The Staphylococcal Leukocidin Bicomponent Toxin Forms Large Ionic Channels[†,‡]

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Received March 6, 2001; Revised Manuscript Received May 22, 2001

ABSTRACT: The genes encoding the F and S components of a leukocidin, LukF (HlgB) and LukS (HlgC), a pore-forming binary toxin, were amplified from the Smith 5R strain of *Staphylococcus aureus* both with and without sequences encoding 3′-hexahistidine tags. The His-tagged components were expressed in *Escherichia coli* and purified under nondenaturing conditions. In addition, the two unmodified proteins and the His-tagged versions were produced in an *E. coli* cell-free in vitro transcription and translation system. An SDS-stable oligomer of approximately 200 kDa appeared when both components were cotranslated in the presence of rabbit erythrocyte membranes. Hemolytic activity of the combined components against rabbit erythrocytes was measured for both in vitro- and in vivo-produced polypeptides, yielding similar HC_{50} values of ∼0.14 μg/mL. The pore-forming properties of the recombinant leukocidin were also investigated with planar lipid bilayers of diphytanoylphosphatidylcholine. Although leukocidins and staphylococcal α-hemolysin share partial sequence identity and related folds, LukF and LukS produce a pore with a unitary conductance of 2.5 nS [1 M KCl and 5 mM HEPES (pH 7.4)], which is more than 3 times greater than that of α-hemolysin measured under the same conditions. Therefore, if the leukocidin pore were a cylinder, its diameter would be almost twice that of α-hemolysin. In addition, the leukocidin pore is weakly cation selective and exhibits gating at low positive potentials, while α-hemolysin is weakly anion selective and gates only at high potentials. Taken together, these data suggest that the structure of the oligomeric pore formed by the leukocidin examined here has diverged significantly from that of α-hemolysin.

One family of β-barrel pore-forming toxins (the β-PFTs)[1] includes staphyloccocal α-hemolysin (αHL) (*1*, *2*), the leukocidins (*3*, *4*), *Clostridium perfringens* β-toxin (*5*), *Bacillus cereus* hemolysin II (*6*), and the recently described cytotoxin K (*7*). All are secreted as water-soluble monomeric polypeptides which have similar length chains, but vary in calculated isoelectric points [α-hemolysin, 293 residues, pI = 7.9; LukF (HlgB), 300 residues, pI = 9.2; LukS (HlgC), 286 residues, pI = 9.2; β-toxin, 309 residues, pI = 5.5; hemolysin II, 412 residues, pI = 9.0; and cytotoxin K, 305 residues, pI = 6.1]. Regions of sequence homology are dispersed throughout the aligned polypeptides (ref *3* and unpublished work). Interestingly, the extent of sequence identity in the region corresponding to the transmembrane β-barrel is weakest. The pathophysiological effects of most of these toxins have been ascribed to pore formation on target cells, resulting in cell permeation by Ca^{2+} ions and the release of inflammatory mediators, and, in some cases, cell lysis.

The leukocidins are binary toxins, thereby differing from the other β-PFTs (*3*, *4*, *8*). By the action of two distinct proteins, one component from class F and the other from class S, leukocidins primarily attack polymorphonuclear cells, monocytes, and macrophages. Within each class, F or S, exist at least six different proteins, whose amino acid sequences are between 59 and 79% identical (*3*, *4*, *8*). By contrast, the closest relationships between classes F and S are between proteins with a 20−30% level of identity. No member of either class is more than 30% identical to α-hemolysin.

The crystal structures of the water-soluble monomers of two class F components were recently determined: LukF (HlgB) and LukF-PV, whose amino acid sequences are 70% identical (*9*, *10*). When the amino latch and putative stem regions are excluded, both proteins display folds that are closely similar to the fold of an individual protomer of α-hemolysin in the fully assembled, membrane-inserted heptameric state (*1*). Of the 28 strictly conserved residues

[†] This work was supported by the DOE, NIH, and ONR. G.M. was the recipient of an ASSERT (ARO) award and an MD-PhD fellowship in the Medical Scientist Training Program at The Texas A&M University System Health Science Center.

[‡] This work was presented at the 4th International Workshop on Pore Forming Proteins, Trento, Italy, September 2000.

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[1] Abbreviations: αHL, α-hemolysin; β-PFT, β-barrel pore-forming toxin; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HlgB, γ-hemolysin, B component (LukF); HlgC, γ-hemolysin, C component (LukS); IVTT, in vitro transcription and translation; LB medium, Luria-Bertani medium; LukF, leukocidin F protein; LukF-H6, LukF with the His tag; LukS, leukocidin S protein; LukS-H6, LukS with the His tag; MBSA, MOPS-BSA; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ni−NTA, nickel−nitrilotriacetic acid; NTE, TE supplemented with NaCl; PCR, polymerase chain reaction; rRBC, rabbit erythrocytes; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; TE, Tris-EDTA; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi010454o CCC: $20.00
Published on Web 06/28/2001

among α-hemolysin, LukF, and LukS, the majority are clustered in the interior of the α-hemolysin protomer, and are probably required to preserve the fold of the cap domain (*3*). No structural information is available for a LukS monomer, or for the assembled leukocidin heterooligomer (composed of LukF and LukS). Nonetheless, the structures of the LukF monomers and α-hemolysin heptamer serve as working prototypes for the beginning and end points of *β*-PFT assembly and form the basis for the current version of the mechanism of assembly of α-hemolysin (*9*, *11*, *12*).

The channel forming properties of α-hemolysin have been well characterized. α-Hemolysin passively transports globular molecules with molecular masses of up to 2000 Da (*13*) or large elongated polymers such as single-stranded nucleic acids (*14*). Single-channel conductance measurements (450 pS in 0.5 M KCl, pH 7.4, −40 mV) were used to estimate the effective diameter of the water-filled transmembrane pore as 11−12 Å, assuming a cylinder that was 40 Å in length (*15*). Based on the crystal structure of α-hemolysin (*1*), the diameter of the most constricted region of the pore lumen is 14 Å and lies near the cis (cap) end of the *β*-barrel (*1*). The α-hemolysin pore displays partial rectification at neutral pH ($I_{+80}/I_{-80} = 1.2$; 1 M KCl, pH 7.4) and is weakly anion selective [$V_r = -3.7$ mV; $P_{K^+}/P_{Cl^-} = 0.79$; 1000 mM KCl (cis), 200 mM KCl (trans), pH 7.5].

By contrast with α-hemolysin, limited data exist on the mechanism of assembly and molecular architecture of the leukocidin pore. Several studies have indirectly investigated pore formation. Ion-specific fluorescent probes and flow cytometry were used to illustrate time- and dose-dependent entry of divalent cations into cells exposed to leukocidin (*16*). Permeabilization of small unilamellar vesicles to calcein has also been demonstrated (*17*). Osmotic protection experiments have been used to estimate a diameter of 19−21 Å for the leukocidin pore (*18*). It has also been suggested that the leukocidin oligomer forms a hexameric structure with a 1:1 molar ratio of LukF and LukS (*18*, *19*).

In the study presented here, we have expressed and purified a LukF (HlgB) and a LukS (HlgC) component in *Escherichia coli* and by cell-free in vitro transcription and translation. Both the combined recombinant polypeptides and electrophoretically purified leukocidin oligomers form large conductance, cation-selective pores in planar bilayers and exhibit voltage-induced gating. In addition to extending our knowledge about the assembly of *β*-barrel-forming toxins, these large stable channels will expand our toolbox for membrane protein design (*20*).

