A functional protein pore with a “retro” transmembrane domain

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Abstract

Extended retro (reversed) peptide sequences have not previously been accommodated within functional proteins. Here, we show that the entire transmembrane portion of the β -barrel of the pore-forming protein α -hemolysin can be formed by retrosequences comprising a total of 175 amino acid residues, 25 contributed by the central sequence of each subunit of the heptameric pore. The properties of wild-type and retro heptamers in planar bilayers are similar. The single-channel conductance of the retro pore is 15% less than that of the wild-type heptamer and its current-voltage relationship denotes close to ohmic behavior, while the wild-type pore is weakly rectifying. Both wild-type and retro pores are very weakly anion selective. These results and the examination of molecular models suggest that β -barrels may be especially accepting of retro sequences compared to other protein folds. Indeed, the ability to form a retro domain could be diagnostic of a β -barrel, explaining, for example, the activity of the retro forms of many membrane-permeabilizing peptides. By contrast with the wild-type subunits, monomeric retro subunits undergo premature assembly in the absence of membranes, most likely because the altered central sequence fails to interact with the remainder of the subunit, thereby initiating assembly. Despite this difficulty, a technique was devised for obtaining heteromeric pores containing both wild-type and retro subunits. Most probably as a consequence of unfavorable interstrand side-chain interactions, the heteromeric pores are less stable than either the wild-type or retro homoheptamers, as judged by the presence of subconductance states in single-channel recordings. Knowledge about the extraordinary plasticity of the transmembrane β -barrel of α -hemolysin will be very useful in the de novo design of functional membrane proteins based on the β -barrel motif.

Keywords: α -hemolysin; β -barrel; channel; membrane protein; pore; retro sequence; reversed sequence

Many small membrane-active peptides remain effective when synthesized entirely from D-amino acids (Wade et al., 1990; Chorev & Goodman, 1995; Merrifield et al., 1995; Derossi et al., 1996; Ido et al., 1997). This is often taken as proof that they can assemble

into natural biological membranes without interaction with a receptor or an accessory subunit, which would be the incorrect chirality. Larger polypeptides assembled from D-amino acids are also functional (Milton et al., 1992; Chorev & Goodman, 1995; Schumacher et al., 1996), although in the case of enzymes, e.g., HIV protease (Milton et al., 1992), they should and do turn over substrates of the opposite chirality to those handled by the natural form. More surprisingly, the retroforms (reversed sequence forms) of several membrane-active peptides are active (Merrifield et al., 1995; Ido et al., 1997). However, although it has been suggested that they should fold (Olszewski et al., 1996), conceivably into quasienantiomeric structures (Guptasarma, 1992), extended retro sequences have not been accommodated within functional proteins. Indeed, it has been shown experimentally that the retro forms of three small protein domains do not fold (Lacroix et al., 1998). Further, although dramatic changes in blocks of amino acids have been made in enzymes (e.g., 10 alanines in a row can be accommodated in T4 lysozyme) (Heinz et al., 1992), and the sequences of functionally related polypeptides exhibit dramatic differences across species, active site amino acids in proteins are usually conserved. Nevertheless, we show here that the entire transmembrane

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Abbreviations: α HL, α -hemolysin (α -toxin) of *Staphylococcus aureus*; α HL-RL, α HL gene with a reconstructed central sequence; α HL-RL2, a second version of α HL-RL; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; IASD, 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonate; IVTT, in vitro transcription and translation; K8A, protease-resistant mutant of α HL; MBSA, 10 mM Na MOPS, 150 mM NaCl, pH 7.4, containing 1 mg/mL bovine serum albumin; MOPS, 3-[N-morpholino]propanesulfonic acid; PEG, polyethyleneglycol; PMSF, phenylmethylsulfonyl fluoride; retro-1, α HL with a retro (reversed) transmembrane sequence; retro-2, α HL with a retro transmembrane sequence, but with the WT β -turn retained; rRBC, rabbit red blood cell; rRBCM, rabbit red blood cell membranes; SDS, sodium dodecyl sulfate; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; TCM, a version of α HL in which the transmembrane domain is deleted; TM, transmembrane; WT- α HL, wild-type α -hemolysin.

The α HL pore is capable of transporting molecules of up to $\sim 3,000$ Da across the membrane by passive diffusion (Füssle et al., 1981; Bhakdi et al., 1984). Analysis of the pore size by osmotic protection and penetration by neutral polymers, such as poly(ethylene glycol), are in keeping with a cutoff in this range (Krasilnikov et al., 1992; Korchev et al., 1995; Bezrukov et al., 1996). Single-stranded nucleic acids of much larger mass, presumably in an extended form, can move through the central channel in a transmembrane electric field (Kasianowicz et al., 1996). Single-channel conductance measurements (e.g., 450 pS, 0.5 M KCl, pH 7.0, +40 mV) are consistent with a water-filled transmembrane channel with an effective internal diameter of 11–12 Å (Menestrina, 1986). In reality (Song et al., 1996), the internal surface of the channel is corrugated as a result of the different volumes of the amino acid side chains that project into it (Fig. 1A). The diameter of the most constricted region, which lies internally at the *cis* end of the β -barrel, is 14 Å. The α HL pore exhibits weak anion selectivity ($t_{\text{Cl}^-}/t_{\text{K}^+} = 1.5$, pH 7.0) (Menestrina, 1986). The current-voltage (I-V) curve at pH 5.0 is almost linear, while the pore shows weak rectification at pH 7.0 (Krasilnikov et al., 1988; Walker et al., 1992) (e.g., $I_{+100 \text{ mV}}/I_{-100 \text{ mV}} \sim 1.5$).

