Spontaneous oligomerization of a staphylococcal α-hemolysin conformationally constrained by removal of residues that form the transmembrane β-barrel

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Introduction

The exotoxin α-hemolysin (αHL, α-toxin) is secreted by Staphylococcus aureus as a water soluble, monomeric, bacterial exotoxin, which forms heptameric pores in membranes. The rate determining step in assembly is the conversion of a heptameric prepore to the fully assembled pore in which the central glycine-rich domain of each subunit inserts into the membrane to form a 14 strand β barrel. Barrel formation is accompanied by a conformational change in which each N terminus latches onto an adjacent subunit. In the monomer in solution, the central domain is loosely organized and exposed to solvent. In this study, 25 amino acids of the central domain were removed and replaced with the sequence Asp–Gly, which favors the formation of a type I β-turn, to yield a mutant devoid of hemolytic activity. Within minutes after synthesis in the absence of membranes, the mutant polypeptide spontaneously assembled into heptamers, as demonstrated by atomic force microscopy. Limited proteolysis suggested that the N termini of the subunits in the heptamers were in the fully assembled pore conformation rather than the prepore conformation. Based on these findings, the deletion is proposed to constrain the central domain and thereby force the creation of a shortened β barrel, which in turn induces the additional structural changes that normally accompany pore formation. The truncated pore might make a useful framework for the construction of designed membrane active macromolecules.

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of detailed structural information describing the heptamer and the ability of the polypeptide to self assemble in the absence of cellular machinery, αHL is a prototype with which to study the assembly of a transmembrane protein (Bhakdi et al., 1996).

Four stages in assembly have been defined in a working model that is supported by biochemical, biophysical and molecular genetic evidence (Figure 1) (Walker et al., 1992a; Walker et al., 1995). The first stage is monomeric αHL in solution (Figure 1, 1), which is characterized by a marked sensitivity to proteolysis. Two major cleavage sites have been mapped (Tobkes et al., 1985; Walker et al., 1992a): site 1, in the glycine-rich central domain, which after assembly forms the transmembrane domain, and site 2, near the N terminus. In 1, the central region is solvent exposed and, most likely, spends a substantial fraction of time in a largely disordered state. For example, cysteines placed in the central domain are rapidly modified by hydrophobic sulfhydryl-directed reagents (Krishnasasty et al., 1994), fluorescent probes attached there are located in a polar environment (Valeva et al., 1996) and proteolysis at ‘site 1’ occurs at several positions (Tobkes et al., 1985; Walker et al., 1992a; Thelestam and Blomqvist, 1988). In stage two, the membrane-bound monomer (Figure 1, 2), which is relatively poorly characterized, the central region but not the N-terminus has become resistant to proteolysis (Walker et al., 1992a). In stage three, seven membrane-bound subunits associate to form a non-lytic intermediate or ‘prepore’ (Figure 1, 3). The prepore 3 is sensitive to SDS (Valeva et al., 1996; and unpublished data), but the seven subunits are tightly associated on membranes and do not scramble (unpublished data) when 3 is solubilized in a variety of non-denaturing detergents (Valeva et al., 1996). Although the central domain in 2 is insensitive to proteases, it has not completed its move into the hydrophobic environment of the bilayer as monitored by covalently-attached fluorescent probes (Valeva et al., 1996, 1997b). At this point, therefore, the central domain has either acquired a secondary structure that prevents proteolysis or has become inaccessible to macromolecules in the aqueous phase. By contrast, the N-terminus in 3 remains accessible to proteolysis (Walker et al., 1992a, 1995). Imaging by atomic force microscopy (AFM) indicates that the prepore is oriented with its sevenfold axis perpendicular to the membrane surface (Figure 1) (Fang et al., 1997). In the final stage four (Figure 1, 4), the glycine-rich central regions of the seven subunits penetrate the lipid bilayer (Valeva et al., 1996) to form the lytic, SDS resistant, heptameric pore. Fluorescence energy transfer experiments showed that in 4 residue 130 is trans-located to the trans (intracelluar) side of the bilayer (Ward et al., 1994). Further, upon insertion, the central domain forms part of the channel wall. For example, when five consecutive central amino acids (residues 130–134) were replaced with histidines, current through the channel was blocked with divalent zinc ions added to either the cis or trans side of planar lipid bilayers (Walker et al., 1994). Upon insertion of the central domain to form the transmembrane channel, the
N terminus becomes protease resistant. Crystallographic studies show that each N terminus is buried in the top surface of the heptameric pore where it both latches onto a neighboring subunit and lines the cis (extracellular) entrance to the channel (Song et al., 1996). Despite the fact that they rearrange together, the amino latches (residues 1–20) are distant from the central stem domains (residues 110–148), both in sequence and in space. In the crystal structure, the N terminus lies approximately 80 Å from the center of the membrane-inserted sequence of the same subunit (Song et al., 1996). The movement of the N terminus, when 4 is formed, can also be detected by the appearance of excimer fluorescence from covalently-attached pyrene molecules (Valeva et al., 1997a). Hence, the conformational change at the N terminus and membrane penetration by the central domain appear to be associated events, which may be linked mechanistically by pivotal intervening structural features such as the triangle region, which connects the barrel to the cap of the heptamer, and the crucial residue His35, which contacts the top of the triangle (Song et al., 1996).