MATERIALS AND METHODS

Reagents. All restriction and DNA-modifying enzymes were from New England Biolabs (Beverly, MA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and purified by desalting. All plasmids and DNAs were purified with reagents by Qiagen (Santa Clarita, CA). DNA sequence analysis was performed by Lone Star Labs (Houston, TX). Low-molecular weight markers (catalog no. 161-034) were obtained from Bio-Rad Laboratories (Hercules, CA): phosphorylase *b* (M_r = 97 400), bovine serum albumin (M_r = 66 200), ovalbumin (M_r = 45 000), carbonic anhydrase (M_r = 31 000), trypsin inhibitor (M_r = 21 500), and lysozyme (M_r = 14 400). Radiolabeled markers [^{14}C-methylated proteins (catalog no. CFA626)] were from Amersham Pharmacia Biotech (Buckinghamshire, England): myosin (M_r = 220 000), phosphorylase *b* (M_r = 97 400), bovine serum albumin (M_r = 66 000), ovalbumin (M_r = 46 000), carbonic anhydrase (M_r = 30 000), and lysozyme (M_r = 14 300). Imidazole (catalog no. I-2399) was from Sigma (St. Louis, MO). Unless otherwise noted, additional reagents were from either Sigma or J. T. Baker (Phillipsburg, NJ).

Isolation of Genomic DNA from Staphylococcus aureus. *S. aureus* strains 31889 (Smith 5R) and 49775 (American Type Culture Collection) were grown to saturation from log-phase inocula and stored frozen at −80 °C in 50% glycerol. To isolate genomic DNA, a 1.5 mL portion of a culture grown for 18 h at 37 °C was centrifuged at 700*g*. The pellet was resuspended in 50 μL of buffer P1 [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 100 μg/mL RNase A] (Qiagen) supplemented with 5 μg of lysostaphin (Sigma catalog no. L 0761) in 5 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 10 mM EDTA (1× NTE, 5 μL) and allowed to stand at room temperature for 30 min. Proteinase K (GibcoBRL, Gaithersburg, MD; 3 μL of a 20 mg/mL solution in 1× NTE) was added to this mixture with 10% SDS in 1× TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)] (30 μL). The mixture was then incubated at 37 °C overnight. After phenol/chloroform extraction and ethanol precipitation, the genomic DNA pellet was washed, dried, and redissolved in 1× TE.

Amplification of Leukocidin Genes. The coding sequences for LukF (HlgB) and LukS (HlgC) from both *S. aureus* strains were amplified by using the following primers: LukF (sense), 5′-GCTCAACATATGGCTGAAGGTAAAATAA-CACC; LukF (antisense), 5′-GCAAGCTTCTATTACTATT-TATTGTTTTCAGTTTCTT; LukS (sense), 5′-GCTGAA-CATATGGCTAACGATACTGAAGACATC; and LukS (antisense), 5′-GCTAAGCTTTTATCAATTCTGTCCTTT-CTCCTTGA. The sense primers for both LukF and LukS generated an *Nde*I site (CATATG), containing a new initiation codon, immediately before the first codon of the mature polypeptide. The antisense primers encode two stops and a *Hin*dIII site immediately following the last codon of the genes. Genomic DNA (40−50 ng) in 100 μL of PCR buffer (Promega buffer A from Promega Corp., Madison, WI) containing 60 pmol each of a primer pair, 200 μM dNTPs, 5 units of Taq polymerase (Promega), and 0.01 unit of recombinant Pfu polymerase (catalog no. 600153, Stratagene, La Jolla, CA) was subjected to 25 cycles of amplification with the following program: 94 °C for 30 s, 50 to 60 °C gradient for 30 s, and 72 °C for 60 s. A final extension was carried out at 72 °C for 5 min. Each PCR product was then cloned into the TOPO-TA plasmid (catalog no. K4500-01, Invitrogen, Carlsbad, CA). The LukS plasmid was digested with *Nde*I and *Hin*dIII, and the DNA was inserted into a pT7 expression vector pT7-SC1, derived from pT7-SMC (*12*) and differing only in the stuffer fragment. When two independently amplified clones from the two *Staphylococcus* strains were sequenced, it was noted that residue 282 of LukS should be reassigned to encode glutamic acid rather than the reported valine (GTG → GAG). The revised sequences have been deposited in GenBank with accession numbers AF352555 [strain 31889 (Smith 5R)] and AF352556 (strain 49775). Before the LukF gene could be

subcloned into the pT7 vector, it was necessary to remove two internal restriction sites (see below).

Site-Specific Mutagenesis with in Vivo Recombination. Site-specific mutagenesis with in vivo recombination (*21*, *22*) were used to remove two internal *Nde*I restriction sites in the LukF gene (from ATCC 31889) without altering the encoded amino acid sequence. The sites were simultaneously removed by three separate PCRs followed by three-way, ligation-free recombination. The mutagenic primers were designed to remove the *Nde*I sites without changing the coding sequence with single base changes (lowercase): M1a (sense), 5′-GCTTCCACCCAACgTATGGTAATGAACTC; M1b (antisense), 5′-GAGTTCATTACCATAcGTTGGGTG-GAAGC; M2a (sense), 5′-GAACATTTAAATCAACtTAT-GAAATTGATTGG; and M2b (antisense), 5′-CCAAT-CAATTTCATAaGTTGATTTAAATGTTC. The nonmutagenic primers [NM1a (sense), 5′-GTATTCAACATTTCCGT-GTCGCCCTTATTC; and NM1b (antisense), 5′-GAAT-AAGGGCGACACGGAAATGTTGAATAC] were used for two of the three PCRs. PCR was carried out in a 50 μL mixture containing linearized plasmid LukF-TOPO-TA DNA (20 ng), 200 pmol each of the appropriate primer set, 200 μM dNTPs (Stratagene), and 1.5 units of Taq/Pwo DNA polymerase mixture in PCR buffer 1 (Expand Long Template PCR System, Boehringer Mannheim, Mannheim, Germany). Amplification conditions were as follows: initial heat denaturation at 94 °C for 2 min, followed by 10 cycles of 1 min at 94 °C, a 1 min linear annealing gradient of 45 to 56 °C, 4 min at 72 °C, and a final 5 min extension at 72 °C. Each PCR generated a single band of the expected size, and the DNA was used without further purification. The three resulting DNAs were mixed at roughly equimolar concentrations, and 5 μL of the mixture (approximately 50 ng) was used to transform *E. coli* XL-10 Gold cells. Recombinant plasmids were screened by restriction digestion with *Nde*I and *Hin*dIII. Finally, the desired *Nde*I−*Hin*dIII fragment was subcloned into pT7-SC1 that had been digested with the same enzymes. Two sequences of two independent clones from the same TA construct agreed with the reported coding sequence of LukF (*23*), aside from the two intentional point mutations.

Construction of Leukocidin Genes Encoding a C-Terminal Hexahistidine Tag. A sequence encoding a hexahistidine tag was inserted at the 3′ end of both the LukF and LukS genes to permit the purification of recombinant proteins by metal chelate affinity chromatography. First, a 3′ extension containing an *Xho*I site (in italics) which encodes a Gly/Ser linker was added to each wild-type gene by PCR. pT7-LukF and pT7-LukS plasmids linearized with *Hin*dIII were used as the templates for the following primers: SC001 (sense for both), 5′-CACTATAGGGAGACCACAACGG; RnLukF-*Xho*I (antisense, LukF), 5′-TGC*CTCGAG*TTTATTGTTTT-CAGTTTC; and RnLukS-*Xho*I (antisense, LukS), 5′-C*CTC-GAG*ATTCTGTCCTTTCTCCTTGATTT. The PCR products were cloned into the TOPO-TA plasmid (Invitrogen). The purified plasmids were digested with *Nde*I and *Xho*I, and the resulting inserts were ligated into pT7-αHL-H6 (*12*), which had been cut with the same enzymes, to yield pT7-LukF-H6 and pT7-LukS-H6. Each gene contains extensions encoding the *Xho*I site followed by six His residues and two stop codons (Figure 1). Sequences were verified after ligation into the T7 vector.