We are interested in the *de novo* design of functional proteins containing β -barrel domains. To determine the constraints on such structures, we are examining the effects of drastic mutations in the TM region of α HL, such as truncations and extensions (Cheley et al., 1997), as well as point mutations (Walker & Bayley, 1995), and multiple amino acid replacements (Walker et al., 1995; Braha et al., 1997). In the course of these studies, we have determined the properties of a pore with a TM domain formed entirely from a retro sequence.

Results

Construction of the retro-1 gene

A gene was constructed encoding α HL with residues 117–141 in reverse sequence (α HL-retro-1, Fig. 1B). The reverse sequence is centered on Thr129, which is at the turn of the hairpin contributed to the TM β -barrel by each subunit (Song et al., 1996). When aligned, the WT and retro sequences show no sequence identity and very low similarity (Fig. 1B). As expected, both sequences do show the same general pattern of roughly alternating hydrophobicity expected of a transmembrane β -sheet. Other similarities include the charged residues near the turn and the same side chains, in a different order, projecting into the bilayer or the lumen of the channel. Therefore, we ventured that replacement of the TM segment with the retro sequence would not result in a dramatic reorganization of the all-L polypeptide backbone and viewed the manipulation as a domain replacement (Fig. 1A,C), which is now supported by studies of function (see below).

Reduced hemolytic activity of retro-1 is in part due to premature assembly

In a standard hemolytic assay, retro-1 was far less active than WT- α HL or α HL-K8A (Table 1, K8A is a mutant of α HL that is resistant to adventitious proteases). We surmised that this might be the result of premature assembly of retro-1 before the addition of

Table 1. Hemolysis assays with K8A, retro-1, and retro-2^a

	Translation for 1 h	Translation for 8 min	Cotranslation
K8A	11	9	7
retro-1	0	0	6
	1 (2 h assay)	3 (2 h assay)	
retro-2	7	9	8

^aIn the standard hemolysis assay, the IVTT mix was present at a dilution of 1 in 20 in the first well of a microtiter plate, and as a serial twofold dilution in the subsequent wells. The well with 50% lysis after 1 h is recorded in the first two columns. Retro-1 showed activity only when the assay was prolonged for 2 h. In the cotranslation assay, serial twofold dilutions of DNA (800 ng in well 1) were translated in the presence of rRBC. Hemolysis was prevented by the PEG8000 in the translation mix (35 mg/mL final concentration). After 1 h at 37 °C, the mix was diluted 10-fold into MBSA. The well that resulted in 50% lysis is recorded. After preliminary experiments, each assay was performed at least twice with identical results as recorded here.

red blood cells.³ Assembly in solution is a common outcome of mutations in the TM region (Valeva et al., 1996; Cheley et al., 1997) (see Discussion). To test this possibility, retro-1 was prepared by *in vitro* translation. Protease-resistant heptamers were indeed formed, as visualized by SDS-polyacrylamide gel electrophoresis of unheated samples that had been treated with various concentrations of proteinase K (Fig. 2A). K8A made under the same conditions was monomeric and protease sensitive (Fig. 2A). K8A, retro-1, and TCM were then examined by protease treatment as soon as possible after translation *in vitro*. TCM is a version of α HL in which the entire TM region has been removed; TCM undergoes rapid assembly in solution within minutes of synthesis (Cheley et al., 1997). After 16 min, 3% of the retro-1 chains had been converted to the protease-resistant form (Fig. 2B). K8A showed no conversion, while 52% of the TCM sample was protease resistant (Fig. 2B). The lack of activity of retro-1 toward red cells is more pronounced than initially suggested by this measurement, but it is consistent with the idea that retro-1 converts to a heptamer in solution, which is partly SDS-resistant, with the remainder in the form of the SDS-sensitive heptameric prepore. Accordingly, we also found that the hemolytic activity was greater after short translation times. However, the specific activity of retro-1 did not approach that of WT or K8A (Table 1). Finally, retro-1 formed by *in vitro* translation in the presence of target rRBC was highly active, again in keeping with the idea of premature assembly in the absence of cells. With regard to hemolytic activity, the primary defect in retro-1 appears to be in the sequence at the turn. In retro-2, the five residues centered on Thr129 were kept in the order found in WT- α HL, while the remaining 20 amino acids in the TM domain were reversed (Fig. 1B), yielding a protein far more active toward rRBC in the standard assay than retro-1. Nevertheless, retro-2 did lose activity in solution, albeit more slowly than retro-1 (Table 1).

³When WT- α HL is converted to heptamer it is inactive on rRBC (Bhakdi et al., 1981), presumably because it cannot insert into the lipid bilayer by the receptor-mediated pathway. Heptamers do, however, insert readily into planar bilayers. Indeed, this is a convenient way to initiate single-channel recordings (Braha et al., 1997).

