An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch

Barbara Walker, Orit Braha, Stephen Cheley and Hagan Bayley*

Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545-2737, USA

Background: Studies of the mechanisms by which certain water-soluble proteins can assemble into lipid bilayers are relevant to several areas of biology, including the biosynthesis of membrane and secreted proteins, virus membrane fusion and the action of immune proteins such as complement and perforin. The α-hemolysin (αHL) protein, an exotoxin secreted by *Staphylococcus aureus* that forms heptameric pores in lipid bilayers, is a useful model for studying membrane protein assembly. In addition, modified αHL might be useful as a component of biosensors or in drug delivery. We have therefore used protein engineering to produce variants of αHL that contain molecular triggers and switches with which pore-forming activity can be modulated at will. Previously, we showed that the conductance of pores formed by the mutant hemolysin αHL–H5, which contains a Zn(II)-binding pentahistidine sequence, is blocked by Zn(II) from either side of the lipid bilayer, suggesting that residues from the pentahistidine sequence line the lumen of the transmembrane channel.

Results: Here we show that Zn(II) can arrest the assembly of αHL–H5 before pore formation by preventing an impermeable oligomeric prepore from proceeding to the fully assembled state. The prepore is a heptamer. Limited proteolysis shows that, unlike the functional pore, the prepore contains sites near the amino terminus of the polypeptide chain that are exposed to the aqueous phase. Upon removal of the bound Zn(II) with EDTA, pore formation is completed and the sites near the amino terminus become occluded. Conversion of the prepore to the active pore is the rate-determining step in assembly and cannot be reversed by the subsequent addition of excess Zn(II).

Conclusions: The introduction of a simple Zn(II)-binding motif into a pore-forming protein has allowed the isolation of a defined intermediate in assembly. Genetically-engineered switches for trapping and releasing intermediates that are actuated by metal coordination or other chemistries might be generally useful for analyzing the assembly of membrane proteins and other supramolecular structures.

Chemistry & Biology February 1995, **2**:99–105

Key words: assembly intermediate, membrane protein, molecular switch, pore, Zn(II) binding motif

Introduction

Staphylococcal α-hemolysin (αHL) [1] is a 293-residue polypeptide that forms heptameric pores [2] in lipid bilayers. αHL is both an excellent model for investigating how a water-soluble protein assembles into biological membranes [1] and a useful target for protein engineering studies intended to produce new materials [3,4]. αHL can also contribute to the pathogenicity of *Staphylococcus aureus* [1].

A working scheme for the assembly of αHL (Fig. 1a), is supported by an accumulation of biophysical and biochemical evidence, including that presented here. The αHL monomer consists of two domains of approximately equal mass connected by a glycine-rich loop (see Fig. 1a, structure 1) [5,6]. The loop becomes resistant to proteolysis when αHL binds to membranes [5,7] and is likely to line a segment of the lumen of the transmembrane channel in the fully-assembled pore (Fig. 1a, structure 4) [4,8]. The fully-assembled pore might contain a 14-stranded β barrel, by analogy with the porins, which contain 16-stranded barrels [9]. Such a barrel could accommodate [10] the essentially nonselective channel of 1–2 nm internal diameter [11,12]. Two intermediates in assembly have been proposed [7]: a membrane-bound monomer (structure 2) and an impermeable oligomeric 'prepore' (structure 3). Functionally-inactive oligomers, suggestive of a prepore, are formed by wild-type αHL after cleavage by trypsin [13], amino-terminal truncation mutants of αHL [7], mutants in which the central loop is grossly altered [14] and certain point mutants, notably those in which His35 is substituted [15–17]. In cases where it has been examined, the putative prepore possesses characteristic proteinase K cleavage sites near the amino terminus (Fig. 1b) [7,17]. Because evidence for the prepore is based on the examination of defective αHL polypeptides, it has not been determined whether the prepore is a kinetically-competent intermediate in assembly, rather than a dead-end product. Here we demonstrate that the former is the case by trapping the intermediate with a genetically-engineered switch located in the central loop of the mutant αHL–H5, which contains a pentahistidine sequence (residues 130–134) that acts as a binding site for divalent metal ions [4,18].

Results and discussion

Zn(II) blocks assembly of αHL–H5 as an oligomeric prepore

We showed earlier that channels formed by αHL–H5 in planar lipid bilayers are blocked by Zn(II) added from either the *cis* or the *trans* side of the membrane [4]. The simplest explanation of this result is a direct channel block

*Corresponding author.

