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Minireview

Ion channels and bacterial infection: the case of β-barrel pore-forming protein toxins of *Staphylococcus aureus*

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Abstract ***Staphylococcus aureus*** **strains causing human pathologies produce several toxins, including a pore-forming protein family formed by the single-component α-hemolysin and the bicomponent leukocidins and γ-hemolysins. The last comprise two protein elements, S and F, that co-operatively form the active toxin. α-Hemolysin is always expressed by *S. aureus* strains, whereas bicomponent leukotoxins are more specifically involved in a few diseases. X-ray crystallography of the α-hemolysin pore has shown it is a mushroom-shaped, hollow heptamer, almost entirely consisting of β-structure. Monomeric F subunits have a very similar core structure, except for the transmembrane stem domain which has to refold during pore formation. Large deletions in this domain abolished activity, whereas shorter deletions sometimes improved it, possibly by removing some of the interactions stabilizing the folded structure. Even before stem extension is completed, the formation of an oligomeric pre-pore can trigger Ca^{2+}-mediated activation of some white cells, initiating an inflammatory response. Within the bicomponent toxins, γ-hemolysins define three proteins (HlgA, HlgB, HlgC) that can generate two toxins: HlgA+HlgB and HlgC+HlgB. Like α-hemolysin they form pores in planar bilayers with similar conductance, but opposite selectivity (cation instead of anion) for the presence of negative charges in the ion pathway. γ-Hemolysin pores seem to be organized as α-hemolysin, but should contain an even number of each component, alternating in a 1:1 stoichiometry.**

Key words: α-Hemolysin; Bicomponent leukotoxin; γ-Hemolysin; Oligomerization; Ion selectivity; Pore size

1. Introduction

Pathogenicity of bacteria relies on several virulence factors including the secretion of specialized protein toxins. Among these, pore-forming toxins (PFT) are biological weapons that exert a lethal action by forming oligomeric pores in the plasma membrane of target cells [1,2]. They offer an advantage to the bacteria by killing the incoming leukocytes, thus weakening the immune defense of the host and gaining access to nutrients stored in those cells.

Among other pathogens, *Staphylococcus aureus* is quite frequently isolated in human clinics. It is particularly noxious because of the large variety of infections it can originate, the high frequency of antibiotic-resistant strains [3,4], and the large number of toxins it may produce. PFT are present with a peculiar family of proteins forming β-barrel pores, which include two subfamilies: the bicomponent leukotoxins and the single-component α-hemolysin [5,6]. α-Hemolysin is secreted as a monomer, but associates into a heptamer at the surface of the target cells [7]. The active bicomponent leukotoxins, instead, are formed by the association of two distinct and separately secreted proteins, indicated as class S and F [8]. To date, at least six S proteins and five F proteins are known (Table 1). They include γ-hemolysins (as ubiquitous as α-hemolysin in pathogenic strains of *S. aureus*), Panton–Valentine (PVL) and LukE-LukD leukocidins, sometimes associated with other staphylococcal toxins in specific diseases (impetigo and antibiotic-associated diarrhea, respectively).

A precise characterization of the molecular events underlying the biological activity of these PFT is important not only for understanding bacterial virulence, but also to investigate the basic mechanisms of protein–membrane interactions.

2. Genetics and purification of staphylococcal β-barrel PFT

Four leukotoxin operons have been characterized in *S. aureus*: the γ-hemolysin (HlgA and HlgC/B), the PVL (LukS-PV/F-PV) [9], LukE/D [10] and LukM/F-PV-like [11]. The genes are chromosomal and are cotranscribed, the one encoding the S protein being upstream of that encoding the F protein. They are not collected in pathogenicity islands, except for *lukE/D* that is near to the *egc* operon encoding several enterotoxins [10,12], but may be located in the vicinity of operons encoding metabolic functions (e.g. the γ-hemolysin and the biotin operons) [9]. The regulation of the expression level is usually dependent on the regulation complex *agr/sar* [13].