Fig. 2. A: Retro-1 forms an SDS-resistant heptamer in solution. K8A and retro-1 were treated with proteinase K at 25°C immediately after IVTT (37°C, 1 h). The final concentrations of proteinase K were 0 (lanes 1, 5, 9, and 13), 5 (lanes 2, 6, 10, and 14), 50 (lanes 3, 7, 11, and 15), and 500 (lanes 4, 8, 12, and 16) $\mu\text{g}/\text{mL}$. The samples run in lanes 1–8 were not heated. Those in lanes 9–16 were heated. An autoradiogram is shown. The SDS-resistant heptamer in the unheated retro-1 samples migrates with the M_r 200,000 marker. **B:** Kinetics of heptamer formation by K8A, retro-1, and TCM. IVTT was carried out at 37°C for 2, 4, 8, and 16 min before immediate digestion with proteinase K (500 $\mu\text{g}/\text{mL}$). The samples were heated and subjected to SDS polyacrylamide gel electrophoresis. An autoradiogram is shown.

Single-channel properties of retro-1 homoheptamers

Bilayer recordings with retro-1 were first done with the “monomer.” However, after observing the conversion to heptamer in solution, we decided to repeat and complete the experiments with retro-1 heptamers (retro-1₇) that had been purified by electrophoresis (Braha et al., 1997). With the monomer, the channel behavior was, in any case, less predictable. The addition of the monomer to the *cis* chamber often resulted in noisy currents with discrete transitions of low amplitude (60 pS). Occasionally, after several minutes, a quiet, large channel was obtained (~600 pS), similar to those seen with the preformed heptamer, retro-1₇.

By contrast with the monomer, retro-1₇ inserted more quickly into planar bilayers at a transmembrane potential of +40 mV, exhibiting discrete conductance steps, presumed to correspond to single ion channels (Fig. 3A). Small noisy channels were rare. By recording series of insertion events, such as those shown for retro-1₇ (Fig. 3A), for retro-1₇, WT₇, and K8A₇, histograms representing the distributions of single-channel currents were obtained (Fig. 3B). In 1 M NaCl, at pH 7.4, with a transmembrane potential of +40 mV, the mean values were retro-1₇, 25.1 ± 2.2 pA; WT₇, 29.5 ± 1.7 pA; and K8A₇, 30.2 ± 2.0 pA, which correspond to conductances of 628, 738, and 755 pS, respectively. As well as exhibiting a significantly lower conductance, retro-1₇ channels were “noisy” at negative transmembrane potentials.

Single-channel I-V curves were also determined for retro-1₇, WT₇, and K8A₇ in 1 M NaCl at pH 7.4 (Fig. 3C). WT₇ ($I_{+80 \text{ mV}}/I_{-80 \text{ mV}} = 1.19$, SD = 0.018, $n = 5$) and K8A₇ ($I_{+80 \text{ mV}}/I_{-80 \text{ mV}} = 1.12$, SD = 0.015, $n = 5$) show weak rectification, while retro-1₇ is close to ohmic ($I_{+80 \text{ mV}}/I_{-80 \text{ mV}} = 0.96$, SD = 0.036, $n = 4$). All three pairs of differences are statistically significant, $p < 0.001$.

The ion selectivities of retro-1₇, WT₇, and K8A₇ were compared by measuring the reversal potentials with asymmetric KCl solutions. The values for $p_{\text{Cl}^-}/p_{\text{K}^+}$ were retro-1₇, 1.9 ± 0.81 ($n = 5$); WT₇, 2.0 ± 0.51 ($n = 3$); K8A₇, 1.9 ± 0.31 ($n = 5$). There are no statistically significant differences between these values (*t*-test).

Heteroheptamers containing retro-1

To further explore the properties of retro-1, we made heteroheptamers (Braha et al., 1997), incorporating both retro and WT subunits. An additional mutation was made in retro-1 to provide a cysteine at position 292 for derivatization with sulfhydryl-directed reagents. The number of mutant subunits in an SDS-resistant heteromer can be determined by an experiment in which the reaction of each sulfhydryl with a disulfonate reagent, IASD, produces an incremental shift upon analysis by gel electrophoresis (Gouaux et al., 1994; Walker et al., 1995; Braha et al., 1997). To circumvent the rapid conversion of retro-1 to a homoheptamer, retro-1 and

Fig. 3. Single-channel currents from retro-1₇, WT₇, and K8A₇. **A:** Multiple insertions of retro-1₇. The stepwise increase in current is shown as a function of time. The bilayer potential was +40 mV, and both chambers contained 1 M NaCl, 10 mM MOPS, pH 7.4. The protein was added to the *cis* chamber, which was stirred continuously to favor multiple insertions. The current was filtered at 20 Hz to remove noise produced by the stirrer. **B:** Histograms of single-channel currents for retro-1₇, WT₇, and K8A₇. Histograms were constructed from measurements of current steps, such as those shown in A. Data from between 5 to 10 separate recordings were used to construct each histogram. The centers of the fitted Gaussian distributions (mean conductance) and standard deviations are shown. **C:** Current-voltage (I-V) relationships. Left: Single-channel currents at alternating negative and positive potential steps of 20, 40, 60, 80, 100, and 120 mV are shown for retro-1₇, WT₇, and K8A₇. Both chambers contained 1 M NaCl, 10 mM MOPS, pH 7.4. The traces were filtered at 100 Hz with an 8-pole Bessel filter. Right: I-V curves for retro-1₇, WT₇, and K8A₇. The noisy appearance of the retro-1₇ current at negative potentials is due to short-lived substates of lower conductance, which are apparent when the timescale is expanded (data not shown). Therefore, the maximal current at each potential was used to construct the I-V curves and calculate rectification ratios.

