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E. coli α-hemolysin: a membrane-active protein toxin

F.M. Goñi and H. Ostolaza

Grupo Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, Bilbao, Spain

Abstract

α-Hemolysin is synthesized as a 1024-amino acid polypeptide, then intracellularly activated by specific fatty acylation. A second activation step takes place in the extracellular medium through binding of Ca^{2+} ions. Even in the absence of fatty acids and Ca^{2+} HlyA is an amphipathic protein, with a tendency to self-aggregation. However, Ca^{2+}-binding appears to expose hydrophobic patches on the protein surface, facilitating both self-aggregation and irreversible insertion into membranes. The protein may somehow bind membranes in the absence of divalent cations, but only when Ca^{2+} (or Sr^{2+}, or Ba^{2+}) is bound to the toxin in aqueous suspensions, i.e., prior to its interaction with bilayers, can α-hemolysin bind irreversibly model or cell membranes in such a way that the integrity of the membrane barrier is lost, and cell or vesicle leakage ensues. Leakage is not due to the formation of proteinaceous pores, but rather to the transient disruption of the bilayer, due to the protein insertion into the outer membrane monolayer, and subsequent perturbations in the bilayer lateral tension. Protein or glycoprotein receptors for α-hemolysin may exist on the cell surface, but the toxin is also active on pure lipid bilayers.

Key words
- α-Hemolysin
- Bacterial toxins
- RTX toxins
- Lipid-protein interactions
- Calcium-binding proteins
- Model membranes

Correspondence
F.M. Goñi
Grupo Biomembranas
(Unidad Asociada al C.S.I.C.)
Departamento de Bioquímica
Universidad del País Vasco
Apartado 644
48080 Bilbao
Spain
Fax: +34-4-4648500
E-mail: gbzoseth@lg.ehu.es

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Introduction

E. coli α-hemolysin (Figure 1) is a major virulence factor for some pathogenic strains of the bacterium involved in human extraintestinal diseases like urinary tract infections, peritonitis, meningitis and septicemia (1). α-Hemolysin owes its name to the fact that it causes hemolysis, or lysis of red blood cells, and this is indeed the basis for its most commonly used assay. (The prefix α, that is often omitted nowadays, indicated originally the extracellular form of the toxin). In spite of its name, α-hemolysin is widely believed to act mainly by attacking the immune system cells of the host, usually without inducing cell lysis, yet severely impairing their function (2). In any case, cell membranes appear to be the primary targets of the toxin. As done in many other studies of membrane biology, model membranes have been extensively used in the elucidation of the cell membrane effects of α-hemolysin. Figure 2 shows an example of such an application, an experiment in which large molecular weight molecules leak out from phospholipid vesicles under isotonic conditions by the effect of α-hemolysin. The interaction of this toxin with model and cell membranes is the main subject of this review.

Biosynthesis and structure

α-Hemolysin is the prototype of a family

of homologous proteins (mainly toxins) secreted by Gram-negative bacteria, e.g. *Escherichia, Proteus, Morganella, Pasteurella, Actinobacillus, Bordetella* (3-10). These toxins have a number of features in common, conferred by a common genetic structure comprising four linked genes (*hly* in the case of *E. coli* hemolysin) that are essential for toxicity. These encode four proteins termed C, A, B, D. A is the cytotoxin that is primarily synthesized as the inactive protoxin. Its maturation occurs via a post-translational acylation at an internal Lys residue, mediated by C (11). In the case of α-hemolysin, two Lys residues are modified, K564 and K690 (12,13). The mature toxin is then exported through a peculiar secretion pathway involving the protein gene products of B and D, as well as some other membrane components (14-17). The signal for secretion is contained in the C-terminal end of the protein (18), unlike the more common case of proteins exported through the *E. coli sec* system (or equivalent systems in related bacteria), that contain the secretion signal in their N-terminal ends. The use of a unique secretion pathway by this family of toxins has attracted the attention of biotechnologists, who have made use of the *hly* or related export systems for the extracellular transport of the products of genetic constructs involving the C-terminal end of hemolysin A (HlyA) or homologous genes (19-21). Although α-hemolysin is the modified or mature form of the *hly*A gene product, and not the gene product as such, it is still commonly designated in an abbreviated form as HlyA.

An additional important feature of the hemolysin family of proteins is that they contain a remarkable structure, namely a Gly-rich nonapeptide motif that is repeated in tandem between 9 and 42 times (22-27). This repeated motif has earned for the family the name of RTX (Repeat in ToXin) toxins. The nonapeptide repeats constitute a domain that binds Ca^{2+} ions (28-30), as will be detailed below for the case of HlyA.

HlyA consists of a long polypeptide chain (1024 amino acids) with which a number of carbohydrates are associated (31,32). Little is known of the biosynthesis or structure of the sugar moiety, that may be related to the recognition and docking of the toxin to the target membrane. Membrane damage is the direct result of the polypeptide activity, since mutations involving one or two amino acids in certain positions render the protein inactive (33).