Class S and class F proteins share around 30% identity, but within each class, the identity scores are higher (between 55

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Abbreviations: EM, electron microscopy; Et, ethidium; PC, phosphatidylcholine; PEG, polyethylene glycol; PFT, pore-forming toxins; PMA, phorbol myristyl acetate; PMN, polymorphonuclear cells; PVL, Panton–Valentine leukocidins; RBC, red blood cells

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Table 1
Properties of *S. aureus* proteins belonging to the α-hemolysin family of β-barrel PFT

Toxin/protein[a]		Acronym	PDB[b]	GenBank	Number of amino acids	M_r	Leukocytes[c]	Red blood cells[c]	Large unilamellar vesicles[c]
α-Hemolysin		α-HL	7AHL	M90536	293	33 249	++	++	++
Leukocidin S-PV	S	LukS-PV		X72700	286	32 529	++	−	−
Leukocidin F-PV	F	LukF-PV	1PVL		301	34 384			
Leukocidin S-R[d]	S	LukS-R		X64389	286	32 536	++	−	−
Leukocidin F-R[d]	F	LukF-R			299	34 138			
Leukocidin E	S	LukE		Y13225	286	32 304	+	+	−
Leukocidin D	F	LukD			301	34 206			
Leukocidin M	S	LukM		D42144	280	32 010	+	+	−
Leukocidin F-PV like	F	LukF′-PV		D83951	296	33 687			
γ-Hemolysin A	S	HlgA		L01055	281	31 994	++[e]	++	++
γ-Hemolysin B	F	HlgB	1LKF		300	34 121			
γ-Hemolysin C	S	HlgC			286	32 554	++	+	+

[a]Next to bicomponent proteins it is indicated whether they are of the S or F type.
[b]References for the PDB files are as follows: 7AHL [35]; 1PVL [37]; 1LKF [36]
[c]Sensitivity of cells or model membranes: ++, high; +, moderate; −, nil.
[d]LukS-R and LukF-R (X64389) correspond to HlgC and HlgB (L01055) [71].
[e]The upper row refers to the couple HlgA-HlgB the lower row to the couple HlgC-HlgB.

and 81%) [14]. A phylogenetic tree [15] suggests the existence of a common ancestor, from which the bicomponent toxins evolved through duplication followed by complementary mutations, which blocked self-aggregation and allowed heterologous aggregation [16]. Other single-component toxins of the same family have been found in different bacteria, e.g. *Bacillus subtilis* and *Clostridium perfringens* [5,6].

The secreted proteins can be concentrated by precipitation with ammonium sulfate and purified by a combination of ion exchange and hydrophobic interaction chromatography [14,17]. They are thermolabile and cysteineless, they have around 300 amino acids, a net positive charge (p*I* 8.5), solubility up to 1–5 mg/ml, and a predominance of β-structure.

3. Epidemiological and clinical links

All the strains isolated in human clinics produce in vitro more than 1 μg/ml α- and γ-hemolysin [7,17,18]. Around 30% of strains also produce noticeable quantities of LukE/D [9], despite this operon is more frequent. Only 2% of strains possess and express the genes for PVL, which, however, characterize more than 90% of the strains isolated from furuncle [19]. PVL-producing strains may also cause community pneumopathies [20,21] and urogenital infections [22]. Interestingly, about 90% of *S. aureus* strains isolated from antibiotic-associated diarrheas produce LukE/D in association with enterotoxin A [23] and 80% of strains isolated from impetigo contagiosa produce LukE/D and one epidermolysin (A or B) [24]. The LukM/F-PV-like operon has, in contrast, never been isolated in human clinics.

4. Target cells and cellular response

Injection of leukotoxins into the rabbit vitreous humor provokes major inflammatory reactions [25], thus providing an important animal model. The privileged target cells of bicomponent leukotoxins are the monocytes, the macrophages and the polymorphonuclear (PMN) cells [8,9]. HlgA/B and HlgC/B can also lyse erythrocytes [9,26]. α-Hemolysin has a wider spectrum of action adding T lymphocytes [27], platelets [28], epithelial cells [29] and fibroblasts [30].

