BIOORGANIC CHEMISTRY 23, 340–354 (1995)

REVIEW

Pore-Forming Proteins with Built-in Triggers and Switches

HAGAN BAYLEY

Worcester Foundation for Biomedical Research, Shrewsbury, Massachusetts 01545

Received August 21, 1995

The self-assembling, pore-forming protein α-hemolysin is a monomeric, 293-amino-acid, water-soluble polypeptide that forms heptameric pores of 1- to 2-nm internal diameter in lipid bilayers. By genetic engineering and targeted chemical modification, we have produced α-hemolysins in which pore-forming activity can be triggered or switched on and off by biochemical, chemical, or physical stimuli. These remodeled molecules include protease-activated pores, metal-regulated pores, pores that are activated by chemical alkylation, and pores that are turned on with light. Engineered α-hemolysins have potential applications that include acting as components of sensors for various analytes, mediating the controlled release of drugs and forming building blocks for agents that selectivity damage malignant cells.

ENGINEERING OF α-HEMOLYSIN: A SELF-ASSEMBLING PORE-FORMING PROTEIN

> I have argued in vain against the premature use of the word engineering, on the grounds that the engineer knows what he is doing...
>
> J. R. Knowles (1987)

While considerable effort has been put into the engineering of enzymes and antibodies for applications in biotechnology, membrane channels and pores have been largely ignored from this standpoint. In a foray into this area, my laboratory has been examining remodeled versions of the pore-forming protein staphylococcal α-hemolysin (α-toxin, αHL), which is an excellent "model" system with which to confront the problems that can be encountered in this sphere of protein engineering.

Assembly of Staphylococcal α-Hemolysin

αHL is synthesized by *Staphylococcus aureus* as a monomeric water-soluble polypeptide chain, which forms homooligomeric pores in lipid bilayers, each of which contains seven subunits (for a general review of the properties of αHL see Ref. (*1*)). The effective internal diameter of the pore is 1 to 2 nm and molecules of up to 3000 Da can pass through it. Recently, significant progress has been made

0045-2068/95 $12.00

in the elucidation of the assembly pathway (Fig. 1) (see (*2*) and references therein). Notably, αHL binds to the membrane surface as a monomer. The subsequent insertion of individual amphipathic domains into a bilayer would be strongly disfavored; a problem that is averted in the case of αHL by oligomerization prior to what is likely to be the concerted insertion of seven glycine-rich loops into the bilayer. The transmembrane channel thus formed might be a β barrel, by analogy with the porins (*3–5*), but composed here of 14 rather than 16 or 18 strands. The loop has a central location in the polypeptide chain and, in the monomer in solution, it is exposed to solvent as judged by its accessibility to proteases (*6*, *7*) and hydrophilic chemical reagents (*8*). That the rather hydrophilic central loop proceeds to penetrate the bilayer (*9*) and line the lumen of the transmembrane pore (*10*) is remarkable. Indeed, it is astonishing to those of us brought up with the concept of greasy membrane-spanning helices (*11*), the legacy of the pioneering structural studies on membrane proteins carried out with glycophorin (*12*) and bacteriorhodopsin (*13*).

This review focuses on studies of αHL polypeptides with altered properties, produced by protein engineering, that have proceeded in parallel with studies of the mechanism of assembly of the pore. Importantly, these two aspects of our research have shown reciprocity. Experiments designed to elucidate the mechanism of assembly of αHL have inspired the design of pores with new functions. Con-

