The leukocidin pore: Evidence for an octamer with four LukF subunits and four LukS subunits alternating around a central axis

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Abstract

The staphylococcal α -hemolysin (α HL) and leukocidin (Luk) polypeptides are members of a family of related β -barrel pore-forming toxins. Upon binding to susceptible cells, α HL forms water-filled homoheptameric transmembrane pores. By contrast, Luk pores are formed by two classes of subunit, F and S, rendering a heptameric structure displeasing on symmetry grounds at least. Both the subunit stoichiometry and arrangement within the Luk pore have been contentious issues. Here we use chemical and genetic approaches to show that (1) the predominant, or perhaps the only, form of the Luk pore is an octamer; (2) the subunit stoichiometry is 1:1; and (3) the subunits are arranged in an alternating fashion about a central axis of symmetry, at least when a fused LukS-LukF construct is used. The experimental approaches we have used also open up new avenues for engineering the arrangement of the subunits of β -barrel pore-forming toxins.

Keywords: β -barrel; chemical cross-linking; concatameric subunits; leukocidin; pore-forming toxin; staphylococcal α -hemolysin; subunit stoichiometry; subunit arrangement

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α -Hemolysin (α HL) and leukocidin (Luk) are β -barrel pore-forming toxins (β PFT), which are secreted by *Staphylococcus aureus* as water-soluble monomers (Alouf and Freer 1999; Bhakdi et al. 2000; Menestrina et al. 2001; Montoya and Gouaux 2003; Kaneko and Kamio 2004). Upon binding to susceptible cells, they assemble into transmembrane pores that cause cell permeation and, in some cases, lysis. The proteins are pathogenic factors in various diseases (König et al. 1997; Prévost et al. 2001, 2003).

α HL is a 293-residue polypeptide, which assembles on biological membranes, on lipid bilayers, and in detergent to form homoheptameric pores (Gouaux et al. 1994; Song

et al. 1996; Fang et al. 1997; Krasilnikov et al. 2000). Under certain circumstances, a fraction of the oligomer may be hexameric (Czajkowsky et al. 1998). The sensitivity of cells to attack by α HL varies over many orders of magnitude, suggesting the existence of a receptor that facilitates assembly (Hildebrand et al. 1991). The receptor on red blood cells remains unidentified, but caveolin may play a role with other cell types (Pany et al. 2004; Vijayavargia et al. 2004a,b). Upon binding to membranes, α HL monomers first form an inactive heptameric prepore (Walker et al. 1992, 1995; Olson et al. 1999; Kawate and Gouaux 2003). The prepore then inserts into the lipid bilayer to form the active heptamer. The crystal structure of the α HL pore has been solved at 1.9 Å resolution and currently serves as a prototype for the end point in the assembly of β PFT (Song et al. 1996). The α HL pore is emerging as a useful tool in biotechnology (Bayley and Cremer 2001; Bayley and Jayasinghe 2004). For example, it has been extensively engineered for stochastic sensing,

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by which a wide variety of analytes is detected at the single molecule level through the modulation of the current flowing through a single pore. Because of their importance in medicine and technology, it is important to understand the assembly and structure of α HL and related β PFTs in detail.

Unlike α HL, leukocidins are bicomponent toxins and the co-assembly of one class F component with one class S component is necessary to form a functional hetero-oligomeric pore (Montoya and Gouaux 2003; Kaneko and Kamio 2004). There are at least six class F proteins (LukF-PV, LukF-R, LukD, LukF'-PV, HlgB, and LukF-I) and seven class S proteins (LukS-PV, LukS-R, LukE, LukM, HlgA, HlgC, and LukS-I) associated with various strains of *Staphylococcus aureus* (Alouf and Freer 1999; Prévost et al. 2003; Guillet et al. 2004). The F and S proteins share a common ancestor (Kaneko and Kamio 2004). Proteins within each class (F or S) share ~70% identity at the amino acid level, while the identity drops to < 27% between members of the two different classes (Prévost et al. 2001, 2003). Members of neither class are > 30% identical to α HL (Gouaux et al. 1997; Prévost et al. 2003).

Although the structure of a Luk oligomer is yet to be solved, the structures of two water-soluble class F monomers and one class S monomer have been determined. The LukF (HlgB) structure has been solved at 1.8 Å and 2.3 Å resolution and the fold resembles that of the α HL protomer in the heptameric pore, with the exception of the amino latch and pre-stem domains, which are involved in intersubunit interactions and the formation of the transmembrane barrel, respectively (Olson et al. 1999). The LukF-PV structure has been solved at 2.0 Å resolution and is almost identical to LukF (Pédélecq et al. 1999). The LukS-PV structure has been determined recently at 2.0 Å resolution (Guillet et al. 2004). Although most of the fold of LukS-PV is similar to that of LukF, the rim domain shows a significantly different conformation. The lack of a crystal structure for the oligomeric state has limited the use of Luk in protein engineering studies. The similarity between Luk and α HL would allow such studies to move ahead in the absence of a structure if two issues were settled: (1) the number of subunits present in the oligomer from each of class F and class S and (2) the arrangement of those subunits around the central axis of the pore.