The response of human neutrophils is multiple and leads to the secretion of granular mediators of cytotoxicity, chemotactism and vasodilatation [31–33], and to the release of interleukins, enzymes and oxidizing elements [34]. α-Hemolysin also induces the degranulation of platelets with a reduction of clotting time [28]. Some *S. aureus* strains produce up to seven different bicomponent proteins, carried by the loci *hlg*, *lukS/F-PV* and *lukE/D*. Given the close similarity between these components, the possibility arises that new toxins are formed combining the products of different operons. In principle, any class S component (HlgA or HlgC or LukS or LukE) could give an active toxin with any class F component (HlgB or LukF or LukD). Indeed, many non-classical couples have been found to be active on cellular systems, e.g. HlgA-LukF and LukS-HlgB [9,26]. Interestingly, the cell specificity of the new couples is different from that of the classical ones, suggesting that this combinatorial mechanism may give an adaptive advantage to the microorganism.

5. Three-dimensional structure

The structure of the pore formed by the α-hemolysin heptamer was obtained by co-crystallization with a detergent [35]. The overall shape resembles a mushroom, therefore, the three structural domains that compose it were called: cap, the external part, rim, the domain anchoring to the membrane, and stem, the β-barrel that perforates the membrane (Fig. 1). The cap has a diameter of 100 Å and is composed of a β-sandwich of 2×6 antiparallel β-strands for each monomer, representing 55% of the residues. Together with the rim it forms the core of the protein. The N-terminal extremity detaches from each monomer and interacts with the next two protomers around the entrance of the pore, forming a latch. The monomer/monomer interfaces involve a series of contacts between residues of adjacent units, e.g. His35-Ser99, His48-Asp24, Lys37-Asp100. The 37 consecutive residues of the stem (from Glu111 to Lys147) are organized in a β-hairpin, connected to the cap by the triangle region. Seven hairpins associate to form a transmembrane β-barrel of 52 Å length and 26 Å average diameter. Charged residues exposed inside the lumen of the stem are coupled to form two rings of alternating charges,

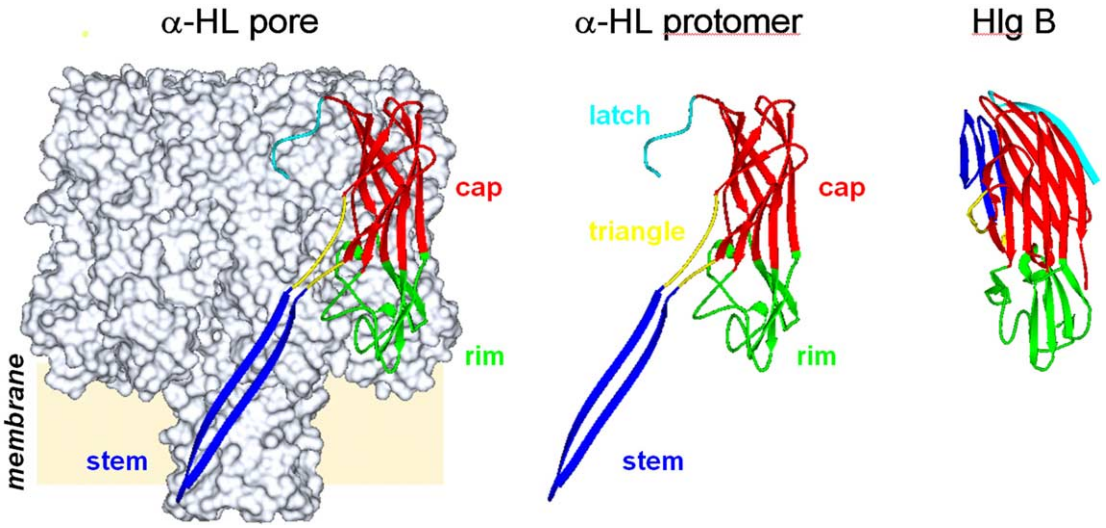

Fig. 1. Crystal structure of the α-hemolysin (α-HL) heptameric pore and the HlgB monomer based on the PDB files 7AHL [35] and 1LKF [36]. A cartoon representation of the three-dimensional structure of one α-HL protomer is highlighted in color and either superimposed on the molecular surface representation of the whole pore (left), or extracted and represented alone (center). The same representation is given of the monomeric HlgB molecule (right). The surface and the cartoon representations were generated by the program PyMOL v.0.86 [72].