 ISSN 1074-5521

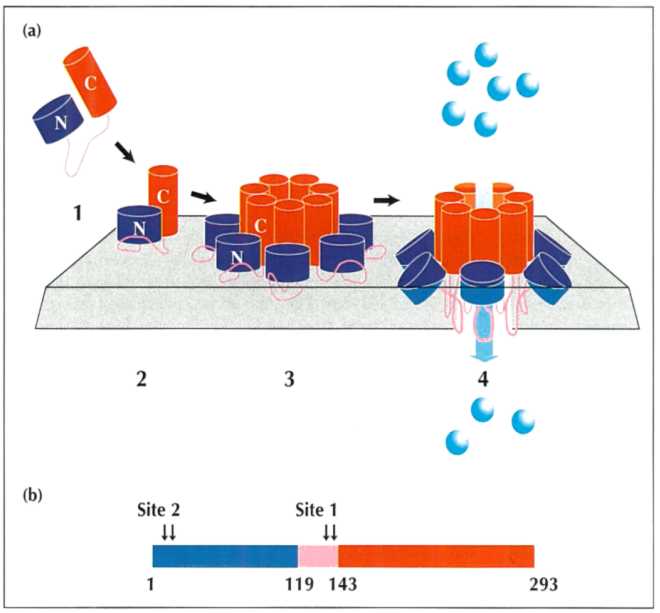

Fig. 1. Structural models for staphylococcal α-hemolysin. **(a)** Working scheme for assembly of the transmembrane pore. The amino- and carboxy-terminal domains (amino-terminal domain, N, blue; carboxy-terminal domain, C, red) of soluble monomeric αHL (structure 1) are separated by a glycine-rich loop (pink). αHL first binds to the bilayer as a monomer (structure 2), which aggregates to form an oligomeric prepore complex (structure 3). The prepore converts to the fully-assembled heptameric pore (structure 4), which allows molecules of up to 3 kDa (blue spheres) to pass through it. **(b)** Positions of cleavage by proteinase K in the primary sequence of α-hemolysin as deduced previously [7] and as reported here. The color scheme matches that in Fig. 1a. Site 1, in the central loop, is exposed only in the monomer in solution (a, structure 1). Site 2 is exposed in the monomer in solution and the monomeric and oligomeric assembly intermediates (a, structures 1–3), but not in the fully assembled pore (a, structure 4).

(Fig. 2, structure 4 → structure 5), although reversal of the proposed final step of assembly (Fig. 1a, structure 4 → structure 3) could not strictly be eliminated. We have now made a definitive examination of this issue by determining the conformation of oligomeric structures on rabbit erythrocytes (rRBC) by limited proteolytic digestion. αHL–H5 was allowed to assemble on rRBC for 1 h at 20 °C in the presence of 100 μM Zn(II), which prevents hemolysis. Under these conditions at least 75 % of the polypeptides form oligomers as determined by nondenaturing SDS-PAGE (αHL oligomers are stable in SDS if not heated). A portion of the sample was subjected to limited proteolysis with proteinase K, while another portion was treated with an excess of the Zn(II) chelator EDTA (500 μM). After 1 h, during which lysis of the rRBC occurred, the latter portion was further subdivided. One part was treated with proteinase K, while Zn(II) was added to the other part for a total concentration of 600 μM. After a further 1 h, this last portion, which had now been taken through a Zn(II) → EDTA → Zn(II) cycle, was also treated with proteinase K. Proteinase K, acting at site 2 (Fig. 1b, and see below), removed fragments of ~1.3 and 1.8 kDa from αHL–H5 assembled in the presence of Zn(II) (Fig. 3, top, lane 2), whereas wild type αHL assembled under the same conditions was completely protected from digestion (Fig. 3, bottom, lane 2). The addition of EDTA to αHL–H5 assembled in the presence of Zn(II) resulted in the formation of a proteinase K resistant species (Fig. 3, top, lane 3), with the mobility of undigested αHL–H5 (lane 6). Subsequent addition of excess Zn(II) did not lead to the regeneration of proteinase K sensitivity (Fig. 3, top, lane 4). αHL–H5 treated with 100 μM Zn(II) alone for the 3-h duration of the experiment did not become resistant to proteolysis (Fig. 3, top, lane 1). Neither did αHL–H5 treated for 3 h with 600 μM Zn(II)/500 μM EDTA (Fig. 3, top, lane 5), the same final concentrations of Zn(II) and EDTA that are obtained after the Zn(II) → EDTA → Zn(II) cycle, which establishes considerable resistance to proteinase K (Fig. 3, top, lane 4). These data suggest that αHL–H5 assembled in the presence of Zn(II) forms the proposed nonlytic prepore (Fig. 1a, structure 3), which is converted to the fully-assembled pore (structure 4) upon the addition of EDTA. Further, the addition of Zn(II) to the assembled pore results in simple channel block (Fig. 2, structure 4 → structure 5) [4] rather than conversion back to the prepore.