Here we describe a truncated channel mutant (αHL-TCM) with an engineered conformational constraint produced by deleting 23 residues of the central domain. Newly synthesized αHL-TCM heptamersize rapidly in the absence of membranes. The N terminus is protease resistant, indicating that the N terminal conformational change accompanies oligomerization and that a water-soluble ‘pore’, resembling 4, has been formed. Therefore, the constraint introduced by the internal deletion must force the central domain into a truncated version of the β barrel found in the fully assembled wild-type pore. Barrel formation in turn drives the N terminal conformational change that is normally associated with membrane insertion of the central domain.

Materials and methods

pT7-SMC vector

The αHL gene was removed with NdeI and HindIII from pT7SF1A, the pT7 vector developed previously for work with αHL (Walker et al., 1992b), and replaced with a 125 bp NdeI–HindIII stuffer fragment. The resulting plasmid was digested with ClaI, filled in with Klenow fragment and religated yielding pT7-SMC.

Reconstruction of the αHL gene

For mutagenesis of the glycine-rich central domain of αHL, the gene was partly reconstructed with synthetic oligonucleotides to introduce eight unique restriction sites (HpaI, BsiWI, BsrEI, SpeI, StuI, ApaI, AvrII and AflII) spanning codons 116–147. The reconstructed gene (αHL-RL) was generated by PCR of 5’ and 3’ segments of the αHL-K8A gene (Walker and Bayley, 1994) with the following primers: 5’-CGGATCCTAATACGACTCACTATAGGG (5’ half; sense), 5’-CGCTAAGGTAACTCGTTGTAACGTTAAGCTTTCGCA (5’ half; antisense), 3’-GGTCTACGTTAAGCTTTAGGA (3’ half; sense), and 5’-AACATCATTTCGACCTCTT (3’ half; antisense). K8A proteins are resistant to adventitious lysine-directed proteases at the N terminus, but remain sensitive to proteinase K (Walker and Bayley, 1994). The PCR product representing the 5’ segment of the reconstructed gene was digested with NdeI and BsrEI and the 3’ segment with BsrEI and HindIII. Three-way ligation of the digested PCR products with pT7-SMC, digested with NdeI and HindIII, yielded a plasmid containing an αHL gene with a deletion in the central region. This construct was then digested with BsrEI and AflII and ligated with a synthetic DNA duplex prepared from phosphorylated 5’-GTAACTTACCTGGTATGATCAGTATAG TAA AATTGAGGCTTATTGAGGCCCATGGTCCCTATAGGTCACTACAC (sense) and phosphorylated 5’-TTAGTGTACCTAGGAGAAACCTGGCCCAATAAGGCTTC CCAATTTTACTGATATCCACAGTAAG (antisense) to yield the reconstructed αHL gene. DNA sequencing of the entire gene revealed two deletions and one substitution in the region represented by the synthetic insert. These errors were repaired by replacing the DNA between the BsrEI and StuI sites with a synthetic duplex generated from phosphorylated 5’-GTAACCTTACTGGTATGATCAGTTAA AATTGAGGCTTATTGAGGCCCATGGTCCCTATAGGTCACTACACAGTAAG (sense) and phosphorylated 5’-CCTCCTAATTTTACTGATATCCACAGTAAG (antisense) yielding the corrected reconstructed αHL gene in the T7 vector (pT7αHL-RL). The corrections were verified by DNA sequencing of the entire reconstructed gene. Of the eight new restriction sites present in αHL-RL, four (HpaI, BsiWI, StuI and AflII) were silent and four (BsrEI, SpeI, ApaI and AvrII) resulted in conservative amino acid replacements: V124L, G130S, N139Q and I142L.

Construction of an αHL gene with a deletion in the region that encodes the glycine-rich central domain

DNA encoding central residues 116–147 was removed from pT7αHL-RL with HpaI and AflII and replaced with a synthetic duplex prepared from phosphorylated 5’-AGATGGTATTGGTCATACAC (sense) and phosphorylated 5’-TTAGTGTATGAGCAATCATCATGCTCCAG (antisense) to yield pT7-TCM. Because central domain residues in αHL-RL that were altered by conservative replacement are either absent in TCM (residues 124, 130 and 139) or changed back to the wild-type residue (Ile–142), the αHL gene is identical to K8A except for the deleted residues (117–141), which were replaced by Asp–Gly in TCM. The TCM gene was sequenced in it’s entirety. No sequence differences from K8A were observed, other than those intended in the deleted region.

Construction of αHL genes with 3’ extensions encoding a poly-histidine tag

Attempts to purify recombinant TCM from E.coli cell lysates under non-denaturing conditions were unsuccessful due to extensive protein aggregation. Accordingly, a procedure was
devised that could be carried out under denaturing conditions by employing metal chelate affinity chromatography (Hochuli et al., 1988) followed by renaturation (see below). For this purpose, the TCM gene was modified at the 3’ end with an extension encoding a Gly/Ser-rich linker sequence followed by a stretch of six histidines. First, an extended wild type (WT) gene was synthesized by PCR using pT7-NPH8S (Walker et al., 1992a; Walker and Bayley, 1994) linearized with EcoRI as the template with the following primers: 5'-CGGATCTCT-1144ATTACGACTCATATAGGG (sense), 5'-GCGCAAGCTTCAATTACCATCAGGCCACAGATGATCCGGACACCAGTATTGTCATTCTTTTTCTCCTTTTTCC (antisense). The PCR product was directly inserted into the pCR vector (TA cloning kit; Invitrogen no. K2000-01), and potential recombinants were selected by DNA sequencing through the extended region. After sequence verification, the vector containing the extended αHL gene was digested with NdeI and HindIII and the resulting fragment was ligated into pT7-SMC, which had been cut with the same enzymes, to yield pT7-WT(3'-EXT). DNA sequencing was performed on the entire extended WT gene. No sequence changes were observed other than those intended for the C-terminal extension, which encodes:

$$\text{XhoI}$$

Arg Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser

$$\text{HindIII}$$

GCT GGT TCG GGC TCA TCT GGT GGC TCG AGT GGA TAA TGA AGC TT

Prior to the addition of codons encoding a hexahistidine tract, hemolysis assays were performed on the extended αHL polypeptide after in vitro synthesis (see below). The extended αHL retained full activity. The plasmid pT7-WT(3’-EXT) was then digested with XhoI and HindIII and ligated with duplex DNA prepared from phosphorylated 5’-TCTGATCACCACCATCACCACATTAATAGGA and phosphorylated 5’-AGCTT-CATTAATGATGTTGATGGTTGAC to yield pT7-WT-H6. The tagged construct was verified by DNA sequencing through the altered region. The entire new tail consisted of the sequence:

$$\text{Arg Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser Gly * *}$$

$$\text{CGT GGT TCG GGC TCA TCT GGT GGC TCG AGT GGA TAA TGA AGC TT}$$

To generate a TCM gene with an identical C-terminal extension encoding a Gly/Ser-rich linker sequence followed by an αHL tract, hemolysis assays were performed on the extended αHL gene was digested with NdeI and HindIII and the resulting fragment was ligated into pT7-WT(3’-EXT). DNA sequencing was performed on the entire extended WT gene. No sequence changes were observed other than those intended for the C-terminal extension, which encodes:

$$\text{XhoI}$$

Arg Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser Gly * *

$$\text{HindIII}$$

GCT GGT TCG GGC TCA TCT GGT GGC TCG AGT GGA TAA TGA AGC TT

In vitro transcription and translation

Mutant αHL polypeptides were synthesized in vitro by coupled transcription and translation (IVTT) in an E.coli S30 extract (Promega, T7 S30 no. L114A) supplemented with rifampicin (20 μg/ml) as previously described (Walker et al., 1992b). Radiolabeled polypeptides were synthesized using amino acid pre-mixes minus methionine or cysteine in the presence of [35S]methionine (Dupont-New England Nuclear, 1200 Ci/ mmol), or [35S]cysteine (Amershams, 1300 Ci/mmol). For hemolysis assays, IVTT was carried out with a complete amino acid medium.

Protein expression and purification

WT-αHL monomer from Staphylococcus aureus Wood strain 46 (American Type Culture Collection) was purified by a variation based on procedures previously described (Lind et al., 1987; Walker et al., 1992b; Palmer et al., 1993). All operations were conducted at 4°C. Briefly, monomer was concentrated from culture supernatants by precipitation with ammonium sulfate (500 g added per L of culture supernatant). After centrifugation at 10 000 g for 30 min, pellets were dissolved in 10 mM sodium acetate (pH 5.2) containing 500 μM phenylmethanesulfonyl fluoride (PMSF) and dialyzed overnight against 40 volumes of the same buffer without PMSF. Precipitated protein was removed by centrifugation at 12 000 g for 30 min. The supernatant was loaded onto S-Sepharose FF cation exchanger (Sigma no. S-1264, bed volume, 1% of culture volume) and eluted with a linear gradient of 0 to 400 mM NaCl in 10 mM sodium acetate (pH 5.2). Fractions (10 ml) were collected and analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed against 10 mM Tris–HCl (pH 8.0) containing 150 mM NaCl.

WT heptamer formation was carried out at room temperature by treatment of purified monomer with deoxycholate (DOC) as detailed elsewhere (Bhakdi et al., 1981; Tobkes et al., 1985; Walker et al., 1992b). A solution of 100 mM DOC in 100 mM Tris–HCl (pH 8.3) was added dropwise, with stirring, in five equal portions at 10 min intervals, to a final concentration of 6.25 mM DOC. After concentration by ultrafiltration with a Diaflo YM-10 membrane filter (62 nm, Amicon) to ~10 mg/ml, protein solutions were stored in 10 ml aliquots at ~20°C. Gel filtration was then carried out to separate heptamer from remaining monomer. The concentrate (10 ml) was loaded onto a 2.5×100 cm column containing Sephacryl S-300 HR gel filtration medium (Pharmacia no. 17-0599-01) that had been equilibrated with chromatography buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.25 mM DOC). Fractions (10 ml) were collected and analyzed by SDS-PAGE. Peak fractions were quantitated by measuring absorbance at 280 nm (OD_{280} of 1.0 mg/ml solution = 1.95) and pooled. The purity was generally >95%. Yields were ~5 mg purified heptamer per liter of culture.

