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Department of Medical Biochemistry and Genetics

Faculty Search Committee,
c/o Professor James Glazier,
Department of Physics,
University of Indiana,
Swain Hall West 117,
Bloomington, IN 47405-7105

Ref: Opening for tenure-track faculty

September 25th, 2003

Dear Chair of the Search Committee,

I have enclosed a full application for an opening as an Assistant Professor within your outstandingly equipped department. My application includes the following documents:

1. Extended CV + List of publications
2. Five representative publications
3. Research plan/interests
4. Summary of past research
5. List of six references
6. Statement of teaching philosophy

My expertise covers areas of membrane biophysics, biophysical chemistry and electrophysiology, including single-channel recording techniques in planar lipid bilayers. Specifically, my laboratory will use single-molecule detection techniques, membrane protein engineering and other biochemical and biophysical methods to characterize the interaction of polypeptides or other biopolymers with large transmembrane β-barrel channels.

I look forward to hearing from you,

With kind regards,

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Summary of past research

Liviu Movileanu, PhD
The Texas A&M University System Health Science Center

A. Interrogating transmebrane protein pores with tethered and untethered polymers - Fundamental science and nanobiotech

Staphylococcal α-hemolysin (αHL) has been a useful model system with which to test new approaches for engineering membrane proteins. The αHL monomer of 293 amino acids assembles on lipid bilayers to form a mushroom-shaped heptameric pore. We have been able to use single-channel electrical recording to observe current fluctuations associated with the attachment of a single polymer, a 3 or 5 kDa polyethylene glycol (PEG) molecule[1;2]. In addition to the protein engineering, we considered that this work is of interest in two areas: single molecule detection and the development of new biosensors. In the area of biosensors, significant progress has been made in developing protein channels and pores as sensor elements. According to this concept, analyte molecules modulate the ionic current passing through the pores under a transmembrane potential. For example, binding sites can be engineered into pores expressly for capturing analyte molecules, which act as partial channel blockers. The approach yields both the concentration and identity of an analyte, the latter from its distinctive current signature[2]. The procedure that we developed represents a major step towards using responsive polymers for stochastic sensing (see summary of research plan).

We were able to tether a 3.4 kDa PEG chain at a defined site within the lumen of the transmembrane protein pore formed by αHL. The free end of the polymer was covalently attached to a ligand, in this case a biotin molecule (Fig. 1).

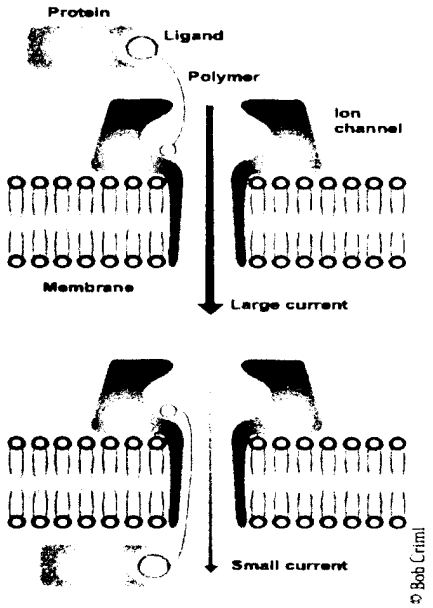

Figure 1 *When the modified pore is incorporated into a lipid bilayer, the biotinyl group moves from one side of the membrane to the other, as detected by reversible captures with a mutant streptavidin. We have observed the capture events as changes in ionic current passing through single pores in planar bilayers. The modified pore represents a new type of biosensor element with which proteins in solution can be detected at nanomolar concentrations.*

Thus, the newly engineered pore comprises an unusual nanostructure with a moveable part. The system we have developed also has potential for the examination of the

dynamics of polymers other than PEG at the single molecule level, including biological molecules such as polynucleotides, oligosaccharides and peptides. Furthermore, we have shown that pores with tethered functionalized polymers can be used as components for stochastic sensing of macromolecules in solution[2]. The approach has advantages over other kinetic measurements, such as those obtained by surface plasmon resonance, which have lower time resolution.

We have also combined cysteine scanning mutagenesis and chemical modification with sulfhydryl-reactive polymers to locate the constriction in the lumen of the αHL pore, a model protein of known structure[3]. In addition, we discovered that PEG chains gave greater reductions in pore conductance when covalently attached to the narrower regions of the lumen, permitting further definition of the interior of the pore. The reagents might be applied to a variety of pores including additional pore-forming toxins, the outer membrane porins of gram negative bacteria and components of the immune system such as perforin and the membrane-attack complex of complement. The reagents might also be used to examine the vestibule geometry of ligand- and voltage-gated ion channels.