Numerous experiments have been carried out to determine the stoichiometry and the subunit arrangement of Luk pores (comprising the F component, HlgB or LukF, and the S component, HlgC or LukS) and the closely related γ -hemolysin pore (γ HL, comprising the F component, HlgB or LukF, and the S component, HlgA or γ HLII). Densitometric data from immunoblots of γ HL assembled on human erythrocyte membranes and run on SDS-polyacrylamide gels, suggested a 2:1 molar ratio of LukF to LukS components (Ozawa et al. 1995; Kaneko

et al. 1997). Ring-shaped structures of γ HL were later observed by electron microscopy on human erythrocytes (Sugawara et al. 1997). Densitometric analysis performed on the heat-dissociated rings, isolated from a sucrose density gradient and run on SDS gels, revealed a 1:1 molar ratio of the LukF to LukS components. Based on these findings, a hexameric structure was proposed for the γ HL pore with very little justification (Sugawara et al. 1997). Similar ring-shaped structures of Luk oligomers were then observed on human polymorphonuclear leukocytes and rabbit erythrocytes (Sugawara et al. 1999). In this case, densitometric analysis and immunoblotting data again suggested a 1:1 molar ratio of LukF to LukS. Kinetic data on membrane permeabilization were subsequently used to justify hexameric structures for both Luk and γ HL pores assembled on lipid vesicles (Ferrerias et al. 1998). Quantification of the heat-dissociated subunits after SDS-polyacrylamide electrophoresis of the oligomers revealed a 1:1 molar ratio of the F and S components (Ferrerias et al. 1998).

Contradictory heptameric and hexameric model structures later accompanied the crystal structures of monomeric LukF and LukF-PV, respectively (Olson et al. 1999; Pédélecq et al. 1999). Later, however, gel-shift electrophoresis and single-channel recording experiments, in which the subunits were counted by in situ chemical modification, provided clear evidence for an octameric pore containing four subunits each of LukF and LukS (Miles et al. 2002b). Nevertheless, images of purified γ HL pores on human erythrocyte membranes obtained by electron microscopy were interpreted as heptamers (Sugawara-Tomita et al. 2002). The results of cross-linking experiments with glutaraldehyde were taken in support of a 3:4 or 4:3 molar ratio of the F to S components. High-resolution electron microscopy of pores with glutathione S-transferase fused to S or F subunits revealed a heptameric arrangement with six of the subunits in an alternating configuration. A model was proposed for the assembly of heptamers, in which an open heterohexamer with an alternating arrangement of F and S subunits is first formed. This is transformed into a closed circular form upon binding of the seventh (S or F) subunit (Sugawara-Tomita et al. 2002). However, the images were not entirely convincing. Because a heptameric arrangement must tolerate some identical neighbors, it seems likely that the full spectrum of subunit combinations should be permitted in this case, including homo-oligomers.

The assembly of LukF and γ HLII to form the γ HL pore on erythrocyte membranes has also been investigated at the single-molecule level with fluorescently labeled subunits (Nguyen et al. 2003). Fluorescence resonance energy transfer (FRET) between acceptor and donor dyes attached to the two monomers revealed the formation of LukF- γ HLII heterodimers and subsequently tetramers, but not homo-

dimers of either LukF or γ HLII. The investigators again favored a hexameric or heptameric pore.

In the present paper, we present convincing evidence that the Luk pore, formed by the class F component HlgB (LukF) and the class S component HlgC (LukS) (Cooney et al. 1988, 1993) and assembled on rabbit red cell membranes, is an octamer. We further show that there are four LukF and four LukS subunits. Finally, at least when a tandem LukS-LukF construct is used, the subunits are arranged in an alternating fashion around the central axis of the pore.

Results and Discussion

Possible permutations of subunits around the central axis of the Luk pore

If the total number of subunits in a hetero-oligomeric pore containing two types of subunit is n , and the number of one type is m , the number of the other type is $(n - m)$. If assembly occurs by random sequential addition of subunits, the total number of linear arrangements of subunits is $n! / m! (n - m)!$. When these linear forms are closed to form a circular arrangement, the total number of permutations is $(n - 1)! / m! (n - m)!$, where n is odd. However, where n is even, the ways of opening the structures with higher symmetry than C_n is less than n . In this case, the simplest way to calculate the number of permutations and the probability that they will occur is by using a probability tree. We constructed such a tree for the Luk pore by assuming that it is an octamer with four subunits each of LukF and LukS (Miles et al. 2002b) and that the subunits assemble in a random fashion. The probability of adding an F, or an S, subunit in each step was $p = 0.5$. It was also assumed that subunit additions were reversible until closure, so that linear forms with more than four F or four S subunits would break up, allowing reuse of the components. In this case, the total number of linear arrangements is 70 and the number of circular forms is 10 (Fig. 1A; Table 1). Importantly, the forms of higher symmetry have a reduced probability of formation because they correspond to a lower number of linear forms (see Electronic Supplemental Material for details).

However, because of the 1:1 ratio of the F and S subunits (Miles et al. 2002b), it can be argued, as follows, that all the assembled pores will be in the most symmetrical arrangement J (Fig. 1A). Unlike α HL, neither LukF nor LukS forms homo-oligomeric pores (Alouf and Freer 1999; Sugawara et al. 1999). Therefore, it is reasonable to assume that neither F nor S subunits can interact with their own type. If LukF interacts only with LukS as in arrangement J, there are only two distinct interfaces between the two types of subunit (Fig. 1B, b-c and d-a). If two LukF or two LukS