For expression of recombinant TCM-H6 and WT-H6, a T7 promoter-based expression system was used. Escherichia coli JM109(DE3) (Promega), freshly transformed with pT7-TCM-H6 or pT7-WT-H6, was used to inoculate 10 ml of Luria-Bertani medium containing 100 μg/ml ampicillin (LB/amp). After 6 h of growth at 37°C, the starter culture was added to a shaker flask containing 500 ml LB/amp and grown overnight at 28°C to an OD_{600} of 2.0. Cells were harvested by centrifugation (5000 g for 20 min at 4°C) and the pellet resuspended in 5 ml of ice cold 50 mM Tris–HCl (pH 8.0) which was then passed through a French press (pre-chilled on ice) at 8000 p.s.i. One half of the cell lysate was made 6 M in guanidine hydrochloride by addition of the solid salt and then centrifuged at 200 000 g for 30 min. The supernatant was loaded onto a metal chelate affinity column (0.5 ml, NiII) NTA Agarose, Qiagen no. 30210) equilibrated in loading buffer: 100 mM Na phosphate, 8 M urea, 200 mM NaCl, 20 mM imidazole, 10 mM Tris–HCl (pH 8.0). The column was washed with 10 bed volumes of the same buffer and the bound protein was then released with four bed volumes of elution buffer: 100 mM Na phosphate, 6 M urea, 200 mM NaCl, 250 mM imidazole, 10 mM Tris–HCl (pH 8.0). The eluate was diluted twofold with water and dialyzed at 4°C against 10 l of 10 mM sodium acetate (pH 5.2). Precipitated protein was removed by centrifugation at 16 000 g and the supernatant dialyzed at 4°C against 10 l of 10 mM Tris–HCl, pH 8.0. Precipitated protein was again removed by centrifugation. Radiolabeled TCM polypeptide was partially purified before...
gel filtration experiments. A solution of 1% (v/v) polyethyleneimine (PEI) in water, pH 8.0 (Gegenheimer, 1990), was added slowly with mixing to 35S-labeled TCM in IVTT mix (100 µl) until a concentration of 0.2% PEI (v/v) was reached. After 10 min on ice, the PEI-treated extract was centrifuged at 16 000 g for 10 min at 4°C. The supernatant was collected and gently mixed with a 50% (v/v) slurry of SP Sephadex C50 cation exchanger (Sigma, no. SP-C50-120) (50 µl) that had been equilibrated with 10 mM Tris–HCl (pH 8.0). The mixture was applied to a 0.2 µm cellulose acetate microfilter-fuge tube (2 ml capacity, Rainin no. 7016-024), which was centrifuged for 2 min at 16 000 g. The filtrate was diluted 10-fold in 10 mM sodium acetate (pH 5.2) and mixed with a 50% (v/v) slurry of S-Sepharose FF cation exchanger (Sigma, no. S-1264) (60 µl) that had been equilibrated with the same buffer. After gentle rotation for 1 h at 4°C, the mixture was applied to another microfilterfuge tube and centrifuged as before to remove unbound protein. The spin filter containing the resin was inserted into a new microfilterfuge tube. Bound TCM was released by mixing the resin with four bed volumes (120 µl) of elution buffer (100 mM Tris–HCl, 200 mM NaCl, pH 8.0) and collected by centrifugation. The recovery with this procedure is usually ~50%.

**Hemolysis assays**

To assay hemolysis in microtiter wells, IVTT mixes were centrifuged at 16 000 g for 10 min. A portion of the supernatant (10 µl) was immediately diluted 10-fold in MBSA: 10 mM 3-[N-morpholino]propane sulfonic acid (MOPS), 150 mM NaCl, pH 7.4, containing 1 mg/ml bovine serum albumin. After twofold serial dilution of the diluted mix in MBSA, which left 50 µl in each well, an equal volume of washed rabbit red blood cells (rRBC, 1% in MBSA) was added to each well. Lysis was recorded for 1 h at 20°C by monitoring the decrease in light scattered at 595 nm with a Bio-Rad microplate reader (Model 3550-UV).

**Gel electrophoresis in the presence of SDS**

Samples were dissolved in gel loading buffer containing SDS (Laemmli, 1970) and either heated at 95°C for 5 min, or not heated, before electrophoresis in 12% SDS–polyacrylamide gels (Laemmli, 1970). Radiolabeled markers were [14C]methylated proteins (Gibco BRL): myosin heavy chain (Mr 290 000), β 2-Microglobulin (Mr 18 400) and lysozyme (Mr 14 300).

**Gel electrophoresis in the presence of deoxycholate**

Polyacrylamide gel electrophoresis in deoxycholate (DOC–PAGE) was carried out with a variation of the Laemmli procedure for SDS (Laemmli, 1970). Separating gels of 7.5% (w/v) polyacrylamide were prepared by mixing a stock solution (10 ml) consisting of 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide (Protogel, National Diagnostics, no. EC-890) with 4X gel buffer (10 ml; 1.5 M Tris–HCl, 5.0 mM DOC, pH 8.8) and water (19.5 ml). Polymerization was initiated by the addition of ammonium persulfate [0.475 ml of a 10% (w/v) solution] and N,N,N',N'-tetramethylethylenediamine (0.025 ml). A stacking gel approximately 2.5 cm in height but with a final acrylamide concentration of 4.5% (w/v) was prepared with the same stock solutions, including the pH 8.8 buffer. Samples were mixed with 2X gel loading buffer containing DOC (50 mM Tris–HCl, 25 mM DOC, 8% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% bromophenol blue, pH 8.0) and electrophoresed with a running buffer identical to that used for SDS–PAGE (Laemmli, 1970), except for the replacement of SDS with 1.25 mM DOC. Gels were run at 200 V (constant voltage) until the tracking dye reached the bottom of the gel.

**Oligomer formation on rabbit red blood cell membranes**

Rabbit red blood cell membranes (rRBCM) were prepared by hypotonic lysis of washed rabbit erythrocytes and suspended in MBSA at a concentration of ~5 mg protein/ml. To induce oligomer formation, IVTT mix containing a 35S-labeled αHL polypeptide (5 µl) was mixed with 35 µl MBSA and 10 µl rRBCM. After 1 h at 20°C, the membranes were recovered by centrifugation at 16 000 g for 5 min and washed twice at room temperature with MBSA (500 µl).