Control experiments showed that the enzymatic activity of proteinase K was unaffected by the various concentrations of Zn(II) and EDTA. In particular, wild-type αHL was resistant to proteinase K under all the conditions examined (Fig. 3, bottom). By contrast, and in keeping with the proposed scheme for assembly (Fig. 1a), the nonlytic mutant His35Asn (B.W. and H.B., manuscript in preparation) was digested at site 2 (Fig. 1b) under all conditions (Fig. 3, center). Similar results were obtained when the experiment with αHL–H5 was carried out on rRBC membranes, or on intact rRBC in 30 mM dextran (M_r ~4000), in which the cells are protected from lysis [19]. Therefore, the integrity of the cells does not affect the activity of proteinase K. When unheated samples of wild-type αHL or αHL–H5 are subjected to SDS-PAGE after assembly on rRBC membranes and limited proteolysis, oligomers and monomers are observed, in a ratio of about 3:1. As expected, the proteolytic fragments that we observed with heated samples (Fig. 3) were largely derived from oligomer, because similar results were obtained when oligomers were isolated from proteolyzed samples by electrophoresis,

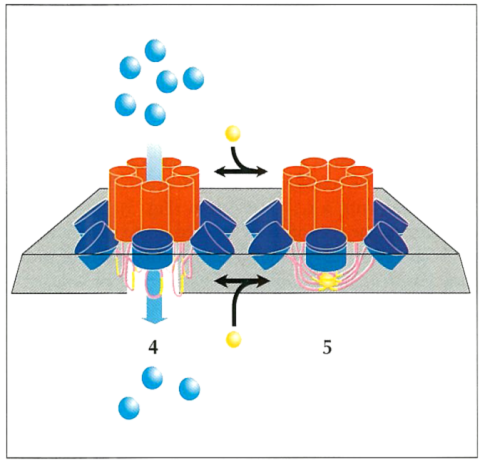

Fig. 2. Working scheme for blockade of the αHL–H5 pore by Zn(II). The formation of the fully-assembled pore (structure 4) proceeds in an identical manner to that of wild-type αHL (see Fig. 1a). The five histidine residues (yellow) introduced into the protein at residues 130–134 probably form part of the transmembrane pore (structure 4). Hence, the pore can be reversibly blocked (structure 5) by Zn(II) (yellow spheres) added from either the *cis* or *trans* side of the membrane.

heated and rerun in a second gel (data not shown). By contrast, the monomers that remained after assembly were cleaved at site 2 under all conditions examined (data not shown), in keeping with the assembly scheme (Fig. 1a).

The amino terminus of αHL becomes occluded during the final step in assembly

The position of the site 2 cleavage in αHL–H5 was confirmed by limited proteolysis of αHL–H5/Ser3CamCys and αHL–H5/Thr292CamCys, which are single Cys mutants of αHL–H5 derivatized with iodoacetamide. These polypeptides were labeled with [^{35}S]Cys or [^{35}S]Met. The latter is incorporated at seven positions throughout the polypeptide chain. After assembly on rRBC membranes in the presence of 100 μM Zn(II), treatment with proteinase K cleaved both polypeptides at site 2, as determined by examining the [^{35}S]Met polypeptides by SDS-PAGE. The label was cleaved from [^{35}S]Cys αHL–H5/Ser3CamCys, but not from [^{35}S]Cys αHL–H5/Thr292CamCys under the same conditions, demonstrating that site 2 is near the amino terminus. The predilection of proteinase K for cleavage at hydrophobic residues suggests that the 30.8 and 30.3 kDa bands (Fig. 3) are formed by cleavage after Ile7 and Ile14 respectively. These data firmly establish that the amino terminus of αHL becomes occluded during the final step in assembly (Fig. 1a, structure 3 → structure 4).

The oligomeric prepore is a heptamer

The oligomeric state of the prepore complex was investigated by gel-shift electrophoresis [20] of SDS-resistant αHL oligomers containing two classes of subunit. The heteromers were generated by chemical modification of a single-cysteine mutant with mixtures of iodoacetamide (IAM) and 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonate (IASD) [2]. Each polypeptide chain modified with the dianionic sulfhydryl reagent IASD increases the electrophoretic mobility of an αHL oligomer in SDS, while IAM modification has no effect [2]. The single cysteine mutant αHL–H5/Thr292Cys was allowed to assemble on rRBC in the presence of 100 μM Zn(II). Samples were modified with various ratios of IAM to IASD. The resultant αHL heteromers were then separated by extended SDS-PAGE. If each oligomer contained n subunits, one would expect to see n + 1 bands, representing all possible combinations of the two classes of subunit. The αHL–H15 heteromers yielded eight bands, whether they were prepared in the presence of Zn(II) (Fig. 4) or EDTA (data not shown), suggesting that the prepore (Fig. 1a, structure 3), like the fully-assembled pore (structure 4), contains seven subunits. A similar result was obtained when αHL–H5 and