Glu111-Lys147 at one entrance and Asp127-Lys131 at the other.

The three-dimensional structures of the monomeric, soluble form of HlgB and LukF-PV were also determined [36,37]. Their overall arrangement is very similar to that of α-hemolysin (Fig. 1) though the rim domains are rotated by 11° and 15° (for HlgB and LukF-PV respectively), and their N-terminal extremity sticks to the β-sandwich body forming an additional strand. Most importantly, the stem and the triangle region are folded against the core of the protein in three antiparallel β-strands covering a hydrophobic interface of 2750 Å^2. These major differences give some hints on the structural modifications occurring during pore formation, e.g. the change of folding of the stem.

6. Mode of action

The interaction of these *S. aureus* PFT with biological membranes has a common, multi-step pathway which includes: binding, oligomerization, insertion and assembly of a β-barrel which forms the functional pore [38].

6.1. Binding

Within the bicomponent toxins, the S protein is the one that attaches first to the target cell, thereby allowing the secondary binding of the F protein and the expression of biological activity [39]. The binding of LukS-PV and HlgC is diminished by treating cells with phorbol myristyl acetate (PMA), a stimulator of the regulation pathway of protein kinase C, and is maximal with staurosporine, a suppressor of the same system [40]. These observations may suggest the participation of a still unidentified receptor to the binding. LukS-PV and HlgC compete for the same binding sites, but HlgA and LukE do not. HlgA has, besides a specific binding, also an unspecific binding [41]. γ-Hemolysins can permeabilize even synthetic membranes containing only phosphatidylcholine (PC) and cholesterol [26,41]. Other leukotoxins, e.g. the PVL, aggregate on the same membranes, but do not form a functional pore. Therefore, the composition of the host membrane can modulate not only the binding, but also the transition to the functional pore. α-Hemolysin is also active on PC/cholesterol vesicles [42], yet, at variance with the leukotoxins, its action on T lymphocytes is stimulated rather than depressed by PMA [39].

6.2. Oligomerization of the monomers

Evidence from electron microscopy (EM), molecular and structural biology, indicated the formation of heptamers by α-hemolysin [35,43], though the presence of hexamers was also noted [44]. During oligomerization, monomer–monomer interactions are established, which involve His35. Using acrylodan, a fluorescent probe sensitive to environmental polarity, it was shown that the stem domain does not unfold until an oligomeric pre-pore is formed [45]. Residue Thr28 of LukS-PV and HlgC, or the corresponding Thr30 of HlgA, is also sensitive to substitutions and affects the secondary binding of the F component [41]. This position corresponds to His35 of α-hemolysin, and most probably participates in interface contacts between bicomponent monomers [37].

In the case of γ-hemolysins the stoichiometry of the pore is still debated. Early analysis of EM pictures of lesions in red blood cells (RBC) and PMN led Sugawara et al. to suggest the presence of a hexamer containing an equimolar ratio of each component [46,47]. A study of pores formed in lipid vesicles came to the same conclusion [26] based on two lines of evidence: on average, the lesions contained equal amounts of each component, implying the presence of an even number of protomers; the apparent MW was around 200 kDa, compatible with the assembly of six monomers. It could not be excluded, however, that these toxins may form an equally populated mixture of two types of pores containing 4:3 and 3:4 combinations of the two components. Indeed a new, more thorough examination of EM pictures led Sugawara's group to the conclusion that cell lesions are equimolar mixtures of two iso-probable heptameric forms, with 4:3 and 3:4 ratios of the two components [48]. A careful inspection of the pub-