The penetration of neutral, flexible, water-soluble polymers into pores from dilute solution is of fundamental importance for a variety of practical applications including ultrafiltration, gel permeation chromatography, and gel electrophoresis. We examined the behavior of PEG chains by analyzing the rates of reaction of monomethoxy-poly(ethylene glycol)-*o*-pyridyl disulfide (MePEG-OPSS) reagents with cysteine sulfhydryl groups in the lumen of the αHL pore[4]. We have studied the dependence of the rate on polymer mass for the reaction of four sulfhydryl-directed PEG reagents with cysteine residues located in the lumen of the staphylococcal αHL pore. The logarithms of the apparent rate constants for a particular site in the lumen were proportional to N, the number of repeat units in a polymer chain. The measured reaction rates yield an estimate of the diameter of the pore and might be applied to determine the approximate dimensions of cavities within other similar proteins[4].

Finally, we employed single-channel electrical recording to probe the partitioning of single unmodified PEG molecules, at concentrations near the dilute regime, into the transmembrane β barrel of individual protein pores formed from staphylococcal αHL[5] (Fig. 2). The association rate constants (k_{on}) were strongly dependent on polymer mass, and values of k_{on} ranged over two orders of magnitude. By contrast, the dissociation rate constants (k_{off}) exhibited a weak dependence on mass, suggesting that the polymer must be largely folded at the barrier for entry into the pore. The values of k_{on} and k_{off} were used to determine partition coefficients (Π) for the PEGs between the bulk aqueous phase and the pore lumen. The low values of Π are in keeping with a negligible interaction between the PEG chains and the interior surface of the pore, which is independent of ionic strength. We have shown that PEG molecules partition into the αHL pore with a dependence on mass that is at least approximated by a simple scaling law. For PEG molecules larger than ~ 5 kDa, Π reached a limiting value suggesting that these PEG chains cannot fit entirely into the β-barrel region of the pore. In this work, we set out to reconcile the results obtained from studies with free PEGs at high concentrations and chemical modification with sulfhydryl-directed PEGs, by examining the interaction of dilute free polymers with the αHL pore by using single channel-recording at high time resolution.

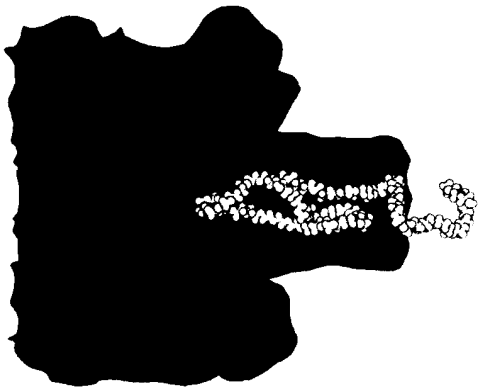

Figure 2 *A cross-sectional picture of the αHL protein pore with a single PEG-2.0kDa chain located in the transmembrane region of the protein.*

B. Kinetics of double-stranded DNA probed at the single molecule level

Duplex formation by complementary RNA or DNA strands is a fundamental biochemical process. A single oligonucleotide was covalently attached to a genetically engineered subunit of the heptameric protein pore αHL to allow DNA duplex formation inside the pore lumen[6] (Fig. 3). We have employed single-channel current recording to study the properties of the modified pore. Upon addition of an oligonucleotide with a sequence complementary to the tethered DNA strand, current blockades with durations of hundreds of milliseconds occurred representing as hybridization events of individual oligonucleotides to the tethered DNA strand.

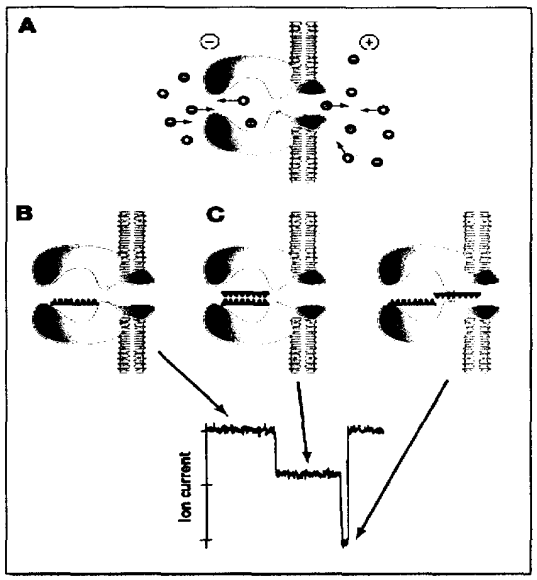

Figure 3 *Attachment of charged DNA polymers in the vestibule of the αHL nanopore: (A) ions move across the nanopore following an application of a voltage bias through the membrane. (B) a small DNA oligomer is attached within the large vestibule of an αHL nanopore. (C) a complimentary DNA strand is added in the cis bath and forms a duplex DNA assembly in the nanopore. The right-hand panel illustrates the dissociation of the anti-sense DNA strand from the tethered oligonucleotide*[7]