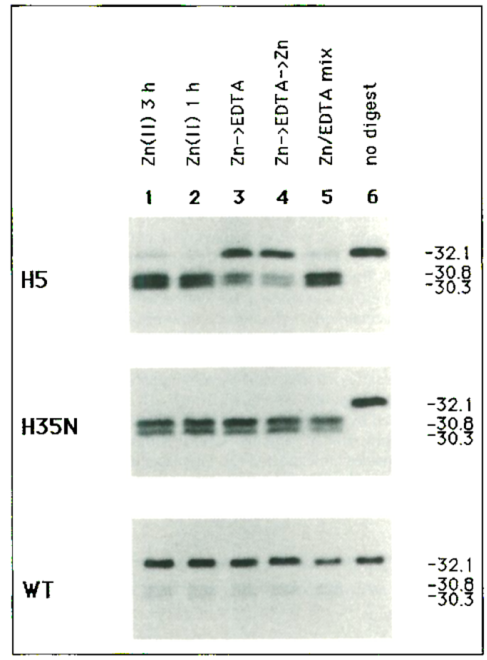

Fig. 3. Limited proteolysis of αHL–H5 (H5), the His35Asn mutant (H35N) and wild-type αHL (WT) after assembly on rRBC under various conditions. Top, αHL–H5; center, His35Asn; bottom, wild-type αHL. ^{35}S-labeled αHL was digested with proteinase K on membranes after assembly in the presence of: lane 1, 100 μM Zn(II) for 3 h; lane 2, 100 μM Zn(II) for 1 h; lane 3, 100 μM Zn(II) for 1 h, followed by 500 μM EDTA with 100 μM Zn(II) for 1 h; lane 4, 100 μM Zn(II) for 1 h, then 500 μM EDTA with 100 μM Zn(II) for 1 h, and finally 500 μM EDTA with 600 μM Zn(II) for a further 1 h; lane 5, 500 μM EDTA with 600 μM Zn(II) for 3 h; lane 6, undigested αHL polypeptides. The masses of the polypeptides, determined by comparison with ^{14}C-labeled standards, are indicated: uncleaved αHL, 32.1 kDa; proteolytic fragments, 30.8 and 30.3 kDa.

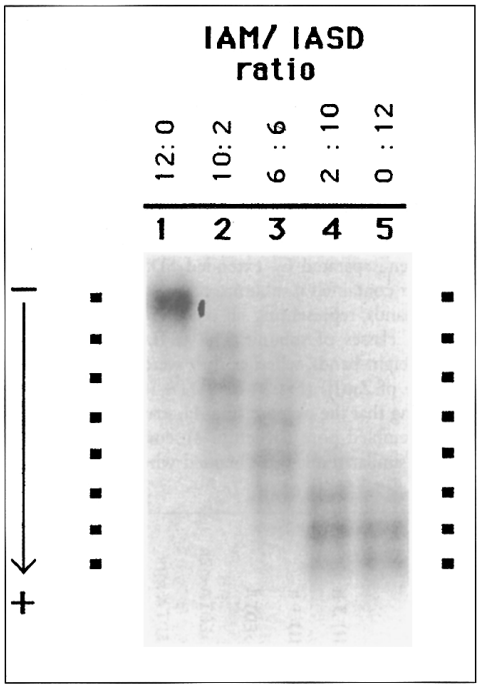

Fig. 4. The nonlytic oligomer formed by αHL–H5 in the presence of Zn(II) is a heptamer. αHL oligomers were generated on rRBC from ^{35}S-labeled αHL-H5/Thr292Cys in MBSA containing 100 μM Zn(II). The cells were lysed and portions of the recovered membranes were subjected to modification with iodoacetamide (IAM) and 4-acetamido-4'-((iodoacetyl)amino)stilbene-2,2'-disulfonate (IASD) that had been premixed in the ratios indicated above each gel lane. Each IASD modification introduces two additional negative charges and thereby causes an increase in electrophoretic mobility of the oligomer [2]. The heteromers were separated by non-denaturing SDS-PAGE and visualized by autoradiography. For an oligomer with n subunits, n + 1 electrophoretic species are expected [2]. Eight species are seen, indicating that the αHL–H5 oligomers contain seven subunits when they are assembled in the presence of Zn(II).

αHL–H5/Thr292Cys were mixed in various ratios, allowed to assemble on rRBC and then modified with IASD alone.

The assembly of the His35Asn mutant is arrested at the prepore step (Fig. 1a, structure 3 and Fig. 3). Accordingly, the analysis of heteromers formed from the His35Asn mutant and the double mutant His35Asn/Thr292Cys in the absence of Zn(II) or EDTA also suggested heptamer formation (data not shown).