lished pictures, however, highlights a strong asymmetry of the analyzed images, the part of the structure containing four protomers being smaller than the part with the other three. This asymmetry, also recognized by the authors [48], makes it difficult to reconcile the reported EM images with a perfectly axially symmetrical structure such as that observed with α-hemolysin (see Fig. 1) and suggests they may rather represent an intermediate on the pathway to form a completely developed pore. In fact, heptamers were declared to be only the most abundant form in the EM images [48]. Another group, analyzing the conductance levels of pores in which a single cysteine was introduced in one component and modified with a thiol-specific negatively charged reagent, came to the conclusion that the reconstituted functional pores contain four copies of each component, being in fact octamers [16]. A way to reconcile these different findings is to assume that hexamers, heptamers and octamers may actually co-exist on the membrane. However, it would remain to be established if all these forms are functional and which one is the most important in vivo.

6.3. Insertion of the hairpins

The insertion of the stem hairpins of α-hemolysin in the membrane of rabbit RBC or liposomes was investigated by the transfer of acrylodan-labeled newly introduced single cysteines [49]. In the final state, the lateral chains of stem residues are oriented alternately one towards the membrane and the other towards the lumen, and create an amphiphilic β-barrel. The process of insertion is rather slow, and the first evidence of a leukotoxin, and possibly also α-hemolysin activity, is a quick entry of Ca^{2+} through toxin-activated endogenous cell channels [50–52]. This activity, detected by Ca^{2+} indicators, is selectively inhibited by econazole (a blocker of Ca^{2+} channels [50]), is independent of the presence of functional toxin pores and can follow an even incomplete stem refolding. Ethidium (Et^{+}) entry, instead, indicates that the formation of toxin pores occurs later and is reduced by high concentrations of Ca^{2+}. In LukF-PV (associated with LukS-PV) the substitution Gly130Cys, or the deletion ΔGly127–Ser129, considerably decreases Et^{+} entry, but leaves enough Ca^{2+} activation to induce the secretion of interleukin-8 [53,54]. Interestingly, this modified PVL is incapable of starting dermonecrosis in the rabbit eye, demonstrating that the ability to form pores is important for pathogenesis. The reason is probably that cell lysis, caused by pore formation, liberates larger amounts of inflammatory molecules, releases nutrients useful for the bacteria and creates cell ghosts that misdirect the host immune system.

To study stem refolding and insertion, a set of mutant LukF-PV proteins were produced with variable deletions in this region [54]. Larger deletions completely abolished cellular activity, probably leaving a stem too short to span the membrane. Smaller deletions, instead, retained Ca^{2+}-activating and Et^{+}-entry properties. Some of these, e.g. ΔIle124–Asn126 and ΔSer125–Gly127, were actually more potent than the wild-type, demonstrating that stem refolding is a crucial event,

