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Triggers and Switches in a Self-Assembling Pore-Forming Protein

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Abstract Protein engineering is being used to produce a collection of pore-forming proteins with applications in biotechnology. Knowledge provided by investigations of the mechanism of self-assembly of staphylococcal α-hemolysin has allowed the design of genetically and chemically modified variants of the protein with pore-forming activities that can be triggered or switched on-and-off by chemical, biochemical and physical inputs. Examples include α-hemolysins that are activated by specific proteases and α a-hemolysins whose activity is controlled by divalent metal ions. These proteins have potential value in drug delivery as components of immunotoxins that can be activated at the surfaces of target cells. Further applications are likely in improved encapsulation techniques for drugs, enzymes and cells.

Key words: biotherapeutics, drug delivery, encapsulation, genetic engineering, immunotoxin, liposome, membrane, monolayer, pore, protein engineering

A goal of our laboratory is to use genetic engineering and chemical modification to produce membrane pore proteins with properties tailored for applications in biotechnology. The primary target of our studies has been the α-hemolysin of *Staphylococcus aureus* (αHL) [Bhakdi and Tranum-Jensen, 1991]. αHL is a hydrophilic 293-amino acid polypeptide, folded largely into β-sheet secondary structure, which is secreted by the bacterium as a monomer. The molecule forms pores by oligomerizing both in natural membranes, including the plasma membranes of eukaryotic cells, and in protein-free artificial bilayers, such as those of phospholipid vesicles. The diameter of the αHL pore is between 1.1 nm, as estimated from measurements of the single channel conductance, and 2.5 nm, as estimated from the permeability of the channel to nonelectrolytes. Molecules of two or three thousand daltons can pass through it.

αHL is especially suitable as a component of biomolecular materials [Krishnasastry et al., 1992]. The αHL pore consists of six identical subunits and is extraordinarily robust, surviving heating to 65°C in detergents that denature most membrane proteins. The short sequence of the αHL polypeptide can be conveniently manipulated by using recombinant DNA technology. It contains no cysteine and this residue, which is selectively modified by a variety of chemical reagents, can be introduced where desired as a linkage point for functionalities that do not occur in the repertoire of the natural amino acids. The αHL pore is open under normal circumstances and the channel hardly distinguishes between negative and positive ions. Therefore, the pore constitutes a "blank slate" for the introduction of selective permeability (control of the size or charge of the molecules that can pass) or gating (the ability to open and close in response to a stimulus).

Self assembly is critical for the manufacture of nanostructures from biomolecular materials. Importantly then, the αHL pore can assemble from monomer without the participation of cellular organelles or enzymes. For example, the addition of deoxycholate, a mild detergent, initiates oligomerization [Bhakdi et al., 1981]. Furthermore, a higher level of self assembly occurs; the pores can aggregate spontaneously in lipid bilayers or at the air–water interface into extended crystalline two-dimensional arrays [Bernheimer et al., 1972].

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Two-dimensional arrays of pores have many potential uses in biotechnology including applications as components of biomaterials [Krishnasastry et al., 1992]. Monolayers or multilayers of αHL pores might be bonded to support matrices to prepare ultrafiltration devices. In sensor technology, a coating of an αHL lattice might be used to select species of predetermined size or charge for access to the surface of a detector. αHL lattices might also be used to immobilize organic or biological molecules in regular arrays, e.g., by attachment to strategically placed cysteine residues. Conceivably, the pores in arrays might be endowed with enzymatic activity or even the ability to actively transport selected molecules. Furthermore, it may prove possible to produce lattices with useful mechanical properties, such as the ability to expand and contract laterally in response to various stimuli. Active or "smart" arrays will be particularly useful at interfaces between devices and tissues. Several applications of two-dimensional protein arrays are being reduced to practice in the case of bacterial S-layers; the reader is referred to the accompanying paper by Uwe Sleytr [Sleytr et al., 1993]. This report focusses on a second aspect of our work that concerns attempts to use protein engineering to produce αHL with built-in triggers and switches so that assembly or channel activity can be modulated by external stimuli.