The trapped prepore is a kinetically competent intermediate in the assembly of αHL–H5

To determine whether the prepore might be an intermediate in the assembly of αHL–H5, the kinetics of formation of the fully-assembled pore (Fig. 1a, structure 4) from the prepore (structure 3) were examined using the built-in Zn(II) dependent switch. αHL–H5 was first allowed to assemble on the surface of rRBC membranes in the presence of 100 μM Zn(II). After 1 h at 20 °C, when binding and oligomerization is complete [4], excess EDTA was added (t = 0 min) and the conversion to the fully-assembled pore was monitored at room temperature by rapid proteolysis of samples removed over a series of timepoints. The transformation of structure 3 to structure 4 occurred with a $t_{1/2}$ of ~8 min (Fig. 5). In a parallel experiment, the lysis of rRBC treated in the same way was 50 % complete in ~20 min. The transformation of structure 3 to structure 4 could also be observed for wild-type αHL, although it could not be triggered with EDTA. Wild-type αHL was allowed to bind to rRBC membranes for 1 min at 0 °C. The membranes were washed rapidly and resuspended in buffer at room temperature (t = 0 min). Samples were removed for proteolysis as before. The protease-resistant species appeared with a $t_{1/2}$ of ~12 min (Fig. 6). The lysis of rRBC treated in parallel occurred at ~22 min under these conditions, where less wild-type αHL is bound to the cell surface than in a standard assay due to the short initial incubation period. The His35Asn mutant, which lyses rRBCs extremely slowly (B.W. and H.B., manuscript in preparation), yielded only a trace of protease-resistant polypeptide after 24 h incubation with rRBC (data not shown).

The oligomeric prepore formed by αHL–H5 in the presence of Zn(II) appears to be a *bona fide* intermediate in the assembly of αHL–H5 (Fig. 7). Hence, Zn(II) acts on αHL–H5 both to prevent progression of the prepore to the fully assembled pore (the conversion of structure 3 to structure 4 in Fig. 7) and to block the assembled pore (structure 4 → structure 5). Only the channel blockade is reversed by EDTA [4]. Conversion of the prepore to the fully assembled pore can be the rate-determining structural transition *in vitro* (Figs 5, 6). Like the fully-assembled pore (structure 4) [2], the prepore (structure 3) is an SDS-resistant heptamer. It differs from the fully-assembled pore

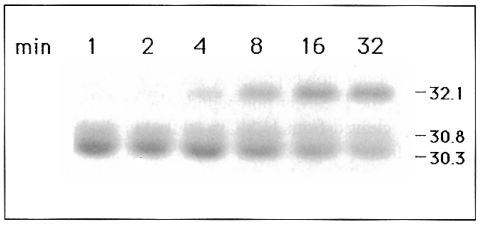

Fig. 5. The nonlytic oligomer formed by αHL–H5 in the presence of Zn(II) is converted to a lytic pore of altered structure upon treatment with EDTA. αHL–H5 was allowed to assemble in the presence of 100 μM Zn(II) and, at t = 0 min, treated with 500 μM EDTA. Samples were removed over a series of timepoints, as indicated above the gel lanes, and digested with a high concentration of proteinase K for 1 min. Protease-insensitive αHL–H5 (32.1 kDa) representing the fully-assembled pore (Fig. 1a, structure 4) appears with a $t_{1/2}$ of ~8 min, whereas the protease-sensitive fraction representing the prepore (Fig. 1a, structure 3) decreases with time (30.8- and 30.3-kDa bands).

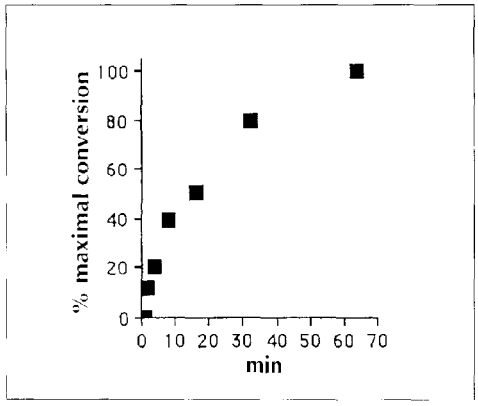

Fig. 6. A protease-sensitive species is a transient intermediate during pore formation by wild-type αHL. Wild-type αHL was allowed to assemble on ^{32}P-RBC membranes for 1 min at 0 °C in MBSA containing 500 μM EDTA. The membranes were recovered by centrifugation and resuspended in buffer at room temperature at t = 0 min. Over a series of timepoints, portions were removed and digested for 1 min with proteinase K. The mean relative intensity of the protease-insensitive fraction, as determined by phosphorimager analysis of SDS gels from two experiments, is plotted versus time. The extent of conversion did not increase appreciably after the 64 min timepoint.

in its lack of permeability to small molecules, which is probably due to the failure of the transmembrane channel to form. Because the central loop lines part of the lumen of the channel [4,8], and because protease sites at the amino terminus become occluded as the prepore becomes fully assembled, as shown here, we suggest that the loop and the amino terminus cooperate in the structural change required for the final step of pore assembly. A prepore structure with similar properties is a transient intermediate in the assembly of wild type αHL (Fig. 6) and the point of arrest for several mutant αHL polypeptides, such as the His35Asn mutant.