We were able to estimate the kinetic constants for DNA duplex formation at the single molecule level from the analysis of "on" and "off" events detected by electrophysiological measurements. These results are in excellent agreement with macroscopic kinetic data, demonstrating profound implications of DNA-conjugated nanopores for kinetic studies. DNA duplex formation in a single modified αHL pore also allows the exploration of the macroscopic concept of melting for individual DNA

molecules[6]. By investigating the temperature dependence of DNA duplex formation at the single molecule level, the standard entropy and enthalpy of the interaction could be inferred.

C. Plasticity of oligomer assembly in membranes studied by chemical modification and gel shift electrophoresis

Staphylococcal leukocidin pores are formed by the obligatory interaction of two distinct polypeptides, one of class F and one of class S, making them unique in the family of β-barrel pore-forming toxins (β-PFTs). By contrast, other β-PFTs form homooligomeric pores; for example, the staphylococcal αHL and *Bacillus Cereus* hemolysin II are homoheptameric pores. We were able to demonstrate the subunit composition and stoichiometry of a leukocidin transmembrane pore by two independent methods: gel shift electrophoresis and site-specific chemical modification with methanethiosulphonate reagents (MTS) during single-channel electrical recording[8]. Four LukF and four LukS subunits co-assemble to form an octameric transmembrane pore (Fig. 4). This result in part explains properties of the leukocidin pore, such as its high conductance compared to the αHL pore.

Knowledge of the subunit composition of the leukocidin pore raises interesting questions about the assembly process. In the case of αHL, monomers first form a heptameric prepore. Nothing is known about intermediates in heptamer formation. For example, the subunits may come together through random collisions or through the successive addition of individual subunits until a ring is completed.

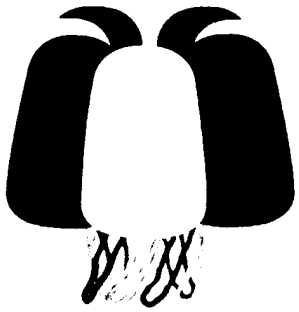

Figure 4 *Stoichiometric model of a bi-component transmembrane protein pore of Leukocidin. Knowledge of the subunit composition also opens up new prospects for the engineering of β-PFTs.*

D. Probing DNA thermostability by difference Raman spectroscopy

We have developed procedures for the determination of thermodynamic parameters governing base stacking (ΔG_{st}), van't Hoff premelting (ΔH_{vH}^{pm}, ΔS_{vH}^{pm}) and van't Hoff melting (ΔH_{vH}, ΔS_{vH}, $\Delta G_{vH(25°C)}$, $\langle n_{melt} \rangle$) transitions in DNA of defined base sequence using the data of temperature-dependent Raman spectra[9;10]. The Raman melting profiles were recorded with a spectrometer of high spectral precision and sensitivity. We have shown that temperature-dependent Raman spectra of poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) have the capability to distinguish contributions of A and T bases and of backbone moieties to the thermodynamic stability of *B* DNA[10]. The results affirmed and quantified melting and premelting phenomena in DNAs containing $(dA)_n \cdot (dT)_n$ and $d(AT)_n$ tracts. The findings suggested that A and T melting events may be less coupled in the homopurine and homopyrimidine tracts of poly(dA)·poly(dT) than in the alternating A/T tracts of poly(dA-

dT)·poly(dA-dT). The results delineate three molecular mechanisms contributing to DNA stability: base stacking, which is highly cooperative and extensively perturbs vibrational states of base and sugar-phosphate moieties; base pairing, which perturbs vibrational states of localized base sites and is also coupled to base stacking; and backbone conformational ordering, which is highly uncooperative and dominates the premelting interval. We anticipate that the procedures developed in this project should be applicable to other DNA sequences and to specific DNA-ligand complexes.