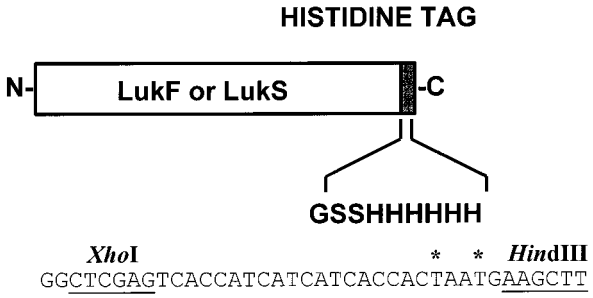

FIGURE 1: His-tagged LukF and LukS constructs. A sequence encoding a hexahistidine tag was inserted at the 3′ end of each gene. First, an *Xho*I site was added to the end of each gene by PCR. The resulting construct was then cut with *Nde*I and *Xho*I, and subcloned into the pT7-αHL-H6 vector, which contains an *Xho*I site followed by six histidine codons.

Expression and Purification. Recombinant LukF-H6 and LukS-H6 were expressed in a T7 promoter-based expression system. *E. coli* JM109(DE3) cells (catalog no. P9801, Promega) were freshly transformed with pT7-LukF-H6 or pT7-LukS-H6 and used to inoculate 1 L of Luria-Bertani medium (Difco) containing 100 μg/mL ampicillin. The culture was grown at 30 °C for 21 h to an OD_{600} of 4.0. Cells were harvested by centrifugation (5000*g* for 25 min at 4 °C) and the pellet resuspended in 12 mL of ice-cold lysis-wash buffer [10 mM Tris-HCl, 20 mM imidazole, and 600 mM NaCl (pH 8.0)] containing 0.5 mM AEBSF (catalog no. 101500, Calbiochem Biosciences Inc., La Jolla, CA) and passed three times through a prechilled French press at 10 000 psi. The lysate was centrifuged at 20000*g* for 15 min at 4 °C to pellet bacterial debris. The supernatant was immediately combined with metal chelate affinity matrix [1 mL, Ni(II) NTA agarose, catalog no. 30210, Qiagen] and mixed overnight at 4 °C. The suspension was poured into a disposable column (Econo-Pac column, catalog no. 732-1010, Bio-Rad), the flow-through was collected for analysis and the column washed with 10 bed volumes of the lysis-wash buffer. Bound protein was then removed with 4 bed volumes of elution buffer [10 mM Tris-HCl, 250 mM imidazole, and 150 mM NaCl (pH 8.0)] and dialyzed at 4 °C against 4 L of 10 mM Tris-HCl and 150 mM NaCl (pH 8.0). The protein concentration of the dialysate was determined in triplicate by measuring the absorbance at 280 nm (*24*). Fractions from each stage of the purification were solubilized in Laemmli sample buffer and run in a 12% SDS−PAGE gel (*25*).

In Vitro Transcription and Translation (IVTT). Wild-type and His-tagged versions of the LukF and LukS polypeptides were synthesized in a cell-free in vitro transcription and translation (IVTT) system using an S30 extract from *E. coli* (T7 S30, catalog no. L114A, Promega) supplemented with rifampicin (20 μg/mL) (*26*). Radiolabeled polypeptides were synthesized by using an amino acid premix without methionine in the presence of [^{35}S]methionine (1175 Ci/mmol) (ICN Biomedicals, Inc., Irvine, CA). In some experiments, varying concentrations (1 to 500 μM) of AEBSF-HCl (Calbiochem) or a protease inhibitor cocktail [product no. P2714; containing AEBSF, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA; Sigma] were added at the start of an IVTT reaction.

Quantitative Hemolysis Assay. LukF and LukS proteins, synthesized by IVTT with the complete amino acid mix, or the recombinant proteins purified from *E. coli* were diluted into MBSA [10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, catalog no. AB1270, American Bioanalytical, Natick, MA) and 150 mM NaCl (pH 7.4) containing 1 mg/mL bovine serum albumin (catalog no. 4503, Sigma)] and subjected to twelve 2-fold serial dilutions in the same buffer in microtiter wells (final volume of 50 μL). An equal volume of 1% washed rabbit erythrocytes (rRBC) in MBSA was quickly added to each well, beginning with the most diluted toxin or combination of toxins. Hemolysis was followed for 4 h at 20 °C by monitoring the decrease in light scattering at 595 nm with a Bio-Rad microplate reader (model 3550-UV) and using Microplate Manager 4.0 software.

Oligomer Formation during Translation and Subsequent Purification. Radiolabeled leukocidin oligomers were prepared by cotranslating LukF or LukF-H6 with LukS or LukS-H6 in the presence of [^{35}S]methionine, complete amino acid mix, and rRBC membranes (10 μL, 3.0 mg/mL, centrifuged and allowed to dry for 15−30 min prior to use) (*27*). The total reaction volume was 125 μL, or the equivalent of five normal IVTT reactions. Membranes were prepared by hypotonic lysis of washed rabbit erythrocytes in 5 mM NaH_2PO_4 and 1 mM EDTA (pH 8.0). The membrane protein content was assayed with the Bio-Rad DC Protein Assay Kit (catalog no. 500-0116, Bio-Rad) with bovine serum albumin as the standard. The assay was carried out in 10 mM Tris-HCl (pH 8.0) and 1% Genapol X-080 (Fluka, Milwaukee, WI). The following oligomers were produced by IVTT by using equimolar amounts of template DNA: LukF−LukS, LukF-H6−LukS-H6, LukF-H6−LukS, and LukF−LukS-H6. After IVTT for 1 h at 37 °C, the mixture was centrifuged and the supernatant discarded. The membrane pellet was washed by resuspension in MBSA (800 μL). Membranes were recovered by centrifugation and redissolved in 1× Laemmli sample buffer (100 μL) without heating. The entire sample was loaded into the well of an 8% SDS−polyacrylamide gel and electrophoresed overnight at 50 V. Unfixed gels were vacuum-dried without heating onto Whatman 3 mm filter paper, which was then exposed to X-ray film overnight. Using the autoradiogram as a template, the leukocidin oligomer band was excised from the gel. The gel slices were rehydrated with water (200 μL), crushed with a sterile pestle, and agitated at 4 °C overnight. Using a 0.2 μm cellulose acetate spin filter (catalog no. 7016-024, microfilterfuge tube, Rainin, Woburn, MA), the eluted protein was separated from the polyacrylamide gel debris by centrifugation of the solution at 16000*g* for 10−20 min. The filtrate was stored in 20 μL portions at −80 °C and used for bilayer recordings without further purification. The sample was rerun on a gel to verify the integrity of the oligomer.

Oligomer Formation on Rabbit Erythrocytes. [^{35}S]Methionine-labeled LukF and LukS (5 μL of an IVTT reaction mix for each) were incubated, either individually or together, with 30 μL of washed 10% rRBC in MBSA for 1.5 h at room temperature. The erythrocyte membranes were collected by centrifugation and resuspended in 5 mM NaH_2PO_4 and 1 mM EDTA (pH 8.0) to lyse any remaining intact cells. After two washes with the same buffer, the membranes were solubilized in Laemmli sample buffer and subjected to electrophoresis in a 10% SDS−PAGE gel.