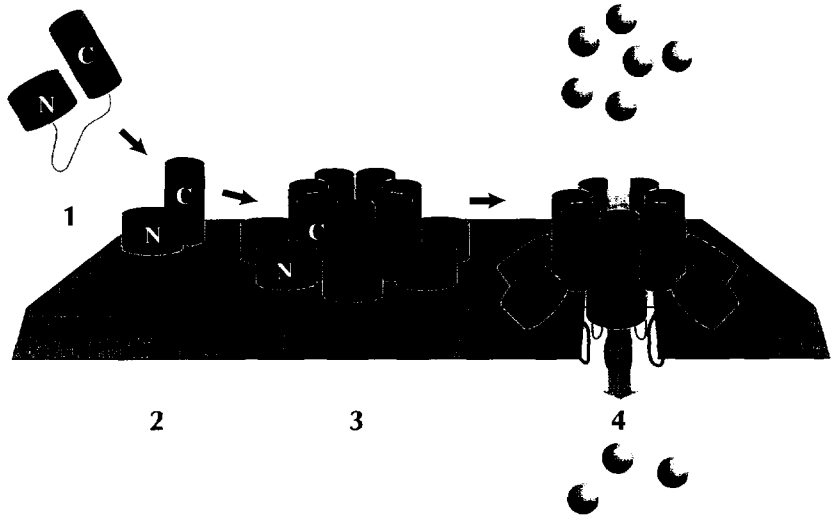

FIG. 1. A recent refinement of a working model for the assembly of α-hemolysin (2) that has provided a useful framework for thinking about the design of pore-forming polypeptides with triggers and switches. The latter have in turn provided information with which to refine the model. α-Hemolysin is secreted by *Staphylococcus aureus* as a protein with a single polypeptide chain **(1)**, which binds to lipid bilayers **(2)** where it assembles into a prepore complex of seven subunits **(3)**. The central loops of the polypeptide chains then penetrate the bilayer where they contribute to the formation of the transmembrane channel **(4)**. Reproduced from Ref. (*2*) by permission of Current Biology Ltd.

versely, pores designed to have applications in biotechnology have provided key information about assembly, which is in turn providing answers to a basic question in biology: "How does a water-soluble protein get into a membrane?" It must be stressed that the assembly pathway (Fig. 1) upon which our attempts at protein engineering have been based remains a working model. Further, the model was by no means as well developed when we started the work as it is now.

Staphylococcal α-Hemolysin as a Favorable Target for Protein Engineering

In terms of remodeling, αHL can be regarded as a large empty warehouse, while mammalian ion channels seem more like cluttered Victorian mansions. The pore formed by αHL is only weakly selective for anions versus cations (*14*) and allows the passage of quite large molecules (*15*, *16*). Further, it is open in normal circumstances (*14*). Therefore, the opportunity exists to introduce preferences for the transport of molecules of defined charge, size, and shape without first removing preexisting appendages responsible for extreme substrate specificity. Again, with a polypeptide such as αHL, it should be possible to introduce gating (as physiologists term the ability of a channel or pore to open and close in response to a stimulus) without first having to eliminate the features that control, say, voltage- or ligand-dependent activation.

αHL has additional advantages for protein engineering with a view to applications in biotechnology (*17*, *18*). It is readily produced in gram amounts and 1 g would form a two-dimensional sheet (which might be used in biosensors as described below) 300 m^2 in area. The polypeptide chain contains just 293 amino acids with no cysteine and few histidine residues (*19*). Therefore, these amino acids can be introduced as sites for targeted chemical modification. While the rate of assembly of the αHL pore is enhanced on certain natural membranes, notably those of the rabbit erythrocyte (RBC) (*20*), self-assembly will occur on phospholipid vesicles (*21*), on planar bilayers (*14*), and in solutions of the detergent deoxycholate (*22*). Self-assembly is crucial for the fabrication of nanostructures (*23*). Further assembly, to form two-dimensional sheets of pores, can take place at high protein to lipid ratios (*24–26*). In comparison to most multisubunit membrane proteins, the pore formed by αHL resists denaturation (e.g., by sodium dodecyl sulfate at up to 66°C (*27*, 28)), although this property might well be improved by chemical crosslinking or genetic selection of yet more robust molecules.

Goals for Engineering α-Hemolysin

An initial goal was to modify the size and selectivity of the αHL pore. Gating was more interesting in terms of biotechnology but we thought it would be tricky in terms of protein engineering. Indeed, we had no idea how it might be achieved, while we had reasonable plans for remodeling the transmembrane channel, once the residues that compose it had been identified. However, by good fortune we quickly learned how to form a biochemical trigger, and this gave us the impetus to examine many other potential built-in triggers and switches. Indeed, we are only just resuming work on size and selectivity.

Here, triggers are defined as built-in devices for turning a protein on in response

to an external stimulus. While a *trigger* mediates an irreversible response, the change brought about by the operation of a *switch* can be reversed. Three modes of triggering and switching are discussed here, namely those mediated by biochemical, chemical, and physical means. All three approaches have succeeded with αHL and although all have precedent in other areas, notably enzymology, we have made innovations, in some cases to tackle the difficulties presented by the lack of a three-dimensional protein structure. The mere use of the term protein engineering has been reprimanded on the grounds that the biochemist lacks the sophisticated analyses available to the true engineer (*29*). Accordingly, though far more modest in their goals, the present studies have been executed in the spirit of the cathedral builders of medieval Europe, many of whose efforts fell.

Engineered pore-forming proteins with triggers and switches have potential applications in basic science, therapeutics, sensor technology, and materials science. There is no section on applications here. Instead, specific examples are introduced throughout the text.