**Limited proteolysis of mutant αHL polypeptides in solution and on membranes with proteinase K**

Proteinase K (Sigma, no. P-0390) solutions (5.0, 0.5 and 0.05 mg/ml in water) were prepared by dilution of thawed enzyme stocks (10 mg/ml in water) and used immediately. For limited proteolysis in solution, 35S-labeled polypeptide from an IVTT mix (1 µl) was diluted 40-fold with MBSA and divided into four tubes (9 µl per tube) to which were added diluted enzyme or water (1 µl). Limited proteolysis was also performed on membrane-bound αHL polypeptides (see above) that had been resuspended in MBSA (40 µl) and divided into four tubes (9 µl per tube). After 5 min at room temperature, the reactions were stopped by treatment with PMSF (2 mM final) for 5 min at room temperature, followed by the addition of 2X gel loading buffer. The samples (either heated at 95°C for 5 min, or not heated) were subjected to electrophoresis in 12% SDS–polyacrylamide gels.

**Conformational changes immediately after protein synthesis as determined by proteinase K digestion**

Protein synthesis by IVTT was initiated in a total volume of 125 µl at 37°C with prewarmed reagents. After 2, 4, 8 and 16 min, a portion (5 µl) was removed and added to a digestion mix at 25°C [20 µl: MBSA (812.5 µl), proteinase K (10 mg/ml in water, 62.5 µl), chloramphenicol (1 mM in water containing 1% methanol, 125 µl)]. The final concentration of proteinase K was 0.5 mg/ml. After 5 min, PMSF (2 µl, 200 mM in isopropanol) was added, followed after a further 5 min by 2X Laemmli loading buffer (27 µl). Samples were heated for 5 min at 95°C, before electrophoresis in a 12% SDS–polyacrylamide gel. In a separate experiment (‘– protease’), the proteinase K solution was replaced with water.

In a second experiment, designed to examine conformational changes at 0°C, protein synthesis by IVTT was initiated in a total volume of 33.3 µl at 37°C with prewarmed reagents. After 3 min, synthesis was blocked with 100 µM chloramphenicol (1 mM, 3.7 µl) and the mix was immediately placed on ice. After 0.25, 7.5, 15, 30, 60 and 120 min, a portion of the mix (4 µl) was added to proteinase K (0.56 mg/ml, 36 µl) that had been prewarmed to 25°C. After 5 min, PMSF (2 µl, 200 mM in isopropanol) was added, followed after a further 5 min by 5X Laemmli loading buffer (10.5 µl). The sample was heated for 5 min at 95°C, before electrophoresis in a 12% SDS–polyacrylamide gel.

**Gel filtration**

For chromatographic analysis, partially purified, 35S-labeled TCM was mixed with purified wild type αHL heptamer (30 µl of 4 mg/ml) and blue dextran (20 µl of 2.5 mg/ml, Sigma no.
**Results**

**TCM is inactive towards rRBC**

In TCM and TCM-H6, 25 amino acids have been deleted from the central glycine-rich domain, which forms the transmembrane channel of the native heptamer (Valeva et al., 1996; Song et al., 1996), and replaced with two amino acids preferred by type I β turns (Asp–Gly) (Hutchinson and Thornton, 1994; Quinn et al., 1994) (Figure 2). The four αHL polypeptides, K8A, TCM, WT-H6 and TCM-H6, were assayed for hemolytic activity against rRBC after in vitro synthesis in E. coli S30 extracts (IVTT) (Figure 3a). TCM and TCM-H6 were inactive. When purified from E. coli, TCM-H6 also lacked hemolytic activity (Figure 3b). By contrast, WT-H6, made by IVTT or purified from E. coli, was fully active when compared with its untagged counterpart, made by IVTT (Figure 3a) or purified from S. aureus supernatants (Figure 3b). The hemolysis data are consistent with the idea that the loss of lytic activity in TCM and TCM-H6 is due to the absence of essential pore-forming residues present in the central glycine-rich domain of αHL. In addition, the data demonstrate that the C-terminal hexahistidine tag and the spacer that precedes it have little effect on hemolytic activity.

**Conformational state of TCM as revealed by limited proteolysis**

By using proteinase K as a probe for protein conformation, earlier studies have shown that structural changes at the N-terminus accompany membrane insertion of the central domain (Walker et al., 1992a, 1995), despite the fact these sequences are located approximately 80 Å apart in the fully assembled pore (Song et al., 1996). For example, deletion of just two amino acids at the N terminus leads to the accumulation of a non-lytic, oligomeric ‘prepore’ (Figure 1, 3) on rRBC (Walker et al., 1992a). In another approach, the assembly of an αHL mutant (αHL-H5) containing a divalent metal ion-binding site of five consecutive histidines in the central domain (residues 130–134) was arrested in the inactive prepore state by low concentrations of Zn(II) (Walker et al., 1995). In this state, the N-terminus remained accessible to proteinase K.