A WORKING MODEL FOR THE ASSEMBLY OF αHL

Biochemical and molecular genetic studies have led to a working model for the assembly of αHL (Fig. 1) [Walker et al., 1992; Bayley et al., 1993]. The model is not to be taken too literally, but it does serve to summarize an expanding collection of experimental data, which includes studies involving chemical crosslinking (the pore is a hexamer), circular dichroism spectroscopy (both the water-soluble monomer and the assembled pore are largely β-structure), conformational analysis by limited proteolysis (αHL contains a central glycine-rich loop that becomes occluded upon assembly), deletion and point mutagenesis (assembly intermediates such as a membrane-bound monomer and a hexameric prepore can be trapped) and dominant negative mutants (certain mutants inhibit the assembly of wild-type protein in a manner predicted by the scheme).

THE CENTRAL LOOP IS CRUCIAL FOR CHANNEL FORMATION

In the present discussion, I focus on the glycine-rich central loop (residues 119–143) that connects the N- and C-terminal halves of the polypeptide chain. After αHL has bound to target membranes, the loop is not available to pro-

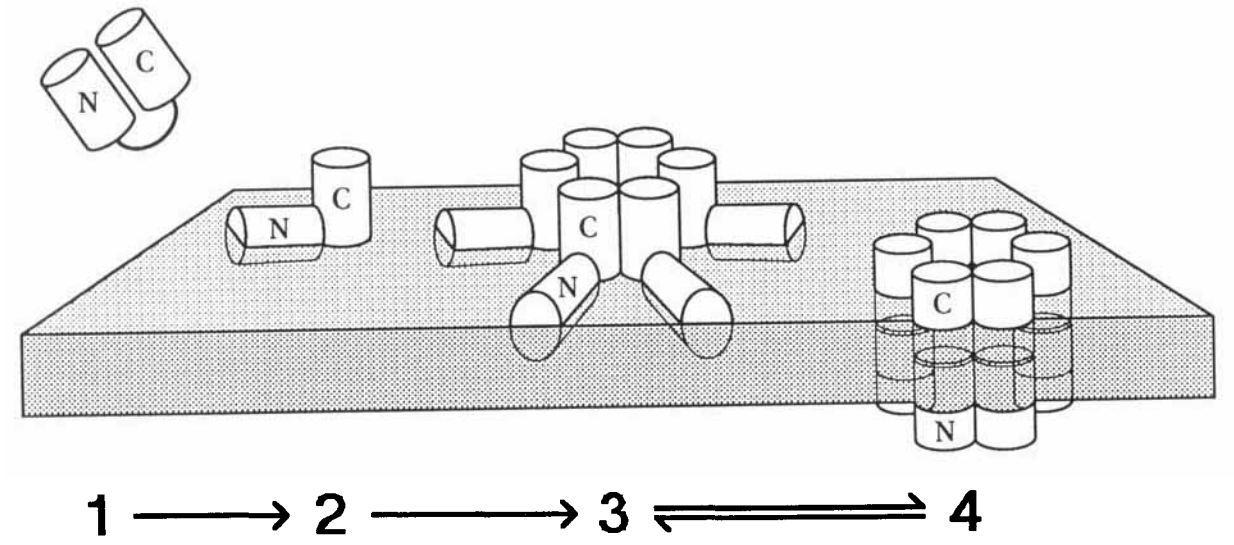

Fig. 1. Working model for assembly of the αHL pore. The model provides a summary of experimental findings and is not to be taken literally. The αHL monomer in solution (1) comprises two domains linked by a glycine-rich loop. αHL binds to the cell surface as a monomer in which the loop is occluded (2). A nonlytic oligomer consisting of six subunits is then formed (3). The subunits then further penetrate the membrane to form the lytic pore (4). The interconversion of 3 and 4 may be reversible. Recent findings suggest that the central loop lines at least part of the transmembrane channel in 4. [Adapted from Walker et al., 1992.]

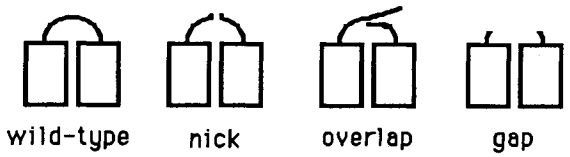

Fig. 2. Two-chain complementation mutants of αHL.

