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***E. coli* Alpha Hemolysin and Properties**

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1. Introduction

Protein toxins are prominent virulence factors in many pathogenic bacteria. While toxins of Gram-positive bacteria do not generally require activation, many toxins of the Gram-negatives are translated into an inactive form and require a processing step.

The most common such step involves a proteolytic cleavage to generate the active form, especially in those toxins with enzymatic activity. Toxins are activated by proteolysis in a variety of ways: As examples, the anthrax toxin is proteolyzed after its interaction with the receptor on the target cell to promote the formation of a prepore (van der Goot & Young, 2009); the toxic subunit of the *Vibrio cholerae* toxin (CT) is posttranslationally modified through the action of a *V. cholerae* protease that generates two fragments, one containing the toxic activity and the other serving to interact with the binding domain (Sanchez & Holmgren, 2011); finally, the toxins that are synthesized as a single polypeptides must be separated by proteolytic cleavage to generate a catalytic, a transmembrane, and a receptor-binding domain—a salient example here being the diphtheria toxin (Murphy, 1996).

Another processing step involves the acylation of proteins, which substitution is achieved by various mechanisms that differ according to the particular fatty acid transferred, the modified amino acid, and the fatty-acyl donor. Myristate and palmitate are the most common fatty acids cross-linked to proteins. Proteins sorted to the bacterial outer membrane or to the eukaryotic plasma membrane undergo processing in which an acyl group is attached to the N-terminal amino acid. In prokaryotes, acyltransferase, lipases, or esterases use catalytic mechanisms involving ester-linked acyl groups attached to serine and cysteine residues; while eukaryotic proteins utilize ester-linked palmitoylation and ether-linked prenylation of cysteine residues for membrane sorting and protein-protein interaction (Stanley *et al.*, 1998).

The pore-forming **α -hemolysin (HlyA)** of *Escherichia coli*, a member of the RTX toxins, represents a unique class of bacterial toxins that require for activation a posttranslational modification involving a covalent amide linkage of fatty acids to two internal lysine residues (Stanley *et al.*, 1998). In general, protein acylation is divided into labile modifications of internal regions and stable modifications at the N and C termini. By contrast, the mechanism of stable internal acylation of HlyA represents a unique example among prokaryotic proteins, thus generating interest in its study and discussion. After

introducing HlyA, its synthesis, posttranslational modification, secretion, and activity; this chapter will focus on the role that covalently bound fatty acids play in the toxin's mechanism of action.

In recent decades, scientific advances have permitted the manipulation of toxins by using different strategies for directing toxic moieties to diseased cells and tissues. The end of the chapter will involve a discussion of this so-called *toxin-based therapy* and the potential use of HlyA in that modality.

2. The alpha-hemolysin (HlyA) of *E. coli*

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is the causative agent of at least 80% of all uncomplicated urinary-tract infections (UTIs), which pathologies currently rank among the most common of infectious diseases worldwide (Marrs et al. 2005), (Foxman & Brown, 2003). ExPEC strains that cause a UTI are called uropathogenic *E. coli* (UPEC). This unique group of *E. coli* strains can reside in the lower gastrointestinal tract of healthy adults (Foxman et al., 2002), (Yamamoto et al., 1997), but upon entry into the urinary tract can ascend to and colonize the bladder, causing cystitis. The infection may be confined to the bladder, or bacteria may ascend into the ureters to infect the kidneys and cause pyelonephritis. In severe cases, bacteria can further disseminate across the proximal-tubular and capillary endothelia to the bloodstream, causing bacteremia (Mobley et al., 2009.). A significant proportion of UTIs occur in patients with no known abnormalities of the urinary tract—the so-called *uncomplicated UTIs*. Certain host characteristics, however, such as a congenital defect in urinary-tract anatomy, are considered complicating factors for UTI and accordingly increase susceptibility to this infection as well as affect its diagnosis and management (Foxman, 2002.). Finally, colonization of the bladder in high numbers may occur without eliciting symptoms in the host, a condition known as asymptomatic bacteriuria (Hooton et al., 2000.). In recent years, an enormous amount of information has accrued through sequencing the genomes of several ExPEC patients. These data, together with epidemiological analyses, have confirmed that different ExPEC pathotypes share many known as well as putative virulence factors. These latter include a number of secreted toxins, iron-acquisition systems, adhesins, and capsular antigens (Wiles et al., 2008). Secreted toxins—which proteins include **HlyA**, the cytotoxic necrotizing factor-1 (CNF-1), and the secreted autotransporter—can alter host signaling cascades, disrupt inflammatory responses, and induce host-cell death; whereas bacterial siderophores like aerobactin, bacteriocin, and enterobactin allow the ExPEC to sequester iron away from the host (Guyer et al., 2002), (Wiles et al., 2008). Adhesive organelles can mediate ExPEC interaction with, and entry into, host cells and tissues; while the expression of encapsulation may enable ExPEC to more effectively avoid professional phagocytes (Wiles et al., 2008), (Dhakal et al. 2008).

Experiments in murine and cell-culture model systems have demonstrated that high levels of HlyA can cause the osmotic lysis of host cells, while sublytic concentrations of this pore-forming toxin can modulate host-survival pathways by interfering with phagocyte chemotaxis (Wiles et al, 2008),(Jonas et al., 1993), (Cavalieri & Snyder, 1982), (Chen et al., 2006). Both HlyA and CNF-1 may in addition stimulate the breakdown of tissue barriers and the release of nutrients (Smith et al. 2008), (Bauer & Welch, 1996), but through the use of the

zebrafish infection model phagocytes were found that appeared to be the primary targets of these toxins (Wiles *et al.*, 2009).

HlyA represents the prototype of the first RTX family of proteins characterized by Rodney Welch (Welch 1991). Produced by a variety of Gram-negative bacteria, these proteins exhibit two common features: The first is the presence of arrays of glycine- and aspartate-rich nonapeptide repeats, which sequences are located at the C-terminal portion. The second is the unique mode of secretion via the type-I system (an ABC-binding-cassette transporter). This first group of RTX toxins consists of toxins – mostly exhibiting cytotoxic pore-forming activity – that often are first detected as a hemolytic halo surrounding bacterial colonies grown on blood-agar plates (Muller *et al.*, 1983), (Welch, 1991), (Felmlee *et al.*, 1985). Recently, a subgroup of very large RTX toxins (>3200 residues) were discovered with multiple activities, such as protease and lipase. These pathogens were named the multifunctional autoprocessing RTX toxins, with the *Vibrio cholerae* toxin being the prototype of this group. In summary, the RTX proteins form a large and diverse family with a broad spectrum of biological and biochemical activities (Linhartova, *et al.*, 2010).

2.1 Synthesis and structure of HlyA

The synthesis, maturation, and secretion of *E. coli* HlyA are determined by the *hlyCABD* operon ((Felmlee *et al.*, 1985), (Issartel *et al.*, 1991), (Koronakis *et al.*, 1997), (Nieto *et al.*, 1996)). The membrane-associated export proteins are synthesized at a lower level than the cytosolic HlyC and pro-HlyA, in part because of transcription termination within the *hlyCABD* operon (Felmlee *et al.*, 1985). This termination is suppressed by the elongation protein RfaH and a short 59-bp, *ops* (operon polarity suppressor) (Bailey *et al.*, 1992, 1996), (Cross *et al.*, 1990), (Nieto *et al.*, 1996) that act in concert to allow the transcription of long operons such as *hly*, *rfa*, and *tra* encoding the synthesis and export of extracellular components key in the virulence and fertility of Gram-negative bacteria (Bailey *et al.*, 1992, 1997).

The structural gene *hlyA* produces a single 110-kDa polypeptide. The estimated pI of the toxin is 4.5, with this characteristic being common among the RTX toxins. The N-terminal hydrophobic domain is predicted to contain nine amphipathic α -helices (Soloaga *et al.*, 1999). Using photoactivable liposomes, Hyland *et al.* (2001) demonstrated that the region comprised between residues 177-411 is the one that becomes inserted into membranes. The C-terminal calcium-binding domain contains 11-17 of the glycine- and aspartate-rich nonapeptide β -strand repeats. Although the membrane interaction of HlyA is assumed to occur mainly through the amphipathic α -helical domain, that both major domains of HlyA are directly involved in the membrane interaction of HlyA has recently been proposed, with the calcium-binding domain in particular being responsible for the early stages of the HlyA's docking to the target membrane (Sanchez-Magraner *et al.*, 2007).

The topic of the existence of a receptor for the toxin in erythrocytes remains quite controversial. Nevertheless, Cortajarena *et al.* (2003) observed that a short sequence from the C-terminal domain (between residues 914–936) was the main HlyA segment that bound to the glycoporphin A on erythrocytes.

The last 60 C-terminal amino acids consist of 2 α -helices separated by 8-10 charged residues. This domain is implicated in the transport of the toxin to the extracellular medium (Hui *et al.*, 2000). Fig. 1 shows a scheme of the HlyA structure.

