Subunit Dimers of α-Hemolysin Expand the Engineering Toolbox for Protein Nanopores* [S]

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Staphylococcal α-hemolysin (αHL) forms a heptameric pore that features a 14-stranded transmembrane β-barrel. We attempted to force the αHL pore to adopt novel stoichiometries by oligomerizing subunit dimers generated by in vitro transcription and translation of a tandem gene. However, in vitro transcription and translation also produced truncated proteins, monomers, that were preferentially incorporated into oligomers. These oligomers were shown to be functional heptamers by single-channel recording and had a similar mobility to wild-type heptamers in SDS-polyacrylamide gels. Purified full-length subunit dimers were then prepared by using His-tagged protein. Again, single-channel recording showed that oligomers made from these dimers are functional heptamers, implying that one or more subunits are excluded from the central pore. Therefore, the αHL pore resists all structures except those that possess seven subunits immediately surrounding the central axis. Although we were not able to change the stoichiometry of the central pore of αHL by the concatenation of subunits, we extended our findings to prepare pores containing one subunit dimer and five monomers and purified them by SDS-PAGE. Two half-chelating ligands were then installed at adjacent sites, one on each subunit of the dimer. Single-channel recording showed that pores formed from this construct formed complexes with divalent metal ions in a similar fashion to pores containing two half-chelating ligands on the same subunit, confirming that the oligomers had assembled with seven subunits around the central lumen. The ability to incorporate subunit dimers into αHL pores increases the range of structures that can be obtained from engineered protein nanopores.

Protein pores have been devised for a variety of applications (1–3). For example, α-hemolysin (αHL)³ from Staphylococcus aureus has been developed for the controlled permeabilization of cells (4, 5), stochastic sensing (6), nucleic acid detection and sequencing (7, 8), the examination of single molecule chemistry (9), and the construction of “prototissues” based on droplets connected by bilayers (10, 11). To approach these goals, engineered αHL pores are essential, and they have been prepared by site-directed mutagenesis with natural and unnatural amino acids and by both noncovalent and covalent chemical modification (12, 13). The wild-type (WT) αHL pore is a homoheptamer (14, 15), and engineered pores have been prepared in both homo- and heteroheptameric form (12). However, in the case of heteromers, the permutation of the subunits around the central axis of the pore has not been controlled. For example, where two of the seven subunits have been altered, there are three possible permutations (Fig. 1A) (16). One goal of the present study was to demonstrate such control.

The staphylococcal αHL pore contains a 14-stranded β-barrel that spans the lipid bilayer (15). Two strands are contributed by each of the seven subunits. Under certain conditions, a fraction of the pores may be hexamers (17). By contrast, the structurally similar leukocidin pore is an octamer comprising four of each of two classes of subunits, both of which have a low extent of sequence identity with αHL (18, 19). Again, the protective antigen of anthrax toxin, which is structurally similar to the αHL pore but lacks sequence similarity, exists in both heptameric and octameric forms (20, 21). These results suggest that it might be possible to manipulate the subunit stoichiometry of the αHL pore, and this was attempted in this study.

One means to control subunit stoichiometry and permutation is to use concatenated subunits produced by genetic engineering, which has most often been used with ion channels (22). The structure and function of K⁺ channels (23), GABA_A receptors (24), nicotinic acetylcholine receptors (25), and large conductance mechanosensitive channels (26) have been investigated in this way. Subunit dimers (S-F) of the F and S subunits of the leukocidin pore have been used to show that these subunits alternate around the central axis of the octamer (19). Because the N and C termini of the subunits in the αHL pore lie on the same side of the lipid bilayer, we felt that a similar approach might be used to create concatemers of αHL, with two goals in mind. First, we wanted to investigate whether subunit dimers of αHL would assemble to give oligomers with new stoichiometries (Fig. 1B). A second goal was to increase the scope for genetic and chemical modifications of the pore. By using subunit dimers, we aimed to form heptamers in which two modified subunits are adjacent to each other. Although we failed to accomplish the first goal, the latter was successful.

EXPERIMENTAL PROCEDURES

Construction of the Tandem Gene αHL-(nSG)-αHL—All αHL genes were assembled in the pT7-SC1 expression vector.
TTCTTC-3'; HL13SG/47, 5'-TGAGGAGGCTGATCCACCC-ACCTGCTCCAGGCTGTTGATTTTTTCTC-3'; and HL15SG/47, 5'-TCCACCTGAGGCGCTTGACCCAC-ACATCCATCCACTGAGCCTGTCATTGTCATTCTT-3'. Homologous recombination of the two PCR products in *Escherichia coli* (19, 28) yielded the plasmid containing the tandem gene αHL-(nSG)-αHL. The integrity of the entire gene was verified by DNA sequencing.

**Preparation of Additional Tandem Genes**—The tandem genes αHL-(10SG)-αHL-D8, αHL-(10SG)-αHL-D8H6, αHL-(10SG)-αHL-H6, αHL-(10SG)-αHL(117C), and αHL(145C)-(10SG)-αHL(117C) were prepared as described above but by using different template plasmids in addition to pT7-SC1 containing the WT αHL gene. These plasmids contained genes encoding αHL-D8 (18), αHL-D8H6, αHL-H6, αHL(T117C), and αHL(T145C). αHL(T117C) and αHL(T145C) had been generated from αHL(RL3) (29), by using the QuikChange kit (Stratagene) according to the manufacturer's instructions to perform site-directed mutagenesis. The mutagenic primer pairs were as follows: T145C_FWD, 5'-GCAATGGTTCGAGTGTCTTAAGTATGTTCAACC-3' and T117C_REV, 5'-GGTTGAACATCTAAAGACAATGACC-AATCGAAGAATTTCG-3' and T117C_FWD, 5'-GATACTAAAAGAGTGATAGTACTTTTGTTATATGTTCAACC-3', and T117C_REV, 5'-CGGTTGAAATCTCATAACATAAGTACTACTACTCTTGTATC-3'.