A BIOCHEMICAL TRIGGER

Protease-Activated Triggers from Complementation Mutants

Our first success in controlling of the activity of αHL was to construct a protease-activated trigger (*30*). The concept stemmed from studies of the effects of mutagenesis on the function of the central loop of αHL. We had surmised that the loop was important in pore formation because it became completely protease resistant when αHL bound to membranes (*7*). Deletion mutagenesis is often used to make a preliminary linear map of the functional attributes of a protein. However, small truncations at either the N or C terminus of αHL destroy the pore-forming activity of the protein (*7*). Therefore, this approach was of no value for examining the central loop. Furthermore, internal deletions in proteins often cause improper folding. Therefore, we used molecular complementation mutagenesis to generate nicks, gaps, and overlaps in the loop region (Fig. 2A). Molecular complementation had been used as early as the 1950s by Richards in his classic studies of ribonuclease (*31*), but it is now greatly facilitated by modern techniques of gene manipulation. The results with αHL are described in detail elsewhere (*32*). Relevant to issues

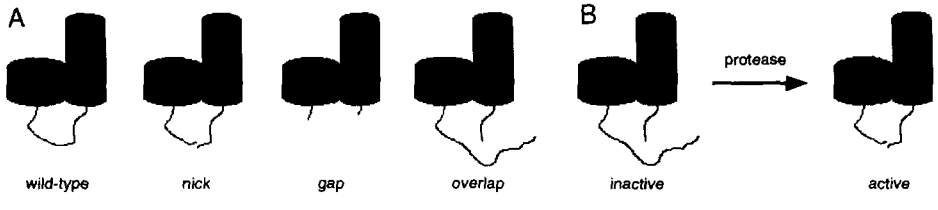

FIG. 2. (A) Schematic depicting nicks, gaps, and overlaps in the central loop of αHL. (B) A biochemical trigger: activation of an overlap mutant by limited proteolysis.

considered here, we found that nicks near the center of the loop did not affect the pore-forming activity of αHL, while other alternations destroyed activity, although the mutant proteins often retained the ability to oligomerize. Therefore, we realized that overlap mutants might be activated by removing the redundant pieces of polypeptide chain from the loop region by limited proteolysis (Fig. 2B). It can be noted that various natural toxins are activated by proteolysis, although the details of the mechanisms differ from that designed into αHL. For example, the pore-forming aerolysin is activated by removal of approximately 45 amino acids from the C terminus (*33*).

Selective Activation by Proteases

Selective activation of αHL was investigated for proteases that cleave after lysine and arginine, residues with positively charged side-chains (*30*). First the unwanted Lys-8 was converted to alanine by point mutagenesis. Cleavage after this residue inactivates αHL (*7*, *30*), most likely by interfering with a conformational change at the N terminus that occurs in concert with insertion of the central loop into the bilayer (Fig. 1) (*2*, *7*). In a second step, overlaps of 11 to 53 amino acids were generated in the central loop and contained Lys or Arg at position 131. Lys-131 is the naturally occuring residue and, besides Lys-8, is the only site of 38 positions with Lys or Arg that is readily accessible to proteases in wild-type αHL. The Lys-131 overlap mutants were activated for lytic activity toward rabbit RBC by endoproteinase Lys-C, a lysine-specific protease, but unaffected by clostripain, an arginine-specific protease. The complementary pattern of activation was found for Arg-131 overlap mutants. Trypsin, which cleaves after both Lys and Arg, activated both classes of mutants. Therefore, we have been able to introduce enzyme-specific protease-activated triggers into αHL.

Polypeptide Chain Ligation by Enzyme-Mediated Transpeptidation

During the course of these studies, we found that protease-treatment of certain overlap mutants resulted in coupling of the two halves of the polypeptide chain in an efficient transpeptidation reaction to form a single polypeptide with the length of the wild-type molecule (*30*, *34*). We are currently exploiting this finding to couple halves of αHL that have been labeled with different spectroscopic probes. For example, by labeling two residues with different fluorescent probes, the distances between the two sites might be determined at various stages of assembly.

Potential Applications of Protease-Activated α-Hemolysin

Protease activation of engineered αHL has several potential applications. For example, a combinatorial cassette placed in the overlap can be used as an alternative to phage display (*35*) for the elucidation of protease-recognition sites (R. G. Panchal, unpublished data). Hemolysins activated by proteases might be used for the selective permeabilization of malignant cells, e.g., metastatic cancer cells, which secrete tumor

proteases. The permeablized cells would be rendered susceptible to cytotoxic drugs unable to penetrate normal cells. Combinatorial cassette mutagenesis might be used to select hemolysins that are more readily or more selectivity activated by tumor proteases. Were it not for the striking lack of progress in cancer therapy over the last 50 years (*36*), such an approach might be regarded as unduly speculative. However, there is clearly a need for alternative therapies based on biological molecules.