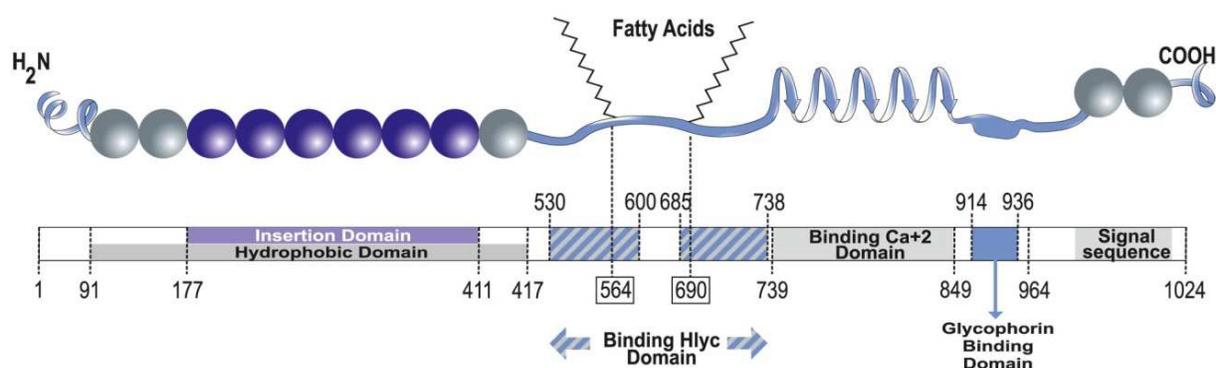


Fig. 1. A scheme of the HlyA structure.

The more relevant domains of HlyA are indicated.

2.2 The posttranslational activation of HlyA

The proHlyA protoxin is matured in the cytosol to the active form by HlyC-directed fatty acylation before export from the toxin-producing bacteria. This process consists in a posttranslational modification of the ϵ -amino groups of internal lysine residues by covalent attachment of amide-linked fatty-acyl residues. This reaction is catalyzed by the HlyC acyltransferases expressed together with the protoxins (Goebel & Hedgpeth, 1982). The mechanism of this novel type of protein acylation was extensively analyzed for HlyA (Issartel *et al.*, 1991), (Stanley *et al.*, 1994). HlyC uses the fatty-acyl residues carried by acyl-carrier protein (ACP) to form a covalent acyl-HlyC intermediate, which species then transfers the fatty-acyl residues to the ϵ -amino groups of the Lys 564 and Lys 690 residues of proHlyA (Worsham *et al.*, 2001, 2005). ACPs carrying various fatty-acyl residues – including palmitate (16:0) and palmitoleate (16:1), the most common in *E. coli* – could be efficiently used *in vitro* as acyl donors for the modification of HlyA ((Issartel *et al.*, 1991), (Trent *et al.*, 1998)). *In vivo*, however, HlyC exhibits a high selectivity for myristic acid (14:0), which species was found to constitute about 68% of the acyl chains covalently linked to Lys 564 and Lys 690 of the native HlyA (Lim *et al.*, 2000). Contrary to expectations, the extremely rare odd-carbon saturated fatty-acyl residues 15:0 and 17:0 were found to constitute the rest of the *in-vivo* acylation of HlyA in two different clinical *E. coli* isolates (Lim *et al.*, 2000). Both acylation sites in the HlyA genome function independently of one another with respect to the kinetics of their interaction with acyl-HlyC (Langston *et al.*, 2004). By using deleted protoxin variants and protoxin peptides as substrates in an *in-vitro* maturation reaction dependent on only HlyC and acyl-ACP, two independent HlyC-recognition domains were identified on the HlyA protoxin, each of which spanned one of the target lysine residues (Stanley *et al.*, 1996). Each domain required 15 to 30 amino acids for basal recognition and 50 to 80 for full wild-type acylation, but HlyC recognized a large topology rather than a linear sequence. The loss of the Lys 564 acylation site either by mutation or structural deletion affected the thermodynamics of the acylation reaction at Lys 690, implying an undefined connectivity between the two acylation sites (Worsham *et al.*, 2005). Nevertheless, the intact acylation at Lys 690 is essential for HlyA activity.

No other HlyA sequences are required for toxin maturation, including the immediately C-terminal Ca^{+2} binding repeats. Indeed, *in vitro*, Ca^{+2} ions prevent acylation at both sites (Stanley *et al.*, 1996). The extreme sensitivity of the proHlyA activation reaction to free Ca^{+2} supports the view that intracellular Ca^{+2} levels in *E. coli* are too low to affect toxin activity and that Ca^{+2} binding does not occur until the toxin is outside the cell.

This posttranslational modification is remarkable because the behavior of the protein is changed by lipid modification from a benign protein to a frank toxin—part of this transformation being an exclusive mechanism in prokaryotes since in only a few eukaryotic proteins is this type of acylation found (for example, in the nicotinic acetylcholine receptor; the insulin receptor; and cytokines such as TNF- α , IL-1 α and IL-1 β) (Stanley *et al.*, 1998). In the following section we discuss the role that these covalently bound fatty acids play in the toxin's mechanism of action.

2.3 The secretion of HlyA into the extracellular medium

Maturation increases the hydrophobicity of the protein, but that property is not required for export (Ludwig *et al.* 1987). *E. coli* HlyA-related toxins are all secreted across both membranes by the type-I export process employing an uncleaved C-terminal recognition signal (Nicaud *et al.*, 1986), (Stanley *et al.*, 1991), but no N-terminal leader peptide (Felmlee *et al.*, 1985) or periplasmic intermediate (Felmlee & Welch, 1988), (Koronakis *et al.*, 1989). The HlyA secretory apparatus comprises HlyB (an inner-membrane traffic ATPase, the ATP-binding cassette), HlyD (a membrane-fusion protein), and TolC (an outer-membrane protein) (Schulein *et al.*, 1992), (Wandersman & Delepelaire, 1990), (Wang *et al.*, 1991). In *E. coli* and most other pathogens, TolC is encoded by a separate gene from *hlyCABD*. As mentioned before (*cf.* Section 2.0) the type-I-secretion-signal sequences have been located within the last 60 C-terminal amino acids, consisting of 2 α -helices separated by 8-10 charged residues (Hui *et al.*, 2000).

The mechanism of exportation of HlyA is as follows: The trimeric accessory protein HlyD has been proposed to form a substrate-specific complex with the inner-membrane protein HlyB, which latter species subsequently recognizes the C-terminal signal peptide of HlyA. Upon the binding of HlyA, the HlyD trimer interacts with the trimeric TolC protein of the outer membrane, inducing a conformational change and the consequent export of HlyA. This assembly between the complex HlyB-HlyD with TolC very likely occurs because, as has been demonstrated by X-ray crystallography, the trimeric complex of TolC is very similar in size to the trimeric structure of HlyD, thus facilitating the formation of a continuous transperiplasmic export channel through which HlyA can pass (Koronakis *et al.*, 2000). This complex appears to be transient, with it disengaging and reverting to a resting state once the substrate has been transported (Thanabalu *et al.*, 1998). The energy necessary for the secretion process depends not only on ATP hydrolysis mediated by HlyB but also on the proton motive force exerted on the inner membrane (Koronakis *et al.*, 1991, 1995). Type-I secretion is generally assumed to involve the translocation of unfolded proteins (Young & Holland, 1999), although Pimenta *et al.* (2005) have suggested that contact with HlyD directly or indirectly affects the folding of HlyA either during the latter's transit through the translocator or afterwards.

In the last decade many researchers have been interested in this type of secretion machinery because of its potential use in the export of chimeric proteins and in vaccine production (Gentshev *et al.*, 1996, 2002).

Although HlyA has its own machinery for export from the bacteria, the presence of a physiologically active HlyA in the outer-membrane vesicles (OMVs) of clinical-hemolytic (Balsalobre *et al.*, 2006) as well as laboratory-recombinant strains of *E. coli* (Herlax *et al.*, 2010) has recently been demonstrated.

OMVs are constantly being discharged from the surface of Gram-negative bacteria during bacterial growth. All Gram-negative bacteria studied to date, including *E. coli*, produce OMVs; and their release is increased when the bacteria are exposed to stressful conditions such as antibiotics or serum. Even though the release of OMVs could not be demonstrated *in vivo*, the presence of particles resembling those vesicles has, in fact, been detected in plasma from patients with different infectious processes (Beveridge, 1999). OMVs serve as secretory vehicles for the proteins and lipids of Gram-negative bacteria and in this manner play roles in establishing a colonization niche for carrying or transmitting virulence factors into host cells or otherwise modulating the host defense and response, thus acting as well as long-range virulence factors that can protect luminal cargo from extracellular host proteases and so penetrate into tissues more readily than the larger bacteria (Kuehn & Kesty, 2005). In addition to toxin-protein delivery, other roles have been characterized for OMVs—namely, interspecies interaction and communication during multispecies infections plus DNA uptake and transfer (Mashburn-Warren & Whiteley, 2006). In the particular example of HlyA, we have demonstrated that the toxin secreted in this way is transferred to the target cell in a concentrated manner and as such is more hemolytically efficient than the free HlyA (Herlax *et al.*, 2010). Moreover, Balsalobre *et al.* (2006) demonstrated that the HlyA associated with OMVs is protected from the attack of proteases, thus facilitating the survival of the toxin within the adverse medium of a patient's plasma.

2.4 The mechanism of action of HlyA

HlyA belongs to one class of a wide range of host-cell-specific toxins. HlyA acts on a variety of cell types from several species—*e. g.*, red blood cells, embryo and adult fibroblasts, granulocytes, lymphocytes, and macrophages (Cavaliere *et al.*, 1984)—and also binds to and disrupts protein-free liposomes (Ostolaza *et al.*, 1993).

The host environments encountered by the ExPEC are extremely nutrient-poor; and the function of HlyA has generally been thought to be primarily the destruction of host cells, thereby facilitating the release of nutrients and other factors, such as iron, that are critical for bacterial growth. The lytic mechanism of HlyA is a complex process. Three stages seem to be involved that ultimately lead to cell lysis: binding, insertion, and oligomerization of the toxin within the membrane.