**Protein Expression by Coupled in Vitro Transcription and Translation**—All proteins were prepared by coupled in vitro transcription and translation (IVTT) using an *E. coli* T7-S30 expression system for circular DNA (Promega) (27). The amino acid mixture minus methionine and the amino acid mixture minus cysteine (both 1 mM) were mixed in equal volumes to give a working mixture of all 20 amino acids. The T7-S30 extract was supplemented with rifampicin (1 μl of 500 μg/ml rifampicin per 150 μl) to inhibit transcription by the endogenous *E. coli* RNA polymerase. For a 25-μl reaction, premix solution (10 μl) was combined with the complete amino acid mixture (2.5 μl), plasmid DNA (4 μl, 400 ng/μl), T7 S30 extract (7.5 μl), and [35S]methionine (1 μl, MP Biomedicals, 1174 Ci/mmol, 10 mCi/ml). The reaction was incubated at 37 °C for 60–90 min and then centrifuged for 8 min at 4 °C at 25,000 g to pellet any insoluble proteins. The supernatant was removed and stored at −80 °C. Protein samples were run on 10% BisTris polyacrylamide gels in MOPS running buffer (both from Bio-Rad).

**Protein Chemical Modification**—The dimer 145C-(10SG)-117C was chemically modified with the N-propyliminodiacetic acid (PIDA) group as described previously (30).

**Protein Purification by Ni-NTA Affinity Chromatography**—The His-tagged dimers αHL-(10SG)-αHL-H6 and αHL-(10SG)-αHL-D8H6 were purified to remove truncated proteins. Ni-NTA magnetic beads (200 μl, Promega) were washed three times with wash buffer (1 ml of 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM imidazole), resuspended in wash buffer (200 μl), and mixed with IVTT protein (200 μl). The beads were incubated for 2–4 h at 4 °C on a rotator. The beads were then immobilized with a magnet and washed twice with wash buffer (200 μl). The protein was removed from the beads with elution buffer (300 μl) and dialyzed against wash buffer (300 μl) for 4 h. The elution buffer contained 300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM imidazole, and 20% glycerol. Protein concentrations were determined using the BioRad Protein Assay kit (BioRad) with BSA as standard.

**Protein Cleavage**—The uncleaved protein was diluted in wash buffer (100 μl) to a concentration of 10 mg/ml and incubated on ice for 30 min. The following protease was used: trypsin (1 μl of 1 mg/ml, Promega); trypsin (1 μl of 2 mg/ml, Promega) for the low yield sample; trypsin (1 μl of 2 mg/ml, Promega) for the high yield sample; trypsin (1 μl of 2 mg/ml, Promega) for the high yield sample with 30% glycerol; trypsin (1 μl of 2 mg/ml, Promega) for the high yield sample with 30% glycerol and 10 mM benzamidine; and trypsin (1 μl of 2 mg/ml, Promega) for the high yield sample with 30% glycerol and 10 mM benzamidine and 1 mM benzamidine. The reaction was incubated at 37 °C for 30 min. The reaction was stopped by adding 1 μl of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 20% glycerol. The reaction was then frozen at −80 °C.

**Tandem Gene Structures**—The tandem gene structure was designed as follows: αHL-(nSG) indicating the number of subunit monomers in the pore; n, indicates the number of subunit dimers of αHL and/or αHL-D8 (figure adapted from Ref. 42). αHL-(nSG)-αHL(145C)-117C was chemically modified with the N-propyliminodiacetic acid (PIDA) group as described previously (30).
buffer (150 μl of 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 500 mM imidazole). The protein solution was then passed through a Micro Bio-Spin 6 gel filtration column (Bio-Rad) equilibrated with 50 mM Tris-HCl, pH 8.0, to remove the imidazole.

**Protein Oligomer Formation and Purification**—IVTT proteins (20–100 μl) were mixed with rabbit red blood cell membranes (rRBCM, 5 μl, ~4 mg of protein/ml) in MBSA buffer (50 μl of 10 mM MOPS, titrated to pH 7.4 with NaOH, 150 mM NaCl, 1 mg/ml bovine serum albumin). Oligomer formation proceeded over 1 h at 37 °C. The membranes containing the assembled oligomers were recovered by centrifugation at 25,000 × g for 8 min. The pellets were resuspended in MBSA (50 μl) and again recovered by centrifugation. Each membrane pellet was solubilized in 1 × sample buffer (50 μl) without heating, loaded onto a 5% SDS-polyacrylamide gel, and subjected to electrophoresis at 50 V for 14 h with TGS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% w/v SDS, pH 8.3). The gel was dried without heating onto paper (Whatman 3MM Chr) under a vacuum, and an autoradiograph was obtained. The protein oligomer bands were cut from the dried gel. After rehydration in buffer (150–200 μl of 25 mM Tris-HCl, pH 8.0), the paper was removed. The gel was then crushed using a pestle, and the slurry was filtered through a QIAshredder column (Qiagen) by centrifugation at 25,000 × g for 10 min. The protein solution (∼5–20 ng/ml) was stored frozen at −80 °C.