Figure 1. Assembly of hetero-oligomeric pores. (A) Possible permutations of the subunits in Luk pores containing four LukF (yellow) and four LukS (red) subunits (see Electronic Supplemental Material; Table 1). (B) Interfaces between subunits in the Luk pore. An alternating arrangement of subunits (structure J in A) requires only two different interfaces between the LukF and LukS subunits (b-c and d-a, left). There are two interfaces, not one, because the subunits are asymmetric objects, not disks as shown. A random arrangement of subunits (structures A–I in A) requires four interfaces (b-c, d-a, b-a, d-c, right).

subunits can interact with each other, as in any other arrangement (Fig. 1A, A–I), there are four interfaces (Fig. 1B, b-c, d-a, d-c, and b-a). If the d-c and b-a interfaces exist, it seems likely that both LukF and LukS should form homo-oligomeric pores. Against this, it might be argued that the F and S subunits each take up a slightly different fraction of space around the central axis and that four of each in any arrangement are required to close the circle at 360° . It should also be noted that there are other oligomeric membrane proteins in which a particular subunit is arranged in more than one way, e.g., the α subunit of the pentameric nicotinic acetylcholine receptor, which occurs in two copies and is flanked (clockwise from the top) by either the γ and β subunits or the δ and γ subunits (Karlin 1993).

Subunit arrangement of the leukocidin pore by chemical cross-linking of adjacent subunits

To determine whether the leukocidin pore has an alternating arrangement of subunits, we performed a cross-linking experiment. Previous attempts with glutaraldehyde gave an $\sim 50\%$ yield of cross-linked subunits (Sugawara-Tomita et al. 2002). In an effort to improve the yield, we sought to make cross-links between Cys residues introduced at specific sites on adjacent subunits. We used the structure of the hemolysin heptamer to identify Asp-2 and Arg-56 as proximal residues in adjacent subunits. Arg-56 is on a

Table 1. Statistical analysis of Luk subunit arrangement and cross-linking

Oligomer arrangement (Fig. 1)	Frequency of occurrence (n)	Potential crosslinks per oligomer (p)	Frequency of potential crosslinks ($n \times p$) / (4 × 70)
A	8/70	1	8/280
B	8/70	2	16/280
C	8/70	2	16/280
D	8/70	2	16/280
E	8/70	2	16/280
F	8/70	3	24/280
G	8/70	3	24/280
H	4/70	2	8/280
I	8/70	3	24/280
J	2/70	4	8/280
Total			160/280 = 57.1%

For details, see the Electronic Supplemental Material and Figures 1 and 2. The potential number of cross-links per oligomer (p) was estimated for each circular permutation of the leukocidin octamer containing four LukF and four LukS subunits (Figs. 1A, 2E). The frequency of occurrence of each circular permutation was determined by counting the number of linear permutations (n) giving rise to each circular permutation. With 70 linear permutations and four pairs of subunits in each circular permutation, the percentage of potential cross-links in the entire ensemble is the sum of the $(n \times p \times 100)/(4 \times 70)$ values = 57.1%. This is the maximum percentage of Luk subunits that can be converted to dimers in the cross-linking experiments described in this work when the subunits of the Luk pore are arranged in a random fashion as shown in Figure 1A.

central strand of a β -sheet of the cap domain and probably has limited mobility. Therefore, we chose Asp-2 on the flexible amino latch as the site to complete the cross-link (Fig. 2A). Then we identified the corresponding residues in LukF and LukS, Lys-55 and Asn-2, by analyzing the sequence homology between α HL, LukF and LukS, and examining the crystal structures (Fig. 2B; Song et al. 1996; Olson et al. 1999; Pédelacq et al. 1999; Guillet et al. 2004). Based on unpublished experiments, we also knew that LukS-Y113H assembles more efficiently and shows higher hemolytic activity when oligomerized with either wild type LukF or LukF-Y117H (data not shown). According to the crystal structures, Tyr-113 in LukS and Tyr-117 in LukF correspond to Tyr-118 in α HL. Therefore, where indicated, we used the double mutant LukS-N2C/Y113H to obtain the improved oligomerization yield required for success in the cross-linking experiments.

The two cysteine mutants, LukF-K55C and LukS-N2C/Y113H, were oligomerized in the presence of rabbit red blood cell membranes (rRBCM) and the oligomer was purified by SDS–polyacrylamide gel electrophoresis (Materials and Methods; Fig. 2C, step A). Various attempts to cross-link the cysteine residues were made with the intact oligomer (Materials and Methods; Fig. 2C, step B). Noncovalent intersubunit interactions were then disrupted by heating the oligomer in SDS. If the

LukF and LukS subunits alternated, we expected to see LukS-LukF dimers at this stage by SDS–polyacrylamide gel electrophoresis performed in the absence of thiols (Fig. 2C, step C). We first used the disulfide-forming Cu-orthophenanthroline (Cu-OPA) as the cross-linking agent (Lee et al. 1994; Hamdan et al. 2002). In this case, < 50% of the oligomer was converted to dimer and the rest to monomer, according to phosphorimager analysis (Fig. 3A, lane B). We then conducted experiments with 4-sulfophenylarsine oxide (SPA0) as the cross-linking agent. Trivalent arsenic compounds had not previously been used to form intersubunit cross-links in proteins. They have, however, been used in many studies to explore closely spaced protein thiols within individual subunits. For example, phenylarsineoxide binds to thiols in the active site of human thioredoxin with $K_d = 370$ nM (Donoghue et al. 2000) and methylarsenous acid binds to two thiols in α -helical polypeptide chains with $K_d = 30$ –200 nM (Cline et al. 2003). In agreement with the experiments with Cu-OPA, we also observed a dimer after treatment with SPA0 and heat dissociation (Fig. 3B, lane B). Phosphorimager quantification revealed that the yield of dimers had been improved to ~65%. As expected, the -S₂- or -S-As-S- bonds were cleaved by treatment with dithiothreitol (DTT) (Fig. 2C, step D) to yield the monomeric constituents, LukS and LukF, in a ratio of 1:1 (Fig. 3A,B, lane C).