Studies that have explored the binding of HlyA to membranes and the characterization of a putative toxin-specific receptor have produced contradictory results. First, the lymphocyte function-associated antigen (LFA-1) (CD11a/CD18; $\alpha_1\beta_2$ integrin), was reported to serve as the receptor for HlyA on polymorphonuclear neutrophils (Lally *et al.*, 1997) and HlyA was found to recognize and bind the N-linked oligosaccharides to their β_2 -integrin receptors (Morova *et al.*, 2008). This finding raises the possibility that the initial binding of the toxin to various cells might occur through the recognition of glycosylated membrane components, such as glycoproteins and gangliosides. Recently, Cortajarena *et al.* (2001) found that HlyA binds to the glycoporphin of horse erythrocytes and that this binding was abolished by a

trypsinization of the membranes. In addition, these authors found that the glycophorin purified from erythrocyte ghosts and reconstituted in liposomes significantly increased liposomal sensitivity to HlyA. Amino acids 914-936 of HlyA were subsequently hypothesized to be responsible for binding to the ghost receptor (Cortajarena *et al.*, 2003).

Other studies, however, indicated that the binding of HlyA to cells occurred in a nonsaturable manner and that the toxin did not interact with a specific protein receptor either on granulocytes or erythrocytes (Valeva *et al.*, 2005). Nevertheless, HlyA produces protein-free liposome disruption. Ostolaza *et al.* have reported that HlyA causes the release of fluorescent solutes following a so-called *all-or-none* mechanism. Using large unilamellar vesicles of different lipid compositions, the authors found that the vesicles composed of phosphatidylcholine, phosphatidylethanolamine, and cholesterol at a molar ratio of 2:1:1 were the most sensitive (Ostolaza *et al.*, 1993). These results demonstrated that the presence of a receptor was not necessary for hemolysis to occur. These contradictory findings regarding the presence or absence of a toxin-specific receptor might be related to the different amounts of toxin and/or the different types and animal species of target cells used in the various studies. At all events, the interaction of HlyA with a target-cell membrane devoid of any specific proteinaceous receptor appears to occur in two steps: an initial reversible adsorption of the toxin that is sensitive to electrostatic forces followed by an irreversible membrane insertion (Bakás *et al.*, 1996), (Ostolaza *et al.*, 1997). Studies with the isolated calcium-binding domain of HlyA revealed that that part of the protein may be adsorbed onto the membrane during the early stages of HlyA-membrane interaction (Sanchez-Magraner *et al.*, 2007).

The next step in the hemolytic process is the insertion of the toxin into the membrane. Hyland *et al.* (2001) demonstrated that the major region of HlyA that inserts into the membrane is located between residues 177 and 411. The insertion is furthermore independent of membrane lysis since HlyA-protein mutants that are completely nonlytic can insert into lipid monolayers (Sanchez-Magraner *et al.*, 2006). In addition, a binding of calcium to the toxin was shown to induce a protein conformational change that made the insertion process irreversible (Sanchez-Magraner *et al.*, 2006), (Bakás *et al.* 1998). Once the toxin is inserted, an oligomerization process occurs. We previously found that the fatty acids covalently bound to the toxin induce conformational changes that expose intrinsically disordered regions so as to promote protein-protein interactions. Thus, the oligomerization process of the toxin is facilitated by microdomains within the membrane (Herlax & Bakas, 2007), (Herlax *et al.*, 2009).

The HlyA pore that is formed is highly dynamic because the size depends on both the interaction time and the concentration of the toxin (Welch, 2001). We recently demonstrated that the pore is of a proteolipidic nature since the conductance and membrane lifetime are dependent on membrane composition (Bakas *et al.*, 2006).

Nevertheless, what is not clear is how often HlyA reaches levels that are high enough to lyse host target cells during the course of an infection. In fact, sublytic concentrations of HlyA may even be more physiologically relevant. Indeed, recent studies have demonstrated that sublytic concentrations of a number of pore-forming toxins can modulate a variety of host signaling pathways, including the transient stimulation of calcium oscillations, the activation of MAP-kinase signaling, and the alteration of histone-phosphorylation and -

acetylation patterns (Hamon *et al.*, 2007), (Ratner *et al.*, 2006). In addition, sublytic concentrations of HlyA have been recently found to potently stimulate the inactivation of the serine/threonine protein kinase B (PKB), which enzyme plays a central role in host cell-cycle progression, metabolism, vesicular trafficking, survival, and inflammatory-signaling pathways (Wiles *et al.*, 2008). These findings may help to explain previously published results implicating sublytic concentrations of HlyA in the inhibition of chemotaxis and in bacterial killing by phagocytes in addition to the HlyA-mediated stimulation of host apoptotic and inflammatory pathways (Cavaliere & Snyder, 1982), (Koschinski *et al.*, 2006), (Mansson *et al.*, 2007), (Tran Van Nhieu *et al.*, 2004), (Uhlen *et al.*, 2000).

3. Role of the fatty acids covalently bound to HlyA

In general, lipid moieties play central roles in protein function—*e. g.*, the targeting into membranes, an increase in the affinity for biological membranes, and an enhancement of protein-protein interactions (Stanley *et al.*, 1998), (Chow *et al.*, 1992).

After a brief introduction to the general aspects of HlyA in the following section, we will describe the role that covalently bound fatty acids play in the mechanism of action of the toxin, from its initial activation to its final functioning in the target cell. This posttranslational modification must be critical since the presence of fatty acids transforms the innocuous proHlyA into the virulent toxin HlyA.

3.1 Exposure of intrinsically disordered regions

After the initial activation of HlyA by acylation, the toxin is exported into the extracellular medium by the type-I secretion system and by OMVs. None of the secretion routes are acylation-dependent, although the extracellular transport yield was found to be lower for proHlyA compared to that for HlyA. In addition, a high concentration of ProHlyA was found in inclusion bodies (Sanchez-Magraner *et al.*, 2006). For comparative studies where acylated and nonacylated proteins were used, proHlyA was obtained from *E. coli* DH1—it having been transformed by a recombinant plasmid, pSF4000 Δ BamHI, in whose DNA a portion of the *hlyC* gene had been deleted. This strain secreted a full-length, but inactive hemolysin. Fatty acids were not necessary for the secretion of the toxin by OMVs, or by the bacteria's own export machinery; but they were essential for the toxin's hemolytic activity (Boehm *et al.*, 1990).

Several steps are involved in the lytic mechanism of the toxin: a binding of calcium previous to the toxin's interaction with membranes, the binding to and insertion into membranes, and the oligomerization of the toxin to form the final lytic pore. We will discuss below to what extent covalently bound fatty acids influence the different steps.

In the extracellular medium, HlyA must associate with calcium in order to bind to membranes in the lytically active form (Ostolaza & Goñi 1995), (Bakás *et al.*, 1998). This second activation step is acylation-dependent because the calcium-binding capacity is lower in the unacylated protein (Soloaga *et al.*, 1996). Once HlyA is calcium-activated, the toxin appears to have a two-stage interaction with membranes: first, a reversible adsorption that is sensitive to electrostatic forces; and second, an irreversible insertion (Bakás *et al.*, 1996). The inserted HlyA behaves as an integral protein because this form of the toxin cannot be extracted without the use of detergents (Soloaga *et al.*, 1999).

Nevertheless, proHlyA, though nonacylated, also interacts with membranes. This observation is not surprising because the amino-acid sequence of the polypeptide shows amphipathic helices in the 250–400 amino-acid region. Despite the amphipathic stretches known to be essential for lytic activity, however, proHlyA is unable to alter the bilayer permeability (Soloaga *et al.*, 1999). Experiments on protein adsorption at an air-water interface suggested that the fatty acids present in HlyA, unlike those in proHlyA, did not modify the surface-active properties of the protein and that the main difference between the precursor and the mature protein was that the proHlyA was virtually unable to insert itself into lipid monolayers (Sanchez-Magraner *et al.*, 2006). Furthermore, we found that the presence of two acyl chains in HlyA confers on this protein the property of irreversible binding to membranes, which feature is essential for the lytic process to take place (Herlax & Bakas, 2003). In summary, although fatty acids covalently bound to HlyA help the toxin to bind calcium in order to adopt a competitive conformation for interaction with membranes, the absence of these fatty acids does not modify that interaction of the toxin, so that these fatty acids must play some other relevant role. The answer is that the fatty acids expose intrinsically disordered regions of the toxin that are involved in a different step within the mechanism of action.

HlyA has a molten-globule conformation promoted by the presence of acyl chains, as demonstrated by a lower denaturing concentration of guanidinium-chloride. Other characteristics demonstrating this conformation were the binding of a higher number of 8-anilinonaphtalene-1-sulfonate (ANS) molecules to HlyA with a weaker affinity, a higher efficiency of energy transfer from tryptophan to the bound ANS, and a faster digestion of HlyA with trypsin compared to the same reactions with proHlyA (Herlax & Bakas, 2007).

The acylated protein was more stable in the absence of denaturant than the unacylated form, as demonstrated by the higher $\Delta G^\circ_{\text{H}_2\text{O}}$ value for HlyA compared to proHlyA. Acyl chains covalently bound to the protein, however, promote a steric hindrance that contributes to a more relaxed structure, which acylated form can thus be denatured at a lower guanidinium-chloride concentration.

ANS binding to ordered regions can be distinguished from the binding to molten-globule-like regions by differences in the apparent binding constant. The exceptionally high value of ANS bound to HlyA and proHlyA might result from amphipathic regions in both forms, but the presence of fatty acids has been observed to double this value because of the molten structure those lipids impart. The binding of a large number of ANS molecules in a weak manner is characteristic of the loose structure of the molten conformation. ANS binding to pockets in ordered or molten-globule proteins operationally gives apparent K_d values that differ by more than a factor of 5; thus, despite the uncertainties involved, these apparent K_d values serve as a diagnostic probe to distinguish ordered from molten proteins (Bailey *et al.*, 2001). This structural difference was also observed between the HlyA and proHlyA K_d values, demonstrating by an independent means that the fatty acids on the former induce a molten structure. Moreover, the higher fluorescence-transfer efficiency for HlyA compared to that for proHlyA indicated that the quenching of tryptophan fluorescence was more effective when the binding of ANS to the molten-globule conformation took place, where the accessibility of both the surface and inner tryptophan residues was increased. Thus, the capability of ANS to quench tryptophan fluorescence was seen to be correlated with the ANS-binding behavior.