**Hemolytic Activity Assay**—Monomers and dimers of αHL (5 μl, 100–400 ng/μl) were prepared by IVTT, diluted with MBSA (95 μl), and then subjected to 2-fold serial dilutions across the 12-well row of a microtiter plate, such that the final volume in each well was 50 μl. An equal volume of 1% washed rabbit erythrocytes in MBSA was quickly added to each well, starting with the most dilute sample. Hemolytic activity was recorded for 1 h by monitoring the decrease in light scattering starting with the most dilute sample. Hemolytic activity was recorded for 1 h by monitoring the decrease in light scattering starting with the most dilute sample. Hemolytic activity was recorded for 1 h by monitoring the decrease in light scattering starting with the most dilute sample.

**Limited Proteolysis Assays**—Proteinase K (Sigma) was dissolved in water (5 mg/ml) and used immediately. Limited proteolysis was performed on gel-purified oligomers. The enzyme (2 μl, or water for the control reactions) was mixed with the protein (18 μl in 20 mM Tris-HCl, pH 8.0) and incubated at 21 °C for 6 min. The reaction was quenched by the addition of phenylmethylsulfonyl fluoride (2 mM) and the slurry was filtered through a QIAshredder column (Qiagen) by centrifugation at 25,000 × g for 10 min. The protein solution (∼5–20 ng/ml) was stored frozen at −80 °C.

**Single Channel Recordings**—Single channel recordings were carried out by using folded planar lipid bilayers, as described previously (16, 31). Both chambers contained 1 ml of recording buffer (2.0 mM KCl, 2 mM succinic acid, pH 4.0, or 2.0 mM KCl, 10 mM MOPS, pH 7.0). The protein was added to the grounded cis chamber. A potential difference of −50 mV was applied through a pair of Ag/AgCl electrodes, which were set in 2% agarose containing 3.0 mM KCl. β-Cyclodextrin was added from stock solutions to the trans chamber. The single channel current was amplified by using a patch clamp amplifier (Axopatch 200B, Axon Instruments), filtered with a low pass Bessel filter (80 dB/decade) with a corner frequency of 1 kHz, and then digitized with a Digidata 1320 A/D converter (Axon Instruments) at a sampling frequency of 5 kHz, giving a time resolution of about 300 μs. The acquisition software was Clampex 10.2 (Molecular Devices).

**RESULTS AND DISCUSSION**

**Design and Expression of Subunit Dimers of αHL**—The termini of the individual subunits in a concatemer are usually linked by short peptide sequences. Such linkers are kept as short as possible to prevent the formation of secondary structure in the linker and to prevent the formation of undesired oligomeric structures. Undesired oligomers include those with interspersed subunits (Fig. 2A, structure 2) and those with concatenated subunits that are incorporated into separate oligomers (structure 3). As yet, no interspersed subunits have been recognized in channels or pores formed from concatenated subunits, but a subunit dimer can offer one subunit to each of two acetylcholine receptors if the linker between the subunits is sufficiently long (25). However, if the linker between the two...
subunits is short, then one subunit is excluded from the central pentameric pore of a receptor.

The same principle could apply to concatemers of αHL; for example, a very short linker might force the exclusion of subunits from the central pore (Fig. 2A, structure 4). In a PyMOL model, we used the crystal structure of the αHL heptamer (Fig. 2B) to estimate the length of a desirable linker between two adjacent subunits. The distance from the C terminus (Fig. 2B, blue) of a specified subunit (red) to the N terminus (green) of the next anticlockwise subunit (yellow), via Phe-42 (cyan) and Thr-12 (purple) on the protein surface, is 37 Å. A suitable linker should be longer than this to cover the uneven protein surface and to prevent strain of the linker. This model for the linker assumes that the N terminus of one subunit tethers tightly onto a second, as seen in the crystal structure of the WT heptamer. However, work in our laboratory has revealed that the N terminus of αHL is not critical for the formation of oligomers, and up to 17 residues can be deleted while retaining the ability to form pores (32). Furthermore, the related Vibrio cholerae cytolsin has no such N-terminal domain (33). We therefore had to consider the possibility that the N terminus of the second subunit (yellow, Fig. 2A) of an αHL subunit dimer is flexible, thereby shortening the required length of the linker. For this reason, the optimal length of the linker was explored experimentally. A tandem gene encoding a subunit dimer in a pT7 expression vector (Fig. 3A) was prepared by PCR followed by homologous recombination in E. coli. The two subunit genes were separated by a sequence encoding a serine/glycine linker. Such linkers have been employed to link subunits of the leukocidin pore (19) and are thought to be conformationally flexible (34). We designed four different linkers from 5 to 15 amino acids in length (Fig. 3B). In PyMOL models of the elongated sequences, the lengths between the terminal Ca atoms are 16 to 51 Å (Fig. 3C).

Subunit dimers of αHL were prepared by coupled in vitro transcription and translation. SDS-PAGE analysis of the radio-labeled proteins (Fig. 4A) revealed a polypeptide that migrated slightly faster than the 69-kDa marker, consistent with a dimer of 66 kDa. The expression levels of all the dimer constructs were two to five times lower than the WT monomer, as seen in the diminished intensities of the bands (Fig. 4A). Upon incubation with rRBCMs, all of the dimers formed oligomers, which appeared as two closely spaced diffuse bands of oligomers formed by the subunit dimer preparations are indicated by arrows. C, hemolytic activity assay with rabbit erythrocytes. Monomers and dimers of αHL were synthesized by IVTT and serially 2-fold diluted across the row of a microtiter plate. Pore formation was initiated by adding washed rabbit erythrocytes, and the rate of hemolysis was monitored by observing the decrease in light scattering at 595 nm over 1 h.