Significance

The structural changes that occur when proteins pass through or assemble into membranes remain poorly understood. Examination of this issue is important for understanding the biosynthesis of secreted, compartmentalized and integral membrane proteins, the actions of pore-forming immune proteins, the fusion of viral envelopes with target membranes and the protein–protein interactions on membranes that occur during cell signaling and vesicle fusion. Many of these events are facilitated by additional proteins. Nevertheless, it is unlikely that the assembly of polypeptides into membranes involves the catalyzed threading of individual amino acids into the lipid bilayer. It seems more reasonable that domains of proteins are prefolded and then inserted into the bilayer. Therefore, there should be much to gain by studying model membrane proteins, such as α-hemolysin (αHL), that can translocate into the bilayer spontaneously. One method of dissecting the assembly process, to allow the examination of individual steps in assembly in isolation, is demonstrated in this paper.

Chemical triggers and switches have been used previously, for example to control the activity of enzymes by redox reactions [21], metal cation association and dissociation [22,23] and photochemistry [24,25]. Here we show that a Zn(II)-modulated switch, introduced as a pentahistidine sequence by site-directed mutagenesis, can be used to control the assembly of the membrane protein, α-hemolysin. Assembly is arrested by Zn(II) after the formation of an oligomeric prepore. Upon the addition of EDTA, pore formation proceeds to completion. As an example of the utility of the technique, we demonstrate that the trapped prepore is a heptamer. By using the

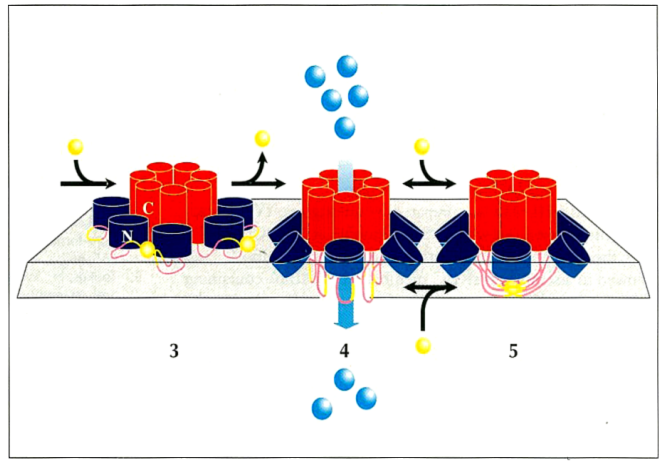

Fig. 7. Working scheme for the effect of Zn(II) on the hemolysin mutant, αHL–H5. Zn(II) (yellow spheres) that is present on the external (*cis*) side of the membrane in the early stages of pore formation can trap αHL H5 in a prepore conformation (structure 3) by binding to the His residues. Removal of Zn(II) by a chelating agent allows the transformation of the prepore to the fully assembled pore (structure 4). This transition is irreversible. The fully assembled pore can be reversibly blocked by the addition of Zn(II) to either side of the membrane.

Zn(II)-switch, it may be possible to obtain high-resolution structural information on the prepore and to monitor the structural changes that occur during conversion to the functional pore. Further, genetically-engineered switches for trapping and releasing intermediates should be generally useful for analyzing the assembly of supramolecular structures.

Materials and methods

Conformational changes of αHL on red cell membranes examined by limited proteolysis with proteinase K

^{35}S-labeled αHL was prepared by *in vitro* transcription and translation (IVTT) in an *Escherichia coli* S30 system with [^{35}S]methionine at 1200 Ci mmol^{-1} [7,26]. A portion of the mix (10 µl) was diluted into 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.4, 150 mM NaCl, 1 mg ml^{-1} bovine serum albumin (MBSA buffer), followed by the addition of Zn sulfate to 100 µM and washed rRBC to 5 % (final volume 100 µl). After 1 h at 20 °C, the cells were washed twice by centrifugation in MBSA containing 100 µM Zn sulfate and then resuspended in the same buffer (100 µl). Two portions (20 µl) were now removed. One was digested immediately (Fig. 3, lane 2) by the addition of water (4 µl) and proteinase K (1 µl, 1 mg ml^{-1}). Digestion was for 5 min at room temperature, followed by treatment with 20 mM phenylmethanesulfonyl fluoride (PMSF) (1 µl) for 5 min at room temperature, the addition of 2x gel loading buffer [27] and heating for 5 min at 95 °C. The other portion was incubated for a further 2 h and then digested (Fig. 3, lane 1). 10.5 mM EDTA (3 µl) was added to the remaining solution (60 µl). After 1 h at room temperature, a portion (21 µl) was removed and digested by the addition of water (3 µl) and proteinase K (1 µl, 1 mg ml^{-1}) (Fig. 3, lane 3). 11 mM Zn sulfate (2 µl) was added to the remaining solution (42 µl). After a further 1 h at room temperature, a portion (22 µl) was removed and digested by the addition of water (2 µl) and proteinase K (1 µl, 1 mg ml^{-1}) (Fig. 3, lane 4). The remaining solution was not treated with proteinase K (Fig. 3, lane 6). A separate suspension of 5 % rRBC was treated with ^{35}S-labeled αHL in MBSA containing 600 µM Zn sulfate and 500 µM EDTA for 1 h at 20 °C. The cells were washed twice by centrifugation in MBSA with 600 µM Zn sulfate/500 µM EDTA, resuspended in the same buffer for 2 h at room temperature and treated with proteinase K (Fig. 3, lane 5). Samples were subjected to electrophoresis in a 12 % SDS polyacrylamide gel [27], followed by autoradiography. Control experiments showed that no further cleavage of the full-length polypeptide (32.1 kDa) occurred at higher enzyme concentrations or after extended periods of digestion.