Proteins with molten-globule-like regions are included in the category of intrinsically disordered proteins, as recently reviewed elsewhere (Dunker *et al.*, 2001). Most of the disordered regions of proHlyA that were predicted through the use of the predictor of naturally disordered regions (PONDR) were located in the C-terminal half of the protein (Fig. 2). These domains could be related to the different steps in this toxin's mechanism of action from its export from the bacterium to pore formation in the target cell.

HlyA carries a carboxy-terminal-secretion signal located within the last 50–60 amino acids (Jarchau *et al.*, 1994). This region is predicted to be disordered; and although export of the toxin has been observed to be acylation-independent (Ludwig *et al.*, 1987), as mentioned above, the yield from extracellular transport for proHlyA was lower than that for HlyA. Consequently, covalently bound acyl chains can expose these signal regions and thus facilitate transport.

Intrinsically unstructured proteins can bind in several different patterns through a process termed *binding promiscuity*. The intrinsic lack of structure can confer functional advantages, including the ability to bind – perhaps in various conformations – to several different target cells. This binding promiscuity would furthermore explain the previously mentioned ambiguity in experimental determinations of the presence of a specific receptor for HlyA published to date (Lally *et al.*, 1997), (Cortajarena *et al.*, 2001), (Valeva *et al.*, 2005).

Many studies have searched for the presence of a receptor for HlyA in different target cells. For example, CD11a and CD18, the two subunits of β 2-integrin, were identified as cell-surface receptors that mediate HlyA toxicity in the human target cells HL60 (Lally *et al.*, 1997). This receptor was found in most circulating leukocytes (lymphocytes, neutrophils, monocytes, and macrophages). Despite the absence of studies identifying the protein region responsible for the interaction with this receptor, studies on the adenylate-cyclase-containing hemolysin of *Bordetella pertussis* (CyaA) – another RTX toxin – revealed that the main integrin-interacting domain of CyaA is located in its glycine/aspartate-rich repeat region; which stretch is characteristic of all protein members of this family. These results allowed the identification of region 1166–1287 as a major CD11b-binding motif (Azami-El-Idrissi *et al.*, 2003). Because this domain is involved in calcium binding, the authors proposed that CyaA shifts from a disordered structure to an R-helical conformation upon calcium binding to the RTX motifs (Rose *et al.*, 1995); therefore, the speculation that the calcium-binding domain composed of glycine-rich tandem repeats corresponding to amino acids 550–850 of HlyA might be involved in the binding to β 2-integrin is tempting. That these regions also match the disordered regions predicted and that acyl chains might be implicated in the exposure since the calcium-binding capacity of proHlyA is lower than that of HlyA, should also be borne in mind (Soloaga *et al.*, 1996).

As cited above in **Section 2.4**, another protein identified as a receptor of HlyA in horse erythrocytes is the glycoprotein glycophorin (Cortajarena *et al.*, 2001). A glycophorin-binding region between residues 914 and 936 accordingly has been identified (Cortajarena *et al.*, 2003). Previous sequence analyses of several RTX toxins had revealed that this stretch was a conserved region. If this region was deleted, the specific binding of HlyA to the cell-surface receptors on erythrocytes was lost without affecting its nonspecific binding (adsorption) to lipid bilayers. This region was also predicted to be intrinsically disordered.

The role of fatty acids in the exposure of disordered regions is supported by results published for the D12-monoclonal-antibody-epitope reactivity. The D12 epitope maps to amino acids 673–726. Since the D12 monoclonal antibody reacts with HlyA, but not with proHlyA; the acylation of the former is directly responsible for the exposure of the epitope within this region (Pellett *et al.*, 1990), (Rowe *et al.*, 1994).

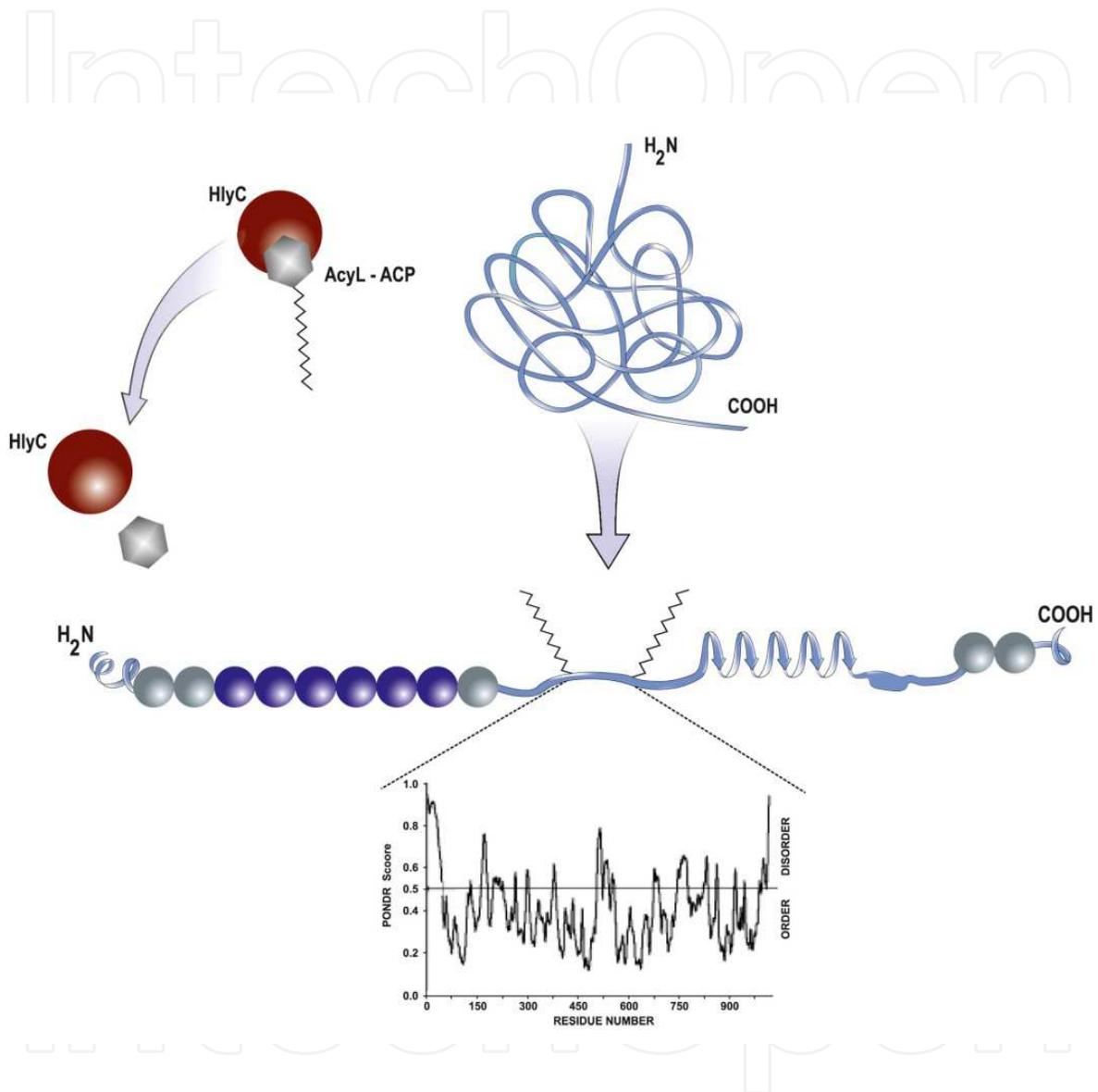


Fig. 2. ProHlyA is activated by acylation.

This process is catalyzed by HlyC, which species transfers a fatty acid from ACP to two internal lysines of ProHlyA (Lys 564 and Lys 690). Once covalently bound, these fatty acids induce a molten-globule conformation in HlyA that exposes intrinsically disordered regions, the existence of which zones was predicted by the predictor of naturally disordered regions PONDRA. The amino-acid sequence is represented on the x -axis, and the prediction of disorder on the y -axis. Peaks >0.5 are strongly predicted to be disordered (Dunker *et al.*, 2005).

3.2 Promotion of protein oligomerization

Lipid binding to proteins can also be a determinant of specific protein-protein interactions such as the assembly of proteins into oligomeric complexes. This circumstance obtains for HlyA, where an oligomer was found at lytic concentrations in sheep-erythrocyte ghosts. In contrast, no oligomeric structure was found for proHlyA (Herlax *et al.*, 2009).