**Figure 3. Design of the serine-glycine peptide linkers.** A, schematic of the subunit dimer gene in a pT7 vector. The 3′ end of one full-length gene of αHL (red) was connected to the 5′ end of a second full-length gene (yellow) through a peptide linker (blue). B, DNA and amino acid sequences of linkers containing from 5 to 15 serines and glycines. C, the lengths of the linkers in B were estimated from PyMOL models of the corresponding peptide sequences by measuring the through-space distance between the Ca atoms at the termini of the elongated peptides.

**Figure 4. Expression and hemolytic activity of subunit dimers of αHL.** A, 10% BisTris polyacrylamide gel showing monomers and dimers of αHL synthesized by coupled IVTT. Lane 1, WT αHL monomer; lane 2, αHL-(13SG)-αHL subunit dimer; lane 3, αHL-(10SG)-αHL dimer; lane 4, αHL-(13SG)-αHL dimer; lane 5, αHL-(15SG)-αHL dimer. B, 5% Tris-HCl SDS-polyacrylamide gel showing oligomers formed by monomers and dimers of αHL on RBCMs. The proteins in lanes 1–5 are the same as those shown in A. The two closely spaced diffuse bands of oligomers formed by the subunit dimer preparations are indicated by arrows. C, hemolytic activity assay with rabbit erythrocytes. Monomers and dimers of αHL were synthesized by IVTT and serially 2-fold diluted across the row of a microtiter plate. Pore formation was initiated by adding washed rabbit erythrocytes, and the rate of hemolysis was monitored by observing the decrease in light scattering at 595 nm over 1 h.
Subunit Dimers of α-Hemolysin

![Diagram](image)

**FIGURE 5.** Limited proteolysis assay to probe the conformation of the N terminus and linkers in oligomers formed from subunit dimers of αHL. SDS-PAGE-purified oligomers of αHL in 20 mM Tris-HCl buffer, pH 8.0, were split into four equal samples and subjected to different treatments. Lane 1, no treatment; lane 2, proteinase K (0.5 mg/ml final) for 5 min, sample not heated; lane 3, no proteinase K, sample heated to 95 °C for 10 min in SDS-containing sample buffer; lane 4, proteinase K (0.5 mg/ml final) for 5 min and heated to 95 °C for 10 min in SDS-containing sample buffer.

35). However, truncation mutants of αHL lacking part of the N terminus (but not the cleavage sites) are sensitive to protease cleavage, presumably because the shortened N termini are no longer occluded within the cap domain (32). Limited proteolysis of oligomers made from subunit dimers should therefore provide insight into the conformation of the linker-internal N terminus sequence. Proteinase K treatment rendered gel-purified αHL oligomers made from subunit dimers (Fig. 4B) more electrophoretically mobile in SDS gels (Fig. 5), but this was not the case for the WT heptamer (Fig. 5). The proteolyzed oligomers made from subunit dimers were also heated in SDS sample buffer at 90 °C to disrupt the intersubunit interactions. Electrophoresis revealed the formation of a protein band with a slightly faster mobility than the WT monomer, implying that cleavage had taken place in the linker-internal N terminus sequence to yield truncated monomers. At lower concentrations of proteinase K, the same experiment yielded several protein bands that correspond to incomplete digestion (supplemental Fig. 2). Because even the shortest linker between the subunit dimers in oligomers is accessible to proteinase K, we conclude that the linker-internal N terminus sequences of all four types of subunit dimer do not bind tightly within the cap domain. Therefore, a shorter linker would be preferable to a longer linker, which could lead to the undesired oligomeric structures mentioned earlier (Fig. 2). However, we chose to proceed with a 10SG linker rather than a 5SG linker, as it offers more possibilities for the introduction of unique restriction sites in the DNA that encodes it. This will ultimately be important for preparing extended concatemers of αHL (subunit trimers, tetramers, etc.), as it will allow us to “cut and paste” mutant subunit genes into pre-made concatemer genes.

**Stoichiometries of Oligomers Made from αHL Subunit Dimers—**

The limited proteolysis experiments described above included a control condition, in which the oligomers were heated but not treated with proteinase K. We expected to recover only dimers of αHL; however, a mixture of dimers (major component) and monomers (minor component) was found (Fig. 5). Closer inspection of the IVTT products (Fig. 4A) revealed weak bands that migrate more slowly than the monomer but faster than the dimer. We suspected that these protein bands represent truncated dimers, generated by either incomplete transcription of the subunit dimer gene or incomplete translation of the mRNA. Mixtures of full-length concatenated protein subunits and truncated protein subunits have been found in oligomers built from concatemers of the P2X receptor and the Kv-1.2/1.1 potassium channel, but their precise origin remains obscure (36, 37). In our experiments, the ratio of truncated dimer to full-length dimer is greater in the heated oligomers than in the original IVTT reaction, suggesting that truncated dimers (which would be structurally similar to monomers) are preferentially incorporated into oligomers. Because subunit dimers cannot form a heptameric central pore, without the exclusion of one or more subunits from the ring, this result provided the first hint that αHL resists all but a heptameric stoichiometry. For example, such a stoichiometry could be achieved by combining three subunit dimers with one truncated dimer (effectively a monomer). At this point, however, it was not clear why the oligomers made from the subunit dimers showed a different electrophoretic mobility compared with the WT heptamer, especially if they actually share the same stoichiometry with respect to the number of subunits surrounding the central pore. However, it is known that oligomers made from certain mutants of αHL can run anomalously fast. For example, the heptamer made from αHL-D8, a mutant that carries an oligoaspartate extension at the C terminus (18), has similar mobility to denatured phosphorylase B (97 kDa) in a 5% Laemmlgel, although its actual molecular mass is 240 kDa.