Planar Lipid Bilayer Recordings. Planar lipid bilayer membranes were formed with 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) on a 150−160 μm diameter orifice in a 25 μm thick Teflon film (Goodfellow Corp., Malvern, PA) separating the cis and trans compartments of a bilayer apparatus (Montal 1972). Prior to formation of the lipid bilayer, the orifice was pretreated with a 1:10 hexadecane (catalog no. 29,631-7; Aldrich)/pentane (Burdick & Jackson) solution and allowed to dry thoroughly. For most measurements, the cis and trans chambers (2 mL each) contained 1 M KCl and 5 mM HEPES (pH 7.4, KOH). Various concentrations of KCl in 5 mM HEPES (pH 7.4) were used for ion selectivity measurements. All protein samples were added to the grounded cis chamber. A positive potential indicates a higher potential in the trans chamber, and a positive current is one in which cations flow from trans to cis. For IVTT-produced polypeptides, 5−10 μL of LukF (or LukF-H6), LukS (or LukS-H6), or a 1:1 mixture of the two components was added directly to the cis compartment to observe multiple-channel insertions. For single-channel experiments, *E. coli*-derived proteins in 1 M KCl and 5 mM HEPES (pH 7.4) were diluted 200-fold into the cis chamber to a final concentration of 0.1−5 ng/mL. To obtain single channels from gel-purified oligomers, the extracts were diluted 10−20-fold and 5−10 μL was added to the cis chamber. All experiments were performed at room temperature (20−25 °C).

A potential difference was applied across the bilayer with freshly prepared Ag/AgCl electrodes in 1.5% agarose (Bio-Rad) saturated with 3 M KCl. Currents were recorded using a Dagan model 3900A patch clamp amplifier (Dagan Corp., Minneapolis, MN) with a model 3910 expander and a built-in low-pass four-pole Bessel filter set at 5 kHz. Data were stored on digital audiotape with a DAS-75 data recorder (Dagan Corp.). For analysis, the signal was filtered at 1 kHz through a low-pass eight-pole Bessel filter (model 902, Frequency Devices, Haverhill, MA) and acquired with a Digidata 1200A A/D board with a sampling time interval of 200 μs. Data were acquired and analyzed with pClamp 7.0 software (Axon Instruments, Foster City, CA). The results are presented using Origin 6.0 (Microcal Software, Northampton, MA). Current/voltage (*I*/*V*) relationships of single leukocidin channels were determined by recording the currents obtained after stepwise increases in applied potential. The ion selectivity ratio (P_{K^+}/P_{Cl^-}) was calculated from experimentally determined reversal potentials (V_r) by using the Goldman−Hodgkin−Katz (GHK) equation (*28*) and the appropriate activity coefficients (*29*). Each experiment was begun with 200 mM KCl in 5 mM HEPES (pH 7.4) in both the cis and trans compartments, and any electrode DC offset was balanced prior to the addition of protein to the cis chamber. After channel insertion, a solution of 1 M KCl and 5 mM HEPES (pH 7.4) was perfused into the cis chamber (2−3 mL/min; 15 mL exchanged) to establish a 5-fold difference in KCl concentration between the chambers. The applied voltage that gave zero current was noted. In addition, *I*/*V* curves were determined from which V_r could be more accurately determined. Symmetrical solutions were then reestablished to evaluate whether any DC offset had built up during the course of the experiment. In all cases, the offset was <1 mV.

RESULTS

Cloning and Expression of LukF and LukS in E. coli Cells and Cell-Free Lysates. We earlier found that α-hemolysin constructed with a C-terminal nine-residue Gly-Ser linker and a hexahistidine tag (WT-H6) retained full hemolytic activity (*12*). Given the similarity in sequence between α-hemolysin and the leukocidins (*3*) and the similarity in structure of the LukF monomer and the subunits in the α-hemolysin pore (*9*, *10*), we deduced that a C-terminal extension to LukF and LukS should be tolerated and would permit the facile purification of both components from *E. coli* (Figure 1). LukF has an eight-amino acid C-terminal extension that is hydrogen-bonded to the backbone of the *β*-sandwich domain but, fortunately, that did not interfere with our plan. LukF-H6 and LukS-H6 were expressed in *E. coli* JM109(DE3) (*30*) and purified under nondenaturing conditions by metal chelate affinity chromatography (Figure 2A). Approximately 20 mg/L recombinant LukF-H6 and 5.0 mg/L LukS-H6 were obtained from the cultures. Both His-tagged proteins were also purified under denaturing conditions, but preliminary attempts at refolding LukF have proven to be unsuccessful (data not shown). In the process of development of a purification scheme, it was noted that both Luk components at concentrations of >2 mg/mL require a NaCl concentration of at least 400 mM to remain soluble.

Recombinant ^{35}S-labeled LukF and LukS were obtained by using pT7-LukF and pT7-LukS as templates in an *E. coli* S30 transcription and translation system. The His-tagged versions were also produced in this way. LukS appears to undergo premature termination of translation, giving rise to bands of reduced molecular mass in SDS−polyacrylamide gels (Figure 2B). Proteolysis does not seem to be responsible, as the addition of various water-soluble protease inhibitors had no effect on the IVTT reaction, nor did it reduce the intensity of the lower bands. When IVTT-produced LukS-H6 was purified by metal chelate affinity chromatography, the intensities of the lower bands were reduced, supporting the possibility of C-terminal truncation.

Oligomerization on Rabbit Erythrocyte Membranes. The binding of LukF and LukS to rabbit erythrocyte membranes (rRBCMs) was examined (Figure 2B). Only when the F and S components were cotranslated in the presence of membranes (or mixed and then added to rRBCMs or rabbit erythrocytes) did a high-molecular mass band, running slightly above the 220 kDa marker, appear upon SDS−PAGE analysis of unheated samples. Similar results were obtained by Western blotting by Kamio and colleagues with both human and rabbit erythrocyte membranes (*18*). As neither LukF nor LukS alone formed the high-molecular mass species, the band most likely contains a LukF−LukS oligomer. The electrophoretic mobility of the oligomer was highly dependent on the polyacrylamide concentration. For example, in preparative gels, which contained 5% polyacrylamide, the band ran beneath the 220 kDa marker (Figure 2C). The band is absent from samples heated at 100 °C for 5 min in 1× Laemmli sample buffer (data not shown). Significantly less of the radiolabeled leukocidin components oligomerize on rRBCMs when cotranslated as compared to the oligomerization of α-hemolysin. By contrast, others have observed a much greater extent of oligomerization of leukocidin components on liposomes (*19*).