Different mutational and structure prediction studies have helped to identify several regions or domains within the HlyA sequence (Figure 1): a) a putative amphiphilic α-helix at the N-terminal end that could interact with the cell membrane (34). Deletion of residues 9 to 37 confers an increased hemolytic activity on the toxin. b) The hydrophobic region (residues 240 to 400). Three separate hydrophobic domains have been predicted in this region in the past. The first one (residues 240 to 260) may contain only one polar residue (Asp243) that would be essential for the lytic activity (35). Substitutions of polar for nonpolar residues in the second (299-327) or third (370-400) of these hydrophobic domains would also lead to inactivity (33,35). A barrel structure formed by 8 α-helices has been proposed for

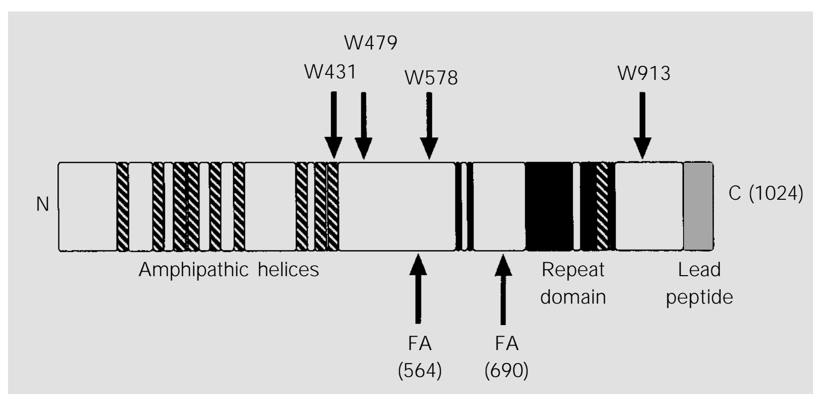

Figure 1 - Schematic representation of the primary structure of E. coli α-hemolysin. The positions of the four tryptophan residues (W) and of the two fatty acids (FA) are indicated. Obtained from data by Felmlee et al. (22), Stanley et al. (89), Ostolaza et al. (37) and Brasseur R (unpublished results). The repeat nonapeptides are represented by black vertical bars, while the striped bars correspond to predicted amphipathic helices.

the hydrophobic region (35). However, the use of more advanced prediction techniques has led to a different picture of the hydrophobic region. Instead of three hydrophobic domains, 9 amphipathic helices are proposed in this region (Figure 1) (Soloaga A, unpublished results). Amphipathic α-helices are ideally suited for interaction with membranes (113). c) Two regions around Lys564 and Lys690 that allow HlyA recognition by HlyC in the process of toxin activation (36). d) The calcium-binding domain (residues 723-872) consisting of 15 repeats of a nonapeptide whose consensus sequence is GGXGX DXUX, where U is a bulky, nonpolar residue and X can be any amino acid. Two additional repeats are located at 615-623 and 633-641, respectively (37). Deletion of three or more repeats leads to a complete loss of activity (33). The three-dimensional structure of this domain is believed to be similar to that of the homologous region of a member of the RTX family, alkaline protease from *Pseudomonas aeruginosa*, whose crystal structure has been resolved by X-ray diffraction (38). e) The secretion signal at the C-terminal end (39).

Also significant from the structural point of view, and particularly because of their usefulness in spectroscopic studies, are four Trp residues located at positions 432, 480, 579 and 914. W432 and W480 are especially important because of their location close to the hydrophobic region.

Membrane-toxin interaction: an overview

In this section we shall discuss briefly the general problem of membrane-toxin interaction, thus setting a frame for the specific discussion on α-hemolysin that follows.

Membrane proteins have been operationally divided into integral and peripheral, according to their mode of interaction with the lipid bilayer, integral (or intrinsic) proteins interacting with the bilayer hydrophobic matrix while peripheral (or extrinsic) proteins are mainly bound to the polar headgroups of phospholipids and to the polar domains of intrinsic proteins (40). Of course this is an equilibrium view of membrane proteins, since proteins are synthesized in ribosomes outside the membranes, and the corresponding polypeptides must then be directed to their proper location in the membrane. Still for most membrane proteins their whole functional lives occur in the membrane, their extramembraneous existence being only a transient step (often the nascent polypeptide begins its membrane insertion before ribosomal synthesis is completed). The situation is different for a number of protein toxins, as well as other proteins that have been described as “amphitropic” (41). These toxins exist as “soluble” proteins, unrelated to membranes, until they bind their target. Only then do they become, for all practical purposes, membrane proteins, often of the intrinsic type.