To avoid producing a mixture of truncated species and dimers by IVTT of the subunit dimer gene, we used a gene with a C-terminal His$_x$ affinity tag. Because only full-length protein carries the tag, Ni-NTA purification should give only subunit dimers of αHL. This was confirmed by SDS-PAGE (Fig. 6A). By comparison, the unpurified dimer contained a truncated protein, in amounts that varied from batch to batch (compare Fig. 4A with 6A). The purified αHL subunit dimer was allowed to oligomerize in the presence of rRBCMs. The band corresponding to the oligomer in a 5% polyacrylamide gel (Fig. 6B) was sharp compared with the band derived from the oligomer made...
from nonpurified dimers. Faint bands could be detected above and below the main band generated from purified subunit dimers, indicating the presence of other types of oligomer. The major band again had a faster mobility than the WT heptamer. The unitary conductance of pores made from His-tagged subunit dimers was gel-purified and heated, only dimers were released (Fig. 6C). Therefore, the truncated proteins seen in Fig. 5 are not the result of proteolytic cleavage during or after oligomer assembly, rather they are IVTT products.

We tried to elucidate the stoichiometry of the oligomers made from purified His-tagged subunit dimers by gel-shift experiments, which had been used successfully to determine the heptameric stoichiometry of the αHL pore (14) and the octameric stoichiometry of the leukocidin pore (18, 19). The most likely oligomers to be formed by subunit dimers of αHL are a hexamer or an octamer (supplemental Fig. 3A). We co-oligomerized αHL-(10SG)-αHL-H6 with αHL-(10SG)-αHL-D8H6, expecting to see a stepladder of four bands for a hexamer or five bands for an octamer, based on the possible combinations of subunits. However, no clear pattern was found (supplemental Fig. 3B). The lack of a clear band pattern for oligomers made from αHL-(10SG)-αHL-H6 and αHL-(10SG)-αHL-D8H6 excludes a hexameric (2,4,6) or octameric (2,4,8) oligomer structure, leaving a heptamer with excluded subunits as a possible structure. It is hard to predict how many subunits would be excluded from the central pore, but because both αHL-(10SG)-αHL-H6 and αHL-(10SG)-αHL-D8H6 alone give rise to minor oligomer bands as well as one major band (Fig. 4B and supplemental Fig. 3), we suspect that several different arrangements can exist (e.g. 2,4,6 and 2,4,8).

Oligomers Made from Subunit Dimers of αHL Form Functional Heptamers with Excluded Subunits—To settle the question of pore stoichiometry conclusively, we carried out single channel recordings of oligomers made from subunit dimers. The unitary conductance of β-barrel pores is extremely sensitive to the pore stoichiometry and mutation. For example, the conductance of the octameric leukocidin pore is more than three times that of the heptameric αHL pore under the same recording conditions (38). Furthermore, systematic mutation of only one residue (Met-113) in homoheptameric αHL pores produces pores with conductance values that differ by as much as 24% (39). We extracted the protein from the major bands of oligomers from an SDS-polyacrylamide gel (Fig. 4B) and allowed multiple pores to insert into planar lipid bilayers in 2.0 M KCl, 10 mM MOPS, pH 7.0, under an applied potential of −50 mV (Fig. 7). By assuming that each current step corresponds to the insertion of a single pore, current amplitude histograms were constructed. WT αHL gave uniform quiet channels with a unitary conductance of 1.71 ± 0.26 nS (n = 50, Fig. 7A). By comparison, the major oligomers made from either nonpurified subunit dimers or purified subunit dimers gave noisier channels with wide distributions of conductance values (Fig. 7, B and C). The histograms indicate that a fraction of the pores made from subunit dimers share the same single channel conductance values as the WT heptamer, suggesting that they might also possess heptameric central pores.

We suspected that either the linkers between the subunit dimers or subunits excluded from the central pore might be responsible for the noise observed in the recordings and that they might also partially block the channel, resulting in lower conductance values. We therefore subjected the oligomers made from purified subunit dimers to limited proteolysis with proteinase K, which we had shown previously would cleave the linkers and release any excluded subunits that might be attached to them. Following proteolysis, the oligomers made from purified subunit dimers gave quiet uniform channels with a mean unitary conductance similar to the WT heptamer, 1.59 ± 0.22 nS (n = 161, Fig. 7D). The unitary conductance values of these pores mirrored those of WT heptamers over potentials ranging from −100 to +100 mV (Fig. 8A).

We also investigated the binding of the molecular adapter β-cyclodextrin (βCD) to these pores (Fig. 8D). The mean dwell time (τoff) of βCD in the αHL pore can vary by up to 5 orders of magnitude between various mutants (39), so it is another sensitive indicator of the pore architecture. The dwell times of βCD in WT pores and pores made from purified subunit
dimers and then subjected to limited proteolysis were the same within experimental error (0.33/0.03 and 0.39/0.03 ms, respectively). We therefore conclude that oligomers made from subunit dimers adopt a heptameric central pore architecture, with one or more excluded subunits.