E. Stochastic pores in a bilayer lipid membrane

Thermally induced oscillations of individual phospholipids, perpendicularly oriented to planar bilayer membranes, are converted into thermal collective motions due to their intermolecular interactions. These thermal collective motions are able to induce local deformations of the lipid bilayer. We demonstrated that, if the amplitude of elastic deformation is equal to the hydrophobic thickness of the monolayer, then a transbilayer stochastic pore could be generated via thermal collective motions. Using the elastic theory of continuous media applied to smectic liquid crystals A, we were able to estimate the pore radius and energetic requirements for its formation[11]. Three types of thermally induced transbilayer pores could be formed across a bilayer lipid membrane: open and stable, open and unstable, and closed. Closed pores are present throughout the cases that we have inspected. As a matter of fact, the formation of closed pores confirms an old hypothesis concerning the existence of "little water threads" into the hydrophobic region of the lipid bilayers. The effect of hydrophobic thickness, polar headgroup size of phospholipids, temperature, surface tension, and phospholipid-phospholipid interactions upon the pore formation was examined in detail[11].

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Plans for Future Research

Macromolecular traffic across transmembrane protein pores

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Abstract

The transport of proteins through a nanoscale pore in a membrane is a fundamental process in biology. While translocation systems have been investigated heavily by molecular biology techniques, biophysical studies have been lacking so far. We propose single-molecule biophysics experiments on protein translocation across a membrane pore of nanoscale dimensions. Specifically, my laboratory will use single-molecule detection techniques (single-channel electrical recording and single-molecule fluorescence microscopy), membrane protein engineering and other biochemical and biophysical methods to characterize the interaction of polypeptides with large transmembrane β-barrel pores. Once a polypeptide molecule enters the pore, the electrical current flowing across is drastically reduced. Signatures of the electrical trace thus represent the current fluctuations through a single nanopore, revealing the protein translocation on- and off-rates describing the kinetics. Our recent studies regarding translocation of other polymers through the pore demonstrate that single-channel electrical recordings of these events are indeed feasible at high timescale resolution. A wealth of options exists for engineering of the exact structure of the pore complex. With our measurements we intend to (1) obtain a detailed understanding of the kinetics describing the steps in protein translocation across a membrane pore; (2) understand the structural changes of the protein for its translocation across the pore; and (3) resolve biophysical features such as the diffusion and partitioning of proteins through membrane pores inferred at the single-molecule level. The outcome of our single-molecule experiments is expected to bridge the gap between the physics of macromolecular traffic through narrow transmembrane pores and the biology of polypeptide transporter systems.

Key words: membrane protein; peptide translocation; single-channel current recording; single-molecule fluorescence microscopy; peptide folding; single-molecule biophysics; protein-protein interaction

Significance

Biomacromolecules can be transported across a membrane by leading them in a linear fashion through a tiny pore of nanoscale dimensions. *How* exactly such large molecules diffuse across transmembrane pores is still a mysterious process. The transport of macromolecules through transmembrane proteins is of fundamental importance in biology. Examples include phage DNA injection into bacteria, protein translocation in the endoplasmic reticulum, the passage of proteins and RNAs through the nuclear pore complex, and the traffic of proteins from the cytoplasm into mitochondria. The importance of macromolecular transport processes is accentuated by a number of associated diseases, such as cystic fibrosis.

Kinetic measurements of protein transport rates through the membrane protein pores suggest several models. Most of them propose that protein translocation across membrane is driven by biased random thermal motion. This so-called "Brownian-ratchet" process depends on the electrochemical asymmetries between both sides of the membrane. Several factors could contribute to biasing the thermal motion of the protein, such as binding and dissociation to the pore lumen, chain coiling induced by pH and/or ionic gradients, disulfide bond formation,

binding and dissociation of chaperonins to the translocating chain etc. A good understanding of the mechanism of translocation through the membrane protein pore is still far away. However, the progress in protein synthesis and purification, the knowledge about the crystal structure of these membrane proteins, and in particular the very recent advances in single-molecule technologies present a major opportunity for the study of polypeptide traffic across a protein pore of nanoscale dimension.

Importantly, protein translocation systems represent a class of transmembrane transporters that have not yet been investigated by single-molecule techniques. Hence, there are exciting opportunities for the discovery of detailed mechanistic information. The wealth of biochemical knowledge about the translocation systems makes them particularly well suited for adaptations of single-molecule technology.

Goal of the project

My goal is to obtain a detailed kinetic profile describing the steps in protein translocation across a transmembrane protein pore. Protein translocation across membranes raises a number of challenging questions. First, what provides the driving force to move the protein across the membrane? Secondly, how does the protein interact with the lumen of the protein-conducting pore? Finally, how does the protein behave inside the pore? I propose to address these issues in my research program.