This change from soluble to integral or

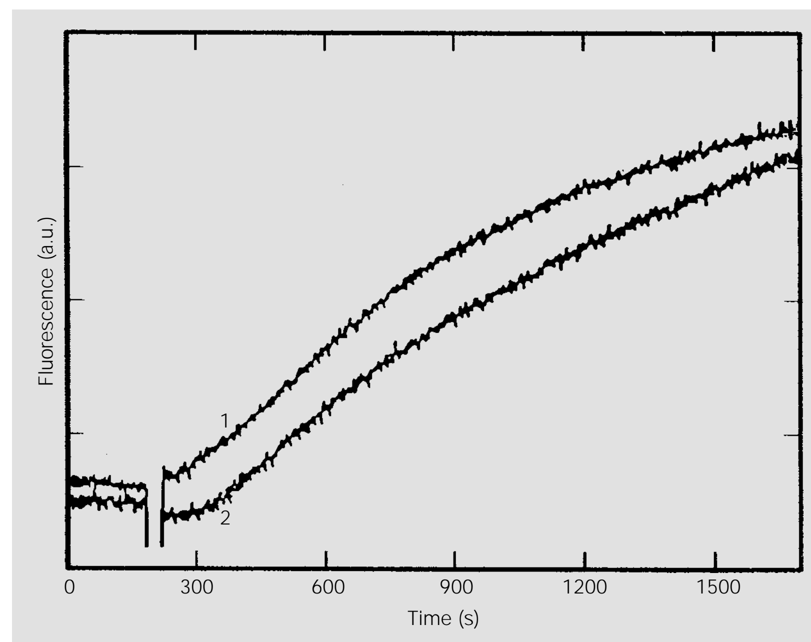

Figure 2 - α-hemolysin-induced release of FITC and FITC-dextrans. Curve 1: Release of FITC-dextran (Mr = 17200). Curve 2: experiment as in curve 1, except that nonfluorescent dextran had been added to the extravesicular buffer until the osmotic pressure exactly matched that of the vesicle contents.

intrinsic membrane proteins is very interesting from the point of view of protein conformation and of membrane-protein interactions. It is certainly a complex process, in which various stages can be distinguished: i) a conformational transition that brings the protein in solution to a state "competent" for insertion, ii) adsorption of the activated protein onto the membrane surface, and iii) insertion into the bilayer (42-48). These stages may not necessarily occur in this order. Individual toxins may act in very different ways, e.g., the competent state may require acidic pH, as in colicin A (49), or neutral pH, as in perfringolysin (50). Colicin A adsorbs only onto negatively charged surfaces (43,51) while perfringolysin or colicin E1 requires electrically neutral bilayers (47). The last stage, insertion, is the least understood. For some toxins, e.g. aerolysin (52) or *S. aureus* α-toxin (53), an oligomerization process appears to be involved.

A conformational change prior to insertion appears to be a rather common phenomenon. The pore-forming domain of colicin A suffers a "collapse of the native tertiary structure", although "a large proportion of the helical secondary structure remains preserved" (43). In *Pseudomonas* exotoxin A, "denaturation-like conformational changes appear to play an important role in membrane insertion" (54). Also in the non-toxin protein α-lactalbumin, that may be either adsorbed to or inserted into membranes (48), a flexible structural intermediate must form in solution for membrane insertion to occur. In these examples, the conformational change is brought about by pH or temperature. In *E. coli* α-hemolysin, as in the adenylate cyclase toxin from *Bordetella pertussis* (55), and perhaps in other members of the RTX family, the process is triggered by Ca^{2+}, as will be discussed in the next section.

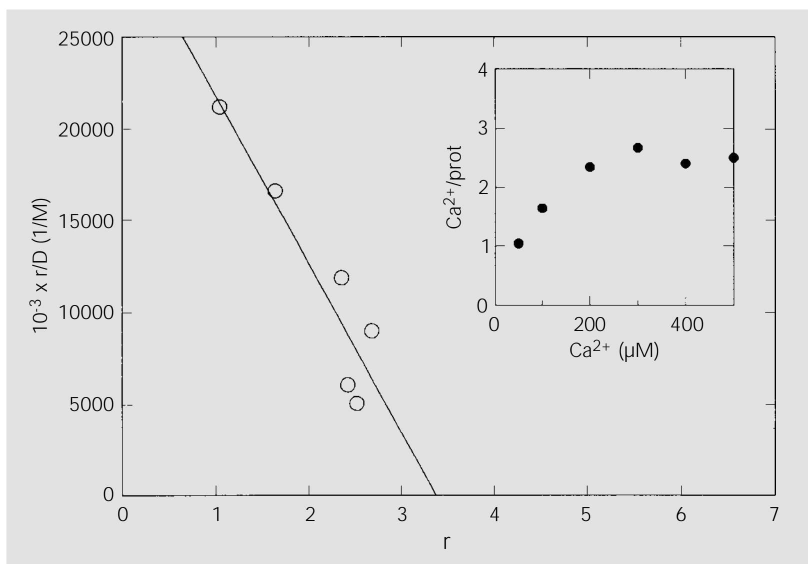

Figure 3 - $^{45}Ca^{2+}$-binding to α-hemolysin. Scatchard plot. The slope corresponds to a Kd = 0.11 mM. Inset, Direct plot. The protein concentration was 1 µM.

Ca^{2+} binding to HlyA and membrane lysis

The presence of Ca^{2+} ions is an essential requirement for the lytic activity of HlyA both in model and cell membranes (31,56). Ca^{2+} concentrations of the order of at least 100 µM are required for optimal activity. Sr^{2+} or Ba^{2+} also support the lytic activity, although at higher concentrations (31). Other divalent cations (e.g., Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}) do not support HlyA activity. The data are in accordance with early observations of Ca^{2+} requirements (57-59). However, other reports appeared to suggest that Ca^{2+} was not required for the hemolytic action (60-62). Nevertheless, careful examination of the experimental procedures in the latter series of studies revealed that, in those cases, cell growth occurred in the presence of millimolar Ca^{2+} concentrations. Boehm et al. (63) had already noticed that the hemolytic activity of culture supernatants containing α-hemolysin was calcium-independent when the growth medium had been supplemented with Ca^{2+}. According to our own data, when 10 mM Ca^{2+} is present either in the cell growth medium or in the purification buffers, or in both, cell and liposome lysis occurs even in the absence of Ca^{2+} in the assay

medium. Only when Ca^{2+} is absent (or present only in micromolar concentrations) throughout the whole procedure, i.e., cell growth, protein purification and lysis assay media, does lysis fail to occur (56).