K8A were cotranslated (37 °C, 1 h) and then added to rRBCM. K8A₇ and retro-1₇ were readily observed when K8A and retro-1 were allowed to assemble individually on rRBCM, although the band of the retro-1 homomer was weaker than that of the K8A homomer (Fig. 4A). Further, while heteroheptamers formed from retro-1 and K8A are observable in SDS gels and hence stable, the

distribution of the heptamers in the gels shows that K8A_{7-n}retro-1_n becomes increasingly less abundant as the number of retro-1 subunits increases, with the exception of retro-1₇ itself, which is relatively abundant (Fig. 4A). WT₇ is stable in SDS at up to 65 °C and additional experiments showed that the heteroheptamers were just as stable (data not shown). Experiments with a large excess of

Fig. 4. Heteroheptamer formation on red cell membranes. **A:** The proteins were obtained by cotranslation (37 °C, 1 h), allowed to oligomerize on rRBCM, treated with the sulfhydryl-directed gel shift reagent IASD, and subjected to SDS-polyacrylamide electrophoresis in a 6% gel. The ratios of template DNAs are indicated. Left: Retro-1 cotranslated with K8A. Right: T292C cotranslated with WT. **B:** The proteins were obtained by cotranslation in the presence of rRBCM (37 °C, 1 h). They were then treated with IASD and subjected to electrophoresis. Left: Retro-1 cotranslated with K8A. Right: T292C cotranslated with WT. **C:** Single-channel currents from heteromeric pores containing retro-1 subunits: K8A₆retro-1₁, K8A₅retro-1₂, and K8A₄retro-1₃. Left: Single-channel currents at alternating negative and positive potential steps of 20, 40, 60, 80, 100, and 120 mV. The results shown are representative of 4, 5, and 3 experiments, respectively. Right: The current traces at -80 mV are shown in an expanded timescale and at higher gain. These traces were filtered at 1 kHz with a Bessel filter.

retro-1 (K8A DNA:retro-1 DNA, 1:20) showed that all eight K8A_{7-n}retro-1_n combinations could be formed under forcing conditions (data not shown). Therefore, either the rate of formation of the high-*n* oligomers was slow relative to the others, or the depletion of retro-1 by diversion into retro-1₇ was responsible for their low relative abundance. When membranes were present during the cotranslation of K8A and retro-1, the abundance of the high-*n* oligomers was greatly increased, favoring the latter hypothesis (Fig. 4B).

Bilayer recordings were made with selected heteromers isolated by electrophoresis (Fig. 4C). Single channels of K8A_{7-n}retro-1_n exhibited increasing "noise" with increasing *n*, but retro-1₇ itself was quieter than expected on this basis. The subunits in K8A₅retro-1₂ and K8A₄retro-1₃ have three and five permutations respectively about the molecular sevenfold axis (Braha et al., 1997). Therefore, the single channels we recorded could have represented any one of these. Nevertheless, the trend toward greater noise with increasing *n* was apparent for the channels that were examined. Molecular graphics suggest that neighboring retro and WT sequences in the TM domain would suffer both steric clashes and unfavorable electrostatic interactions, which might destabilize the β -barrel (Fig. 1C). These interactions would not occur in the K8A₇ and retro-1₇ homomers (Fig. 1C).

Discussion

α HL with a retro TM domain is functional

We are exploring the limits to which a pore-forming protein can be altered by genetic engineering to create functional pores with new properties (Chang et al., 1995; Walker et al., 1995; Panchal et al., 1996; Braha et al., 1997; Cheley et al., 1997). We were provoked to introduce an extended retro sequence into the TM domain of α HL by the extensive literature on antimicrobial and other membrane-active peptides in which the activity of all-D, retro and retro-D versions of all-L peptides has been demonstrated (Chorev & Goodman, 1993, 1995). For example, all four forms of hybrid cecropin-melittin peptides were active under most circumstances (Merrifield et al., 1995). In another example, retro and all-D forms of the 31-amino acid insulin C peptide, which is derived during the processing of proinsulin, were equipotent with the natural C peptide in ameliorating diabetes-associated dysfunctions in rats (Ido et al., 1997). This suggests that stereospecific receptors are not involved in the action of the C peptide and that, perhaps, the C peptide has pore-forming activity.

The single-channel properties of WT₇, K8A₇, and retro-1₇ are similar. Notably, the single-channel conductance of retro-1₇ is only 15% lower than the wild-type pore. The I-V curves and ion selectivity are also similar. Together with the structural constraints imposed by the hemolysin framework, these results strongly suggest that retro-1₇ forms a channel with a TM β -barrel of similar dimensions to the WT barrel, i.e., the retro-1₇ barrel is not collapsed or significantly deformed. The distribution of single-channel conductances is similar for WT₇, K8A₇, and retro-1₇ (Fig. 3B), suggesting that retro-1 is not especially prone to errors in assembly.