If the subunit arrangement were completely alternating (Fig. 1A, arrangement J), a maximum of 100% oligomer to dimer conversion by cross-linking would be possible. The lower value we observed could be due to one or more of the following reasons:

1. The cross-linking efficiency of the reagents are poor or cross-linking is reversed during analysis. We tried to improve the conversion by optimizing conditions such as the pH, the concentrations of the reagents, and the time of incubation. We also decreased the heating time and temperature in the final dissociation of the cross-linked oligomers from 5 min at 95°C to 4 min at 85°C (data not shown). Despite these efforts, we could not improve the dimerization efficiency to >65%. Alternative cross-linking reagents were also used to no avail and included: aqueous iodine (Betanzos et al. 2002), bis-((N-iodoacetyl)piperazinyl)sulfonerhodamine (Corrie et al. 1998), and dibromobimane (Bhattacharjee and Rosen 1996).
2. Some cysteine residues are destroyed during the assembly and purification of the oligomer. We used 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonic acid (IASD) modification to determine the integrity of the cysteine residues (Walker and Bayley 1995). After heating the gel-purified and cross-linked oligomers (Fig. 2C, step C), we treated the samples

Figure 2. Chemical cross-linking of adjacent subunits in the leukocidin octamer. (A) Cysteine mutagenesis for chemical cross-linking. Two adjacent subunits of the α HL pore are shown in ribbon form. The residues Arg-56 and Asp-2 were identified for cross-linking. The corresponding residues were identified as Lys-55 (Arg-56 in α HL; blue) in LukF (represented by yellow α HL subunit) and Asn-2 (Asp-2 in α HL; green) in LukS (represented by red α HL subunit). In α HL, the distance between the C $_{\alpha}$ atoms of Arg-56 and Asp-2 is ~ 11 Å. The model was generated with PyMOL version 0.97. (B) Sequence alignments of α HL, LukS, and LukF. (C) General scheme for the chemical cross-linking of Luk subunits. (A) LukF-K55C and LukS-N2C or LukS-N2C/Y113H are oligomerized on rabbit RBCMs (Fig. 3; Materials and Methods); the oligomer is purified by gel electrophoresis. (B) Cross-linking of adjacent subunits is carried out with Cu-OPA or SPAO; the arrow indicates a possible cross-link. (C) The noncovalent intersubunit interactions in the oligomer are disrupted by heating. (D) When desired, the cross-links in the resulting covalent dimers are broken with DTT to release the constituent monomers. (E) Mechanism of cross-linking of closely positioned cysteines with 4-sulphophenylarsine oxide (SPA). (F) Possible cross-links in different arrangements of the Luk pores containing four LukF (yellow) and four LukS (red) subunits. A cross-link (arrow) involving the Cys residues in the mutants LukF-K55C and LukS-N2C can be formed only when LukS (red) precedes LukF (yellow) in a clockwise rotation, as viewed into the cap of the structure.

with IASD. Upon SDS–polyacrylamide gel electrophoresis, a shift in both the LukF and LukS monomer bands was observed suggesting that they contained free, reactive cysteine residues (Fig. 3C).

3. The initial cross-links in an oligomer might introduce geometrical distortions that prevent subsequent cross-linking. The cysteine residues should be in close proximity for cross-linking to take place. If the formation of two or three cross-links introduces geometrical distortion, it might prevent the formation of a third or a fourth cross-link. In that case, we would expect to see between 50% and 75% conversion to dimer. The value we observed, 65%, lies in this range. Static disorder has been invoked to explain the lack of complete disulfide bond formation when nucleosome arrays are cross-linked (Dorigo et al. 2004). Complete cross-linking is often not observed even in cases that are apparently favorable as suggested

by structural information (Maurer et al. 2000; Jiang et al. 2001), although close to complete conversion has been noted occasionally (Jones et al. 1998).

4. The subunit arrangement is random. The final possibility is that the subunits are assembled in a random manner about the central axis. It is difficult to justify a specific arrangement, other than J (Fig. 1A), based on the arguments made earlier. Therefore, we explored the possibility of a random arrangement of subunits in more detail.

Is the leukocidin subunit arrangement random?

First, we calculated the expected oligomer to dimer conversion if the subunit arrangement of the Luk pore was random (Fig. 2E; Table 1; Electronic Supplemental Material). In the α HL crystal structure, the N termini of the assembled monomers interact with neighboring