Direct measurements of $^{45}Ca^{2+}$ binding to the toxin revealed that each molecule of α-hemolysin has three independent and identical binding sites for Ca^{2+} (Figure 3) (31). These observations must be interpreted in the light of current structural knowledge. Specifically, the three-dimensional structure of the alkaline protease of *P. aeruginosa* has been resolved (38) and it is reasonable to assume that the above experimental data can be interpreted in the light of the structure of the Ca^{2+}-binding C-terminal domain of that protein.

Two particularly relevant properties of the *P. aeruginosa* protein are: a) the parallel ß-roll structure that constitutes the core of the Ca^{2+}-binding domain is built of a succession of nonapeptide motifs, each of them providing two half-sites for Ca^{2+} binding, so that in the protease seven sequence motifs are found, contributing to the binding sites of five Ca^{2+} molecules (two or three additional Ca^{2+} sites are found in other regions of this domain), and b) of the five Ca^{2+} molecules in the central region of the ß roll, two located at sites 6 and 7 (being peculiar in having one or two H_2O ligands in their octahedral structures) can be easily exchanged with Sr^{2+}. Since α-hemolysin has up to 17 nonapeptide repeats, a large number of Ca^{2+}-binding sites is to be expected. In fact, in the presence of 3 M urea, when the protein is presumably in a partially unfolded conformation, the binding of up to 12 $^{45}Ca^{2+}$ molecules/protein molecule has been detected (Soloaga A, unpublished results). We suggest that the three Ca^{2+} sites revealed by Scatchard analysis in the absence of urea correspond to the easily exchangeable Ca^{2+} on α-hemolysin, i.e., they are equivalent to sites 6 and 7 in the *P. aeruginosa* protease (38).

The calcium-binding properties of α-hemolysin have also been studied in other laboratories. Ludwig and co-workers (64) have performed a number of specific mutations in the repeat domain, followed by cell binding competition studies between the native and mutated forms of HlyA. They concluded that the calcium-binding domain is essential for a calcium-dependent binding of HlyA to red blood cells. In particular, Ca^{2+}-binding would be essential for binding of the repeat domain to a receptor located on the red blood cell surface (64). Such a receptor is not essential for HlyA action, since the protein is also active on pure phospholipid liposomes (65), yet several data may suggest the presence of an α-hemolysin receptor in cells. Ludwig et al. (64) found as well that mutants in the repeat domain that could not bind erythrocytes were still capable of forming ion-permeable channels in artificial lipid bilayers in the complete absence of Ca^{2+}. The significance of these observations on the channel-forming capacity of HlyA will be discussed below (see section: "A model for HlyA interaction with membranes"). Boehm et al. (29) had also found that Ca^{2+} was necessary for HlyA binding to erythrocytes according to immunoblotting assays. However, as discussed in the next paragraph, HlyA "bound" in the absence of Ca^{2+} may be interacting with the membranes in a reversible way, thus in danger of becoming "washed out" in the preparation of the immuno-SDS-PAGE gels.

Our data using large unilamellar vesicles consisting of pure phosphatidylcholine demonstrate binding of HlyA both in the presence and in the absence of Ca^{2+}. Binding has been measured directly by a density gradient centrifugation technique, as well as through an increase in the protein intrinsic fluorescence that is attributed to the protein interaction with the lipid bilayer (56). Both procedures revealed similar extents of toxin binding in the presence as well as in the absence of Ca^{2+}. However, calcium ions may be modifying qualitatively, if not quantitatively, the

binding of HlyA to membranes, as discussed in the next two sections.

Reversible adsorption and nonreversible insertion of HlyA in bilayers

By separately measuring toxin binding to membranes and toxin-induced lysis, Ostolaza and Goñi (56) found that HlyA binding to liposomes was not necessarily followed by membrane damage. In particular, HlyA was found to bind bilayers with about the same affinity in the presence or absence of Ca^{2+}. However, only when the protein had been preincubated with this cation did the lytic effect follow toxin binding. In a further study, Bakás et al. (66) applied to α-hemolysin a procedure developed by Tomita et al. (67) that allowed the distinction between reversibly and irreversibly bound HlyA (Figure 4). The method consists essentially of incubating liposomes in the presence of toxin, then washing to remove the non-bound protein. Liposome-bound HlyA is then incubated with red blood cells, for which HlyA has a great affinity, perhaps because these cells contain a receptor on their surface. The protein toxin molecules that are reversibly bound to the liposomal membrane can bind and lyse erythrocytes, while the irreversibly-bound ones cannot.