Fluorescence-Resonance-Energy Transfer (FRET) is a photochemical process whereby one fluorescent molecule or fluorophore, the "donor", upon excitation by an initial photon of light, spontaneously transfers its energy to another molecule, the "acceptor", by a nonradioactive dipole-dipole interaction (Forster, 1959). The distance over which energy can be transferred depends on the spectral characteristics of the fluorophores, but is generally within the 10–100-Å range. Hence, FRET can be used for measuring structure (Lakowicz *et al.*, 1990), conformational changes (Heyduk, 2002), and interactions between molecules (Parsons *et al.*, 2004). Since HlyA does not contain cysteine residues in its sequences, lysine 344 was replaced by a cysteine (HlyA K344C) and the same point mutation introduced into the unacylated protein (proHlyA K344C). The aim of this point mutagenesis was to permit the binding of only one fluorescent probe per protein, where that mutation—hopefully located in the insertion region of the toxin into membranes (Hyland *et al.*, 2001)—would not affect the hemolytic activity of the toxin. To carry out this study, two populations of HlyA K344C mutant proteins, one labelled with donor (Alexa-488) and the other with acceptor fluorophores (Alexa-546), were bound to sheep-erythrocyte ghosts. Our report showed that an oligomer was involved in the hemolytic mechanism of HlyA (Herlax *et al.*, 2009). FRET can be used to study the distribution of molecules in membranes because the average spacing between molecules of interest will depend primarily on their lateral arrangement. Molecules may be within FRET distance either because they are clustered or because they are randomly distributed at such high surface densities that a fraction of them is within FRET proximity. The latter possibility was avoided in our experiments by using a high lipid/protein molar ratio (10^9) to insure that the observed FRET corresponded to oligomerization of the toxin on the erythrocyte surface. In comparison, the absence of FRET in the mutant protein, proHlyA K344C confirmed the participation of the covalently bound fatty acids in the oligomerization process. Fig. 3 shows the fluorescence spectra obtained in the FRET experiments for both proteins. *Prima facie*, this absence of FRET could be attributed to a reduced binding of the mutant protein to the erythrocyte ghosts, but this possibility was discarded because the percentage of binding to the membranes of both proteins was similar. We need to underscore here that fatty acids are essential for hemolytic activity; and considering that they are needed for oligomerization, we can state that oligomerization is necessary for hemolysis. We thus feel tempted to propose that the presence of fatty acids covalently bound to the protein leads to the exposure of regions that are implicated in protein-protein interactions.

In addition, a critical role of acylation in the oligomerization process to form hemolytic pores has been proposed for the adenylate-cyclase toxin from *Bordetella pertussis* (*cf.* **Section 3.1**) (Hackett *et al.*, 1995).

Finally, if we consider that pores formed by HlyA are sensitive to proteases on the *cis* side of the planar lipid membranes (Menestrina *et al.*, 1987), we could propose the possibility that the part of the toxin remaining external to the membrane is involved in the protein-protein interaction responsible for oligomerization and thus participates in pore formation.

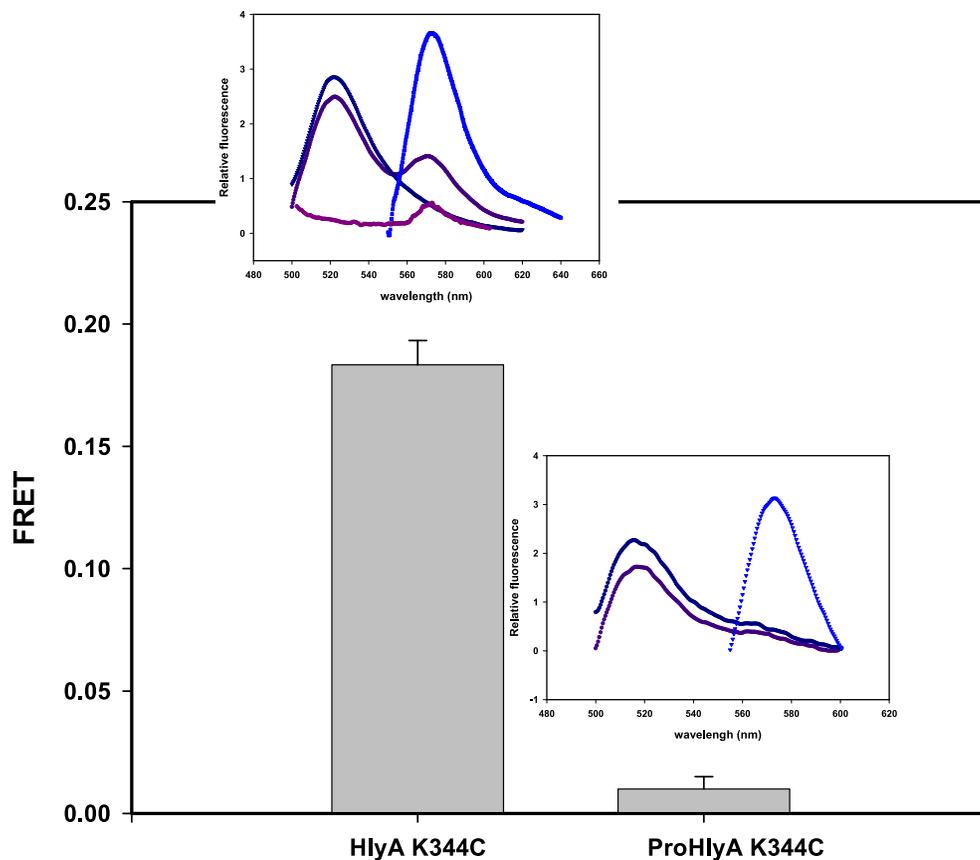


Fig. 3. Oligomerization of HlyA in erythrocyte ghosts. FRET calculated for HlyA K344C and proHlyA K344C bound to erythrocyte ghosts. The lipid/protein ratio was 10^9 . *Left inset*: Example of spectra measured for HlyA K344C. Fluorescence-emission spectrum of erythrocyte ghosts containing donor/acceptor, $F_{D/A}^{(480, \lambda_{em})}$ (excited at 480 nm; blue triangle), emission spectrum of erythrocyte ghosts labelled only with donor, $F_D^{(480, \lambda_{em})}$ (violet circle), emission spectrum of erythrocyte ghosts containing D/A, $F_{D/A}^{(530, \lambda_{em})}$ (excited at 530 nm where only the acceptor absorbs; light blue square), and emission spectrum of erythrocyte ghosts labelled only with acceptor, $F_A^{(480, \lambda_{em})}$ (purple square). *Right inset*: The same emission spectrum as in the left inset but measured for ProHlyA K344C.

3.3 Contrary to expectations, fatty acids do not facilitate the interaction of HlyA with membrane microdomains

A variety of pathogens and toxins have been recognized as interacting with microdomains in the plasma membrane. These microdomains are enriched in cholesterol and sphingolipids and probably exist in a liquid-ordered phase, in which lipid acyl chains are extended and ordered (Brown & London, 1998). Many proteins are targeted to these membrane microdomains by their favorable association with ordered lipids. Interestingly, such proteins are linked to saturated acyl chains, which species partition well into those domains (Pike, 2003). Although covalently bound fatty acids had not been implicated in the targeting of HlyA to membranes, their involvement in the targeting to membrane microdomains was studied (Herlax *et al.*, 2009). For this purpose—and taking into account that these microdomains are enriched in cholesterol and sphingolipids—the hemolytic activity of the toxin on sheep erythrocytes was compared with the activity on cholesterol-depleted

erythrocytes. The hemolysis rate of the cholesterol-poor erythrocytes was lower than that of the control erythrocytes at each HlyA concentration tested, thus pointing to the participation of cholesterol-enriched microdomains in the oligomerization process. For cholesterol-depleted erythrocytes, at low toxin concentrations, the kinetics of hemolysis seemed to be more complex, suggesting that toxin diffusion in membranes is the rate-limiting step. In order to determine if the decrease in the hemolytic rate observed in the cholesterol-depleted erythrocytes was caused by an impairment of toxin oligomerization, we repeated the FRET experiments comparing control and cholesterol-depleted sheep-erythrocyte ghosts. We demonstrated that cholesterol depletion led to a decrease in FRET of 75% compared to the control sheep ghosts. This result indicated that cholesterol-enriched microdomains played a significant role in the oligomerization process. To obtain more information about the effect of cholesterol-enriched microdomains within the oligomerization process, we performed FRET-kinetics experiments. The role of cholesterol was confirmed by the results of FRET kinetics, where the biphasic behavior of FRET suggested the initial formation of small oligomers, followed by their assembly to form multimeric structures (Fig. 4). The concentration of the small oligomers was favored by the cholesterol-enriched microdomains, where the diffusion time in the membrane became diminished. The number of HlyA molecules that became associated to form the

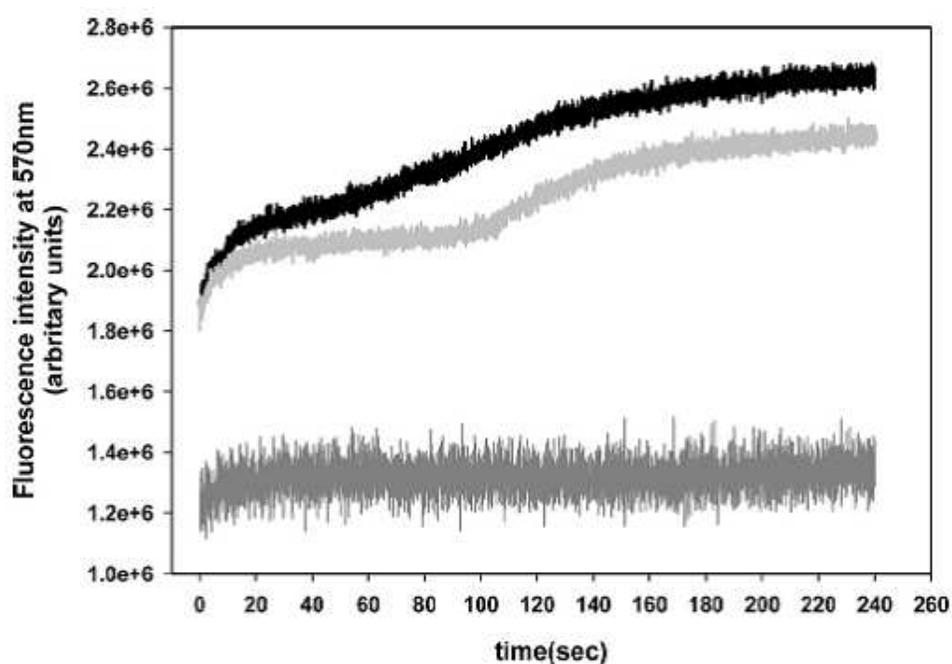


Fig. 4. *FRET kinetics*. Measurement of acceptor fluorescence at 570 nm as a function of time in a mixture composed of HlyA K344C labelled with fluorescent donor and acceptor plus either control erythrocytes (black line) or cholesterol-depleted erythrocytes (light gray line). Measurement of a mixture of unlabelled and labelled with acceptor HlyA K344C with control erythrocytes (*dark gray line*) was done as FRET-negative control. Assays were performed at a ratio of 5 μg of total toxin per 100 μg of phospholipids (as erythrocyte membranes). The excitation and emission monochromators were set at 480 nm and 570 nm, respectively. Alexa-546 emission was measured at a rate of 25 samples/s for 240 s, at 37°C. The *curves* represent the average value of three independent experiments containing five replicates each.