Figure 3. Analysis of LukS-LukF dimers formed by cross-linking Luk oligomers. (A) Chemical cross-linking by Cu-OPA. LukF-K55C and LukS-N2C were oligomerized on rabbit RBCMs. Gel purified oligomers were treated with Cu-OPA (see Materials and Methods). The sample was divided into three equal portions, which were run on a 9% SDS-polyacrylamide gel. (Lane A) Unheated oligomer; (lane B) oligomer heated at 95°C for 5 min; (lane C) oligomer heated at 95°C for 5 min and then treated with DTT; (lane M) protein molecular weight markers (Amersham Biosciences). (B) Chemical cross-linking by SPAO. LukF-K55C and LukS-N2C/Y113H were oligomerized on rabbit RBCMs. Gel purified oligomers were treated with SPAO (see Materials and Methods). The sample was divided into three equal portions, which were run on a 10% SDS-polyacrylamide gel. (Lane A) unheated oligomer; (lane B) oligomer heated at 95°C for 5 min; (lane C) oligomer heated at 95°C for 5 min and then treated with DTT; (lane M) protein molecular weight markers. (C) IASD modification of residual uncross-linked monomers. LukF-K55C and LukS-N2C/Y113H were oligomerized on rabbit RBCMs. Gel-purified oligomers were treated with SPAO, heated at 95°C for 5 min and treated with IASD. (–) No IASD treatment; (+) treated with IASD.

subunits in the clockwise direction only (looking into the structure from the cap side) (Song et al. 1996), and we assume that the situation is the same in the leukocidin oligomer. In our mutants, the cysteine residue of LukS is positioned at a specific site in the N terminus and we expect a dimer only if LukS precedes LukF in a clockwise manner (Fig. 2E). We calculated the frequency of occurrence of each octameric form containing four LukF and four LukS subunits generated by random assembly (Table 1). We then estimated the number of potential dimers that would be formed from each arrangement and from that we evaluated the percentage of the subunits from all 10 forms that would be converted to dimers if cross-linking were complete. The value is 57%. Since the value we obtained, 65%, is higher, it is reasonable to argue that the subunit arrangement of the leukocidin octamer is not random and most likely alternating (with <100% efficient cross-linking). Another possibility is a “semirandom” or biased arrangement. Because of the uncertainty, we performed additional experiments that pertain to this issue.

Confirmation that the oligomers contain an equal number of LukF and LukS subunits

As explained earlier, if the subunit arrangement is random, there should be LukS–LukS and LukF–LukF

interactions in the oligomers, in addition to LukF–LukS interactions (Fig. 1B). If that were the case, it might be possible for more or less than four subunits of each type to be incorporated into the oligomer. To test this possibility, we oligomerized LukF and LukS in widely different starting ratios (Fig. 4A). We then heated the gel-purified oligomers in SDS to disrupt the inter-subunit interactions. The dissociated LukF and LukS

Figure 4. Formation of Luk oligomers beginning with different ratios of LukF and LukS subunits. (A) Five percent SDS-polyacrylamide gel. LukF and LukS oligomerized on RBCMs in different ratios: 15:1, 5:1, 1:1, 1:5, 1:15. (B) Analysis of the ratios of LukF to LukS in each band. Each band in A was gel-purified, heated and rerun on a 12.5% SDS-polyacrylamide gel. Phosphorimager quantification revealed the LukF:LukS ratio in each lane to be close to 1:1 as indicated.

subunits were then separated in an additional gel and their ratios determined by phosphorimager analysis to be close to 1:1 in all cases (Fig. 4B).

The genetic ligation of adjacent subunits in the pore

To further test the likelihood of an alternating arrangement of subunits, we used a genetic ligation approach. We coupled the full-length genes encoding LukF and LukS through a serine/glycine linker (Minier and Sigel 2004). From unpublished experiments, it was clear that the N terminus of LukF can tolerate more changes than that of LukS. Therefore, we decided to place LukS upstream of LukF in the dimer (Fig. 5A). In the hemolysin pore, a 37-Å linker is the minimum length required to connect the N terminus of one subunit to the C terminus of the adjacent subunit, assuming that no rearrangement of the polypeptide chain occurs (Fig. 5B).

Therefore, we decided to use a 15-residue serine/glycine linker (~48 Å) to connect LukF and LukS.

The oligomerization and hemolytic activities of the dimer were evaluated (Fig. 5C,D). Interestingly, the activity of the dimer was found to be at least 50 times higher than that of the wild-type subunits (Fig. 5C), although the extent of oligomerization of the dimer was not notably more efficient than that of the wild-type subunits (Fig. 5D). During the assembly of the wild type Luk pore, the two types of membrane-bound monomers must diffuse on the membrane surface, searching for partners with which to interact in the proper orientation to form the prepore. Linking the two subunits together presumably facilitates this process. The unaltered electrophoretic mobility of the oligomer formed from LukS-LukF suggests the structure X (Fig. 6A). Structures of type Z have been proposed when the β , γ , and α subunits of the epithelial sodium channel (ENaC) have been concatenated as a trimer (β - γ - α) (Firsov et al.

Figure 5. Experiments with a LukS-LukF fusion protein. (A) Gene construction. The 3' end of the full-length gene of LukS (encoding residues 1–286; red) was linked to the 5' end of the full-length gene of LukF (encoding residues 1–300; yellow) through a DNA sequence encoding a 15-residue serine/glycine linker (green). (B) Positions of the N and C termini of two adjacent subunits in the α HL heptamer. The N and C termini of LukF (yellow) and LukS (red) are presumed to assume similar positions as shown here. The model was generated by PyMOL version 0.97. (C) Hemolytic activity of the LukS-LukF fusion protein. (Row A) Wild type LukF with wild type LukS; (row B) LukS-LukF; (row C) LukS-LukF-BacTL; (row D) blank (no protein). Briefly, the proteins were serially two-fold diluted across the row. Pore formation was initiated by the addition of washed rabbit RBCMs and the decrease of light scattering was monitored (see Materials and Methods for details). (D) Oligomerization of LukS-LukF. Luk subunits were allowed to oligomerize on rRBCM. The products were run on a 10% SDS-polyacrylamide gel. (Lane A) Wild type LukS with wild type LukF; (lane B) LukS-LukF dimer; (lane C) LukS-LukF-BacTL; (lane M) protein molecular weight markers.