Timecourse of pore formation by αHL–H5 and wild-type αHL monitored by limited proteolysis

^{35}S-labeled αHL–H5 was prepared by mixing two IVTT reactions, one carried out with [^{35}S]methionine at 1200 Ci mmol^{-1} and the other with [^{35}S]methionine at 0.8 Ci mmol^{-1}, and allowed to assemble on rRBC membranes in MBSA containing 100 µM Zn sulfate (total volume of 200 µl), as described in the section above. After 1 h at 20 °C, EDTA was added to 500 µM and the sample was brought rapidly to room temperature. At each timepoint, a portion of the reaction (19 µl) was removed and treated with proteinase K (1 µl, 10 mg ml^{-1}) for 1 min, followed by the addition of PMSF and loading buffer (see above). Heated samples were subjected to electrophoresis in a 12 % SDS polyacrylamide gel [27], followed by autoradiography. Experiments with wild-type αHL were performed by a similar procedure with the modifications noted in Fig. 6 (legend).

Counting the subunits in the oligomeric prepore by gel shift electrophoresis

The mutant αHL–H5/Thr292Cys contains the pentahistidine sequence at residues 130–134 as well as a single Cys at position 292. The αHL–H5/Thr292Cys gene was prepared by cutting and pasting the αHL–H5 [4] and Thr292Cys [17] genes. 10 % rRBC were treated with ^{35}S-labeled αHL–H5/Thr292Cys (25 µl of IVTT mix) in MBSA containing 100 µM Zn sulfate (250 µl total volume) for 1 h at 20 °C. The cells remained intact and were subsequently lysed by resuspension in 10 mM N-tris[hydroxymethyl] methyl-3 aminopropanesulfonic acid (TAPS), pH 8.5, containing 100 µM Zn sulfate. After recovery by centrifugation, the membranes were divided into portions and treated with 20 mM IAM/IASD mixtures (from stocks in water containing a 100 mM total of the reagents) in 0.25 M TAPS, pH 8.5, containing 100 µM Zn sulfate). IASD was obtained from Molecular Probes (Eugene, OR). After 2 h at room temperature, SDS was added to 1 %, followed by one volume of 2x loading buffer [27] and EDTA to 250 µM. The latter improves the electrophoretic separation. Without prior heating, the oligomers were separated by SDS-PAGE in a 5 % gel according to Laemmli [27], with the modifications described previously [2], and detected by autoradiography. In the experiment shown, sulfhydryl derivatization is incomplete, because prereduction with dithiothreitol [2] was omitted to avoid chelation of Zn(II). This is most apparent after the treatment with IASD alone (Fig. 4, lane 5). Nevertheless, the bottom band in lane 5 comigrates with the oligomer formed after complete modification of αHL–H5/Thr292Cys monomer in solution. Further, there is no scrambling of the subunits after solubilization of the rRBC membranes in SDS. This was demonstrated by mixing membranes carrying oligomers modified with IAM alone and membranes carrying IASD-modified oligomers before solubilization. Immediately prior to chemical modification, the αHL–H5/Thr292Cys oligomers were in the form shown as structure 3 in Fig. 1a, as judged by limited proteolysis of the rRBC membranes as described above.

Acknowledgements: This work was supported by grants from the ONR and DOE.