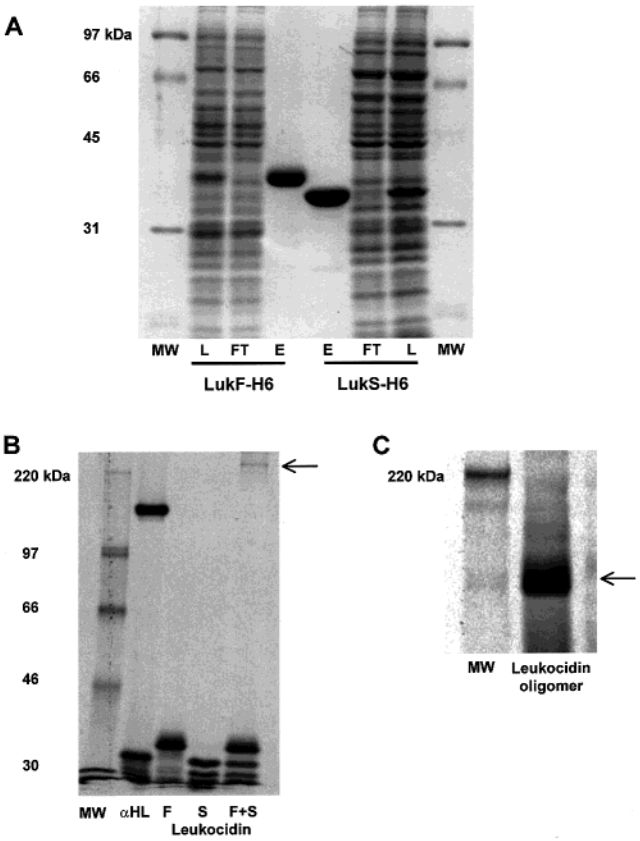

FIGURE 2: Protein purification. (A) Purification of LukF-H6 and LukS-H6 under nondenaturing conditions. A 12% SDS−polyacrylamide gel stained with Coomassie blue is shown: L, lysate; FT, flow-through from Ni−NTA agarose; and E, eluate from Ni−NTA agarose. (B) Untagged ^{35}S-labeled leukocidin polypeptides synthesized by coupled in vitro transcription and translation (IVTT). An S30 extract from *E. coli* was used: αHL, α-hemolysin; F, LukF; S, LukS; and F + S, LukF and LukS cotranslated. Translation was carried out in the presence of rabbit erythrocyte membranes (rRBCMs), which were subsequently washed by centrifugation and resuspension, pelleted, and solubilized with Laemmli sample buffer. LukF and LukS both bind to the membranes, but the high-molecular weight band corresponding to the leukocidin oligomer is seen only when both LukF and LukS are made together in the IVTT reaction. An autoradiogram of a 10% SDS−polyacrylamide gel is shown. α-Hemolysin is included for comparison. The arrow indicates the leukocidin oligomer. (C) Preparative electrophoresis of the untagged leukocidin oligomer. An autoradiogram of part of a 5% SDS−polyacrylamide gel is shown. The arrow denotes the leukocidin oligomer.

Hemolytic Activity of Leukocidin. The hemolytic activities of leukocidins produced by IVTT or in *E. coli* were compared in a quantitative assay. All possible combinations of wild-type and His-tagged leukocidins produced by IVTT were tested (Figure 3A), as well as purified proteins from *E. coli* (Figure 3B). As expected, neither LukF nor LukS alone was hemolytic. Mixtures of LukF and LukS, prepared from His-tagged and wild-type versions made by IVTT, all exhibited the same hemolytic activity, confirming our notion that a C-terminal extension does not affect activity. The concentrations of toxin at which 50% of the rabbit erythrocytes were lysed in 2 h (HC_{50}) were 0.13 *μ*g/mL LukS (the limiting component) for the IVTT-produced material and 0.15 *μ*g/mL for the protein purified from *E. coli* (Figure 3A,B). The value for α-hemolysin is 0.025 *μ*g/mL (*26*). By contrast, a value of 3 *μ*g/mL was obtained for proteins purified from culture supernatants of *S. aureus* Smith 5R and analyzed

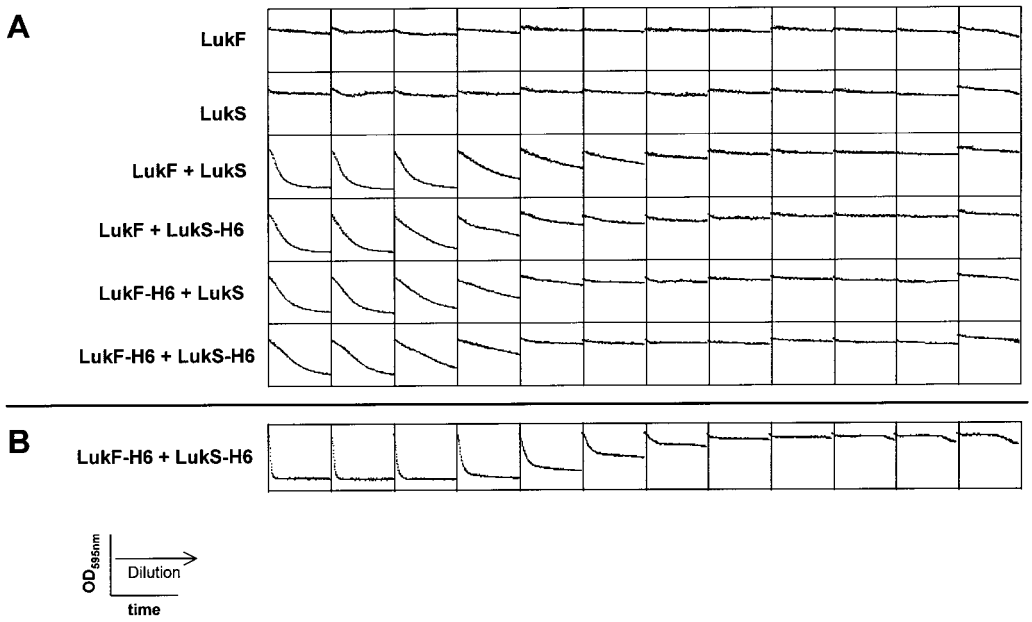

FIGURE 3: Quantitative hemolysis assay. (A) Activity assays (4 h) on various mixtures of LukF and LukS synthesized by IVTT. Two-fold serial dilutions from left to right are shown. The first well of each row contained IVTT mix (5 μL of each component for LukF and LukS; 10 μL for LukF or LukS alone) diluted to a final volume of 100 μL. The concentration of α-hemolysin in the IVTT mix was determined from its known specific hemolytic activity. Using this value and phosphorimager analysis, the concentrations of LukF and LukS were determined taking into account their methionine content. (B) Activity assays (4 h) as described for panel A, but using LukF-H6 and LukS-H6 purified from *E. coli*. Two-fold serial dilutions are shown, with the first well containing a final concentration of 5 μg/mL of each protein.

under similar conditions (*18*). Ferraras and colleagues obtained an HC_{50} value of 0.026 μg/mL for LukF and LukS obtained from *S. aureus* V8 (ATCC 49775) with a slightly different assay (*17*).

Leukocidin Forms Ionic Channels in Planar Lipid Bilayers. The gel-purified oligomeric species of leukocidin obtained by IVTT (Figure 2B,C) readily inserted into planar lipid bilayers and produced discrete stepwise changes in current of approximately 100 pA at a transmembrane potential of −40 mV (Figure 4A, top panel). If each step is taken to correspond to a single pore, the unitary conductance is 2.54 ± 0.06 nS (n = 211) (1 M KCl, 5 mM HEPES, pH 7.4, −40 mV). The channels do not gate at this potential. Similar results were obtained upon addition of equimolar LukF-H6 and LukS-H6 from *E. coli* to bilayers, which yielded channels with a conductance of 2.55 ± 0.05 nS (n = 125) (Figure 4B). Additional combinations of F and S polypeptides produced similar conductance values (Table 1). The addition of LukF-H6 or LukS-H6 alone did not induce channel formation.

Properties of the Leukocidin Pore. Staphylococcal leukocidin pores exhibit a near-linear current/voltage relationship at membrane potentials from −160 to +160 mV (Figure 5A,B), with very weak rectification (LukF−LukS oligomer, I_{+80}/I_{-80} = 1.08; LukF-H6 and LukS-H6, I_{+80}/I_{-80} = 1.10) (Table 1).

The ion selectivity of the leukocidin pore was determined by measuring the reversal potential of single-channel currents in an asymmetric KCl solution [1000 mM KCl (cis) and 200 mM KCl (trans) (in 5 mM HEPES, pH 7.4)] by using the oligomer obtained from wild-type LukF and LukS polypeptides generated by IVTT (Figure 6) or purified LukF-H6 and LukS-H6 monomers from *E. coli*. The charge selectivity ratio was calculated from the reversal potential (V_r) and the GHK equation (*31*). In both cases, the leukocidin pore was found to be weakly cation selective: P_{K^+}/P_{Cl^-} = 1.6 ± 0.1 (wild type, n = 3), P_{K^+}/P_{Cl^-} = 1.5 ± 0.1 (tagged monomers, n = 2).

