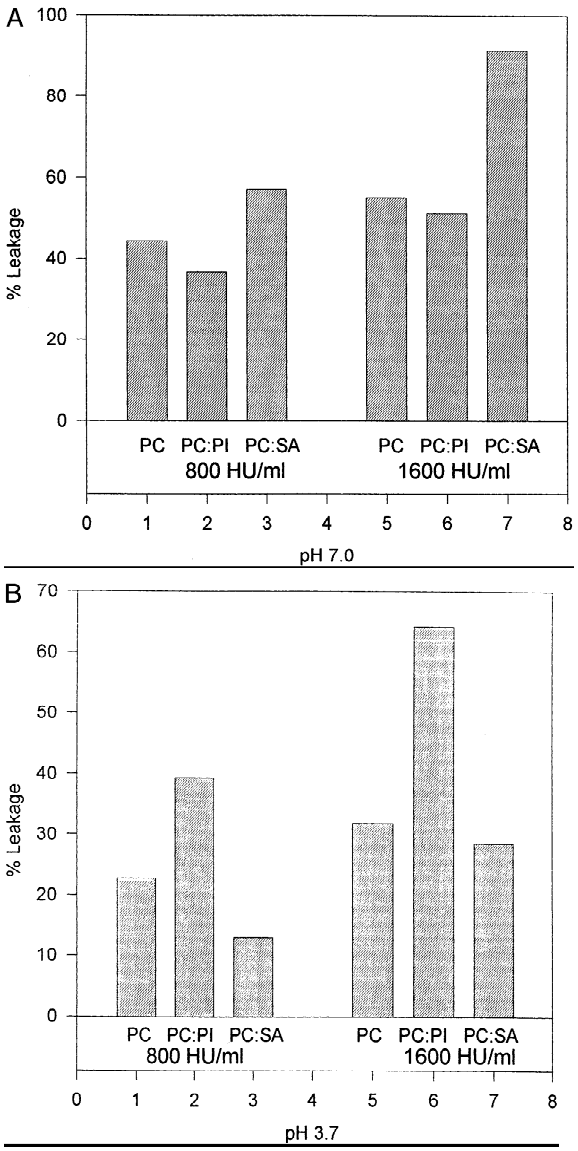

Fig. 6. The effect of surface charge on HlyA-induced liposomal leakage (after 15 min toxin addition). (*A*) pH 7.0. (*B*) pH 3.7. Lipid concentration was 0.1 mM. Results with 800 and 1600 U/ml haemolysin are shown.

membranes respectively (Fig. 6*A*). At the high protein concentration the neutral and the negatively charged bilayers become equally sensitive to the toxin. Conversely, at pH 3.7, when the protein has a net positive charge, PC:PI (9:1) bilayers are more sensitive than the neutral or positively charged ones (Fig. 6*B*). In summary, a surface charge opposite to that of the protein enhances leakage, while a bilayer surface charge of the same sign impairs the lytic action (although the latter effect may be abolished by high protein concentration). These results can also be interpreted in the light of both hydrophobic and electrostatic forces acting together on

Table 3. Effect of bilayer surface charge on HlyA binding to vesicles and HlyA-dependent vesicle leakage

Bilayer composition	pH	Net surface charge	Net protein charge	Bound protein[a]	Leakage (%)[b]
PC	7.0	0	−	1.87	55
PC/PI (1:1)	7.0	−	−	1.00	51
PC/SA (9:1)	7.0	+	−	3.26	91
PC	3.7	0	+	0.74	32
PC/PI (1:1)	3.7	−	+	1.85	64
PC/SA (9:1)	3.7	+	+	n.d.	29

[a] Vesicle-bound protein, expressed as protein:lipid molar ratio $\times 10^4$.
[b] Percent leakage after 15 min. Hemolytic activity 1600 U/ml. Lipid concentration: 0.1 mM.

α-haemolysin insertion. The nonreversible character of toxin insertion may explain why unfavorable electrostatic interactions (PC:PI bilayers at pH 7.0, PC:SA bilayers at pH 3.7) may be overcome by higher toxin concentrations.

The role of bilayer surface charge on toxin binding is clearly demonstrated by the data in Table 3. The amount of bound protein is obviously related to the net surface charge. However, as in the previous cases, an increased amount of bound protein does not necessarily lead to an increased leakage, see e.g., the case of PC and PC:PI vesicles at pH 7.0 (Table 3).

Vesicles composed of PC:PI (9:1) and (3:1) showed only quantitative differences with respect to those consisting of PC:PI (1:1). Vesicles containing more than 10 mol% stearylamine were not stable, thus they were not used in these studies.

Discussion

Electrostatic *vs.* Hydrophobic Interactions

An essential element for understanding the process of α-haemolysin interaction with membranes is the fact that α-haemolysin, being an amphipathic protein, occurs in aqueous media in the form of large aggregates, much as detergents exist in micellar form (Ostolaza et al., 1991). Mature α-haemolysin contains fatty acyl residues (Issartel et al., 1991; Stanley et al., 1994) but the nonacylated precursor prohaemolysin also forms aggregates (Soloaga et al., 1996). α-Haemolysin appears to interact with the membranes as a monomer (Bhakdi et al., 1986; Ostolaza et al., 1991), thus the following equilibria appear to exist: aggregate ⇄ monomer (in the absence of vesicles) and aggregate ⇄ monomer ⇄ membrane-bound (in the presence of liposomes). In the absence of vesicles, the equi-

librium is shifted towards aggregation by high ionic strength conditions, by high pH (6–8) and by the absence of net charges in the protein, i.e., at the isoelectric point (Tables 1, 2, Fig. 3). The monomeric species involved in aggregation are probably different in nature according to the aggregation inducing agent: (*a*) the observation of increased aggregation under high ionic strength conditions suggests that dispersion and/or hydrophobic forces are involved in the process; (*b*) aggregation at the isoelectric pH is also typical of soluble proteins, and indicative of the repulsive electric charges, and (*c*) the complex shape of the ''aggregate size *vs.* pH'' plot of Fig. 3, with a maximum at pH ≈ 4.1 (i.e., the isoelectric point) and a new and larger increase in size at neutral pH is interpreted by us as a pH dependent conformational change.