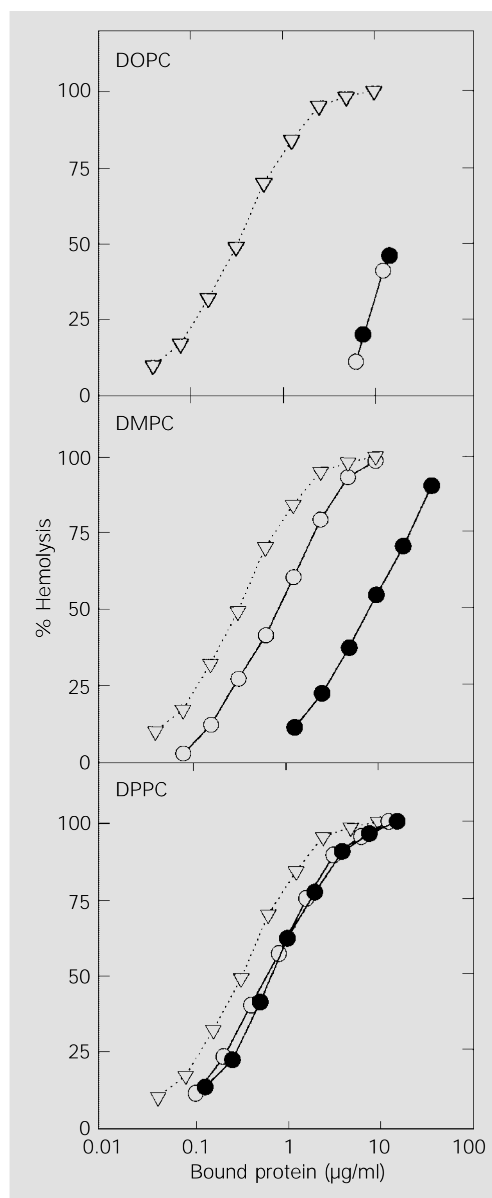

Figure 4 - Accessibility of liposome-bound α-hemolysin to red blood cells. HlyA was incubated for 30 min at 0ºC (open circles) or 37ºC (closed circles) with multilamellar liposomes of dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), or dipalmitoylphosphatidylcholine (DPPC), as indicated. The resulting liposome-protein complexes were further incubated with horse erythrocytes and the hemolytic activity was recorded. Dotted line: Hemolytic activity of native toxin, i.e., preincubated in the absence of liposomes and then added to the erythrocyte suspension.

Using this procedure, Bakás et al. (66) were able to show that α-hemolysin may bind the membranes in at least two ways, a reversible adsorption and an irreversible insertion. Comparison of irreversible binding and cell lysis results demonstrated that not even irreversible insertion leads necessarily to membrane damage. A further phenomenon appears to be required, that occurs only in the presence of Ca^{2+}.

Measurements of HlyA insertion into bilayers formed by a variety of single phospholipids, or binary mixtures of phospholipids, or of phospholipid and cholesterol, allowed Bakás et al. (66) to reach the conclusion that irreversible insertion of the toxin was favored by fluid over gel states, by low over high cholesterol concentrations, by disordered liquid phases over gel or ordered liquid phases, and by gel over ordered liquid phases. These results are relevant to the mechanism of action of α-hemolysin, and provide new insights into the membrane insertion of large proteins. Jain and Zakim (68) have pointed out the requirement that hydrophobic regions of the bilayer become transiently exposed to the aqueous phase for

protein incorporation to occur. Such transient exposure would be favored by overall changes in the bilayer properties, such as fluidity (69), or by particular properties of a localized microenvironment, or defects (68). The requirement of fluid bilayers for insertion is such that, even if gel and fluid phases may coexist, the proportion of irreversibly bound protein decreases with the fluid phase fraction (66). This is in agreement with the preferential partitioning of gramicidin A' in fluid phospholipid phases coexisting with gel phases (70). The uneven distribution of a transmembrane peptide into different coexisting lipid domains has also been described by Zhang et al. (71). Moreover, Polozov et al. (72) described the insertion of two amphipathic peptides in zwitterionic phospholipid bilayers in the fluid, but not in the gel state; thus the preference for fluid bilayers by the peptides to be inserted appears to be a rather general and predictable phenomenon.

Defects and intrinsic instabilities in the bilayer may promote protein insertion because defect sites may accommodate a protein molecule without inducing additional energetically unfavorable general disorder, desolvation, or lateral compression (68). Local defects may arise from lipid mixtures (73), or from the presence of impurities (e.g., detergents) or other proteins, etc. The results reported by Bakás et al. (66) show that conditions under which local defects are likely to occur (e.g., disordered liquid state) do favor irreversible protein insertion. The overall effect of cholesterol is to make protein insertion more difficult, probably because its rigid structure does not help to accommodate the rough protein surface. Note also that cholesterol tends to increase bilayer thickness (74,75) and integral proteins tend to partition into domains of a given thickness (76). The properties of cholesterol are evident in the cholesterol-rich ordered liquid phases, which support very little insertion (66). Almeida et al. (77) have explained this phenomenon in terms of cholesterol occupying free volume in the bilayer.

Moreover, the irreversible binding data are very similar for the ordered liquid L_o phase at 10°C, whereas the recovery of hemolytic activity is much higher at the higher temperature (66). Bretscher and Munro (76) have proposed that changes in cholesterol concentration in cell membranes may induce segregation of domains with high and low cholesterol concentrations, and this idea has received support from the biophysical studies of Virtanen et al. (78) on model membranes. If this were the case, segregation of domains would be favored by low temperatures, and HlyA would partition into domains of a particular composition, irrespective of the average cholesterol content in the mixture.