Figure 6. Analysis of oligomers containing the LukS-LukF fusion protein. (A) Possible arrangements of the LukS-LukF dimer in the oligomer. (X) The two linked subunits are adjacent to each other (four dimers per oligomer). (Y) Only one subunit of each dimer participates in pore formation (eight dimers per oligomer). This is the extreme case of subunit exclusion from the central ring. (Z) The two linked subunits are in non-adjacent positions in the oligomer (four dimers per oligomer). (B) Possible permutations of subunits in pores formed from LukS-LukF and LukS-LukF-BacTL, assuming an alternating arrangement of F and S subunits. (C) Five percent SDS-polyacrylamide gel electrophoresis of oligomers formed on rRBCMs from various ratios of LukS-LukF and LukS-LukF-BacTL. (Lane A) 1:0 (lane B) 1:15; (lane C) 1:5; (lane D) 1:1; (lane E) 5:1; (lane F) 15:1; (lane G) 0:1. The five different bands are presumed to correspond to the five different permutations (P, Q, R, S, and T; Fig. 6B), as indicated. (D) Oligomer bands consist of LukS-LukF and LukS-LukF-BacTL. The oligomer bands in lanes A, F, D, B, and G of Figure 6C were heated to disrupt noncovalent intersubunit interactions and rerun on a 12.5% SDS-polyacrylamide gel. The two bands correspond to LukS-LukF and LukS-LukF-BacTL.

1998). In our case, a linker of at least 60 Å would be required to place the LukS-LukF dimer in the structure Z (Fig. 6A, based on the dimensions of the αHL pore).

It was possible that linking the two subunits together causes one or more of them to be excluded from the central ring. In structure Y (Fig. 6A), the extreme case is shown where eight subunits are excluded. In other cases where concatenated subunits have been examined, “excluded” subunits have been observed. For example, in the study of ENaC stoichiometry, the three subunits of one β-γ-α trimer formed functional channels by incorporating the α subunit of a second β-γ-α trimer, but excluding the γ and β subunits of the latter (Firsov et al. 1998). Similar exclusion of subunits has been observed in a pentameric neuronal acetylcholine receptor (Groot-Kormelink et al. 2004).

Presumably a structure such as Y (Fig. 6A) would result in an altered electrophoretic mobility of the oligomer. Nevertheless, we decided to perform a gel shift experiment to count the number of LukS-LukF dimers

in the oligomer formed from them by using normal dimers and dimers with an extended polypeptide chain. This approach works well with αHL monomers, but produced less clear results with LukF and LukS monomers (Miles et al. 2002b). We attached the same 94-residue polypeptide sequence from *Bacillus cereus* hemolysin II (BacTL) that had been used in these cases at the C terminus of the LukS-LukF dimer. Like the unmodified LukS-LukF dimer, the extended construct, LukS-LukF-BacTL, displayed enhanced hemolytic activity (Fig. 5C, row C, and 5D, lane C). We mixed the unextended and extended dimers in different ratios and allowed them to assemble on rabbit RBCMs, which was followed by examination by SDS-polyacrylamide gel electrophoresis. With the dimer arrangement X (Fig. 6A), we expected to separate five different oligomers corresponding to the five possible combinations of subunits (Fig. 6B, P,Q,R,S,T). We did indeed observe five distinct bands (Fig. 6C), which comprised LukS-LukF and LukS-LukF-BacTL in different

ratios as shown by heating oligomers before electrophoresis (Fig. 6D). These results clearly demonstrate that the oligomer contains only four LukS-LukF dimers, which provides additional strong evidence for the octameric stoichiometry of the leukocidin pore and an alternating arrangement of subunits.

In summary, our study proves (1) there are equal numbers of F and S subunits in the Luk pore (by assembly in different starting ratios; Fig. 4); (2) there are four of each type of subunit in the pore (subunit counting experiment [Miles et al. 2002b]; the tandem construct forms an “octamer” that is of the same mobility in an SDS-gel as the wild type Luk pore [Fig. 6]); (3) the F and S subunits *can* be in an alternating arrangement about a central axis (tandem gene experiment; Figs. 5, 6); and (4) when the Luk pore is formed from individual F and S subunits on red cells, the structure is at least biased toward the alternating arrangement (cross-linking experiment; Figs. 2, 3).

Future prospects for engineering

Knowledge of the crystal structure paved the way to precise engineering of the α HL pore (Bayley and Jayasinghe 2004) and prospective applications, for example in biosensor technology (Bayley and Cremer 2001). Leukocidin is also an attractive target for protein engineering, with properties that differ from α HL:

1. Leukocidin forms pores that are physically larger than the α HL pore and, even after taking this into account, have an unexpectedly high conductance (Miles et al. 2001). It may be possible, for example, to accommodate larger molecular adapters within the Luk pore than is possible with the α HL pore, γ CDs rather than β CDs perhaps.
2. Leukocidin is a bicomponent system. The presence of two different subunits offers flexibility in protein engineering that cannot be readily achieved with α HL; it is possible to engineer one subunit without affecting the other. In the case of α HL, it is straightforward to make homoheptamers and heteroheptamers containing one mutant subunit (Fig. 7A). However, other combinations of subunits have more than one permutation about the central axis (Braha et al. 1997). In the case of the Luk pore, the new knowledge about the subunit arrangement will allow four of the eight subunits to be altered and distributed symmetrically around the central axis (Fig. 7B). Eight alterations of two types can also be introduced in an alternating arrangement (Fig. 7C). The α HL, LukS, and LukF genes can be aligned (Gouaux et al. 1997), permitting mutations in the LukF and LukS polypeptides to be placed at the same or different levels within the β barrel of the assembled pore.