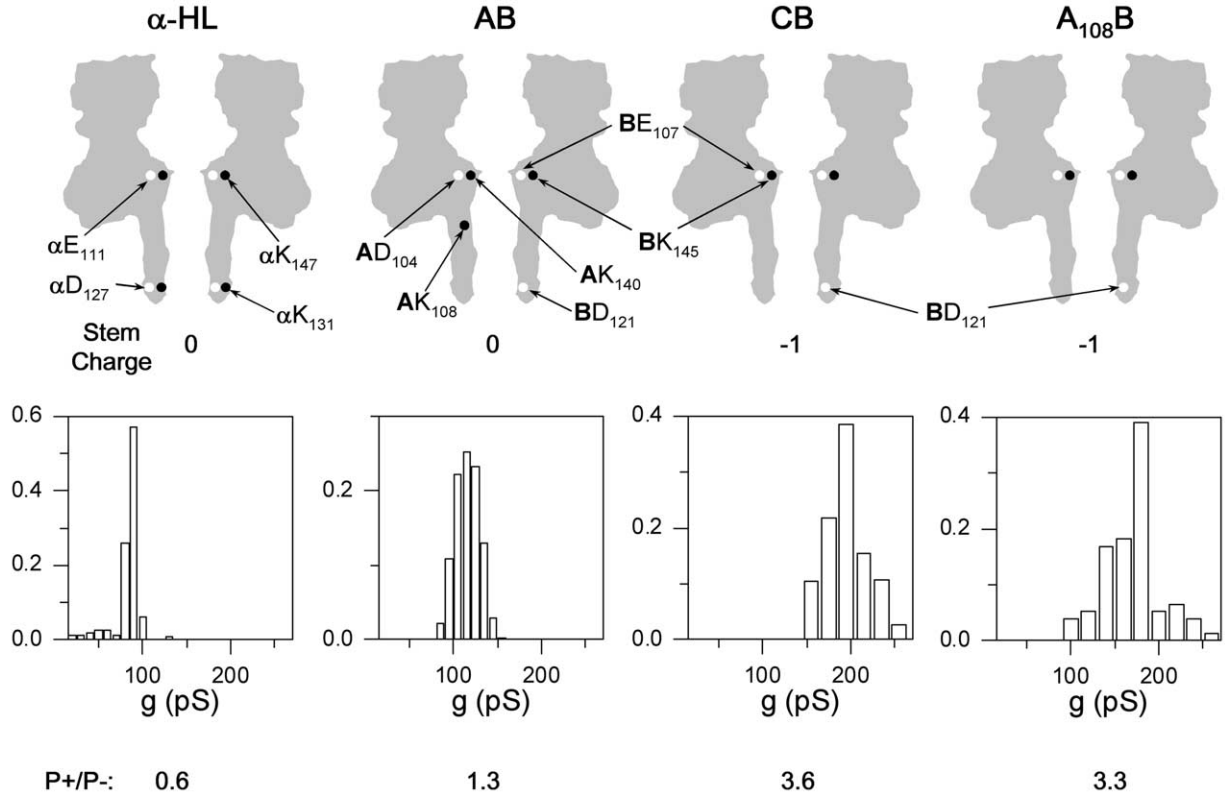

Fig. 2. Electrical properties of α-hemolysin and γ-hemolysin pores. Top: Schematic of the fixed charges which are present in the lumen and at the two entrances of the stem region of the pore. Three natural toxins and the $A_{108}B$ mutant, in which the positive charge K108 of HlgA has been replaced with the uncharged threonine, are reported. Middle: Single-channel conductances, reported in cumulative probability histograms, for the four toxins analyzed. Bottom: Selectivity of the channels, reported as the cation/anion permeability ratio ($P+/P-$), calculated according to the Goldman–Hodgkin–Katz equation. Data are taken and adapted from [63]. Abbreviations: α-HL, α-hemolysin; A, HlgA; B, HlgB; C, HlgC.

which may be facilitated by small deletions that remove some of the intra-chain interactions stabilizing the folding of the soluble form.

6.4. The final pore

According to crystal data [35], the diameter of the heptameric α-hemolysin pore is not constant and varies along its lumen. It is around 30 Å at the entrance, a maximum of 38 Å in the vestibule (inside the cap), a minimum of 14 Å at a constriction formed by Lys145 at the beginning of the transmembrane stem and between 20 and 22 Å along the rest (Fig. 2). Similar entrance diameters were also deduced from low-resolution EM images [55,56], or atomic force microscopy [43,44], or from the differential permeability of sugar molecules of different sizes through pores reconstituted in artificial membranes [57]. In the EM, leukotoxin lesions appear remarkably similar to α-hemolysin, with an entrance diameter between 20 and 30 Å [46,47].