In α-toxin of *S. aureus* (67), as well as in certain pore-forming cytolysins (79), insertion is followed by oligomerization. In the case of HlyA, no oligomer has been isolated up to now, although circumstantial evidence in its favor has been produced (80,81). The fact that protein insertion itself helps to promote new structural defects is important because this would support a cooperative process of insertion, which in turn would be related to the putative oligomerization of HlyA (80,81). Pott and Dufourc (82) have underlined the creation of defect structures in the membrane upon the addition of melittin that might modify the overall elastic properties of the membrane, and Monette et al. (83) proposed that high cholesterol concentrations induce tight lipid packing, which in turn prevents penetration of melittin into the bilayer. In our system, even the gel state is more prone to HlyA insertion than the liquid ordered one, probably because of the structural defects that are known to exist in liposomes below T_c.

More on the role of divalent cations

Our studies have shown that the protein

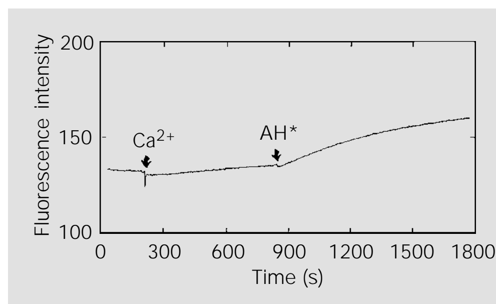

Figure 5 - α-Hemolysin requirements of calcium prior to protein binding to membranes. The protein was incubated for 15 min with PC LUV loaded with l-amino-naphthalene-1,3,6-trisulfonate/p-xylenebispyridinium bromide (ANTS/DPX) in the absence of calcium, and 10 mM $CaCl_2$ was then added (arrow, Ca^{2+}). After some time, the same amount of protein, this time preincubated with 10 mM $CaCl_2$, was added (arrow, AH*).

had to bind Ca^{2+} prior to its interaction with the membrane for its lytic action to develop (Figure 5) (56). This suggests that Ca^{2+} induces in the protein in solution a state such that the toxin becomes capable of producing membrane lysis. The same liposome-red blood cell competition assay for the irreversibility of Hly binding was applied by Bakás et al. (84) in a new series of experiments to study the effects of Ca^{2+} on toxin binding irreversibility and subsequent membrane damage, with the result that Ca^{2+} facilitates the irreversible bilayer insertion of the protein. Looking in more detail into the HlyA-calcium system, the above authors observed that, even in the absence of lipid bilayers, Ca^{2+} binding had significant effects on α-hemolysin. In particular, binding to Ca^{2+} exposes new hydrophobic residues on the protein surface, as indicated by the increased binding of the fluorescent probe aniline naphtholsulfonate (ANS), that is known to bind with high affinity hydrophobic molecules or molecule regions (85,86), and by the increase in size of the protein aggregates that α-hemolysin forms in aqueous media.

It should be noted that Ca^{2+} binding does not elicit significant changes in the protein secondary structure, judging from circular dichroism measurements (84). However, other techniques, namely studies of thermally induced change of intrinsic fluorescence, and trypsin digestion under controlled conditions, are compatible with a Ca^{2+}-induced change in conformation (84). Calcium ions would then induce a change in tertiary structure that would result in surfacing hydrophobic portions of the protein. The increased hydrophobic surface would undoubtedly favor either membrane binding (through hydrophobic forces) or self-aggregation.

According to our measurements (37), about three exchangeable calcium ions per protein molecule must be bound for optimal activity. Very similar calcium requirements and calcium binding data have been found for another member of the RTX family, the adenylate cyclase toxin from *Bordetella pertussis* (87). In this case, a calcium-dependent conformational change has been detected by a variety of techniques, that appears to imply modifications in the tertiary structure (87). Similar changes may be required for the activation of HlyA by Ca^{2+}, and the case of the adenylate cyclase toxin shows that changes in the Ca^{2+}-binding domain may be transmitted to distant regions in the protein structure.

Once the superficial hydrophobic area is increased as a result of Ca^{2+}-binding, the next step in HlyA-dependent cell lysis is membrane insertion (in competition, or maybe in equilibrium, with self-aggregation). Our previous studies (66) have shown the difference between adsorption and insertion, and the requirement of a fluid ("liquid disordered") bilayer for irreversible insertion. The more recent data by Bakás et al. (84) demonstrate that Ca^{2+}-binding is an additional requirement for irreversible insertion. This kind of insertion appears to be essential for the lytic process to go on (through yet unknown steps). Thus the requirement of Ca^{2+} ions for HlyA-dependent hemolysis refers at least to the requirement of Ca^{2+} for a proper (irreversible) insertion into the membrane. Such an insertion, that converts the toxin into an intrinsic membrane protein (84), is undoubtedly favored by the calcium-dependent change in tertiary structure leading to the exposure of hydrophobic patches. Calcium ions may be playing in α-hemolysin or in the *B. pertussis* toxin (87) the role of pH or temperature in the pre-insertion conforma-

tional changes that are observed in colicin A (49) or in *Pseudomonas* exotoxin A (54).