Figure 7. Implications for engineering of β PFTs. (A) One or seven mutations can be introduced readily inside the α HL pore. (B) By mutating only one type of subunit, four mutations can be introduced in a symmetrical arrangement within the Luk pore. (C) By mutating both subunits, eight mutations (either the same or two different types) can be introduced inside the Luk pore. (D) With fused subunits (LukS-LukF), two different mutations can be introduced in adjacent subunits.

3. The ability to chain together the Luk subunits opens up new prospects for engineering of β PFTs. In the simplest case, mutations in two adjacent subunits can be placed in the assembled Luk pore (Fig 7D). Clearly, this approach might be extended to longer chains of subunits and mixtures of them, or to the α HL pore. Active concatamers might also be useful in studying the assembly of β PFTs (Walker et al. 1995; Olson et al. 1999; Nguyen et al. 2003) because they might resemble assembly intermediates.

Materials and methods

Leukocidin mutants

All constructs were made in the pT7-SC1 expression vector (Cheley et al. 1997) and verified by DNA sequencing of the entire genes. Genes encoding the point mutants LukS-N2C, LukS-N2C/Y113H, and LukF-K55C were generated by PCR mutagenesis and in vivo recombination as described elsewhere (Jones 1995; Howorka and Bayley 1998). The LukS-LukF gene was generated by the same technique by using overlapping primers to form the linker region. The entire LukS gene was used, while the initial methionine residue of LukF was omitted. The two genes were connected by DNA encoding a 15-residue linker with the amino acid sequence GGSSGGSGGSGSSGG. The LukS-LukF-BacTL gene was generated by fusing the LukS-LukF gene to DNA encoding the 94-residue C-terminal extension of *Bacillus* hemolysin-II (amino acid residues 289–382) (Baida et al. 1999; Miles et al. 2002a), obtained from the LukF-TL gene (Miles et al. 2002b). Synthetic LukS and LukF genes were used

in this work and were provided by George Miles (G. Miles, unpubl.). The codon usage in these constructs differs from the natural genes, but the encoded amino acid sequences are unchanged.

Coupled in vitro transcription and translation

All proteins were generated by coupled in vitro transcription and translation (IVTT) by using an *E. coli* T7-S30 extract system for circular DNA (Promega). The complete 1 mM amino acid mixture minus cysteine and the complete 1 mM amino acid mixture minus methionine were mixed in equal volumes to obtain the working amino acid solution required to generate high concentrations of the proteins. The amino acids (2.5 μ L) were mixed with premix solution (10 μ L), [35 S]-methionine (1 μ L, MP Biomedicals, 1175 Ci/mmol, 10 mCi/mL), plasmid DNA (4 μ L, 400 ng/ μ L) and T7 S30 extract (7.5 μ L) supplemented with rifampicin (20 μ g/mL) (Cheley et al. 1997). Synthesis was carried out for 60 min at 30°C to generate 25 μ L of IVTT protein. To form hetero-oligomeric proteins containing LukF wild type and LukS wild type, or LukF-K55C and LukS-N2C or LukS-N2C/Y113H, plasmid DNAs encoding each component were mixed in equal amounts, and a portion of the mixture (4 μ L) was used for IVTT. In the cases of the proteins from LukS-LukF and LukS-LukF-BacTL, 4 μ L of plasmid DNA was used.

Purification of oligomers for cross-linking experiment

35 S-labeled heterooligomers containing LukF-K55C and LukS-N2C or LukS-N2C/Y113H were generated by IVTT (200 μ L total volume) in the presence of rabbit erythrocyte membranes (Cheley et al. 1999). The washed membrane pellet was solubilized with sample buffer (Laemmli 1970) and subjected to SDS–polyacrylamide electrophoresis in a 5% gel. An autoradiogram was made from the unfixed gel after it had been dried at room temperature. Using the autoradiogram as the template, the Luk oligomer band was cut from the gel and the fragment placed in 200 μ L elution buffer (50 mM Tris.HCl [pH 6.8], containing 1 mM DTT for SPAO cross-linking and 50 mM sodium phosphate [pH 7.3], containing 1 mM DTT for Cu-OPA cross-linking). The rehydrated gel was crushed using a sterile pestle and agitated overnight at 4°C. The suspension was then transferred to a 0.2 μ m cellulose acetate Microfilter-fuge tube (Rainin) and centrifuged at 21,000g for 30 min (Miles et al. 2001). The eluted protein solution was transferred to a Microcon YM-10 centrifugal filter device (Millipore) and centrifuged at 14,000g for 20 min. The concentrated sample in the filter device was diluted twice with the desired buffer (300 μ L) and reconcentrated (30 min, 14,000g). Finally, the sample was recovered by centrifugation of the inverted filter device (75 μ L final volume).