This is a big diameter when compared to a water molecule (2 Å), yet remains a very small cavity, where the solute movement is different from the bulk solution and can be dominated by local electrostatic fields generated by the charged amino acids [58,59]. When the ion conductance properties of the pores are directly compared in model membranes, some striking differences appear. The α-hemolysin pore is anion-selective and has a conductance of 90 pS in 0.1 M NaCl and 775 pS in 1 M KCl [60,61] (Fig. 2). On the other hand, the selectivity of the leukotoxin pores is cationic, as indicated by the transport of Et^+, K^+ and Na^+ in target cells [53,62], and by the reversal voltages in synthetic membranes [63]. The conductance of the HlgA-HlgB pore in 0.1 M NaCl is 115 pS, that of HlgC-HlgB is 190 pS (and 2500 pS in 1 M KCl) [63,64]. These values are markedly higher than those of α-hemolysin, and could suggest a larger pore diameter, in contrast with structural indications.

A direct method to assess the hydrodynamic radius of the pore formed by these toxins in RBC is to determine the rate of colloid osmotic hemolysis in the presence of neutral solutes of different sizes. We did this using a series of oligosaccharides or polyethylene glycols (PEGs) of increasing size (see Fig. 3). The relative permeability of each osmolyte was derived and found to be inversely related to its size. The data were arranged in a Renkin plot (see [65]), providing an estimate of pore radius. This radius was independent of the toxin concentration (Fig. 3), though this dictates the absolute rate of hemolysis. However, different values were obtained using the PEG or the oligosaccharide series (for α-hemolysin 14 Å and 9 Å, respectively, Fig. 3). This could be due to the fact that PEGs are polydisperse, or that they can assume a non-spherical shape squeezing inside pores narrower than their theoretical diameter. Accordingly, estimates with oligosaccharides should be considered more reliable. The values observed for other bicomponent couples were as follows: 14.2 Å and 9.8 Å for HlgA-HlgB; 14.7 Å and 9.3 Å for HlgC-HlgB; 14.3 Å and 9.2 Å for LukE-LukD (where the first number always refers to the PEGs and the second to the oligosaccharides). It therefore appears that the pore sizes of the different toxins are in fact quite similar. The small differences observed (bicomponent pores up to 10% bigger than α-hemolysin pore) could be due either to a larger stoichiometry (octamers instead of heptamers), or to the absence of the constriction at the entrance of the stem, since Lys147 of α-hemolysin is replaced by less bulky residues in the bicomponent toxins (either Asn or Ser).