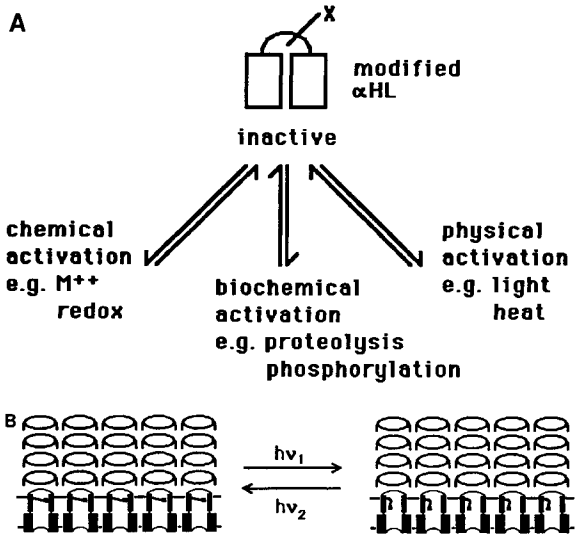

Fig. 3. Potential triggers and switches in αHL. **A:** Inactive genetically engineered and/or chemically modified αHL proteins are being designed that can be activated by chemical, biochemical or physical means. **B:** Speculative depiction of a photogated monolayer of αHL pores. A photoisomerizable group is linked to a cysteine residue that has been introduced at a single site in αHL by site-directed mutagenesis. On illumination, the group, which blocks the pore in the dark, isomerizes. The shape change permits ions to flow. The gate closes when illuminated with light of a different wavelength. [Adapted from Krishnasastry et al., 1992.]

teases [Walker et al., 1992] and cysteine residues introduced into this region become less accessible to chemical modification with hydrophilic reagents (Krishnasastry M: unpublished work). The precise fate of the loop upon pore assembly has not yet been ascertained [Bayley et al., 1993], although recent data favor direct involvement in transmembrane channel formation. Indeed, it seems likely that the loop constitutes the lining of at least a section of the channel (see below).

The importance of the integrity of the loop for pore formation has been studied by complementation mutagenesis [Walker et al., 1993]. Genetically engineered αHL can be synthesized as two polypeptide chains resulting in gaps, nicks or overlaps in the loop (Fig. 2). A collection of these mutants was assayed for their ability to form hexamers and to form pores. Mutants with drastically altered loop regions, including structures with large gaps and overlaps, were able to oligomerize but only those nicked near the midpoint (but not the edges) of the loop could form pores efficiently. These experiments suggested that it might be possible to control the activity of αHL by manipulation of the loop.

TRIGGERS AND SWITCHES FOR CONTROLLING THE ASSEMBLY AND CHANNEL ACTIVITY OF αHL

Mutagenesis and chemical modification have been used to produce enzymes with switches and triggers that respond to chemical and physical stimuli. For example, nucleases and proteases that are turned on-and-off by divalent metal ions have been made by the introduction of cysteine or histidine residues to act as ligands [Corey and Schultz, 1989; Higaki et al., 1992]. Reducing agents [Matsumara and Matthews, 1989] and light [Mendel et al., 1991] have been used to trigger the activity of chemically modified mutant lysozymes. Biochemical activation can be added to the list of potential effectors. For example, phosphorylation and dephosphosphorylation can drive relaxation–contraction cycles of synthetic elastin-based peptide polymers [Patta-

naik et al., 1991]. Extensions of these ideas are now being applied to αHL (Fig. 3A), where activity can be modulated at the level of assembly (Fig. 1) or through control of channel function (Fig. 3B).

Recently, we have shown that a biochemical trigger, proteolytic cleavage, can be used to activate remodeled αHL proteins [Walker and Bayley, 1994]. Complementation mutagenesis was used to produce inactive αHLs with overlap sequences in the central loop (Fig. 4A). Upon proteolysis, the redundant sequences are removed and pore-forming activity is restored. To obtain such mutants, αHL was remodeled by three steps of genetic engineering. First, a protease recognition site near the N terminus was removed by point mutagenesis. Cleavage at this site inactivates αHL. Second, overlap mutants containing a recognition site in the central loop for lysine-directed proteases were constructed by complementation mutagenesis. Finally, the protease recognition sequence in the loop was manipulated by additional point mutagenesis allowing *selective* activation. For example, one mutant is activated by endoproteinase Lys-C and not by clostripain, while another is activated by clostripain and not by endoproteinase Lys-C. Protease-activated triggers can act at early (Fig. 1, **2** → **3**) or late (Fig. 1, **3** → **4**) steps in assembly depending on the precise nature of the mutation in αHL [Walker and Bayley, 1994].