While the leukocidin pore remains completely open at negative potentials up to −160 mV, it exhibits voltage-induced gating at positive potentials (Figure 7). Short-lived substates of lower conductance can be seen at +40 mV, while much longer, more pronounced, and frequent gating patterns are evident at +80 and +120 mV (Figure 7B,C). Three to four distinct subconductance states are seen at +120 mV. The addition of either the preformed oligomer or both monomers to a lipid bilayer produces similar results.

DISCUSSION

Biochemical and biophysical methods were used in this work to characterize leukocidin, a bicomponent pore-forming toxin secreted by *S. aureus*. We expressed both components, LukF and LukS, in *E. coli* and purified them to homogeneity. The two components were also prepared by cell-free in vitro transcription and translation. The hemolytic activities (HC_{50}) of the combined proteins from either source were closely similar at ~0.14 μg/mL for the limiting subunit in the assay, LukS, which is higher (less potent activity) than the value found for α-hemolysin (0.025 μg/mL), without attempting to correct for the fact that there are fewer LukS subunits in the leukocidin pore than α-hemolysin subunits in the α-hemolysin pore. This is in keeping with our finding that only small fractions of the leukocidin polypeptides form SDS-stable oligomers under our assay conditions (Figure 2).

The wild-type leukocidin oligomer obtained by in vitro translation and extracted from SDS−polyacrylamide gels readily inserted into lipid bilayers, producing channels of large unitary conductance (2.54 nS at −40 mV, in 1 M KCl, 5 mM HEPES, pH 7.4). Nearly identical results were

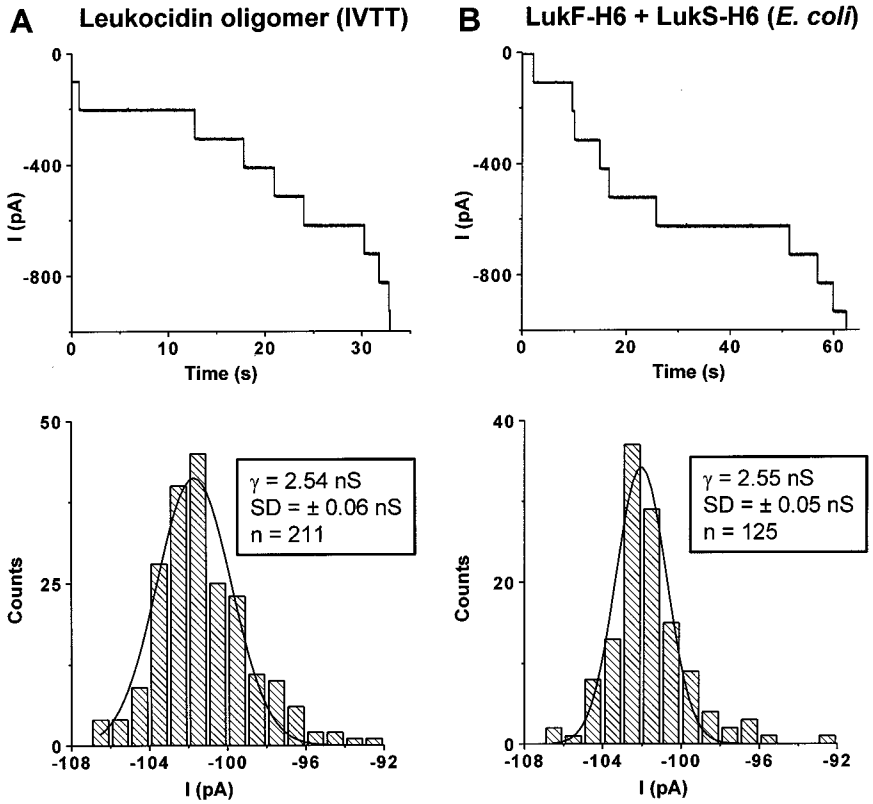

FIGURE 4: Individual insertions of the leukocidin pore into planar lipid bilayers. (A) Leukocidin oligomer prepared by IVTT in the presence of rRBCMs and purified by SDS−polyacrylamide gel electrophoresis. (B) A 1:1 molar mixture of LukF-H6 and LukS-H6 purified from *E. coli*. Stepwise changes in current are shown as a function of time. Proteins were added to the cis chamber of the bilayer apparatus. Both chambers contained 1 M KCl and 5 mM HEPES (pH 7.4), and the applied potential was −40 mV. Histograms of the current steps are displayed and represent a compilation from at least 10 independent recordings. Each histogram was fitted to a Gaussian function. The mean conductance values and standard deviations are shown.

Table 1: Conductance Values and Rectification Ratios for Leukocidin Oligomers

protein	g (pS)[a]	n	I_{+80}/I_{-80}
LukF−LukS (untagged)	2540 ± 60	211	1.08
LukF−LukS-H6	2550 ± 50	115	1.08
LukF-H6−LukS	2550 ± 50	118	1.06
LukF-H6−LukS-H6	2540 ± 50	109	1.08
LukF-H6 and LukS-H6[b]	2550 ± 50	125	1.10

[a] Recordings were made at −40 mV, in 1 M KCl and 5 mM HEPES (pH 7.4). [b] Recombinant monomers from *E. coli.* Gel-purified oligomers from protein obtained by IVTT were used for all other entries.

obtained with a 1:1 molar mixture of monomeric LukF-H6 and monomeric LukS-H6 purified from *E. coli*. The value of 2.54 nS is the highest for all the β-PFTs for which measurements have been taken. By contrast, α-hemolysin forms pores of 775 pS (1 M KCl, 5 mM HEPES, pH 7.4), while the cation-selective channels of *C. perfringens* β-toxin have states with approximately 23 and 43% of the conductance of leukocidin (60 and 110 pS, in 100 mM NaCl, 10 mM HEPES, pH 7.4, −40 mV) (*5*). The recently described CytK of *B. cereus* has a conductance of 627 pS (1 M NaCl, 5 mM HEPES, pH 7.2) (*32*), and that of *B. cereus* hemolysin II is 637 pS (1 M KCl, 5 mM HEPES, pH 7.4, −40 mV) (G. Miles, unpublished results). In terms of conductance, the leukocidin pores are considerably smaller than the *E. coli* mechanosensitive channel (MscL), which is one of the largest of any channels yet described, with a conductance of 3.8 nS (200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH 7.2) (*33*), with the exception of the very large pores formed by streptolysin O and its relatives (*34*).

The conductance of leukocidin is more than 3 times that of α-hemolysin and related pores ($g_{Luk}/g_{\alpha HL} = 3.3$). If the two pores were cylinders, the diameter of leukocidin would be $3.3^{1/2}$ (=1.8) times that of α-hemolysin (*15*, *31*). Rough estimates of the diameter of the leukocidin pore have been performed previously by osmotic protection experiments (*18*, *35*). Incubation of leukocidin with rabbit erythrocytes in the presence of 1000 Da poly(ethylene glycol) (PEG 1000) significantly inhibits hemolysis, whereas ≥1500 Da PEGs completely prevent the activity of the toxin. These data suggest that the leukocidin pore has a functional diameter of 19−21 Å, which are the respective hydrodynamic diameters of PEG 1000 and PEG 1500 (*36*). However, similar approaches give a range of results with α-hemolysin (*13*, *36*−*40*), suggesting that the concept of a functional diameter is simplistic and, further, that identical procedures would have to be applied to the leukocidin and α-hemolysin pores to allow a meaningful comparison.