A Single Subunit Dimer Can Be Incorporated into a Heptameric \( \alpha \)-Pore—Although we were not able to change the stoichiometry of the \( \alpha \)-pore by the concatenation of subunits, a second aim was to incorporate a single modified dimer into a heptameric pore. Because we had learned that oligomers made from subunit dimers readily incorporate monomeric subunits, we set out to make mixed oligomers. The assembly on rRBCMs of different ratios of subunit dimers and monomers that carried a C-terminal D8H6 extension resulted in a stepladder of oligomeric bands upon SDS-PAGE analysis (Fig. 9A, bands B–F). As expected from previous experiments with (\( \alpha \)-HL-D8),\(_7\) (18), the (\( \alpha \)-HL-D8H6), oligomer showed the fastest electrophoretic mobility. With increasing amounts of subunit dimer, the oligomer bands shifted upwards, suggesting that untagged subunit dimers are incorporated into the oligomers in various ratios. To analyze the composition of the mixed oligomers, we separated them by electrophoresis in a preparative 5% gel (Fig. 9B) and extracted the bands for further analysis. After heating the oligomers from bands 1 to 4 (Fig. 9B) in SDS to disrupt the intersubunit interactions, we separated the constituent polypeptides in a second gel and determined the ratio of monomer to dimer (Fig. 9C). Band 2 (Fig. 9, B and C) was expected to contain an oligomer that includes one subunit dimer and five monomers (21157, Fig. 9D; expected 29% dimer, 71% monomer; observed 31, 69%). Similarly, band 3 (Fig. 9, B and C) includes two dimers and three monomers (22137, Fig. 9D; expected 57, 43%; observed 55, 45%). Band 4 (Fig. 9, B and C) was initially thought to include three dimers and one monomer (23117). However, after heating, it was found that this oligomer contains a higher percentage of monomers than expected (expected 86, 14%; observed 74, 26%). A second possibility is an oligomer with two monomers and three dimers, with one subunit excluded from the central pore (23127, Fig. 9D; expected 75, 25%).

Introduction of Functionality across Two Subunits of the \( \alpha \)-Pore—To demonstrate the utility of a subunit dimer in a heptameric pore, we introduced the half-chelating ligand PIDA (Fig. 10A) on each of the two subunits within a dimer of 21337.
pore. We have shown previously that two PIDA ligands on the same subunit can come together to form a complex with a single divalent metal ion (30). This requires the ligands to be in close proximity, which can be achieved by placing them on two adjacent subunits in the heptamer but not on two nonadjacent subunits. We prepared two types of modified pore, containing one or two PIDA ligands, by adapting the method published previously. PPIDA/H11032 has a single PIDA ligand at position 117 on the second subunit of the subunit dimer and is similar to the original PPIDA pore, which has a single PIDA ligand at position 117 on one of the seven monomeric subunits (30). The P(PIDA)2/H11032 pore has one PIDA ligand at position 145 on the first subunit of the dimer (red) and a second PIDA ligand at position 117 on the second subunit of the dimer (yellow) (Fig. 10B). The distance between the two PIDA ligands in the P(PIDA)2/H11032 pore is similar to that in the original P(PIDA)2 pore, which had one PIDA ligand at each of positions 117 and 143 on one of the seven monomeric subunits (the distances between the two sets of Ca atoms are both 8.5 ± 0.1 Å in the WT heptamer structure, Protein Data Bank code 7AHL).

The P(PIDA)2 and P(PIDA)2 pores were characterized by single channel recording in planar lipid bilayers, and their properties were compared with the PPIDA and P(PIDA)2 pores from the original study. The PPIDA/H11032 pore carried a single channel current of 86.1 ± 1.6 pA at 50 mV in 2.0M KCl, 2 mM succinic acid, pH 4.0, which is larger than the value of 75.7 ± 1.6 pA reported for the PPIDA pore under similar conditions. The recordings were performed at low pH because Zn2+ binds very tightly to P(PIDA)2 at neutral pH values (30). The addition of Zn2+ to the trans side of the PPIDA/H11032 pore resulted in the fluctuation of the ionic current between two discrete levels separated by $I_{\text{on}} = 2.7 ± 0.1$ pA, where $\Delta I$ is the current difference between the level partially blocked by Zn2+ and that of the unoccupied pore (Fig. 10C, upper panel). A kinetic analysis of the binding events gave $k_{\text{on-mono4}} = 1300 ± 400$ M$^{-1}$ s$^{-1}$ and $k_{\text{off-mono4}} = 24 ± 6$ s$^{-1}$ to yield an overall formation constant of $K_{f,\text{mono4}} = 52 ±$
Subunit Dimers of α-Hemolysin

FIGURE 9. Composition of oligomers containing monomers and subunit dimers of αHL. A, mixtures of monomers and dimers of αHL were assembled on rRBCM and subjected to electrophoresis in a 5% SDS-polyacrylamide gel. The monomers carried a D8H6 tail to allow separation of the oligomers. Lane A, dimer/monomer ratio 5:0; lane B, dimer/monomer 4:1; lane C, dimer/monomer 3:2; lane D, dimer/monomer 2:3; lane E, dimer/monomer 1:4; lane F, dimer/monomer 0:5. B, the experiment in A was repeated on a larger scale, and the samples with different ratios of monomer to dimer were combined ("Mix"). C, the oligomers in bands 1–4 from B were excised from the gel and heated to 95 °C for 10 min to disrupt the subunit interactions. The polypeptides were then subjected to electrophoresis in a 12% SDS-polyacrylamide gel to resolve the constituents, and the relative intensities of the bands were measured from an autoradiogram by using Quantity One software (Bio-Rad). The predicted intensities of the bands is given in parentheses for the following are: lane 1, 2,1,7; lane 2, 2,1,7; lane 3, 2,1,7; lane 4, 2,1,7. D, proposed stoichiometries of the oligomers 1–4 in B.