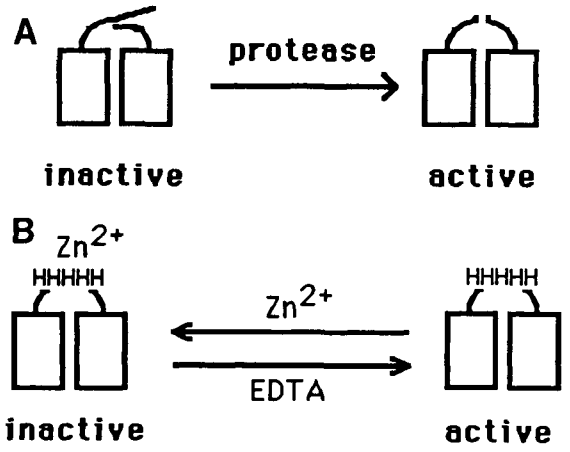

Fig. 4. Examples of a trigger and a switch in αHL that have been reduced to practice. **A:** Activation of an overlap mutant of αHL by proteolysis. An inactive overlap mutant was generated by complementation mutagenesis. Pore-forming activity can be activated by proteases that remove the redundant peptide in the central loop. **B:** The mutant αHL-H5 contains a binding site for divalent metal ions in the central loop. Pore-forming activity is blocked by <10 μM Zn^{2+}. The inhibition is reversed by chelating agents such as EDTA.

A binding site for divalent metal ions has also been introduced into the central loop and provides reversible control of pore activity (B. Walker, unpublished work). Because the conformation of the loop is unknown, a string of five consecutive histidines was used to generate the binding site. This sequence should extend at least two imidazole rings in an orientation suitable for metal ligation whether the loop is largely unstructured or whether it forms α-helix, β-strand or a turn [Arnold and Haymore, 1991]. The mutant, αHL-H5, both forms hexamers and pores in the absence of metal ions. Pore-forming activity, but not hexamer formation, is inhibited by micromolar concentrations of divalent Co, Ni, Cu, and Zn (Fig. 4B). Open αHL-H5 channels are blocked by these metal ions, no matter from which side of the bilayer they are added, suggesting that the loop lines a section of the transmembrane channel (J. Kasianowicz, unpublished work). The inhibition of pore activity is reversed by chelating agents. Therefore, the pentahistidine sequence constitutes a chemically-regulated switch (as opposed to an irreversible trigger), which acts in the assembled pore (Fig. 1, **4**).

A third goal is to build triggers and switches into αHL that can be activated by physical inputs (Fig. 3A). One approach, which is being tested, is to attach photosensitive groups [Adams and Tsien, 1993] to the loop region at single cysteine residues that have been introduced by site-directed mutagenesis. Photoremovable protecting groups will be used to trigger αHL activity and photoisomerizable groups will be used as switches (Fig. 3B).

Each charged residue in αHL has now been individually changed to cysteine by scanning mutagenesis (Walker B, unpublished work). Biochemical and electrical analysis of these mutants, modified when necessary on cysteine, is being carried out to pinpoint residues, other than those in the loop, that contribute to assembly or pore activity. This information will be used to further extend the biotechnological utility of αHL.

PROSPECTS

Potential applications of pore-forming proteins with triggers and switches in biotherapeutics and biomaterials can be illustrated by two current investigations from this laboratory. Despite developments in surgery, radiation therapy and chemotherapy, there has been little improve-