By contrast, divalent cations that cannot support the lytic activity of HlyA, of which Zn^{2+} is a representative example (56), have also a different effect on HlyA structure and on its interaction with membranes. At variance with Ca^{2+} (or Sr^{2+}, or Ba^{2+}), Zn^{2+} binding does not elicit an increase in HlyA intrinsic fluorescence, nor does it enhance the protein tendency to self-aggregation. Still a rather large number of zinc ions, about 14, appear to bind (37). As a consequence, a large conformational rearrangement may occur (though different from the one elicited by Ca^{2+}), so that HlyA binds the membranes as an extrinsic protein and, as such, it may be removed, at least partially, by high ionic strength solutions (84). Finally, the zinc effects appear to be somehow reversible since, in the presence of 10 mM Ca^{2+}, at least part of the protein that had been incubated with liposomes in the presence of 1 mM Zn^{2+} can move onto the red blood cell surface and produce cell lysis there.

In summary, calcium binding to HlyA appears to constitute a sort of second, exocellular, activation (the first one being fatty acylation of Lys residues) that would induce a 'competent state' in the protein, making it capable of inserting into and lyse the membrane.

Electrostatic *vs* hydrophobic interactions in HlyA insertion

An essential element for understanding the process of α-hemolysin interaction with membranes is the fact that α-hemolysin, being an amphipathic protein, occurs in aqueous media in the form of large aggregates, much as detergents exist in micellar form (31). Mature α-hemolysin contains fatty acyl residues (88,89) but the nonacylated precursor prohemolysin also forms aggregates (90). α-Hemolysin appears to interact with the membranes as a monomer (31,91), thus the following equilibria appear to exist: aggregate ⇄ monomer (in the absence of vesicles) and aggregate ⇄ monomer ⇄ membrane-bound (in the presence of liposomes). A wealth of experimental evidence supports the involvement of mainly hydrophobic interactions in the self-aggregation and membrane insertion of α-hemolysin. However, considering the large size of the toxin, 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 (Figure 1), it is to be expected that hydrophobic forces are not the only ones involved in HlyA-membrane interaction.

In a recent study, Ostolaza et al. (92) have tested the effect of changes in pH, ionic strength and bilayer net electric charge on the self-aggregation, membrane binding and lysis properties of HlyA. In the absence of vesicles, the monomer ⇄ aggregate equilibrium 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. 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 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 Ostolaza et al. (92), with a maximum at pH ≈ 4.1 (i.e., the isoelectric point) and a new and larger increase in size at neutral pH was interpreted as a pH-dependent conformational change.

The observed changes in intrinsic fluorescence (92) can be due either to a protein conformational change or 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 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 a 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-hemolysin binding to bilayer is maximal (92): 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 experiments in which a net surface charge is introduced in the bilayer, by including negatively charged (phosphatidylinositol) or positively charged (stearylamine) lipids in the liposomal compositions (Table 1) (92). 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. Benz et al. (93), 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 (94) described that α-hemolysin is more active at low pH (<6) against PC:PS bilayers, and that the toxin produces a large 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 (94) uses small unilamellar vesicles, structures whose inherent metastability is well known (95), and that in his case the insertion of a toxin monomer is sufficient 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.

Table 1 - Effect of bilayer surface charge on HlyA binding to vesicles and HlyA-dependent vesicle leakage.

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

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

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 by Ostolaza et al. (92) show how the opposite electric charge in the PC: PE 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 (48,96).

In the context of the physiological conditions under which α-hemolysin is supposed to act, it should be noted that the protein will have a net negative charge, at pH ≈7.4, and that the net charge of biomolecules on 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-hemol-

ysin monomers could bind prior to their adsorption to the membrane surface.

The mode of insertion of α-hemolysin in lipid bilayers

In the last decade, the crystal structures of several integral membrane proteins have been published and all of them have been found to extend their peptides across the bilayer, i.e. they are transmembrane proteins. However, this may not always be the case, and there are instances in which biochemical and biophysical studies suggest that certain integral proteins bind only a single monolayer in the membrane, e.g. cytochrome b_5 (97). In the case of *E. coli* α-hemolysin, a number of prediction studies and experimental results (Soloaga A, unpublished results) are best interpreted in terms of α-hemolysin being embedded in the host lipid bilayer as an intrinsic protein not traversing the membrane, but rather occupying only the outer monolayer.

A large variety of physical techniques have been used in the past to characterize intrinsic proteins, several of them having also been applied to the study of lipid-HlyA interaction by Soloaga A, Veiga MP, García-Segura LM, Ostolaza H, Brasseur R and Goñi FM (unpublished results). The fact that α-hemolysin widens the gel-fluid transition of saturated phosphatidylcholines, decreasing their associated ΔH with little change in T_m has long been recognized as typical of intrinsic proteins (98,99). So is the effect of HlyA increasing DPH polarization in fluid bilayers (100-102). Equally the quenching of the intrinsic fluorescence of the protein by quenchers located deep inside the bilayer lipid matrix is indicative of an integral protein, as is the case of bromoacyl phospholipids (103) that quench a large proportion of α-hemolysin tryptophanyl fluorescence. Finally, Triton X-114 has been shown to solubilize membranes at 0°C and to separate into a detergent-rich phase, containing the integral membrane proteins, at 30°C (104,105). This is the way in which α-hemolysin behaves (Soloaga A, unpublished results).