The susceptibility of TCM or TCM-H6 to cleavage by proteinase K was compared with K8A or WT-H6 (Figure 4A). The proteolytic patterns for K8A and WT-H6 from [35 S]-labeled IVTT mixes revealed similar, pronounced susceptibilities to proteinase K at the two major cleavage sites near the N-terminus and in the central domain, consistent with the expected pattern for monomeric αHL in solution (Figure 1, 3) (Walker et al., 1992a). By contrast, TCM was highly resistant to proteinase K, even at the highest final concentration of 500 µg/ml. The major proteinase K cleavage sites in the glycine-rich region of αHL are before Val140 and Ile136 (Walker et al., 1992a) and are therefore absent in TCM. However, the major N-terminal sites, which are after Ile7 and
Ile14 in αHL (Walker et al., 1992a, 1995), are present but apparently occulted in TCM. TCM-H6 was cleaved by proteinase K, which most probably removed the C-terminal tag (Figure 4a). Nevertheless, this result raised the possibility that cleavage was occurring at the N-terminus of untagged TCM but produced no shift in electrophoretic mobility. Therefore, αHL−S3C and αHL−S3C/TCM were generated by IVTT in the presence of [35S]cysteine to produce polypeptides specifically radioabeled at position 3. This experiment confirmed that the N terminus of the WT protein [S3C is active (Walker and Bayley, 1995b) is cleaved, while TCM is not attacked under identical conditions (Figure 4b).

K8A, H35N and TCM were also treated with proteinase K after they had bound to rαHL. H35N is an inactive mutant that becomes arrested as the prepore (Walker and Bayley, 1995a) (Figure 1, 3). Under these conditions, K8A and TCM were protease resistant, while H35N was cleaved at the N-terminus, as expected (Figure 4c). This experiment shows that TCM can bind to membranes, where it retains the protease resistance characteristic of the fully assembled pore.

Finally, the protease sensitivity of WT-H6 and TCM-H6 expressed in E.coli were examined. WT-H6 showed the pattern expected of monomer in solution, while TCM-H6 was cleaved, most probably at the C terminus (Figure 4d), as seen with TCM-H6 made by IVTT.

Protease resistant TCM is formed within minutes of synthesis, even at 0°C

When samples were taken during the translation of TCM at 37°C and digested with proteinase K at 25°C, the protease-resistant form was seen within 4 min (Figure 4e). Interestingly, after just 2 min a significant amount of TCM had been synthesized, but it was completely protease sensitive, suggesting that an unfolded or partly folded form of the protein was present, which is in keeping with the idea that in prokaryotes folding follows polypeptide chain completion (Netzer and Hartl, 1997). TCM was also generated by IVTT for 3 min and afterwards the concentration of the protease-resistant state was monitored by SDS–PAGE and autoradiography. At 0°C (Figure 4f), the resistant form appeared with a $t_{1/2}$ of ~30 min. At 37°C (not shown), the $t_{1/2}$ was ~15 min. We have not performed a full analysis of the rate of folding and subsequent conformational changes. However, it is clear that even at low TCM concentrations (short translation time, low methionine concentration), the protease-resistant form appears quickly, even at 0°C.

TCM oligomerizes in the absence of membranes

The resistance of TCM to cleavage at site 2 by proteinase K suggested that the polypeptide had spontaneously oligomerized into a pore-like conformation (Figure 1, 4) soon after protein synthesis. Wild-type pores are resistant to SDS at <65°C, but oligomers of TCM were not detected in SDS gels, suggesting that if TCM oligomers did exist they were unstable in this detergent. Therefore, the oligomeric state of TCM in the absence of SDS was investigated.

A less denaturing gel system was devised, in which DOC was substituted for SDS and used to analyze [35S]-labeled αHL polypeptides made by IVTT. Both K8A and H35N appeared as ‘streaks’ running downward from near the top of the gel while TCM appeared as a discrete band near the top (Figure 5a). After heating, TCM also appeared as a streak on the gel. DOC can induce oligomerization of WT-αHL (Bhakdi et al., 1981) but this requires a much higher concentration of αHL than that present in an IVTT mix, which is in the range of 10–50 μg/ml (Walker et al., 1992a). Thus the slow, discrete migration of TCM in DOC gels, together with its resistance to proteolysis at site 2, strongly favors the idea that TCM spontaneously assembles soon after synthesis in the absence of membranes or lipids.

Because estimates of molecular weight cannot be obtained by electrophoresis in DOC gels, the subunit stoichiometry of the TCM oligomer was examined by gel filtration. Prior to chromatography, TCM was partially purified from an IVTT mix (see Materials and methods) so that the absorbance of molecular weight markers could be followed without interference from S30 proteins. [35S]Methionine-labeled TCM was mixed with unlabeled, purified WT-αHL heptamer and blue dextran ($M_r$ 2 000 000) and subjected to gel filtration on Sephacryl S300 (Figure 5B). The [35S]-labeled TCM as detected by SDS–PAGE emerged just before the WT heptamer. Based on the elution volume, TCM would be predicted to contain 10.3 ± 1.8 αHL subunits.

**TCM drives the formation of SDS-stable heteromers containing WT-αHL in the absence of membranes**

While the TCM homoligomer is unstable in SDS, we sought to determine whether co-oligomerization with WT protein might lead to the formation of SDS-stable heteromers. Purified WT-αHL monomers, present during the synthesis of TCM, were indeed incorporated into SDS-stable heteromers that had a similar electrophoretic mobility to homooligomeric K8A produced by oligomerization on rαHL (Figure 6a). The material at the top of the gel lanes containing radiolabeled unheated TCM is likely to be aggregated TCM formed when the oligomer breaks down in SDS. A similar effect is seen when WT-αHL heptamers are heated in SDS to just above the dissociation temperature, but no higher. By contrast with TCM, no SDS-stable heteromers were detected when K8A was synthesized in the presence of WT (Figure 6a). Heteromers were also obtained when WT and TCM were cotranslated. In this case, TCM was selectively labeled by translating TCM/T292C in the presence of [35S]cysteine (Figure 6b). SDS-stable heteromers were detected with TCM: WT template ratios.