Cross-linking experiments

A portion of the purified oligomer (25 μ L) was kept as a control sample (A). The remainder (50 μ L) was incubated with either Cu-OPA (20 μ L of 15 mM CuSO₄; 45 mM 1,10-phenanthroline; 50 mM Na phosphate [pH 7.3]; 10% [w/v] glycerol) (Lee et al. 1994) or 35 mM SPAO (20 μ L in 35 mM MOPS [pH 8.0]) (Oneto 1938; Shin et al. 2002) for 10 min at room temperature. After the addition of gel-loading buffer (70 μ L) (Laemmli 1970), the samples were heated at 95°C for

5 min and divided into two portions of 70 μ L each (B and C). Sample B was not further treated, while sample C was treated with DTT (10 μ L, 100 mM) for 10 min at room temperature to cleave the cross-links. Samples A, B, and C were subjected to SDS–polyacrylamide gel electrophoresis in 9% or 10% gels, as indicated (Fig. 3A,B).

Cross-linking with aqueous iodine (Pakula and Simon 1992; Betanzos et al. 2002), bis-((N-iodoacetyl)piperazinyl)-sulfonerhodamine (Corrie et al. 1998), and dibromobimane (Bhattacharjee and Rosen 1996) was carried out by similar procedures. In these cases, the proteins were obtained as indicated above in 50 mM Tris.HCl (pH 6.8), containing 1 mM DTT (75 μ L). A portion of this (25 μ L) was kept as a control (A). The remaining protein (50 μ L) was treated with (1) aqueous iodine: 20 μ L of 10 mM aqueous iodine for 15 min at room temperature; (2) bis-((N-iodoacetyl)piperazinyl)sulfonerhodamine (Molecular Probes): 20 μ L of a 5 mM solution in dimethylformamide for 20 min at room temperature; (3) dibromobimane (Molecular Probes): 20 μ L of a 10 mM solution in dimethylformamide for 20 min at room temperature. After cross-linking, the samples were treated and analyzed as indicated earlier.

IASD modification

Dimers of LukF-K55C and LukS-N2C/Y113H were generated by cross-linking of oligomers formed on red cell membranes with SPAO as described above (sample B). After heating at 95°C in gel loading buffer to dissociate the oligomers, the sample was divided into two portions. One half was kept as the control and the other half was incubated with 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonic acid, disodium salt (IASD, Molecular Probes) for 10 min at a final concentration of 10 mM (Fig. 3C). The samples were subjected to SDS–polyacrylamide gel electrophoresis in 10% gels.

Luk hetero-oligomers formed from different ratios of LukF and LukS monomers

Hetero-oligomers containing wild type LukF and wild type LukS were prepared by mixing in various molar ratios the plasmids containing the F and S genes prior to IVTT in the presence of rabbit RBCMs (Cheley et al. 1999). The washed membrane pellets were subjected to SDS–polyacrylamide gel electrophoresis in 5% gels (Fig. 4A). Oligomers were obtained from the gels as described (Miles et al. 2001), concentrated using a vacuum centrifuge, and heated for 10 min at 95°C in the presence of sample buffer (Laemmli 1970). The heated samples were subjected to SDS–polyacrylamide gel electrophoresis in 12.5% gels (Fig. 4B).

Hemolytic assay and oligomerization of Luk dimers

Wild type LukS, wild type LukF, LukS-LukF dimers, and LukS-LukF-BacTL were obtained by IVTT. The protein (25 μ L or 12.5 μ L each of wild type LukS and wild type LukF in the case of Fig. 5C, row A) was diluted with MBSA buffer (75 μ L; 10 mM MOPS titrated with NaOH, 150 mM NaCl [pH 7.4], containing 1 mg/ml bovine serum albumin) in the first well of each row of a microtiter plate (Fig. 5C). MBSA (25 μ L) was used as the blank (Fig. 5C, row D). The proteins were then

subjected to two-fold serial dilution with the same buffer across each row, leaving 50 μ L in each well. Hemolysis was initiated by the addition of an equal volume of washed rabbit RBCs (1% in MBSA) to each well and monitored by observing the decrease in light scattering at 595 nm with a Bio-Rad microplate reader (model 3550-UV) (Cheley et al. 1999).

Oligomerization was examined on rabbit RBCM. Wild type LukS and wild type LukF proteins obtained by IVTT were mixed in an equal ratio. This mixture (12.5 μ L), LukS-LukF dimer (12.5 μ L of IVTT), and LukS-LukF-BacTL (12.5 μ L of IVTT) were each incubated with washed rabbit RBCM (5 μ L, 4.2 mg/mL of protein) in MBSA (50 μ L) for 1 h at 30°C. After centrifugation, the resulting membrane pellets were washed with MBSA and subjected to SDS-polyacrylamide gel electrophoresis in 8% gels (Fig. 5D) (Cheley et al. 1999).

Subunit stoichiometry examined with Luk dimers

Hetero-oligomers containing the LukS-LukF and LukS-LukF-BacTL fusion proteins were prepared by mixing in various molar ratios plasmids encoding two polypeptides, prior to IVTT in the presence of rabbit RBCMs (30 μ L total volume) (Cheley et al. 1999). After centrifugation, the resulting membrane pellets were resuspended in MBSA, recovered by centrifugation and dissolved in electrophoresis sample buffer (60 μ L). A portion of each (40 μ L) was subjected to SDS-polyacrylamide gel electrophoresis in a 5% gel (Fig. 6C). The remainders (20 μ L) were heated for 10 min at 95°C and subjected to SDS-polyacrylamide gel electrophoresis in a precast 7% Tris-acetate gel (Bio-Rad).

Electronic supplemental material

The possible circular permutations of four copies each of two types of protein subunit are described.

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