pore was uncertain; nevertheless, the assumption that several molecules could oligomerize to form a pore was not unreasonable. An extension of this reasoning suggested that at high doses a progressive oligomerization of HlyA leads to the fusion of the pore and rapid destruction of the cell membrane with little time for activation of the central apoptotic pathway. By contrast, at lower concentrations, the pores would be smaller and fewer in number so that the cells, though injured, would survive long enough for apoptosis to be observed (Lally *et al.*, 1997). These results can explain why toxin association with erythrocytes at 0–2°C is characterized as a prelytic state, whereas following a shift to 23°C – and after a lag period – lysis begins (Moayeri & Welch, 1997). In conclusion, the fusion of oligomers may be the rate-limiting step in pore formation, and the integrity of the cholesterol-enriched microdomains is necessary for the concentration of HlyA to induce hemolysis. This notion agrees with the findings of Moayeri and Welch (Moayeri & Welch, 1994), who observed that the degree of osmotic protection of erythrocytes afforded by protectants of varying sizes depended on the amount of toxin applied and the duration of the assay. These authors suggested that HlyA creates a lesion with a very small initial size that then increases in apparent diameter over time. Consequently, the larger the oligomer is, the bigger the pore size becomes.

That the terms "membrane microdomains" and "detergent-resistant microdomains" (DRMs) are not synonymous is essential to remember because the two have different origins and conceptual meanings (Lichtenberg *et al.*, 2005). The DRM technique, though, is widely used in the current literature to investigate the interaction between a protein and membrane microdomains. This technique takes advantage of the selective solubilization of different lipids that occurs when a biomembrane is submitted to the action of a nonionic detergent such as Triton X-100. When erythrocyte ghosts were incubated with HlyA and the DRMs were separated by sucrose-gradient ultracentrifugation, the immunoblot analysis revealed that most of the ghost-associated HlyA was localized in the DRMs, indicating that the binding of HlyA to the erythrocyte membranes was mediated by membrane microdomains that served as concentration platforms for the toxin's oligomerization. That proHlyA colocalizes with HlyA and flotillin (a microdomain protein marker) in DRMs emphasizes our hypothesis that the main role of the saturated acyl chain covalently bound to HlyA is a participation in the oligomerization process, and not the targeting to cholesterol-enriched membranes (Fig 5).

A key feature of cholesterol-enriched microdomains is the tight packing of lipid acyl chains in the liquid-ordered phase, where the lipid acyl chains are extended and ordered (Brown & London, 1998). Because of the difficulty in packing membrane-spanning helices into the ordered lipid environment, some proteins are linked to saturated acyl chains and partition well into those microdomains (de Planque & Killian, 2003). Shogomori *et al.* (2005) found, however, that acylation did not measurably enhance microdomain association, and they concluded that the acylated linker for the activation of T-cell transmembrane domains had a low inherent affinity for cholesterol-enriched microdomains. The possible inference is that acylation is not sufficient for the targeting of any transmembrane protein and that therefore a second mechanism – such as protein-protein interactions for microdomain associations – is required (Fragoso *et al.*, 2003), (Cherukuri *et al.*, 2004).

To conclude, we propose that fatty acids covalently bound to HlyA and membrane microdomains are implicated in the hemolysis process. Fatty acids are essential because they

induce the exposure of intrinsic disordered regions in the toxin so as to enhance protein-protein interactions in order to form the oligomer, while the membrane microdomains act as platforms for the concentration of the toxin during the oligomerization process.

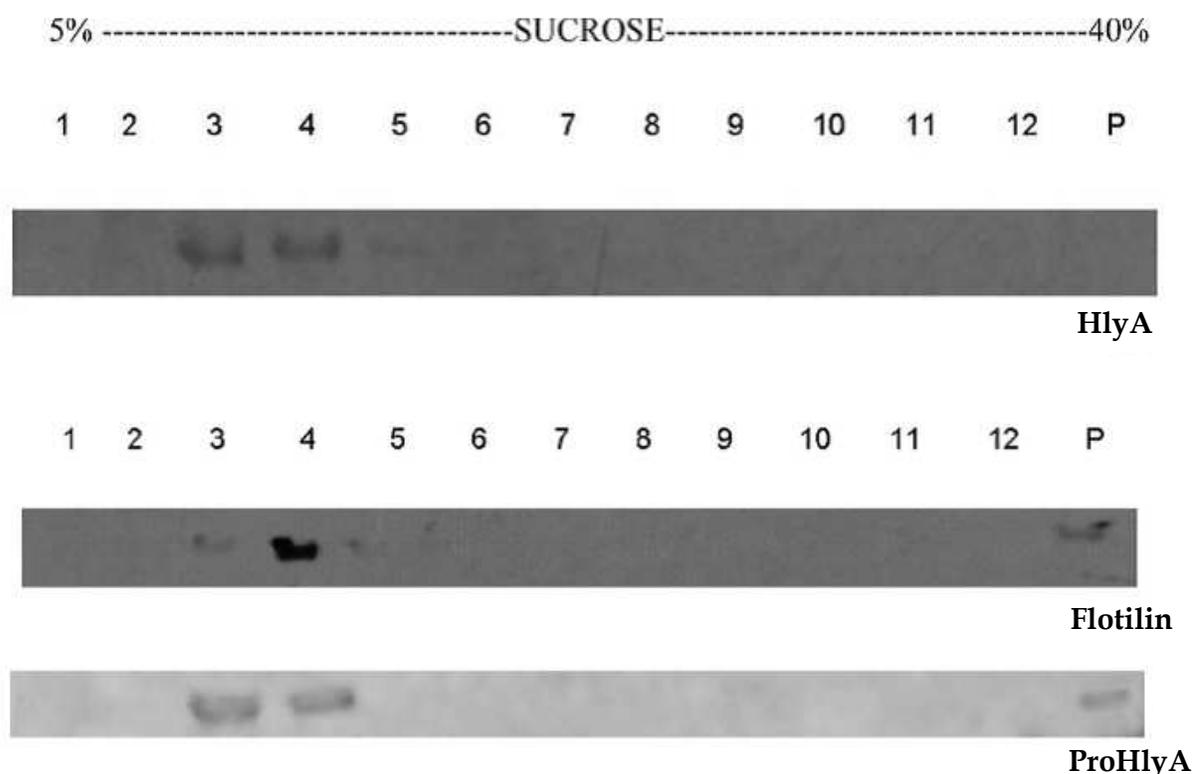


Fig. 5. *Interaction of HlyA with DRMs.* Thirty μg of HlyA were incubated with 100 μl of erythrocyte ghosts for 30 min at 37 $^{\circ}\text{C}$. Cells were lysed with 1% (v/v) Triton X-100 and insoluble cell components separated by sucrose-density-gradient centrifugation. The gradient fractions were analyzed by immunoblotting with anti-HlyA antibodies. HlyA was present in fractions 3 and 4. Gradient fractions were also analyzed by immunoblotting with anti-Flotillin-1 antibodies. Flotillin-1 appears mainly in fractions 3 and 4. ProHlyA was incubated under the same conditions as HlyA. The gradient fractions were analyzed by immunoblotting with anti-HlyA antibodies. ProHlyA colocalizes with HlyA.

In summary, fatty acids covalently bound to HlyA induce a molten-globule structure in the toxin, exposing intrinsically disordered regions involved in the different steps in the toxin's mechanism of action. Fatty acids expose specific regions that induce protein-protein interaction in the oligomerization process that takes place within the membrane microdomains of erythrocytes. The irreversibility of the toxin's membrane binding promoted by fatty acids might result from the formation of the HlyA oligomeric structure (Herlax & Bakas, 2003,2007), (Herlax *et al.*, 2009).

4. Toxin-based therapy

Bacterial toxins have been defined as "soluble substances that alter the normal metabolism of host cells with deleterious effects on the host" (Schlessinger & Schaechter, 1993). Nonetheless, during the last decade, taking advantage of advances in toxin research,

investigators have sought ways of obtaining benefits from toxins. In the present section we will discuss these toxin-based therapies and the possible relevant use of HlyA.

4.1 Immunotoxins

Conventional cancer treatments such as surgery, chemotherapy, and radiotherapy often fail to achieve complete cancer remission. Moreover, radiotherapy and/or chemotherapy are almost always cause significant—and sometimes long-lasting—side effects. These considerations have prompted the development of many new approaches for the treatment of cancer. One such example involves the use of immunotoxins (Bernardes *et al.*, 2010).