A β -barrel is especially compatible with a retro sequence

Previous attempts to make folded retro proteins have failed (LaCroix et al., 1998). How then have we been able to introduce an

extended retro sequence, 175 amino acids in all, into α HL and retain activity? We propose that retro-1₇ forms a β -barrel with a right-handed twist as seen in WT₇ (Song et al., 1996) (Fig. 1C). A quasienantiomeric left-handed barrel (Guptasarma, 1992) would be unable to form an ordered junction with the top half of α HL in which the remainder of the barrel is right-handed (Song et al., 1996). We believe that we have succeeded because a β -barrel is particularly amenable to retromerization without assuming an enantiomeric backbone and because, in α HL, the barrel is largely autonomous, the remainder of the structure providing a scaffold for barrel assembly. Disregarding the twist of the barrel, each retro hairpin superficially resembles a mirror-image of the WT hairpin (Fig. 1C). However, more careful inspection of the modeled barrel (Fig. 1C) reveals that side chains in the retro barrel are in different environments with respect to their neighbors and hydrogen bonding patterns (Hutchinson et al., 1998).

The felicitous situation with a retro barrel can be compared with other retro secondary structures. For example, reversed α -helical segments of L-amino acids are unlikely to form left-handed helices (Matthews et al., 1974; Nicholson et al., 1989; Middaugh et al., 1993), as has been suggested (Guptasarma, 1992). If right-handedness is maintained, retro helices would have switched N- and C-terminal capping residues (Regan, 1993), altered interactions of side chains with the helix dipole, and altered interactions between side chains because of the orientation of the C α -C β bond toward the N terminus. These changes would significantly affect the bundling of helices, which is especially important in maintaining the structure of helical membrane proteins (MacKenzie & Engelman, 1998).

Therefore, our results suggest that the ability to form a retro domain could be diagnostic of a β -barrel. It might even be possible to make an entire retro protein, such as porin, in this form. In the case of membrane-active peptides, such as antimicrobials, the organization of membrane-bound forms of even the natural peptides is rarely known with certainty. Our work suggests that membrane-active peptides with active nonenantiomeric retro forms may be β -barrels.

Retro-1 undergoes premature assembly

Several α -hemolysins with mutations in the TM region undergo premature assembly in the absence of bilayers. The changes range from simple point mutations (Valeva et al., 1996) to a drastic removal of the entire TM segment, which causes the formation of a heptamer in the fully assembled pore conformation immediately after polypeptide synthesis (Fig. 2B) (Cheley et al., 1997). Recent results suggest that the nascent TM sequence is bent back against the protein surface, and partly disordered in the water-soluble monomeric form of the related protein lukF (Olson et al., 1999). The α HL monomer is very likely to have a similar structure (Olson et al., 1999), and the mutations must weaken the interactions of the TM region with the rest of the monomer to initiate assembly. This situation contrasts with the fully-assembled heptamer, where the TM domain does not contact the remainder of the protein and drastic alterations are tolerated as exemplified in the present work.

Retro-1/ WT heteromers form noisy channels

Molecular modeling suggests that heteroheptamers containing both retro-1 and WT subunits might be less stable than WT₇ or retro-1₇ homoheptamers. When retro-1 and WT subunits are adjacent, ste-

ric clashes and unfavorable electrostatic interactions occur in the TM barrel (Fig. 1C). The barrel is important for stabilizing heptamers to denaturation with SDS, which occurs at 65 °C with the WT protein. By contrast, the prepore, in which the barrel has not formed, is SDS sensitive, although it exists on lipid bilayers as a stable heptamer that does not exchange subunits.⁴ Heteromers containing retro-1 subunits were no less stable than WT₇ as judged by the temperature at which the heptamers dissociated in SDS. However, the channels formed with retro-1 subunits displayed transient partial closures ("noise") at negative transmembrane potentials (Fig. 4C). At negative potentials a less stable barrel would be further destabilized because of the net negative charge near the *trans* tip of each subunit (Asp127, Asp128, Lys131).

Relevance to membrane protein design

The constraints explored in experiments such as those described here will be very useful in the de novo design of functional membrane proteins based on the β -barrel motif. The present work demonstrates the extraordinary structural plasticity of the TM barrel in α HL. Nevertheless, it also shows that unfavorable interactions can develop within the barrel domain that might lead to disintegration of the structure in the absence of a supporting framework, such as that provided by the cap domain. The instability is manifested in single-channel recordings as voltage-dependent partial closures, which might also be regarded as a primitive form of rectification or voltage gating. Heteromeric forms of α HL are useful both for basic studies and in applications such as sensor technology (Braha et al., 1997). In the course of this study, techniques have been developed to circumvent problems associated with the premature assembly of α HL mutants and thereby obtain heteromers that are disfavored under normal circumstances.