19 M⁻¹. The $k_{on}$ value is about half that of the original P(PIDA)₂ pore (Table 1), although the $k_{off}$ values are nearly the same. The percentage block by Zn²⁺ relative to the unblocked pore is 3.1% for P(PIDA)₂ and 2.1% for P(PIDA) (Table 2). Therefore, in this regard, pores that include subunit dimers differ only subtly from similar pores made from monomers alone.

The P(PIDA)₂ pore carried a single channel current of −67.1 ± 2.2 pA under the conditions described above, which is again larger than the −56.4 ± 2.0 pA found for the P(PIDA)₂ pore. After the addition of Zn²⁺ to the trans recording chamber, we observed both individual and complex current blockades (Fig. 10C, lower panel). The individual binding events to either level A (∆I = 1.3 pA) or level B (∆I = 3.1 pA) are consistent with Zn²⁺ binding to one or the other of two different sites. The complex blockades of the P(PIDA)₂ pore included three different current levels and always began as a step from the unoccupied pore to either level A or level B, followed by a step to a new current level, C (∆I = 2.0 pA) (Table 2). As supported by subsequent findings, level C represents a Zn²⁺ cation fully chelated by both PIDA ligands. Current steps to level A or B from level C were observed, but we never detected steps from C directly to the level of the unoccupied pore. The reaction rates for each step in the binding scheme for Zn²⁺ by the P(PIDA)₂ pore (Fig. 10D) were determined by fitting the mean lifetimes of the four conductance states (unoccupied, A, B, and C) to the scheme by using the QuB software package (Table 3). Plots of the rates $v_{A, \text{on}}$ and $v_{B, \text{on}}$ at pH 4.0 versus the Zn²⁺ concentration are of the form $v = k[Zn²⁺]$, where $k$ is $k_{\text{on-A4}}$ or $k_{\text{on-B4}}$ (M⁻¹ s⁻¹), which confirms that both proposed half-chelator-Zn²⁺ complexes are formed in a bimolecular reaction (supplemental Fig. 4). As a further test of the kinetic scheme, we note that at equilibrium the product of the rate constants for clockwise movement around the diamond (Fig. 10D) must equal the product for anticlockwise movement (30, 40). We found that $k_{\text{on-A4}} k_{\text{off-AC4}} k_{\text{off-BC4}} k_{\text{isol-B4}} = 33 ± 9 \times 10⁶$ M⁻¹ s⁻¹ and $k_{\text{on-B4}} k_{\text{off-BC4}} k_{\text{off-AC4}} k_{\text{isol-A4}} = 31 ± 11 \times 10⁶$ M⁻¹ s⁻¹, which are the same within experimental error.

The properties of the P(PIDA)₂ pore are similar to those previously reported for the P(PIDA)₂ pore (30); however, there are some differences. Although level C of the P(PIDA)₂ pore corresponds to the largest current block (∆I), level C of the P(PIDA)₂ pore lies between level A and B. As noted previously, small changes within the lumen of the pore can have unpredictable effects on pore currents (39, 41), i.e. there is no reason to assume that fully complexed Zn²⁺ should always produce the largest current block. Neither is it possible to unambiguously assign level A or level B to one of the two locations of the PIDA ligand in either the P(PIDA)₂ or the P(PIDA)₂ pore, although, based on the observed sequence of transitions and the Zn²⁺ binding kinetics, we can be sure that the two levels correspond to Zn²⁺ binding events. In contrast to the two sites in the P(PIDA)₂ pore, the sites (145 and 117) in the P(PIDA)₂ pore although similar are no longer almost kinetically equivalent. For example, for P(PIDA)₂, $k_{\text{on-A4}}$, $k_{\text{on-B4}} = 2200 ± 100$ M⁻¹ s⁻¹, although similar are no longer almost kinetically equivalent. We note that both $k_{\text{on-A4}}$ and $k_{\text{on-B4}}$ for formation of the half-chelator complexes, as well as $k_{\text{on-AC4}}$ and $k_{\text{on-BC4}}$ for formation of the fully chelated complex, are lower for the P(PIDA)₂ pore by comparison with the corresponding rate constants of the P(PIDA)₂ pore (Table 3). These differences could arise because different buffers were...
The PPIDA (levels A and B), and Zn$^{2+}$, pH 4.0, in both chambers and 500 mV. The measurements were made in 2 M KCl, 2 mM succinic acid, pH 4.0 (PPIDA), or 2.0 M KCl, 10 mM potassium acetate, pH 4.0 (P(PIDA)$_2$), in normal font). A single channel current of ~100 pA corresponds to a unitary conductance of 2 nS. The magnitude of the block is quoted with respect to the unoccupied pore current.

| Protein       | Open pore current | Magnitude of current block by Zn$^{2+}$ | % current block by Zn$^{2+}$ |
|---------------|-------------------|----------------------------------------|-----------------------------|
| P(PIDA)$_2$   | pA                | 2500                                    | 2.0 ± 1.0 s$^{-1}$          |
| P(PIDA)$_2$   | 1.6 ± 0.03 s$^{-1}$ | 2.5 ± 0.2 s$^{-1}$                     |
| P(PIDA)$_2$   | 2.0 ± 0.2 s$^{-1}$ | 3.7 ± 0.1 s$^{-1}$                     |
| P(PIDA)$_2$   | 3.0 ± 0.2 s$^{-1}$ | 6.6 ± 0.1 s$^{-1}$                     |