The term "immunotoxin" classically refers to chimeric proteins with a cell-selective ligand chemically linked or genetically fused to a toxin moiety that can target cancer cells overexpressing tumor-associated antigens, membrane receptors, or carbohydrate antigens. In the 1970s the first therapeutic agents composed of toxins conjugated to antibodies against cell-surface antigens started to emerge as tumor-cell killers (Moolten & Cooperband, 1970), (Moolten *et al.*, 1976). Since then, many hybrid molecules consisting of a toxin coupled to a specific targeting antibody or ligand were developed, with most of these hybrids being directed against tumor cells (Pastan *et al.*, 2007).

First-generation immunotoxins were prepared by chemically conjugating antibodies to natural intact toxin units or to toxins with attenuated cell-binding capability. These constructs, however, were heterogeneous and nonspecific both because multiplicities of potential sites were available for chemical conjugation and since the presence of the cell-binding domain on the toxin led to an intoxication of nontumor cells as well. Immunotoxins of the second generation were also based on chemical conjugation between the targeting moiety and the toxin. Nevertheless, accumulated knowledge on the structure and function of the toxins enabled the removal of their native nonspecific cell-binding domain, thus generating immunotoxins that were much more target-specific when conjugated to monoclonal antibodies. Although more specific and thus better tolerated by animals, immunotoxins from this second generation were still chemically heterogeneous, and their large size hindered them from penetrating solid tumors. In order to avoid this heterogeneity, improve tumor penetration, and reduce production complexity and expense, recombinant-DNA techniques were applied in the production of the third-generation immunotoxins. In these constructs—mostly produced in the bacterium *E. coli*—the cell-binding domain of the toxin is genetically replaced with a ligand or with the Fv portion of an antibody in which the immunoglobulin light- and heavy-chain variable regions are either genetically linked or held together by a disulfide bond (Shapira & Benhar, 2010).

Among the bacterial toxins that were used for the construction of immunotoxins, the most common were the diphtheria toxin and the pseudomonas exotoxin A, which toxins are naturally produced by the Gram-positive, aerobic *Corynebacterium diphtheria* and by the Gram-negative, aerobic *Pseudomonas aeruginosa*, respectively. Clinical trials with different exotoxin A-immunotoxins have already been performed with positive results in leukemia and bladder cancer (Kreitman *et al.*, 2001), (Kreitman *et al.*, 2005), (Biggers & Scheinfeld, 2008).

In spite of the promise shown by bacterial toxin-based chimeric proteins, these hybrids still present several obstacles that limit their clinical application. The toxin part of the fusion

proteins elicits a high degree of humoral response in humans. In addition, in developed countries, where people have become immunized against diphtheria, the patient's serum will have circulating antibodies against the diphtheria toxin that will result in a neutralization of diphtheria toxin-based immunotoxins (Hall *et al.*, 2001). Both the *Pseudomonas* exotoxin and the diphtheria toxin are large molecules and are difficult to humanize. At sufficiently high concentrations these fusion proteins lead to symptoms like the vascular-leak syndrome and thus exhibit a certain degree of nonspecific toxicity.

HlyA as a possible candidate toxin for the synthesis of immunotoxins

Considering all the details of the structure and mode of action of HlyA discussed above, we can state that HlyA can be a good candidate for an effective immunotoxin. Although, certain additional details about the domains implicated in the binding of the toxin to target-cell receptors need to be clarified, we can consider that the domain that comprises amino acids 914–936 should be exchanged for the specific antibody (or ligand) chosen for interaction with the tumor cell of interest. Perhaps some amino acids within the repeat domain that might be involved in the interaction with the β 2-integrins should also be removed in the fusion protein.

The reason for using HlyA in an immunotoxin

An observation deserving emphasis is that the more relevant effects that HlyA produces during an infection are sublytic rather than cytolytic. Among these effects we must bear in mind the one related to the modulation of the host-signaling cascades, where HlyA was found to produce an inactivation of the PKB (*cf.* **Section 2.4**)—a key protein involved in several pathways related to host-cell survival, inflammatory responses, proliferation, and metabolism (Manning & Cantley, 2007), (Fayard *et al.*, 2005). By inactivating PKB, HlyA is able to fine-tune host responses related to the inflammatory- and apoptosis-signaling cascades that are initiated during the course of an infection. PKB inactivation is produced by an extracellular calcium-dependent, potassium-independent process requiring HlyA insertion into the host plasma membrane and subsequent pore formation. Calcium influx induces the activation of host-protein phosphatases that dephosphorylate PKB, inactivating it and finally inducing host-cell apoptosis. Thus, if a ligand directed at a specific tumor-cell receptor is fused with HlyA, that immunotoxin might induce the apoptosis of the desired cell.

The advantage of using HlyA is that the translocation of the immunotoxin into a tumor cell is not necessary, only its binding to the membrane where HlyA can insert itself and form the pore needed to execute its apoptotic action. Of course, these hypotheses are only possibilities that would warrant further investigation.

4.2 Vaccines

In recent years, an increase in the development of vaccination technology has taken place, but the ideal vaccine has not yet been found. In general terms, there are certain criteria that a vaccine must satisfy: it must be capable of eliciting the appropriate immune response; and it should be safe, stable, and reproducible (Perrie *et al.*, 2008).

UTIs caused by UPEC still represent an enormous challenge for the development of vaccines targeted to induce an immunity that can either prevent the infectious agent from attaching

to and colonizing the mucosal epithelium and/or can block the binding and action of microbial toxins, such as HlyA (Holmgren *et al.*, 2003). Since the infections by these bacteria occur at, or take their departure from, a mucosal surface; a mucosal route of vaccination should be selected rather than a parenteral one.

A significant aspect of immune responses at mucosal surfaces is the production of a secretory IgA (S-IgA) and its transport across the epithelium. This S-IgA response represents the first line of defence against the invasion by bacterial pathogens. The mucosal immune system is an integrated network of tissues, cells, and effector molecules that functions to protect the host from those pathogens. Furthermore, mucosal lymphocytes exhibit unique homing receptors, the integrins, that recognize ligands expressed on mucosal endothelial cells so as to allow their retention within mucosal tissues for the delivery of cellular and humoral immune responses (Butcher & Picker, 1996). Because of the presence of specific interconnected mucosal induction and effector sites for eliciting the S-IgA antibody response, the mucosal immune system has been shown to be separated from the peripheral immune system. Thus, the induction of peripheral immune responses by parenteral immunization does not necessarily result in significant mucosal immunity; by contrast, mucosal immunization is capable of inducing immune protection in both the external secretions and the peripheral immune compartments (Kiyono *et al.*, 1992), (McGhee *et al.*, 1992).

The induction of immune responses following mucosal immunization is usually dependent upon the coadministration of the appropriate adjuvants that can initiate and support the transition from innate to adaptive immunity. While a number of substances of bacterial origin have been tested as mucosal adjuvants, the most widely used mucosal adjuvants in experimental animals are the cholera toxin (CT) and the closely related *E. coli* heat-labile enterotoxin (LT). Both CT and LT consist in homopentamers of cell-binding B subunits associated with a single toxically active A subunit. The A subunit enzymatically ADP-ribosylates the Gs protein of adenylate cyclase leading to an increased cAMP production in the affected cells (de Haan & Hirst, 2000). CT and LT can alter several steps in the induction of a mucosal immune response. These effects, which alone or in combination might explain their strong adjuvant action after oral immunization, include: (1) an increased permeability of the intestinal epithelium leading to an enhanced uptake of a coadministered antigen, (2) an enhanced antigen presentation by various antigen-presenting cells, (3) a promotion of isotype differentiation in B cells leading to increased IgA formation, and (4) complex stimulatory as well as inhibitory effects on T-cell proliferation and cytokine production. Finally, CT and LT have been shown not only to avoid an induction of oral tolerance but also to abrogate additional otherwise efficient regimens producing tolerance induction after oral antigen administration (Holmgren *et al.*, 2005).

A number of studies have examined the activity of LT and CT as mucosal adjuvants in vaccines against a variety of bacterial, fungal, and viral pathogens. Representative examples include the tetanus toxoid (Xu-Amano *et al.*, 1993), (Yamamoto *et al.*, 1996), (Cheng *et al.*, 1999), (Xu-Amano *et al.*, 1994), the inactivated influenza virus (Hashigucci *et al.*, 1996), (Tumpey *et al.*, 2001), a recombinant urease from *Helicobacter* spp. (Lee *et al.*, 1995), (Weltzin *et al.*, 1997), (Lee, 2001), and the pneumococcal surface protein A from *S. pneumoniae* (Wu *et al.*, 1997). Many other examples have been reported, and all these studies clearly indicate that both LT and CT have significant potential for use as adjuvants for mucosally administered

antigens. Nevertheless, both LT and CT are potent enterotoxins, and this property has seriously limited the practical use of these molecules (Freytag & Clements 2005). To avoid such toxicity, a number of nontoxic mutant derivatives of CT or LT have been proposed (Douce G, 1997) (Douce *et al.* 1998). In particular, single-amino-acid-substitution mutants of LT (R7K, S63K and R192G) that lack ADP-ribosyltransferase activity have been shown to retain their adjuvant properties (Douce *et al.* 1995).

In contrast, because of their size, plasticity, and safety profile in humans, OMVs are attractive vehicles for vaccine delivery. OMV vaccines for serogroup-B meningococcal disease, consisting in vesicles from *Salmonella typhimurium* and *Pseudomonas aeruginosa* that contain surface antigens native to the pathogens have been shown to exhibit immunogenic properties (Alaniz *et al.*, 2007), (Bauman & Kuehn, 2006). Two vaccines for serogroup-B meningococcal disease currently exist that are formulations comprising bacterial surface antigens that have been naturally incorporated into OMVs (Oster *et al.*, 2005), (Feiring *et al.*, 2006). These OMV-based vaccines represent a novel system where both the antigen and delivery vehicle are derived from the *Neisseria meningitidis* pathogen itself (Claassen *et al.*, 1996), (Arigita *et al.*, 2004). Moreover, genetically engineered OMVs offer an attractive possibility for use as easily purified vaccine-delivery systems capable of greatly enhancing the immunogenicity of low-immunogenicity protein antigens without the need for an added adjuvant.