Materials and methods

Partly reconstructed α HL gene: α HL-RL2

To permit facile mutagenesis of the glycine-rich TM domain of α HL, the α HL gene had been previously reconstructed to introduce eight unique restriction sites encompassed by residues 116–147 (Cheley et al., 1997). Recently, the reconstructed gene (α HL-RL) was further modified by silent mutation to introduce two additional restriction sites (*Sac*II and *Xho*I) to yield α HL-RL2, which was used in the present work. The new sites include codons 103 and 104 (*Sac*II) and 157 and 158 (*Xho*I). Site-specific mutagenesis and recombination (Jones & Howard, 1991; Howorka & Bayley, 1998) was carried out with pT7- α HL-RL as a template by using a *Taq*/*Pwo* DNA polymerase mix (Expand Long Template PCR System, Boehringer Mannheim, Germany). The *Sac*II and *Xho*I sites were introduced sequentially in two separate PCR operations, each followed by ligation-free recombination. The mutagenic primers used for introduction of the *Sac*II site (italics) were: 5' CTGATTACTATCCGCGGAATTCGATTGATAC (forward) and 5' GTATCAATCGAATTCGCGGATAGTAATCAG (reverse) while those used for introduction of the *Xho*I site (italics) were: 5' CAAAACAATTCTCGAGAGCCCAACTG (forward) and 5' CAG

TTGGGCTCTCGAGAATTGTTTTG (reverse). The nonmutagenic primers, 5' ATAAAGTTGCAGGACCACTTCTG (forward) and 5' CAGAAAGTGGTCCTGCAACTTTAT (reverse), were used for both sets of PCR. The sequence of the entire reconstructed gene in a T7 vector (Walker et al., 1992; Cheley et al., 1997) was verified. Like α HL-RL, α HL-RL2 contains the following changes over wild-type α HL: Lys8 $\rightarrow$ Ala and the conservative replacements Val124 $\rightarrow$ Leu, Gly130 $\rightarrow$ Ser, Asn139 $\rightarrow$ Gln, and Ile142 $\rightarrow$ Leu. α HL-RL2 retained full activity compared to wild-type α HL based on hemolysis assays after in vitro synthesis (see below). A map of the construct is available upon request.

Construction of an α HL gene (retro-1) with a retro (reversed wild-type) TM domain

DNA encoding residues that form the transmembrane β -barrel of α HL was removed from pT7- α HL-RL2 with *Hpa*I and *Avr*II, and replaced by using two synthetic duplex DNAs encoding the reversed sequence to yield pT7-retro-1 (Fig. 1B). The new insert contained the wild-type codons in reversed order with the exception of the boundaries where Ser117 was encoded by TCC (WT Ser141 is TCG) and Thr141 was encoded by ACC (WT Thr117 is ACT). DNA encoding the N-terminal half of the replacement sequence was prepared from phosphorylated 5' ATCCGTTAATGCAGGTATCTTGGCGGCATTAAA (sense), and 5' CATCTGTTCTTTAATGCCGCCAAGAATACCTGCATTAACGGAT (antisense), while DNA encoding the C-terminal half of the replacement sequence was prepared from phosphorylated 5' GGAACAGATGATGGTACTGTTAATGGTAACCTCGGATATACC (sense) and 5' CTAGGGTATATCCGAAGTTACCATTAAACAGTACCAT (antisense). The two DNA cassettes share a 10 bp overlap. Three-way ligation of the cassettes with the cut pT7- α HL-RL2 yielded pT7-retro-1. The construct was verified by sequencing the entire cDNA insert. Besides the altered TM domain, retro-1 also differs from α HL-WT at two additional positions, Lys8 $\rightarrow$ Ala and Ile142 $\rightarrow$ Leu. The K8A mutation, which is present in α HL-RL, was introduced to confer resistance to lysine-directed proteases (Walker & Bayley, 1994). As shown here, the mutation at position 8 has a slight effect on the single-channel properties of the α HL pore; for example, it is distinctly less rectifying at low salt concentrations (e.g., 100 mM KCl). Therefore, we have compared retro-1 with both WT and K8A throughout. The I142L mutation was introduced during the construction of α HL-RL to generate the *Avr*II site (Cheley et al., 1997) and produces no observable effect on the single-channel properties of α HL (Braha et al., 1997). This was confirmed by making K8A/I142L. Homoheptamers of this mutant were indistinguishable from those made from K8A; they had the same mean amplitude, same current noise at positive and negative potentials, and the same rectification properties. K8A/I142L was made by cutting pT7- α HL-RL2 in the reconstructed central domain with *Bsi*WI and *Avr*II and replacing the central sequence with phosphorylated synthetic oligonucleotides: duplex 1, 5' GTACGGATTCAACGGTAATACTGGTGATGAT (sense) and 5' TCCTGTATCATCACCAGTAACATTACCGTTGAATCC (antisense); duplex 2, 5' ACAGGAAAATTTGGCGGCCTTATTGGTGCAAATGTTTCC (sense) and 5' CTAGGGAAACATTTGCACCATAAGGCCGCCAATTTT (antisense). In this construct, the amino acids at positions 124, 130, and 139 were as in wild-type α HL.

