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6. AUTHOR(S)
Hagan Bayley

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical Biochemistry and Genetics
440 Reynolds Medical Bldg.
1114 TAMU
Col ge Station, TX 77843-1114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
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Stochastic sensing with engineered pore-forming proteins is a new technology for analyte detection at the single molecule level. The approach has been developed by using divalent metals (M(II)) as analytes. M(II) are essential or toxic components of seawater, effluents, and biological fluids. The sensitivity of stochastic sensing was found to be in the nanomolar range. Further, each analyte provides a distinctive signature allowing its identification, and several related analytes can be distinguished in mixtures. In addition, stochastic sensing was demonstrated to be a versatile technology for the detection of many additional classes of analytes of DoD interest, e.g. organic molecules and proteins. Spin-offs of the work include applications in mammalian cell preservation.

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FINAL REPORT

GRANT #: N00014-97-1-0228

PRINCIPAL INVESTIGATOR: Dr. Hagan Bayley

INSTITUTION: The Texas A&M University System Health Science Center

GRANT TITLE: Genetically Engineered Pores for Sensing Metal Ions

AWARD PERIOD: 1st January 1997- 30 September 2000

OBJECTIVE: To discover engineered variants of a self-assembling pore-forming protein, staphylococcal α-hemolysin (αHL), which can be used as components of sensors for M(II).

APPROACH: Analyte-sensitive pores will be constructed by mutagenesis and targeted chemical modification of αHL. Complete control over pore structure will be achieved by employing a new approach for making hetero-heptameric pores. Prototype M(II)-sensitive pores will be tested for function after incorporation into conventional or stabilized planar bilayers.

ACCOMPLISHMENTS: The ability of single sensor elements to identify and quantify multiple analytes at the same time was examined. Our prototype heteromeric pore for the detection of M(II), $WT_6 4H_1$, contains six wild-type subunits and a seventh subunit with a single M(II)-binding site formed by four engineered histidines. The single channel current of $WT_6 4H_1$ in planar bilayers is modulated by several different divalent metal ions M(II), each of which produces a characteristic signature. Stochastic sensing with single pores $WT_6 4H_1$ has now been used to distinguish and quantify metal ions in mixtures containing up to four different M(II), e.g. Zn(II), Ni(II), Co(II) and Cd(II).

In a spin-off from the sensing technology, metal-sensitive pores have been used for the reversible permeabilization of living mammalian cells (with Mehmet Toner, Harvard Medical School). αHL-H5, a mutant of αHL that is completely (but reversibly) closed by divalent metal ions (such as Zn(II)) was tested and shown to be useful for this purpose. αHL-H5 is now being used by many other groups for cell biology experiments in which the reversible permeabilization of cells is required. An important application will be in cryopreservation, and this aspect has been developed in Toner's laboratory. Cryoprotectants, such as sucrose and trehalose, are allowed to equilibrate with the interior of cells through opened pores that can subsequently be shut with low concentrations of ZnII) before the cells are

frozen. After this treatment, cells show far greater viability upon thawing than cells treated by conventional cryoprotection procedures. Recently, a similar procedure has been used to protect mammalian cells from desiccation.

αHL-H5 was also used in experiments carried out in collaboration with Jie Yang (U. of Vermont) in which the aspects of the assembly of αHL were investigated by atomic force microscopy. When Zn(II) is added to αHL-H5 at the beginning of the assembly process, pore formation becomes arrested at the pre-pore stage. By using this as a strategy, it could be shown that, like the fully assembled pore, the pre-pore is a heptamer with its seven-fold axis perpendicular to the plane of the lipid bilayer.

In collaboration with other groups, various ways of making more robust bilayers for sensor applications have been tested. They include bilayers supported with two-dimensional porous protein crystals, S layers (with U. Sleytr), and bilayers on solid supports (with J. Kasianowicz). Further developments in this area are necessary and the work will be continued.