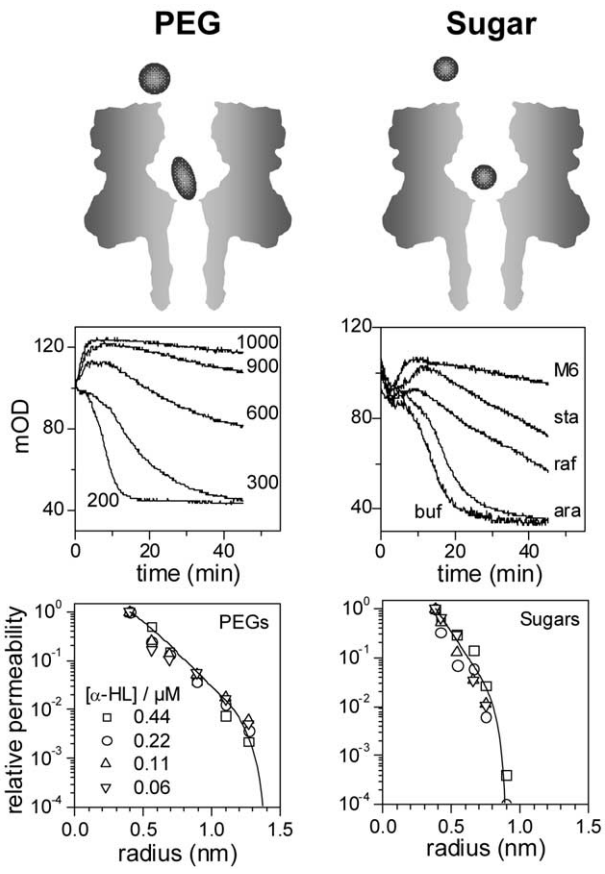

Fig. 3. Osmotic protection experiments. Top: Schematic of the passage of electrically neutral molecules through the α-hemolysin pore. Sugars (right) are considered spherical rigid particles, polymeric PEGs (left) are instead supposed to be more flexible, and therefore prone to adopt elongated shapes, allowing them to pass more easily through the channel constrictions [65]. Middle: Kinetics of human RBC hemolysis caused by α-hemolysin (0.22 μM for PEG and 0.11 μM for sugar experiments) in the presence of 30 mM osmotic protectants in the external buffer. The protectants used are reported next to each trace and were: PEG200 (0.40), PEG300 (0.48), PEG600 (0.69), PEG900 (0.85), PEG1000 (0.89) in the left panel; arabinose (0.38), raffinose (0.66), stachyose (0.75), maltohexaose (M6, 0.90) in the right panel (in brackets is the hydrated radius, in nm [65]). The trace labeled 'buf' is the control without protectant. Bottom: Renkin plot reporting the relative diffusion rates of the osmotic protectants vs. their hydrated radii [65]. Lines through the points are best fits giving a pore radius of 1.40 ± 0.05 nm for PEGs and 0.90 ± 0.05 nm for sugars. Reference molecules were PEG200 (left) and arabinose (right). The larger apparent pore radius obtained in PEG experiments is probably related to the flexibility of PEG molecules, which can more easily change their shape when forced to flow through the pore. For both protectants, radius estimation was independent of toxin concentration (different toxin concentrations are indicated with different symbols), suggesting a stable structure of the pore, which does not change in size with toxin dose.

Ion conductance differences should therefore be due to the influence of charges that are present in the pore lumen (Fig. 2). Inspection of sequence alignments in the stem region indicated interesting differences. The lumen of the stem of the α-hemolysin pore is neutral because of charge compensation between the residues, i.e. Glu111-Lys147 at its entrance and Asp127-Lys131 at its exit. A similar compensation occurs in

the HlgA-HlgB pore between entrance charges (Glu107-Lys145 in HlgB and Asp104-Lys140 in HlgA) as well as internal charges, i.e. Asp121 of HlgB and Lys108 of HlgA. However, in the HlgC-HlgB pore, residue Asp121 of HlgB is not compensated because of the lack of a positive residue such as Lys108 of HlgA. This uncompensated negative charge is conceivably the reason for the larger conductance and higher cation selectivity of the HlgC-HlgB pore. Consistently, we saw that the pore formed by $HlgA_{Lys108Thr}$-HlgB (where the positive charge of Lys108 of HlgA was removed) was as cation-selective and almost as conductive as the HlgC-HlgB pore (Fig. 2). Meanwhile, the pore formed by $HlgA_{Lys108Thr}$-$HlgB_{Asp121Asn}$, where both charges were removed, retrieved a neutral stem lumen and the same conductive properties of wild-type HlgA-HlgB [63]. We concluded that conductance and selectivity of the γ-hemolysin pores depend substantially on the presence and location of charged residues in the channel, suggesting that these pores work as electrostatic filters.

7. Conclusions

Though belonging to a single structural family, α-hemolysin and the bicomponent leukotoxins seem to have been selected, or at least maintained, by *S. aureus* to have a wider range of virulence factors in terms of both cellular specificity and pore properties. Different animal experiments have shown that strains deficient for one or the other of these genes do not achieve the same infectious levels as the wild counterparts [14,66]. The bicomponent leukotoxins introduce an additional, important element of variability, due to the possibility of assembling different toxins by combination of different gene products.

Besides important for understanding the molecular basis of staphylococcal diseases, these studies have led to relevant advances in understanding protein integration in lipid membranes and in proposing pertinent models. They also suggested some conceptually new applications: Bayley and coworkers were able to use suitably modified α-hemolysin channels as stochastic sensors of pollutants, organic molecules or other substrates [67–69], and for the cryopreservation of stem cells [70].

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