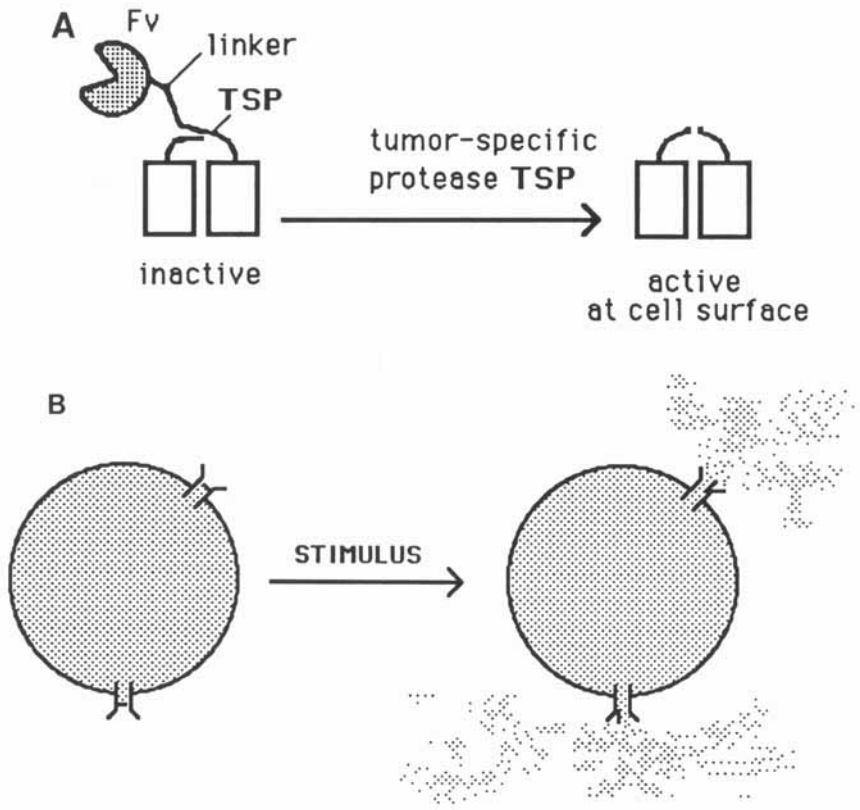

Fig. 5. Potential applications of αHL proteins with triggers and switches. **A:** Destruction of malignant cells with proimmunolysins. A single-chain Fv antibody fragment will be linked to one half of a two-chain complementation mutant using a linker that includes a tumor-protease recognition site. The Fv fragment will target the construct to selected tumor cells. At the target, proteases will clip off the antibody fragment and simultaneously activate the lytic activity of αHL. **B:** Controlled release of a liposome-encapsulated drug by activating the pore-forming activity of an αHL with a built-in switch.

ment in mortality rates for common cancers for decades [Cairns, 1989]. Particularly recalcitrant are metastatic cells that remain after surgery or radiation treatment [Blood and Zetter, 1990]. These cells are often resistant to conventional chemotherapy. Therefore, new therapeutic tools based on biotechnology are being sought. One approach is the use of hybrid cytotoxic agents including immunotoxins, which are toxins delivered to malignant target cells by specific antibodies [Pastan et al., 1992]. Targets may include not only cancer cells but selected cells of the immune system and cells chronically infected with a virus.

We are constructing a new class of immunotoxins, "immunolysins," that incorporate pore-forming proteins such as αHL (Fig. 5A). The constructs are designed to act at the cell surface and disrupt the plasma membrane, killing the cell directly or at least rendering it more permeable to agents such as chemotherapeutics or antisense oligonucleotides. Because many receptor–ligand complexes are poorly internalized by cells (they remain at the cell surface or are shed), immunolysins that act at the plasma membrane would have an important advantage over conventional immunotoxins that must enter the cell. A second tactic, based on the protease-activated trigger described above (Fig. 5B), involves "proimmunolysins" that can be designed for activation by target cells, specifically by tumor-associated proteases [Liotta et al., 1991]. Activation by tumor cells adds a second degree of cellular specificity beyond that conferred by the target-directed antibody. Proimmunolysins attached to irrelevant cells will remain inactive.

A second application that requires control of channel activity in the assembled pore is enzyme or cell encapsulation and controlled drug release [Langer, 1990]. Encapsulated enzymes or cells are being investigated as potential treatments for hormone deficiencies or for metabolic diseases in which the presence of toxic metabolites must be remedied. The encapsulated material

should be immunologically isolated, while small molecules remain free to enter and leave the capsule. We are currently examining the access of substrates to enzymes trapped in liposomes permeabilized with αHL. In principle, the αHL pore is capable of allowing molecules of up to 3000 daltons to pass. By using pores with switches, enzyme output might be controlled. In a related project, attempts are being made to use pores with triggers and switches to release drugs trapped in liposomes.

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