In additional engineering experiments, a version of αHL was made in which the transmembrane domain was deleted. This form of the protein assembled spontaneously into a ring of seven subunits in the same conformation as the subunits in the fully assembled pore. A αHL in which the sequence of amino acids in the transmembrane domain was reversed was also made. In this radical example, the "retrosequences" comprise a total of 175 amino acid residues, twenty five contributed by the central domain of each subunit of the heptameric pore. Remarkably, the properties of wild-type and retro heptamers in planar bilayers are similar. Structural considerations suggest that the ability to form a retro domain could be diagnostic of a beta barrel, explaining, for example, the activity of the retro forms of many membrane-permeabilizing peptides. By contrast with the wild-type subunits, monomeric retro subunits undergo premature assembly in the absence of membranes. A technique requiring translation in the presence of membranes was devised for obtaining heteromeric pores containing both wild type and retro subunits. The technique is generally applicable to heteromers required as sensor elements that must be formed from unstable subunits. These experiments demonstrated extremes with which the transmembrane domain can be engineered and thus increased the versatility of αHL as a target for protein engineering. Work was also initiated on OmpG, a monomeric porin, with the idea of mitigating some of the difficulties associated with the engineering of a heptameric pore.

Recently, stochastic sensing was demonstrated by αHL equipped with a cyclodextrin as an internal non-covalent

molecular adapter, to mediate channel block. The approach is effective for identifying and quantifying a wide range of organic molecules. As in the cases of M(II) detection, a single sensor element can be used to analyze mixtures of organic molecules. Because different adapters can be used and mutant αHLs can be made to accommodate them, the system is programmable. Cyclic peptides are an example of additional adapters that have been demonstrated to work. They may prove to be especially useful because better adapters can be selected from large peptide libraries.

Finally, protein pores modified internally by the covalent attachment of polymers have been assembled. These pores are of great interest for the sensing of macromolecules such as proteins. To achieve this, a ligand was placed at the the free end of the polymer. The ligand could be captured by a target protein in the bulk aqueous phase, thereby "transmitting" a signal to the interior of the protein through the polymer chain. The rather noisy signal stemming from polymer movement inside the pore became quiet after capture, thus forming the basis of a protein detection method that operates at the nanomolar concentration level.

CONCLUSIONS: The proposed work on engineered pores for the detection of M(II) was largely completed. One of these pores could also be used for the reversible permeabilization of mammalian cells, and therefore to help load the cells with sugars to aid in cryoprotection and desiccation. New ways of engineering β barrels were also tested, resulting in dramatic changes that strengthen the use of αHL as a versatile target for engineering. In addition, ways to detect organic molecules by stochastic sensing were developed by using non-covalent molecular adapters. Further, protein analytes were detected by using ligands tendered by the pore at the end of long polymer chains.

SIGNFICANCE: During the course of this project, engineered variants of αHL were explored as components of sensors for M(II), which are essential or toxic components of seawater, effluents, and biological fluids. In addition several other lines of research were initiated that established stochastic sensing as a versatile technology for the detection of many classes of analytes of DoD interest. Sensitivity is in the nanomolar range. Each analyte provides a distinctive signature allowing its identification. Spin-offs of the work are apparent in other areas including cell preservation.

PATENT INFORMATION:

US Patent No. 5,777,078: Triggered pore-forming agents (issued July 7th, 1998)

US Patent No. 5,817,771: Cell-targeted lytic pore-forming agents (issued October 6th, 1998) Continuation filed January 29th , 1998.

US Patent No. 5,824,776: Cell-targeted lytic pore-forming agents (issued October 20th, 1998) Continuation filed January 29th , 1998.

Stochastic sensing mediated by carrier molecules (November 1998, provisional patent filed; November 1999, full patent filed)

Biosensor compositions and methods of use (February 2000, provisional patent filed; February 2001, full patent filed)

AWARD INFORMATION: none

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