References

1. Bhakdi, S. & Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev.* **55**, 733–751.
2. Gouaux, J.E., *et al.*, & Bayley, H. (1994). Subunit stoichiometry of staphylococcal α-hemolysin in crystals and on membranes: a heptameric transmembrane pore. *Proc. Natl. Acad. Sci. USA* **91**, 12828–12831.
3. Walker, B.J. & Bayley, H. (1994). A pore-forming protein with a protease-activated trigger. *Protein Eng.* **7**, 91–97.
4. Walker, B., Kasianowicz, J., Krishnasastry, M. & Bayley, H. (1994). A pore-forming protein with a metal-actuated switch. *Protein Eng.* **7**, 655–662.
5. Tobkes, N., Wallace, B.A. & Bayley, H. (1985). Secondary structure and assembly mechanism of an oligomeric channel protein. *Biochemistry* **24**, 1915–1920.
6. Gray, G.S. & Kehoe, M. (1984). Primary sequence of the α-toxin gene from *Staphylococcus aureus* Wood 46. *Infect. Immun.* **46**, 615–618.
7. Walker, B.J., Krishnasastry, M., Zorn, L. & Bayley, H. (1992). Assembly of the oligomeric membrane pore formed by staphylococcal α-hemolysin examined by truncation mutagenesis. *J. Biol. Chem.* **267**, 21782–21786.
8. Ward, R.J., Palmer, M., Leonard, K. & Bhakdi, S. (1994).

Identification of a putative membrane-inserted segment in the α-toxin of *Staphylococcus aureus*. *Biochemistry* **33**, 7477–7484.
9. Cowan, S.W., *et al.*, & Rosenbusch, J.P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* **358**, 727–733.
10. Murzin, A.G., Lesk, A.M. & Chothia, C. (1994). Principles determining the structure of β sheet barrels in proteins II. The observed structures. *J. Mol. Biol.* **236**, 1382–1400.
11. Menestrina, G. (1986). Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage-dependent inhibition by divalent and trivalent cations. *J. Membr. Biol.* **90**, 177–190.
12. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. & Wellensiek, H.-J. (1981). On the mechanism of membrane damage by *Staphylococcus aureus* α toxin. *J. Cell Biol.* **91**, 83–94.
13. Blomqvist, L. & Theiestam, M. (1988). Oligomerization of ^{3}H-labelled staphylococcal alpha-toxin and fragments on adrenocortical Y1 tumour cells. *Microb. Pathog.* **4**, 223–229.
14. Walker, B.J., Krishnasastry, M. & Bayley, H. (1993). Functional complementation of staphylococcal α-hemolysin fragments: overlaps, nicks and gaps in the glycine rich loop. *J. Biol. Chem.* **268**, 5285–5292.
15. Jursch, R., *et al.*, & Bhakdi, S. (1994). Histidine residues near the N-terminus of staphylococcal alpha-toxin as reporters of regions that are critical for oligomerization and pore formation. *Infect. Immun.* **62**, 2249–2256.
16. Menzies, B.E. & Kernodle, D.S. (1994). Site-directed mutagenesis of the alpha-toxin gene of *Staphylococcus aureus*: role of histidines in toxin activity *in vitro* and in a murine model. *Infect. Immun.* **62**, 1843–1847.
17. Krishnasastry, M., Walker, B., Braha, O. & Bayley, H. (1994). Surface labeling of key residues during assembly of the transmembrane pore formed by staphylococcal α-hemolysin. *FEBS Lett.* **356**, 66–71.
18. Arnold, F.H. & Haymore, B.L. (1991). Engineered metal-binding proteins: purification to protein folding. *Science* **252**, 1796–1797.
19. Bhakdi, S., Muhly, M. & Füssle, R. (1984). Correlation between toxin binding and hemolytic activity in membrane damage by staphylococcal α-toxin. *Infect. Immun.* **46**, 318–323.
20. Creighton, T.E. (1980). Counting integral numbers of amino acid residues per polypeptide chain. *Nature* **284**, 487–489.
21. Matsumara, M. & Matthews, B.W. (1989). Control of enzyme activity by an engineered disulfide bond. *Science* **243**, 792–794.
22. Corey, D.R. & Schultz, P.G. (1989). Introduction of a metal-dependent regulatory switch into an enzyme. *J. Biol. Chem.* **264**, 3666–3669.
23. Higaki, J.N., Fletterick, R.J. & Craik, C.S. (1992). Engineered metalloregulation in enzymes. *Trends Biochem. Sci.* **17**, 100–104.
24. Turner, A.D., Pizzo, S.V., Rozakis, G. & Porter, N.A. (1988). Photoreactivation of irreversibly inhibited serine proteases. *J. Am. Chem. Soc.* **110**, 244–250.
25. Mendel, D., Ellman, J.A. & Schultz, P.G. (1991). Construction of a light-activated protein by unnatural amino acid mutagenesis. *J. Am. Chem. Soc.* **113**, 2758–2760.
26. Walker, B.J., Krishnasastry, M., Zorn, L., Kasianowicz, J.J. & Bayley, H. (1992). Functional expression of the α-hemolysin of *Staphylococcus aureus* in intact *Escherichia coli* and in cell lysates. *J.Biol.Chem.* **267**, 10902–10909.
27. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Received: **18 Jan 1995**. Accepted **30 Jan 1995**.