**TABLE 3**

Kinetic constants for the formation of complexes between the P(PIDA)$_2$ and P(PIDA)$_2$ pores and Zn$^{2+}$

The values were calculated from at least three independent experiments and are quoted as the mean ± S.D. The measurements were recorded at ~50 mV in buffer containing 2.0 M KCl, 2 mM succinic acid, pH 4.0, or 2.0 M KCl, 10 mM potassium acetate, pH 4.0 (P(PIDA)$_2$). The kinetic constants were obtained by using the QuB software package. The formation constants ($K_f$) were calculated by using $K_f = k_{on}/k_{off}$.

| Level A       | Level B       | Level C       | Level C       |
|---------------|---------------|---------------|---------------|
| kon-A         | kon-B         | kon-AC        | kon-BC        |
| 2200 ± 100 M$^{-1}$ s$^{-1}$ | 1400 ± 200 M$^{-1}$ s$^{-1}$ | 29 ± 2 s$^{-1}$ | 25 ± 2 s$^{-1}$ |
| 2500 ± 400 M$^{-1}$ s$^{-1}$ | 2300 ± 200 M$^{-1}$ s$^{-1}$ | 1300 ± 400 M$^{-1}$ s$^{-1}$ | 51 ± 5 s$^{-1}$ | 56 ± 7 s$^{-1}$ |
| kon-B         | kon-AC        | kon-BC        | kon-BC        |
| 24 ± 2 s$^{-1}$ | 23 ± 2 s$^{-1}$ | 42 ± 13 s$^{-1}$ | 22 ± 5 s$^{-1}$ |
| 27 ± 2 s$^{-1}$ | 23 ± 2 s$^{-1}$ | 32 ± 2 s$^{-1}$ | 35 ± 5 s$^{-1}$ |
| kon-AC        | kon-BC        | kon-BC        | kon-BC        |
| 92 ± 11 M$^{-1}$ | 59 ± 9 M$^{-1}$ | 65 ± 22 M$^{-1}$ | 62 ± 19 M$^{-1}$ |
| kon-BC        | kon-BC        | kon-BC        | kon-BC        |
| 92 ± 15 M$^{-1}$ | 100 ± 10 M$^{-1}$ | 150 ± 30 M$^{-1}$ | 160 ± 40 M$^{-1}$ |

**TABLE 2**

Single channel currents through unoccupied αHL pores and after blockade by Zn$^{2+}$

The measurements were recorded at ~50 mV in buffer containing 2.0 M KCl, 2 mM succinic acid, pH 4.0 (P(PIDA)$_2$), or 2.0 M KCl, 10 mM potassium acetate, pH 4.0 (P(PIDA)$_2$). The kinetic constants were obtained by using the QuB software package. The formation constants ($K_f$) were calculated by using $K_f = k_{on}/k_{off}$.

**TABLE 1**

The association and dissociation rate constants for Zn$^{2+}$ with the P(PIDA)$_2$ and P(PIDA)$_2$ pores

The measurements were made in 2 M KCl, 2 mM succinic acid, pH 4.0 (P(PIDA)$_2$), or 2 M KCl, 10 mM potassium acetate, pH 4.0 (P(PIDA)$_2$), at an applied potential of ~50 mV. The kinetic constants were obtained by using the Clampfit 10.2 software package. The formation constants ($K_f$) were calculated by using $K_f = k_{on}/k_{off}$.

| Protein       | kon-min          | kon-max         | koff-min        | koff-max         | $K_f$ |
|---------------|------------------|------------------|-----------------|------------------|-------|
| P(PIDA)$_2$   | 2500 ± 400 s$^{-1}$ | 1300 ± 400 s$^{-1}$ | 29 ± 4 s$^{-1}$ | 24 ± 6 s$^{-1}$ | 85 ± 16 M$^{-1}$ | 52 ± 19 M$^{-1}$ |

**General Implications and Future Prospects**—Although the utility of the concamer approach has been carefully documented, the data from this study reinforce the need for rigorous analysis of translation products to ensure that only full-length concameters are produced. We were able to obtain full-length subunit dimers by Ni-NTA purification of a His$_6$-tagged protein. By placing the affinity tag at the C terminus of the protein, we could ensure that only fully translated proteins would bind to the Ni-NTA column. This approach, however, cannot solve the problem of proteolytic cleavage of the concamer after translation, which has been a problem in other studies (36).

Even though there are several examples of pore-forming proteins that can form oligomers with variable stoichiometries, we have shown that αHL oligomers made from subunit dimers resist all but a heptameric pore stoichiometry. This implies that one or more subunits must be excluded from the central pore. Subunit exclusion is not uncommon for ion channels made from concatenated subunits, when there are more subunits to
be accommodated in the oligomer than in the native protein (25, 26).

Based on our findings, we have developed a method to prepare αHL pores containing one subunit dimer and five monomers, allowing us to selectively introduce mutations and/or chemical modifications on adjacent subunits of the αHL pore. Our metal chelation experiments show that pores containing subunit dimers behave similarly to pores containing only monomeric subunits, showing that they assemble correctly. Subunit concatenation has therefore widened the scope for engineering of the αHL pore and might be expanded to extended concatamers (trimers, tetramers, etc.). Ultimately, the approach could allow us to control the nature and position of every subunit in the pore. For practical reasons, it would be useful to introduce unique restriction sites into each linker at the DNA level, to allow the cutting and pasting of specific subunit genes into the construct.

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