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**Fig. 4.** Conformational state of TCM tested by limited proteolysis. (a) Freshly translated K8A, TCM, WT-H6 and TCM-H6 were treated with proteinase K in solution and subjected to SDS–PAGE and autoradiography. Final concentrations of proteinase K were as follows. Lanes 1, 5, 9 and 13, 0 μg/ml; lanes 2, 6, 10 and 14, 5 μg/ml; lanes 3, 7, 11 and 15, 50 μg/ml; and lanes 4, 8, 12 and 16, 500 μg/ml. (b) Freshly translated S3C and S3C/TCM labeled with either [35S]methionine or [35S]cysteine were treated with proteinase K as in (a). The labeled cysteine at position 3 acts as a marker for the N-terminus. (c) Freshly translated K8A, H35N and TCM were allowed to bind to membranes. The washed membranes were then treated with proteinase K. The samples were heated before electrophoresis to dissociate heptamers. (d) WT-H6 and TCM-H6 were expressed in E.coli and purified as described above with treatment with proteinase K in solution. This gel was stained with Coomasie blue. The additional band that appears in lanes 4 and 8 is proteinase K. (e) K8A and TCM were synthesized by IVTT at 37°C. At the times indicated, synthesis was blocked with chloramphenicol and the sample was digested with proteinase K at 25°C (500 μg/ml) or not treated. (f) TCM was generated by IVTT for 3 min at 37°C. The mix was then cooled quickly to 0°C and the appearance of the protease-resistant form of TCM was followed by rapid proteolysis at the times indicated (proteinase K, 500 μg/ml, 25°C).
Fig. 5. Oligomerization state of TCM. (a) DOC gel electrophoresis. TCM produced by IVTT runs as a discrete band in a DOC–polyacrylamide gel. Lane 1, K8A; lane 2, H35N; lane 3, TCM; lane 4, K8A; lane 5, H35N; lane 6, TCM. The samples in lanes 1–3 were not heated before electrophoresis, while those in lanes 4–6 were heated at 95°C for 5 min. (b) Gel filtration of TCM made by IVTT on Sephacryl S300 HR in 100 mM Tris–HCl, 200 mM NaCl, pH 8.2, containing 25 mM deoxycholic acid. Partially purified, 35S-labeled, TCM was mixed with purified WT-αHL heptamer and blue dextran (M₉ 2 000 000). Fractions were analyzed by their absorbance and by SDS–PAGE. Insert (Top) WT-αHL heptamer (Coomassie blue); (bottom) TCM (autoradiogram).

of 1:1 or lower. By contrast, a control co-IVTT experiment with plasmids encoding T292C and WT polypeptides did not produce SDS-stable heteromers at any DNA ratio tested (Figure 6b). In addition, SDS-stable heteromers were also produced following co-IVTT of TCM with WT, K8A or T292C (data not shown). The formation of SDS-stable heteromers with WT-αHL argues against a possible alternative interpretation of the limited proteolysis data, in which the TCM oligomer is viewed as a protease-insensitive malfolded aggregate, as does the accessibility of TCM-H6 to proteinase K (Figure 4a) and the failure of significant TCM (<10%) to appear in the void volume of a gel filtration column (Figure 5b).

The subunit stoichiometry was examined for SDS-stable heteromers containing WT or K8A and TCM produced by co-IVTT in which both subunits were labeled with [35S]methionine. The oligomers were isolated from SDS gels, dissociated into monomers by heating in sample buffer, and re-electrophoresed in long SDS gels (Figure 6c). Because TCM and K8A monomers are better separated than TCM and WT, the TCM:K8A subunit ratio was determined by phosphorimagery analysis and found to be 2.6 (S.D. = 0.23, n = 4), consistent with the structure TCM₄K8A₃. Because a maximum of two WT subunits are required to stabilize the heptamer, the TCM subunits can exist in stable oligomers with either TCM or WT, or both, as nearest neighbors.

Imaging of TCM by atomic force microscopy
Purified TCM-H6 from E.coli was applied to a freshly cleaved mica surface and allowed to adhere. Images in which all seven subunits were resolved were readily obtained (Figure 7). In previous experiments in which the heptameric prepore was imaged on a fluid-phase supported bilayer, considerable technical problems were encountered in obtaining stable images even when the particles were packed at high densities (Fang et al., 1997). In the case of TCM on mica, widely distributed individual oligomers could easily sustain the probe force. The dimensions of the TCM heptamer were obtained by averaging 44 images selected for their apparent integrity: diameter of central dent, 5.6 ± 0.3 nm; average diameter of heptamer, 12.4 ± 0.6 nm.