In addition to this experimental evidence, the observation that HlyA forms ion channels in black lipid films (106) is equally suggestive of an intrinsic protein. Also, previous work from this laboratory, including indirect evidence for irreversible insertion from liposome lysis studies (37) and fluorescence data showing irreversible insertion of HlyA in fluid bilayers (66) support the notion that *E. coli* α-hemolysin, which behaves as a soluble protein when secreted by the bacterium into the extracellular medium, binds and inserts into phospholipid bilayers, and presumably into cell membranes, becoming an intrinsic protein of the host membrane.

With respect to the suggestion of α-hemolysin as a non-transmembrane protein, the simplest and clearest suggestion that HlyA is not a transmembrane protein comes from the lack of "particles" on the fracture faces of vesicles containing inserted α-hemolysin under conditions that would give rise to vesicle leakage (Soloaga A, unpublished results). In addition to this, quantitative results arising from differential scanning calorimetry and fluorescence polarization studies provide strong support to the hypothesis of the non-transmembrane character of HlyA, only one of the lipid monolayers being occupied by the protein. In fact, both techniques can be used to determine the average number of lipids that are perturbed by the presence of the protein, so that they appear to be removed from the gel-fluid phospholipid transition (100-102,107). In the present case, the figure is of about 400-500 phospholipids per protein molecule, a very large number when compared to transmembrane proteins of equal or even higher molecular mass. However, two proteins associated with the lung surfactant SP-B and SP-C, respectively, that are expected to lie down along the surfactant monolayer, rather than traversing any membrane, are also found to perturb a disproportionately large number of

lipids (108). It can also be intuitively understood that the same mass of peptide, spread over a membrane or crossing it more or less perpendicularly, may cause a very different perturbation, affecting a much larger number of lipids in the first case.

The theoretical studies are also in agreement with this view, since a large number of amphipathic helices, but no transmembrane helices, are predicted (Brasseur R, unpublished results). Amphipathic helices are ideal for anchoring a protein to a single monolayer (109). Calculations of molecular hydrophobicity potentials (MHP) show that the amphipathic helices of HlyA are very similar to those of apolipoproteins (110,111). However, an important difference is the proportion of amino acid residues involved in amphipathic helices, more than 70% for the apolipoproteins, but less than 30% for α-hemolysin. Perhaps this difference explains why apolipoproteins give rise to lipoprotein disks, while α-hemolysin does not solubilize the bilayer, remaining inserted into it.

A model for HlyA interaction with membranes

A preliminary model of the conformation of HlyA inserted into a bilayer is shown in Figure 6. Its main features are: a) the protein is bound to a single monolayer through a number of amphipathic helices, according to the structure prediction studies, and perhaps through the two fatty acyl residues (89); b) there are no transmembrane helices, according to prediction and to freeze-fracture observations (Soloaga A and García-Segura LM, unpublished results); c) the nonapeptide-rich Ca^{2+}-binding domain is located in the water phase outside the membrane, as expected from its water-soluble character (37,38). While in agreement with the available data, this model is to be refined in the light of further experimentation. The binding of HlyA to the bilayer through amphipathic instead of transmembrane helices explains the observed role of polar forces in α-hemolysin insertion (92), that is not commonly found with integral membrane proteins.

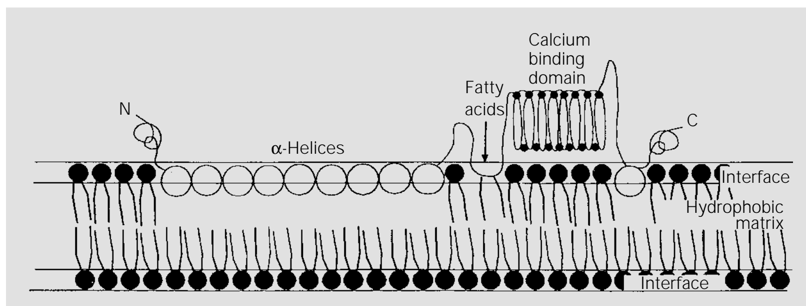

Figure 6 - A model for the insertion of α-hemolysin in a membrane. The ten amphipathic helices are represented as their cross-section. The relative sizes of the helices and phospholipids are according to White and Wimley (113).

Two main consequences derive from the above model. One is a mechanism for vesicle or cell leakage. The insertion of one or more of these molecules into the outer monolayer of the membrane must induce an increase in the lateral pressure of the monolayer lipids. Beyond a certain increase the monolayer will reach a point of transient breakdown (perhaps repaired by a net transfer of lipids to the inner monolayer) with subsequent leakage of contents. Experimental tests of this proposed mechanism are under way. The second consequence is that the present data and calculations are against the idea of *E. coli* α-hemolysin as a pore-forming toxin. The pore hypothesis was published and supported by indirect evidence (61). However, observations from our laboratory (65) and others (112) have provided evidence against the pore concept, particularly since it has been shown that HlyA induces leakage of large molecular weight polymers under isotonic conditions. The many published observations of transmembrane electric currents induced by α-hemolysin, and usually attributed to “pores”, may also be the result of the transient disruptions of the membrane barrier due to the mechanism just discussed. Our view of α-hemolysin as a non-transmembrane intrinsic protein, while providing a putative mechanism for transient bilayer breakdown, would certainly rule out the idea of a protein pore through which solvent and/or solutes could flow.

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