Retro-2, a retro derivative with a WT turn (Fig. 1B), was constructed in the same way as retro-1, except that the four synthetic

⁴Certain mutants that are arrested as prepores (e.g., as judged by limited proteolysis) appear as heptamers on SDS gels. This is because a fraction of these prepores is dissociated by the detergent, while the remainder is converted to fully assembled pores (B. Walker, unpubl. data).

oligonucleotides were: duplex 1, 5' ATCCGTTAATGCAGGTAT TCTTGGCGGCATTGATGAT (sense) and 5' TCCTGTATCAT CAATGCCGCCAAGAATACCTGCATTAACGGAT (antisense); duplex 2, 5' ACAGGAAAAGGTACTGTTAATGGTAACTTCGG ATATACC (sense) and 5' CTAGGGTATATCCGAAGTTACCATT AACAGTACCTTT (antisense).

To generate heteromers containing retro-1 and K8A subunits, a derivative of retro-1 was constructed containing a cysteine at residue 292. pT7-retro-1 was digested with *Nde*I and *Mfe*I and the resulting internal fragment was ligated to pT7- α HL-T292C (Walker & Bayley, 1995), which had been cut with the same enzymes, to yield pT7-retro-1/T292C.

In vitro transcription and translation (IVTT)

Wild-type and mutant α HL polypeptides were synthesized by coupled *in vitro* transcription and translation (IVTT), by using an S30 extract from *Escherichia coli* (Promega, Madison, Wisconsin, T7 S30 no. L114A), as described previously (Cheley et al., 1997). Radiolabeled polypeptides were synthesized in the presence of [³⁵S]methionine (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, 1,200 Ci/mmol) by using amino acid pre-mixes lacking methionine. For hemolysis assays and bilayer experiments (see below), a complete amino acid mix was used.

Hemolysis assays

IVTT mix (10 μ L) was diluted with MBSA buffer (90 μ L; 10 mM Na MOPS, 150 mM NaCl, pH 7.4, containing 1 mg/mL bovine serum albumin), and subjected to twofold serial dilution with the same buffer, which left 50 μ L in each well of a microtiter plate. Hemolysis was initiated by the addition of an equal volume of washed rabbit red blood cells (rRBC, 1% in MBSA) to each well and monitored by observing the decrease in light scattering at 595 nm with a Bio-Rad (Hercules, California) microplate reader (Model 3550-UV).

Kinetics of heptamer assembly as determined by proteinase K digestion

The rate of heptamer assembly was determined as detailed elsewhere (Cheley et al., 1997). Briefly, IVTT was initiated at 37°C with prewarmed reagents in a total volume of 50 μ L. After 2, 4, 8, and 16 min, a portion (5 μ L) was removed and added to 20 μ L of a digestion mix at 25°C, to give a final concentration of 1 mM chloramphenicol and 500 μ g/mL proteinase K in MBSA (Cheley et al., 1997). In a control experiment, proteinase K was replaced with water. After digestion for 5 min, samples were treated with phenylmethylsulfonylfluoride (PMSF) for a further 5 min (200 mM in isopropanol, 2 μ L), followed by the addition of 2X Laemmli sample buffer (Laemmli, 1970). The samples were heated for 5 min at 95°C and run in a 12.5% SDS polyacrylamide gel. The protease-resistant fraction was estimated from the dried gel by using a phosphor-scanner (Cyclone, Packard Instrument Co., Meriden, Connecticut).

Heptamer formation and purification

To generate radiolabeled heteroheptamers, the single cysteine mutants α HL-T292C or α HL-retro-1/T292C were cotranslated with

WT or K8A, respectively. IVTT was initiated with various molar ratios of template DNA (6:0, 5:1, 1:1, 1:5, and 0:6) in the presence of [³⁵S]methionine and the complete amino acid mix. The labeled subunits were allowed to oligomerize on rRBC membranes (prepared by hypotonic lysis of washed rRBCs) for 1 h at room temperature in MBSA containing 1 mM EDTA. The membranes were then washed by centrifugation and resuspension in the same buffer. After a second centrifugation, the pellets were resuspended in 2.0 M TAPS, pH 8.5 (10 μ L), and 10 mM DTT (2 μ L). After 10 min at room temperature, the mixture was treated with 100 mM 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonate (IASD, 4 μ L in water) (Molecular Probes, Eugene, Oregon), for 1 h at room temperature to modify Cys292 on the α HL-T292C or α HL-retro-1/T292C subunits. DTT (100 mM in water, 4 μ L) was then added and the membranes recovered by centrifugation. The pellet was washed with 200 mM TAPS, pH 8.5, and then resuspended in 1.5 $\times$ Laemmli sample buffer (30 μ L) without heating.

Portions (10 μ L) were loaded into the wells of a 40 cm long, 1.5 mm thick, 6% SDS-polyacrylamide gel, and electrophoresed for 20 h at 110 V, with 0.1 mM Na thioglycolate in the cathode buffer. Unfixed gels were vacuum dried without heating onto Whatman 3MM filter paper and exposed to X-ray film. Bands containing heteroheptamers were excised from the gel using the autoradiogram as a template. After rehydration with water (400 μ L), gel slices were thoroughly crushed and then rotated at 37°C for 16 h. The eluted protein was freed from gel debris by centrifugation of the solution through a 0.2 μ m cellulose acetate spin filter (Microfilterfuge tube; Rainin, #7016-024 Woburn, Massachusetts). The recovered filtrate was stored in 25 μ L portions at -80°C. The recovery of protein was analyzed by subjecting one portion to electrophoresis in a 12% SDS-polyacrylamide gel after treatment with Laemmli sample buffer without heating (Laemmli, 1970).