With the development of controlled-release technologies, the engineering of OMVs emerged as a promising strategy for antigen delivery because these vesicles are similar in geometry to naturally occurring pathogens and are readily internalized by antigen-presenting cells, thus avoiding the complex manufacturing steps required to purify and encapsulate antigens into particulate delivery systems such as polymer particles (Singh *et al.*, 2007), immune-stimulating complexes (Morein *et al.*, 1984), liposomes, proteosomes, and related vesicles (Lowell *et al.*, 1988), (Lowell *et al.*, 1988), (Felnerova *et al.*, 2004), (Copland *et al.*, 2005) – all of which processes render these approaches economically unfeasible (Ulmer *et al.*, 2006).

The genetic fusion of the green-fluorescent protein (GFP) as a model subunit antigen with the bacterial hemolysin ClyA resulted in a chimeric protein that elicited strong anti-GFP antibody titers in immunized mice, whereas immunization with GFP alone elicited no such titers. Similar to native unfused ClyA, the chimeric ClyA-fusion proteins were found localized in bacterial OMVs, where they retained the activity of the fusion partners, thus demonstrating for the first time that ClyA can be used to colocalize fully functional heterologous proteins directly in bacterial OMVs. The anti-GFP humoral response in mice immunized with the engineered OMV formulations was indistinguishable from the response to the purified ClyA-GFP fusion protein alone and was equal to the response to purified proteins adsorbed to aluminum hydroxide, a standard adjuvant. Engineered OMVs containing ClyA-GFP were easily isolated by ultracentrifugation, thus effectively eliminating the need for a laborious antigen purification from cell-culture expression systems (Chena *et al.*, 2010). The retention of hemolytic-protein activity indicated that ClyA-antigen fusions maintained their conformations. Although no pathologic effects were observed in mice immunized with ClyA, a detoxification of the toxin through mutation, truncation, or chemical methods may attenuate any possible toxicity while still retaining the hybrid's immunomodulatory capabilities.

On the basis of all these data, HlyA presents many properties that can be considered when designing a vaccine.

Anti- UPEC vaccine: The urinary tract is one of the most common sites of bacterial infection. As mentioned above, over half (53%) of all women along with 14% of men experience at least one UTI in their lifetime (Griebing, 2005), (Griebing, 2005). *E. coli* is the infectious agent in more than 80% of the uncomplicated UTIs (Marrs *et al.*, 2005), (Foxman & Brown, 2003). In addition, the upper UTIs of young children can cause permanent kidney damage. An estimated 57% of children with acute pyelonephritis develop renal scarring (Lin *et al.*, 2003).

In recent years, an increase in the antibiotic resistance of UPEC isolates has been observed (Bours *et al.*, 2010) that imposed an urgent need for alternative treatment and prevention strategies to combat this serious and widespread human pathogen. With this aim, much research has been focussed on the development of vaccines to stimulate protective immunity against UPEC. In those studies, surface-exposed molecules such as P fimbriae, the lipopolysaccharide core, α -hemolysin, and the salmochelin receptor IroN have been utilized as antigens for subunit vaccines (Goluszko *et al.*, 2005), (Russo *et al.*, 2003), (O'Hanley *et al.*, 1991); but the limited success of these strategies prevented any vaccine from being currently available. One consideration is that this vaccine has to generate immune responses at the level of mucosal surfaces.

Large-scale reverse-vaccinology approaches offer an alternative to the traditional vaccine design through applying genomic and bioinformatic methods to identify novel vaccine targets (Pizza *et al.*, 2000). Using this technique, Alteri *et al.* identified a class of molecules involved in iron acquisition as vaccine candidates and reported that intranasal immunization with this UPEC outer-membrane iron receptor generated an antigen-specific humoral response to provide protection from UTI (Alteri *et al.*, 2009). The authors proposed that the targeting of an entire class of molecules instead a single protein would permit the identification of components of a more generally protective UTI vaccine and that this strategy could be used in the development of vaccines to prevent infections caused by other pathogenic bacteria. During this present year, these same authors, using the *in-vivo*-induced-antigen technology, identified a novel UPEC virulence factor (*tosA*, a gene encoding a predicted repeat-in-toxin family member) that could be useful as a potential vaccine target (Vigil *et al.*, 2011). Although this methodology did not identify HlyA as a potential candidate for this vaccine, the introduction of that toxin would be beneficial. First of all, HlyA has been recognized as one of the main virulence factors associated with the pathogenicity caused by UPEC (Wiles, Kulesus and Mulvey 2008); second, the toxin induced an immunity response in host organisms (O'Hanley *et al.*, 1991) and thus is immunogenic in its native state; third, it can also produce focal leaks in intestinal epithelia (Troeger *et al.*, 2007). Focal leaks are small openings within the epithelium where bacterial penetration occurs. HlyA induces such focal leaks in a proinflammatory environment – those being also induced by the secretion of the cytokines TNF α and IL-13. Of relevance to highlight is that HlyA can increase the permeability of the intestinal epithelium so as to lead to an enhanced uptake of a coadministered antigen, thus acting as both a coadjuvant and an antigen in its own right. The dose that induces this effect would naturally have to be extensively investigated.

HlyA can also be used as adjuvants in any other vaccine design against another pathogen. For example, the toxin can be included in any liposomal vaccine in order to facilitate uptake through epithelia for the induction of immunity.

OMV vaccines: Balsalobre *et al* (2006) demonstrated that physiologically active HlyA is associated with the OMVs produced from *E. coli* laboratory strains and also from natural and clinical isolates. In our laboratory, we found that the unacylated toxin (proHlyA) can also be associated with OMVs (Herlax *et al.*, 2010). On the basis of this finding, OMV vaccines can be designed by effecting a fusion of the desired antigen with ProHlyA. In this way, ProHlyA would direct the exposure of the antigen on the surface of the OMVs without inducing any cytotoxic response. An advantage of OMV vaccines is that, because of their size and lipopolysaccharide content, they are able to induce an adequate immune response.

Finally, mention must be made that these hypotheses are just speculative on the basis of what is known about the structure and function of HlyA, whose application in toxin-based therapy still has to be exhaustively investigated and especially the immune response the toxin might evoke.

5. Conclusion

E. coli is one of the predominant species of facultative anaerobes in the human gut and in the majority of the cases is harmless to the host. These strains are mostly commensals but also contain a group called the extraintestinal pathogenic *E. coli* (ExPEC). Usually the ExPEC are also harmless colonizers but under certain circumstances can translocate and cause infection. The main virulence factor responsible for this translocation is the HlyA toxin, which pathogen is mainly associated with severe UTI but in addition with bacteremia and extraintestinal infections. In this chapter an exhaustive description of the toxin has been delineated; including its synthesis, maturation, and export from the bacteria. Effects produced by HlyA in different target organs have also been discussed. The significance of the maturation process for the toxin cannot be understated. The acylation of the protein at two internal lysines gives the toxin its virulence, by exposing intrinsic disordered regions that are essential to different steps of the toxin's mechanism of action. The further exposure of regions involved in the protein-protein interaction within the oligomerization process is responsible for the permeability induced in all the target cells, despite the intracellular signal pathway the toxin induces in each specific organ. This activation is unique to prokaryotic proteins.

Based on the already known structural and functional characteristics of HlyA, we might speculate about its use in toxin-based therapy. Such therapy is a versatile and dynamic research area with a great potential application. Further investigation, however, is required in order to improve the efficiency and safety of toxin-based agents. Investments in the development of delivery and targeting techniques are definitely needed in order to achieve this goal, though the basic research on the structure and mechanism of natural toxins should nevertheless not be abandoned. Topics related to HlyA have still to be clarified concerning the existence of a toxin-specific receptor in target cells and the domains of the toxin involved in its interaction with those putative binding sites. The deeper our knowledge becomes about this unique family of secreted polypeptides, the more easily will we be able to harness their great potential for our own benefit.

6. Acknowledgements

We thank Prof. Norma Tedesco for revising the English grammar; Mario Ramos for the graphic designs; and Dr. Donald F. Haggerty, a retired career biochemist and native English speaker, for editing the final version of the manuscript.

This work was supported by grants from the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, and Agencia Nacional de Promoción Científica Grant PICT N° 647, Argentina.

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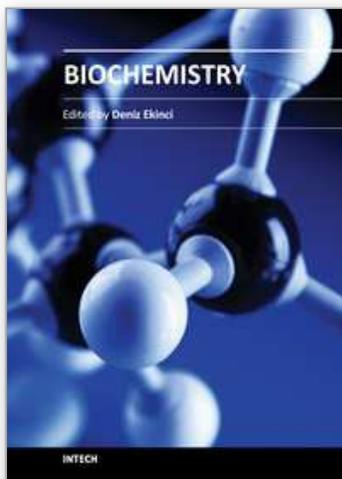
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Biochemistry

Edited by Prof. Deniz Ekinci

ISBN 978-953-51-0076-8

Hard cover, 452 pages

Publisher InTech

Published online 02, March, 2012

Published in print edition March, 2012

Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

How to reference

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Bakás Laura, Maté Sabina, Vazquez Romina and Herlax Vanesa (2012). E. coli Alpha Hemolysin and Properties, Biochemistry, Prof. Deniz Ekinci (Ed.), ISBN: 978-953-51-0076-8, InTech, Available from: <http://www.intechopen.com/books/biochemistry/e-coli-alpha-hemolysin-and-properties>

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