The large conductance of the pore formed by LukF and LukS suggests that the assembly may have diverged significantly from those formed by other family members, either in overall structure or in internal architecture. Despite the similar folds, there exist many differences in primary structure between toxins in the β-PFT family. Importantly, the lowest extent of sequence identity between α-hemolysin, LukF, and LukS is within the β-barrel (α-hemolysin−LukF, 23%; α-hemolysin−LukS, 13%; and LukF−LukS, 35%). In

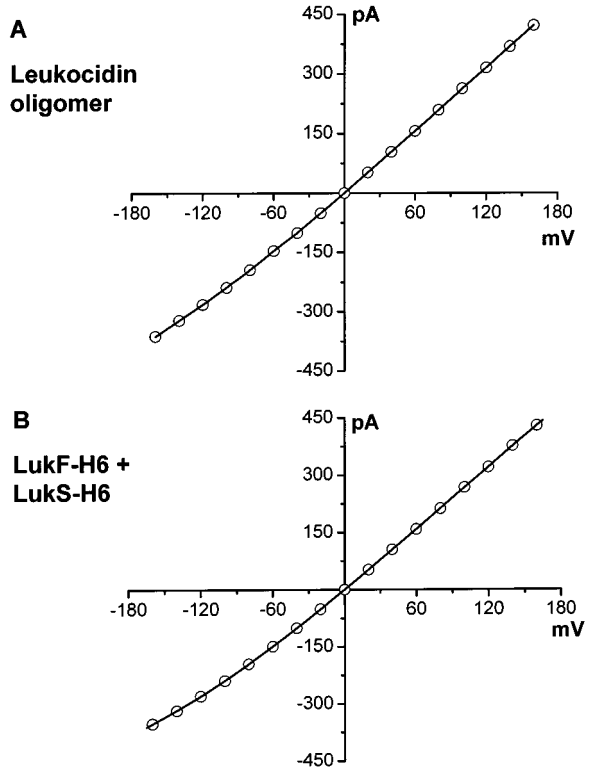

FIGURE 5: Single-channel current/voltage (*I*/*V*) relationships. (A) Leukocidin oligomer (untagged) prepared by IVTT in the presence of rRBCMs and purified by SDS−polyacrylamide gel electrophoresis. (B) A 1:1 molar mixture of LukF-H6 and LukS-H6 purified from *E. coli*. Data averaged from four or more independent experiments are plotted. Standard deviations are within the size of the symbols. The currents were recorded as described in the legend of Figure 4.

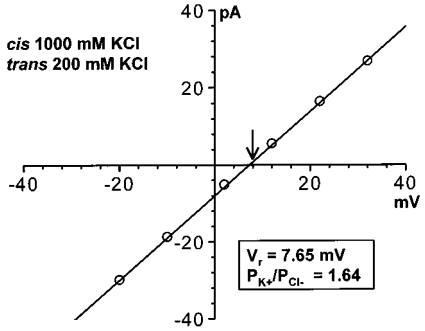

FIGURE 6: Leukocidin is weakly cation selective. The reversal potential (V_r), the applied voltage that gave zero current (arrow), was determined from single-channel current/voltage plots with asymmetrical KCl solutions [1000 mM KCl (cis) and 200 mM KCl (trans), both in 5 mM HEPES (pH 7.4)]. A representative plot obtained with the oligomer formed from in vitro-translated untagged LukF and LukS is shown. The ion selectivity value (inset, P_{K^+}/P_{Cl^-}) was derived from the GHK equation.

α-hemolysin, the narrowest internal diameter is ~14 Å at a constriction formed by the side chains of Glu-111, Lys-147, and Met-113, which project into the lumen of the pore (*1*). Both LukF and LukS contain small uncharged polar residues at these positions (Gln, Gln, and Asn for LukF and Asn, Ser, and Ser for LukS, respectively), suggesting that the leukocidin oligomer lacks the full complement of constricting side chains, which may explain the large conductance of the pore. Previous studies have shown that the leukocidin oligomer contains LukF and LukS in a 1:1 molar ratio, and it has been speculated that the oligomer is a hexamer (*17*, *18*). Indeed, both the leukocidin and α-hemolysin pores can be modeled as such (*10*, *41*, *42*). A leukocidin hexamer would have smaller physical dimensions than heptameric α-hemolysin. However, the conductance measurements argue against a hexamer, and there is no direct evidence to support this claim.

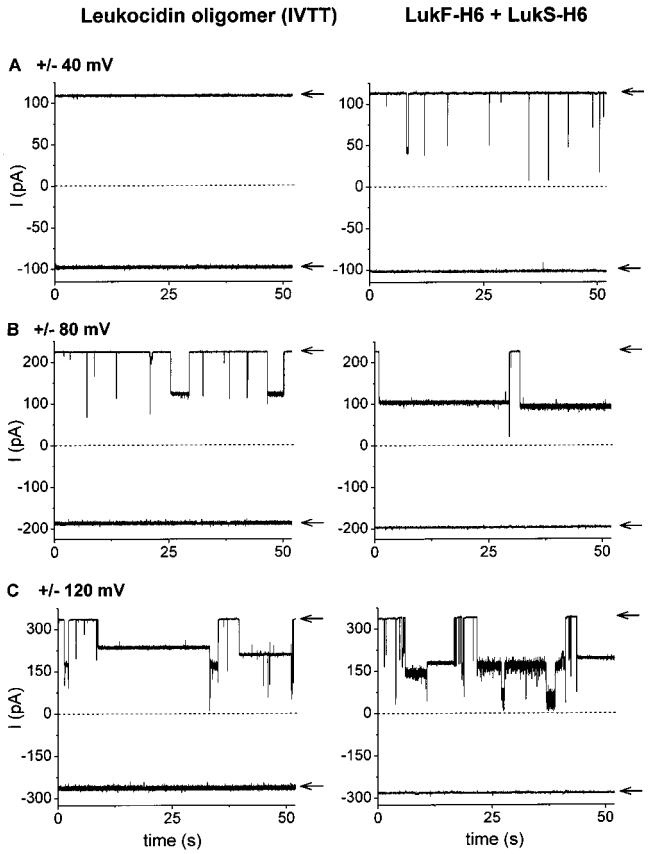

FIGURE 7: Voltage-dependent gating of the leukocidin pore. Panels in the left column are representative single-channel traces from the leukocidin oligomer (untagged) prepared by IVTT in the presence of rRBCMs and purified by SDS−polyacrylamide gel electrophoresis; panels on the right show a single channel obtained from a 1:1 mixture of LukF-H6 and LukS-H6 from *E. coli*. Conditions were the same as those described in the legends of Figures 4 and 5: (A) +40 and −40 mV, (B) +80 and −80 mV, and (C) +120 and −120 mV for the top and bottom traces in each panel, respectively. Arrows indicate the fully open state of the channel.

Like α-hemolysin, leukocidin exhibits weak positive rectification (I_{+80}/I_{-80} = 1.1, 1 M KCl, pH 7.4). While α-hemolysin is weakly anion selective [P_{K^+}/P_{Cl^-} = 0.79, 1000 mM KCl (cis), 200 mM KCl (trans), pH 7.5], leukocidin is weakly cation selective (P_{K^+}/P_{Cl^-} = 1.6, same conditions). Interestingly, only a single negatively charged residue of LukF (Asp-122), and no charged residues of LukS, is predicted to face the lumen of the leukocidin barrel. However, in the cap domain, near the N-termini of LukF and LukS and lining the mouth of the leukocidin pore are several negatively charged side chains that are not present in α-hemolysin and which might be responsible for the cation selectivity.