Homoheptameric proteins (WT₇, K8A₇, K8A/I142L₇, retro-1₇, and retro-2₇) were synthesized, assembled, and purified as described above except that the IASD modification was omitted.

Heteroheptamer assembly during translation

rRBCM (20 μ L of 1.0 mg/mL) were centrifuged and the supernatant removed. The components of an *in vitro* translation mix (total volume 3.75 μ L, amino acids last) were added. After 1 h at 37°C, the membranes were recovered by centrifugation and washed once with MBSA. The pellet was resuspended in 2.0 M TAPS, pH 8.5 (10 μ L), and 10 mM DTT (2 μ L) and treated with IASD as described above.

Channel properties in planar lipid bilayers

Solvent-free planar lipid bilayer membranes were formed by applying diphytanoylphosphatidyl choline (Avanti Polar Lipids, Birmingham, Alabama), dissolved in highly purified pentane (Burdick & Jackson, Allied Signal Inc., Muskegon, Michigan) to a 100–150 μ m diameter orifice in a 25 μ m thick Teflon film (Goodfellow Corporation, Malvern, Pennsylvania) separating the *cis* and *trans* compartments of the apparatus (Montal & Mueller, 1972). The compartments contained 1 M NaCl, 10 mM MOPS (Fluka Chemical Co., Milwaukee, Wisconsin), pH 7.4, or for ion selectivity measurements, various concentrations of KCl with 10 mM MOPS, pH 7.4. The eluted heptameric protein (2–20 μ L) was added to the *cis* chamber to a final concentration of

0.08–0.4 ng/mL. A potential difference was applied across the bilayer with Ag/AgCl electrodes in 1.5% agarose (BioRad) containing 3 M KCl. The *cis* chamber was at ground, a positive potential indicates a higher potential in the *trans* chamber and a positive current is one in which cations flowed from the *trans* to the *cis* chamber. The current was recorded by using a patch clamp amplifier: either a Dagan 3900A with a 3910 expander (Dagan Co., Minneapolis, Minnesota) or an Axopatch 200B (Axon Instruments, Foster City, California). The signals were stored on a DAS-75 data recorder (Dagan Co., Minneapolis, Minnesota). After filtering with a Bessel filter (Model 902, Frequency Devices, Haverhill, Massachusetts) at frequencies specified in the text, the signals were acquired by a personal computer by using a Digidata 1200A A/D board and analyzed with Axoscope software (Axon Instruments). The data are presented using Origin 4.1 (Microcal, Northampton, Massachusetts).

The ion selectivity ($p_{\text{Cl}^-}/p_{\text{K}^+}$) was determined by using the Goldman-Hodgkin-Katz (GHK) equation:

$$V_{\text{rev}} = RT/F \ln \frac{(p_{\text{Cl}^-}/p_{\text{K}^+})[\text{Cl}^-]_{\text{trans}} + [\text{K}^+]_{\text{cis}}}{(p_{\text{Cl}^-}/p_{\text{K}^+})[\text{Cl}^-]_{\text{cis}} + [\text{K}^+]_{\text{trans}}} \quad (1)$$

The reversal potential (V_{rev}) was measured with asymmetrical KCl solutions. Each experiment was started with symmetrical solutions (100 mM KCl in 10 mM MOPS, pH 7.4 both *cis* and *trans*), which allowed any electrode DC offset to be balanced after the addition of protein to the *cis* chamber. After multiple channel insertions, concentrated KCl solution was added to the *cis* chamber to establish the desired asymmetrical condition. The final concentration (157–400 mM) was calculated at the end of each experiment, after measuring the exact volume of solution in the chamber. The applied voltage that gave zero current was noted. In addition, I-V curves were determined from which V_{rev} could be more accurately deduced. At the end of each experiment, symmetrical conditions were re-established by adding concentrated KCl solution to the opposite chamber to determine any DC offset that might have built up during the experiment. These values were usually ≤ 1 mV. Experiments with shifts of more than 2 mV were rejected.

Molecular modeling

The coordinates for the wild-type α HL pore were obtained from the Protein Data Bank (PDB) (7ahl.pdb) (Song et al., 1996). An abbreviated version of the coordinates containing just the barrel (residues 110–148) was made by deleting residues 1–109 and 149–293. The water molecules and hydrogen atoms were also removed to simplify import into Insight II 97.0 (Molecular Simulations, Inc.). The hydrogen atoms were restored in Insight II. The retro TM domain (residues 117–142) was generated by using the replace function in the biopolymer module of Insight II. The modified coordinates were minimized by using the autorotamer function followed by a steepest descent minimization of 150 iterations and a conjugate gradient minimization of 50 iterations, all using an AMBER forcefield. Minimization of the retro barrels produced structures that were similar to WT with respect to the total energy and the appearance of the polypeptide backbone (data not shown). The modified coordinates were exported as PDB files. Molecular graphics were produced using SPOCK (Christopher, 1998) and Adobe Photoshop.

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