Discussion
The TCM mutant of αHL, which has no channel-forming central domain, lacks hemolytic activity but assembles in the absence of membranes to form an SDS-sensitive heptamer. Further, SDS-resistant heteromers of WT and TCM are formed, again in the absence of membranes. The TCM heptamer exhibits the protease resistance normally associated with the conformational change at the N terminus that accompanies insertion of the central domain into the lipid bilayer when the
Spontaneous oligomerization of α-hemolysin

Fig. 6. TCM can drive WT-αHL into an oligomeric state. (a) Increasing amounts of unlabeled WT-αHL were added to the IVTT reaction mixture before initiating translation of K8A or TCM. After translation, unheated samples were subjected to SDS–PAGE. Final concentrations of WT-αHL were as follows. Lanes 2 and 6, 0 µg/ml; lanes 3 and 7, 0.35 µg/ml; lanes 4 and 8, 3.5 µg/ml; lanes 5 and 9, 35 µg/ml. Lanes 1 and 10 display K8A oligomerized on rRBCM. Lane 11 is identical to lane 9, but shown alongside the K8A heptamer. (b) TCM/T292C and WT or T292C and WT were cotranslated in the presence of [35S]cysteine. The ratios of TCM/T292C: WT were in lane 1, TCM/T292C alone; lane 2, 16:1; lane 3, 8:1; lane 4, 4:1; lane 5, 1:4; lane 6, 1:8; lane 7, 1:16. The ratios of T292C: WT were in lane 8, T292C alone; lane 9, 16:1; lane 10, 8:1; lane 11, 4:1; lane 12, 1:4; lane 13, 1:8; lane 14, 1:16. (c) Ratio of subunits in the heteromer. A gel lane displaying 35S-labeled subunits from a TCM + WT heteromer is shown. TCM and WT were cotranslated in the presence of [35S]methionine using a template ratio of 1:1. The oligomer that formed was purified by SDS–PAGE, dissociated by heating in loading buffer at 95°C for 5 min, and rerun in an extended 12% SDS–polyacrylamide gel. An autoradiogram is shown.

fully assembled transmembrane pore is formed by WT-αHL. In one possible sequence of events, residues 116 and 142, which are normally separated in the disordered central domain in the monomer in solution (Figures 1 and 8a), are forced together in TCM, thereby eliciting the formation of a truncated β barrel (Figure 8a). Barrel formation is in turn transmitted to the remainder of the heptamer, which undergoes the additional conformational changes associated with the formation of a pore (Figure 1, 4), in this case a severely truncated version (Figure 8b). These findings lend support to the idea that the membrane insertion of the central region and the N terminal conformational change are associated, perhaps cooperative, transitions. It is interesting that several point mutations in the central domain also promote heptamerization in solution (Valeva et al., 1996). It has not been demonstrated whether these heptamers are in the prepore (Figure 1, 3) or pore (Figure 1, 4) conformation and this point would be worth investigation. In the point mutants, heptamerization must be much slower than it is for TCM as the monomers can be purified, albeit at low temperatures, and then chemically modified. Nevertheless, the existence of these mutants emphasizes the crucial role of the central domain in assembly.

In a previous study, we used AFM to visualize WT-αHL heptamers assembled on supported bilayers (unpublished data). Because assembly was surprisingly slow, we could not be sure whether we were looking at the prepore (Figure 1, 3) or the pore (Figure 1, 4). However, a mutant αHL, αHL-H5, unquestionably arrested as the prepore 3 (Walker et al., 1995), also appeared in the AFM as a heptamer with a diameter (~8.9 nm) (Fang et al., 1997) close to that of the fully assembled pore as determined by crystallography (~8.6 nm) (Song et al., 1996). The mutation examined in this work appears to provide a way to lock αHL into form 4. Therefore, TCM as viewed in the AFM might represent the cap structure of the fully assembled pore (Song et al., 1996). However, the TCM heptamer as imaged by AFM is far larger (diameter
Fig. 7. TCM imaged by atomic force microscopy. Purified TCM-H6 from *E. coli* was used. (a) Field showing TCM deposited on a mica grid. The scale is in nm. (b) A gallery of individual TCM molecules (images a–q) and an image averaged from 44 particles (image r). Each image is $16.15 \times 16.15$ nm.

Fig. 8. Assembly of αHL-TCM. (a) Proposed scheme. The newly synthesized monomer spontaneously assembles in solution into a ‘pore’ with a truncated channel domain. Intermediates in assembly are not shown because there is currently little information about them. (b) Model of TCM based on the crystal structure of the WT pore. Each subunit in the homoheptamer is colored differently. Features in the mauve subunit are indicated. The asterisks mark the triangle region. Beginning at the N-terminal end of the triangle (top left asterisk), the residues marked are: Pro103, Glu111, Lys147, Pro151 and Pro160. The linker comprises the sequence Asp–Gly as described in the text. The crucial His35 is also shown.

~12.4 nm) than expected from the X-ray structure. Tip broadening and compression are unlikely to account for a difference this large. Gel filtration, while less reliable, also suggests an enlarged or open structure, as TCM emerges from the column just before the WT heptamer (Figure 5B). However, at this point, we cannot say whether TCM is distorted by direct adherence to a mica surface (as opposed to a lipid bilayer), whether the deletion itself alters the integrity of the molecule in a way that is not revealed by analysis by limited proteolysis or whether a bona fide ‘open’ conformation of the pore can exist. Each of these possibilities could be related to the formation of a ‘weak’ short barrel or indeed, if our mechanistic speculations are incorrect, the absence of a barrel. The molecular dimensions of a number of other proteins on mica determined by AFM are consistent with crystallographic studies (Yang *et al.*, 1994b).
TCM might provide a useful framework for the design of additional membrane-active macromolecules. For example, amphipathic α-helical peptides or membrane-active organic molecules might be suspended at the base of each truncated subunit by attachment, for example, at single cysteine residues. This is a biochemical version of the TASP (template-assisted synthetic protein) idea, where a backbone structure is used to preorganize synthetic peptides (Pawlak et al., 1994; Montal, 1995). In the case of α-hemolysin, the cap domain would serve to deliver the synthetic molecules to the membrane surface, bring them together and organize them for membrane insertion and, once a barrel or bundle structure is formed in the membrane, prevent its collapse.

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