CHEMICAL TRIGGERS AND SWITCHES

Triggering Protein Activity by Covalent Modification

Targeted chemical modification of cysteine has been used to restore the activity of several mutant proteins. In most cases, the cysteine replaces and thereby neutralizes a charged residue found in the active wild-type protein. Chemical modification can then be used to restore the charge and, in favorable cases, the activity of the protein. For example, Hartman and colleagues restored the activity of ribulosebisphosphate carboxylase by using aminoethylation of cysteine to mimic lysine (*37*) and carboxymethylation of cysteine to mimic glutamate (*38*). In our studies, we have found that treatment of a cysteine residue with iodoacetamide to produce carboxamidomethylcysteine, which mimics histidine, restores pore-forming activity to a mutant αHL.

Triggering αHL Activity by Covalent Modification at Position 35

The cysteine at issue replaces His-35, which is vital for pore-forming activity. While many studies of αHL have concentrated on the glycine-rich central loop, the N-terminal region of the protein has also been implicated in the final step of assembly and undergoes a change in accessibility to proteases as the central loop enters the lipid bilayer (Fig. 1, **3** → **4**) (*2*). Of residues near the N terminus, His-35 is remarkable for its sensitivity to substitution (*8*, *39–41*). Cys-35 in the inactive single-cysteine mutant H35C was found to be highly reactive when the monomeric protein was treated in solution with the water-soluble alkylating agent 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonate (IASD). By contrast, Cys-35 became inert after H35C bound to rabbit RBC membranes, either as monomer or oligomer, suggesting that Cys-35 (and by inference His-35 itself) becomes buried in the bilayer or within the structure of the membrane-bound protein (*8*).

We found that modification of H35C with iodoacetamide generated significant pore-forming activity (*28*). The closely related polypeptides H35N and H35Q had, respectively, no activity and greatly reduced activity. Unmodified H35C is defective in the final step of pore formation (Fig. 1, **3** → **4**) and it is this problem that is remedied after cysteine modification. The modified residue, *S*-carboxamidomethylcysteine, mimics histidine in volume, polarity, and hydrogen-bonding potential. However, its inability to ionize rules out protonation of His-35 as an essential step in pore formation.

Covalent triggering of protein activity might have practical implications. In the

case of αHL, it might be possible to engineer pores as detectors for chemical warfare agents or organomercurials that react selectivity with a cysteine presented in a suitable binding pocket.

αHL–H5, A Hemolysin with a Metal-Actuated Switch

We have also been able to build a metal-actuated switch into αHL (*10*). Knowing that the central loop was essential for proper pore formation, we decided to place five consecutive histidines into it in the hope that divalent cation binding at this site would upset pore-forming activity. A pentahistidine sequence should extend at least two imidazole rings in an arrangement likely to bind Zn^{2+} and certain other divalent cations, no matter what the conformation of this segment of the polypeptide chain (*42*). By using this simple idea, we hoped to forgo the extensive exploration that might be required to introduce two suitably placed histidines by trial and error or combinatorial mutagenesis. Despite the forethought, we were indeed fortunate that the approach worked at the first attempt (*10*). The mutant αHL-H5 (Fig. 3), in which residues 130 to 134 inclusive are each replaced with histidine, has both provided a prototype sensor component and been tremendously helpful in revealing details about the mechanism of assembly of αHL, a gratifying congruity between biotechnology and basic science.

αHL–H5 and the Mechanism of Assembly of the α-Hemolysin Pore

In studies that clarified aspects of the mechanism of assembly of αHL, we found that Zn^{2+} and related divalent cations acted on αHL–H5 in two ways (*2*, *10*). First, Zn^{2+} was able to block the transmembrane channel in the fully assembled pore (Fig. 1, **4**) by acting from either side of the lipid bilayer. This strongly suggests that at least two histidines in the pentahistidine sequence project into the lumen of the channel. Indeed, this experiment provided the first secure identification of channel-forming residues (*10*). Second, Zn^{2+} could block the final irreversible step in assembly and thereby allow examination of the oligomeric prepore (Fig. 1, **3**). For example,

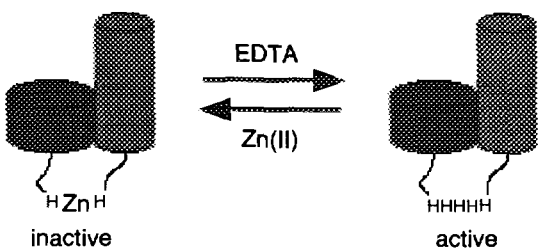

FIG. 3. A metal-actuated switch. When Zn^{2+} is bound to αHL–H5, a mutant hemolysin containing a pentahistidine sequence in the central loop, the protein is inactivated. αHL–H5 can be reactivated by treatment with a chelating agent, such as EDTA. Details of the mechanism of inactivation of αHL–H5 by Zn^{2+} are discussed in the text.