Like most porins and α-hemolysin, leukocidin exhibits voltage-induced gating, but only at positive potentials. Gating is especially evident at applied potentials greater than +60 mV. Unlike the α-hemolysin channel, which exhibits 50% closure at −150 mV in macroscopic current recordings (*43*), the leukocidin channel remains fully open and quiet at

negative potentials. In the major subconductance states seen at a positive potential of 80 mV, which last from milliseconds to more than 30 s, the channel is "half-closed". Multiple subconductance levels are evident when the applied potential is increased to +120 mV. Additional experimentation is needed to understand the differences in gating behavior between α-hemolysin and leukocidin.

The leukocidin pore will be useful in protein engineering studies, and this was one of our motives for undertaking the reported work. The presence of two different subunits, presumably in a defined arrangement, will permit the combinatorial assembly (*44*) of pores with unique channel properties.

ACKNOWLEDGMENT

We thank Li-Qun Gu for his advice on ion selectivity measurements.

REFERENCES

1. Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) *Science 274*, 1859−1865.
2. Gouaux, E. (1998) *J. Struct. Biol. 121*, 110−122.
3. Gouaux, E., Hobaugh, M., and Song, L. (1997) *Protein Sci. 6*, 2631−2635.
4. Prévost, G. (1999) in *The comprehensive sourcebook of bacterial protein toxins* (Alouf, J. E., and Freer, J. H., Eds.) pp 402−418, Academic Press, New York.
5. Shatursky, O., Bayles, R., Rogers, M., Jost, B. H., Songer, J. G., and Tweten, R. K. (2000) *Infect. Immun. 68*, 5546−5551.
6. Baida, G., Budarina, Z. I., Kuzmin, N. P., and Solonin, A. S. (1999) *FEMS Microbiol. Lett. 180*, 7−14.
7. Lund, T., De Buyser, M. L., and Granum, P. E. (2000) *Mol. Microbiol. 38*, 254−261.
8. Szmigielski, S., Prévost, G., Monteil, H., Colin, D. A., and Jeljaszewicz, J. (1999) *Zentralbl. Bakteriol. 1398*, 185−201.
9. Olson, R., Nariya, H., Yokota, K., Kamio, Y., and Gouaux, E. (1999) *Nat. Struct. Biol. 6*, 134−140.
10. Pédelacq, J.-D., Maveyraud, L., Prévost, G., Baba-Moussa, L., González, A., Courcelle, E., Shepard, W., Monteil, H., Samama, J.-P., and Mourey, L. (1999) *Structure 7*, 277−288.
11. Walker, B., Braha, O., Cheley, S., and Bayley, H. (1995) *Chem. Biol. 2*, 99−105.
12. Cheley, S., Malghani, M. S., Song, L., Hobaugh, M., Gouaux, J. E., Yang, J., and Bayley, H. (1997) *Protein Eng. 10*, 1433−1443.
13. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T., and Wellensiek, H.-J. (1981) *J. Cell Biol. 91*, 83−94.
14. Kasianowicz, J. J., Brandin, E., Branton, D., and Deamer, D. W. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13770−13773.
15. Menestrina, G. (1986) *J. Membr. Biol. 90*, 177−190.
16. Finck-Barbançon, V., Duportail, G., Meunier, O., and Colin, D. A. (1993) *Biochim. Biophys. Acta 1182*, 275−282.
17. Meunier, O., Ferreras, M., Supersac, G., Hoeper, F., Baba-Moussa, L., Monteil, H., Colin, D. A., Menestrina, G., and Prévost, G. (1997) *Biochim. Biophys. Acta 1326*, 275−286.
18. Sugawara, N., Tomita, T., Sato, T., and Kamio, Y. (1999) *Biosci., Biotechnol., Biochem. 63*, 884−891.
19. Ferreras, M., Höper, F., Dalla Serra, M., Colin, D. A., Prévost, G., and Menestrina, G. (1998) *Biochim. Biophys. Acta 1414*, 108−126.
20. Bayley, H. (1999) *Curr. Opin. Biotechnol. 10*, 94−103.
21. Jones, D. H. (1995) in *PCR primer: a laboratory manual* (Dieffenbach, C. W., and Dveksler, G. S., Eds.) Cold Spring Harbor Laboratory Press, Plainview, NY.
22. Howorka, S., and Bayley, H. (1998) *BioTechniques 25*, 766−772.
23. Cooney, J., Kienle, Z., Foster, T. J., and O'Toole, P. W. (1993) *Infect. Immun. 61*, 768−771.
24. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci. 4*, 2411−2423.
25. Laemmli, U. K. (1970) *Nature 227*, 680−685.
26. Walker, B. J., Krishnasastry, M., Zorn, L., Kasianowicz, J. J., and Bayley, H. (1992) *J. Biol. Chem. 267*, 10902−10909.
27. Cheley, S., Braha, O., Lu, X., Conlan, S., and Bayley, H. (1999) *Protein Sci. 8*, 1257−1267.
28. Hille, B. (1992) *Neuron 9*, 187−195.
29. Zemaitis, J. F., Clark, D. M., Rafal, M., and Scriver, N. (1986) in *Handbook of aqueous electrolyte thermodynamics: theory and application*, American Institute of Chemical Engineers, New York.
30. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
31. Hille, B. (1991) in *Ionic channels of excitable membranes*, Sinauer, Sunderland, MA.
32. Hardy, S. P., Lund, T., and Granum, P. E. (2001) *FEMS Microbiol. Lett. 197*, 47−51.
33. Cruickshank, C. C., Minchin, R. F., Le Dain, A. C., and Martinac, B. (1997) *Biophys. J. 73*, 1925−1931.
34. Bayley, H. (1997) *Curr. Biol. 7*, R763−R767.
35. Sugawara, N., Tomita, T., and Kamio, Y. (1997) *FEBS Lett. 410*, 333−337.
36. Krasilnikov, O. V., Sabirov, R. Z., Ternovsky, V. I., Merzliak, P. G., and Muratkhodjaev, J. N. (1992) *FEMS Microbiol. Immunol. 105*, 93−100.
37. Bhakdi, S., Muhly, M., and Füssle, R. (1984) *Infect. Immun. 46*, 318−323.
38. Kasianowicz, J. J., Brutyan, R. A., Vodyanoy, I., and Bezrukov, S. M. (1994) *Biophys. J. 66*, A214.
39. Merzlyak, P. G., Yuldasheva, L. N., Rodrigues, C. G., Carneiro, C. M. M., Krasilnikov, O. V., and Bezrukov, S. M. (1999) *Biophys. J. 77*, 3023−3033.
40. Movileanu, L., Cheley, S., Howorka, S., Braha, O., and Bayley, H. (2001) *J. Gen. Physiol. 117*, 239−251.
41. Smart, O. S., Breed, J., Smith, G. R., and Sansom, M. S. P. (1997) *Biophys. J. 72*, 1109−1126.
42. Smart, O. S., Coates, G. M. P., Sansom, M. S. P., Alder, G. M., and Bashford, C. L. (1998) *Faraday Discuss. 111*, 185−199.
43. Korchev, Y. E., Alder, G. M., Bakhramov, A., Bashford, C. L., Joomun, B. S., Sviderskaya, E. V., Usherwood, P. N. R., and Pasternak, C. A. (1995) *J. Membr. Biol. 143*, 143−151.
44. Braha, O., Walker, B., Cheley, S., Kasianowicz, J. J., Song, L., Gouaux, J. E., and Bayley, H. (1997) *Chem. Biol. 4*, 497−505.

BI010454O