The observed changes in intrinsic fluorescence (Fig. 4) can be due either to a protein conformational change or else to local changes in the microenvironment of the tryptophanyl residues. However, the concomitant increase in fluorescence, in aggregate size and in susceptibility to papain digestion at pH >5 (Figs. 3–5) support preferentially the hypothesis of a significant change in protein conformation, that could lead to the exposure of hydrophobic patches. Under these conditions the protein would be in a ''competent state'' for insertion. However, in the absence of membranes those monomers would tend to aggregate.

In the presence of vesicles, or membranes, the availability of highly hydrophobic environment displaces the aggregate ⇄ monomer ⇄ membrane-bound double equilibrium towards the right–hand side, and the fact that the membrane offers, in principle, a nonpolar medium of virtually infinite size, makes still feebler the electrostatic interactions. This explains why at neutral pH, a condition favoring large protein aggregates in the absence of vesicles, α-haemolysin binding to bilayers is maximal (Table 2): the protein has a large exposed hydrophobic surface, and the bilayer provides room for accommodating the toxin.

The presence of acting electrostatic forces, and their ancillary role with respect to hydrophobic interactions, is clearly shown in the experiments in which a net surface charge is introduced in the bilayer (Fig. 6, Table 3): electrostatic attraction favors protein binding, while electrostatic repulsion makes it more difficult. However, when leakage is considered, only electrostatic attraction appears to be significant, repulsion being superseded by the hydrophobic forces (or by the irreversible nature of protein insertion) when protein concentration is high enough (Fig. 6). Benz et al. (1989) in studies carried out on planar bilayers have observed higher conductance values with increasing pH of the solution in agreement with our results. However, Menestrina (1988) described that α-haemolysin is more active at low pH (<6) against PC:PS bilayers, and that the toxin produces a larger release of aqueous contents (i.e., calcein in that case) when bilayers contain negatively charged lipids (PS or PI) at pH 5.0. These results are difficult to interpret in the light of the above hypothesis, but it should be noted that Menestrina (1988) uses small unilamellar vesicles, structures whose inherent metastability is well known (Gershfeld, 1978), and that in his case the insertion of a toxin monomer is enough to break down the vesicle, all of which suggests a mechanism different from the one operating in large unilamellar vesicles and cell membranes, in which toxin oligomerization in the bilayer is believed to be an essential step.

The role of electrostatic attraction in HlyA vesicle binding and lysis is particularly noteworthy at pH 3.7, i.e., when the protein in solution is not in a ''competent state.'' In this case, the data in Table 3 show how the opposite electric charge in the PC:PI bilayer successfully overcomes the protein noncompetent state, so that high binding and lysis follow. Similar cases in which protein conformation and/or activity is modulated by lipid bilayers have been published (Muga et al., 1991, 1993; Bañuelos & Muga, 1996).

Membrane Binding, Protein Insertion and Vesicle Leakage

Membrane disruption by α-haemolysin may be dissected into several steps, as follows: (*a*) adoption, in solution, of a conformationally competent state, (*b*) receptor binding, suggested by some authors (e.g., Benz et al., 1989) although not essential for lysis to occur (Ostolaza et al., 1993), (*c*) binding to the membrane bilayer (Ostolaza & Goñi, 1995), (*d*) unknown intermediate steps, including perhaps oligomerization in the bilayer (Ludwig, Benz & Goebel, 1993; Ostolaza et al., 1993), (*e*) impairment of the membrane barrier function. Ostolaza and Goñi (1995) showed that membrane binding does not automatically lead to lysis; the protein must contain bound Ca^{++} prior to binding the membrane for lysis to occur. In addition, there was no correlation between the amount of membrane-bound toxin and the extent of lysis. Such a disparity has been repeatedly observed in the experiments described in this paper. Obviously, the phenomena occurring between membrane binding and membrane disruption are still poorly understood. Recent results from Bakás et al. (1996) constitute a further step in the dissection of the process. They have shown that under the parameter ''amount of bilayer-bound toxin'' lie two different realities, namely the reversible adsorption of α-haemolysin to the bilayer surface, and the irreversible insertion of the protein into the hydrophobic matrix of the membrane. Insertion, and not the mere adsorption, would be a prerequisite for lysis. A somewhat similar situation was described for α-toxin by Tomita, Watanabe & Yasuda (1992). Although a precise quantitation of

these two processes is not available at present, their existence provides an explanation for the quantitative differences and lack of correlation between bindin (reversible + irreversible) and lysis observed in this (Tables 1–3) and the previous paper (Ostolaza & Goñi, 1995).

In the context of the physiological conditions under which α-haemolysin is supposed to act, it should be noted that the protein will have, at pH ≈ 7.4, a net negative charge, and that the net charge of biomolecules in the cell surface is usually of the same sign. This would also speak in favor of a receptor (e.g., a protonated aminosugar) to which α-haemolysin monomers could bind prior to their adsorption to the membrane surface.

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