When one considers a protein that moves across a nanopore as a result of the thermally driven random walk, one must take into account many factors: the extended geometry and flexibility of the polypeptide chain, the constraint of the nanopore on the entropic configurations of the translocating polypeptide, as well as the electrochemical asymmetries across the membrane. Single-molecule biophysics experiments can be used to address many of these issues. To reveal the kinetics of interactions between a translocated polypeptide and the pore, we will use single-channel electrical recording in planar bilayer membranes. Planar bilayers allow very precise quantitative measurements at the single-molecule level[1-3]. In addition, the electrical recordings in planar bilayers demonstrated the capability to reveal unprecedented detail of polymer behavior in nanopores that is not available with ensemble-average measurements[4-12]. In our work, single-channel current will be measured in the absence and presence of a translocating polypeptide (Fig. 1A-D). The translocation process can be probed by the changes in current that represent multiple steps in a complex kinetic scheme. We have been motivated to use single-channel recording to study polypeptide translocation for the following issues:

(1) Because of its sensitivity, many subconductance states can be detected, which will provide detailed information about the frequency and type of interactions between polypeptides and the pore, residence times for the polypeptides within the pore, "on" and "off" rate constants, etc.

(2) The protein pores that we will investigate are of known three-dimensional structure and exhibit prolonged event-free single-channel currents in the absence of translocated polypeptides (Fig. 1C) for wide external conditions such as electrochemical gradients, pH, temperature etc.

(3) Transport processes can be readily devised, with different potential thermodynamic driving forces for the translocated polypeptides that include transmembrane potential, transmembrane difference in pH, ionic strength, disulfide-thiol exchange or other electrochemical gradients, as both sides of the membranes are fully accessible.

(4) Both the lipid bilayers and the membrane protein pores are stable for long periods (several hours), so a low flux of protein can be adequately quantified.

By contrast, the fine details of transport would be impossible to ascertain with alternative systems, such as proteoliposomes.

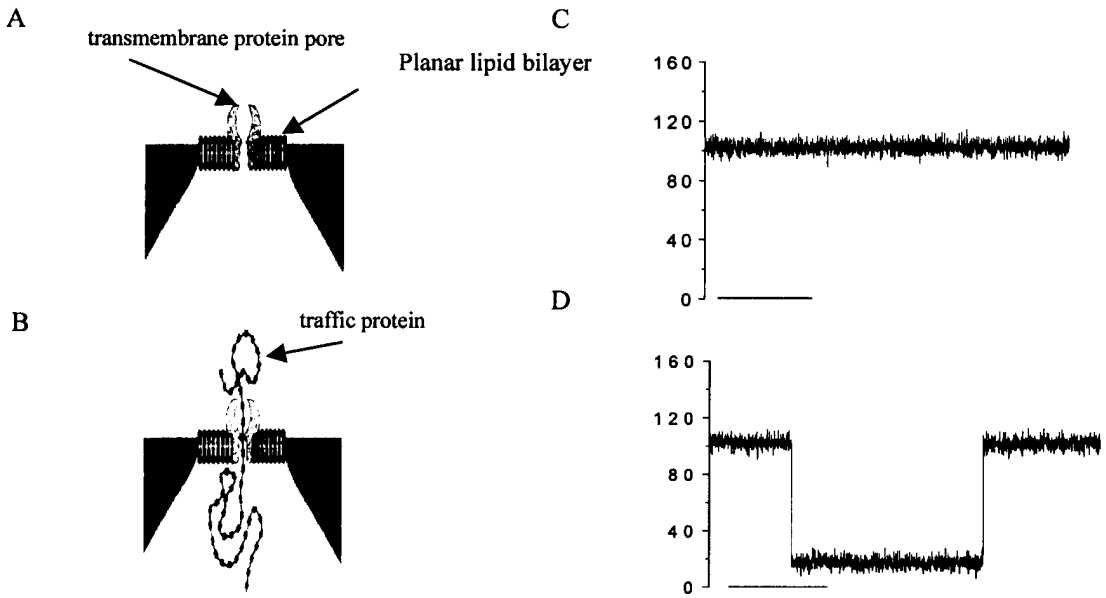

Figure 1 Macromolecular translocation across protein nanopores reconstituted in a planar lipid bilayer, as probed by single-channel recordings. A lipid bilayer forms a nearly perfect insulating membrane between two electrolyte-filled chambers: (A) Reconstitution of a transmembrane protein pore in a planar lipid bilayer; (B) Partitioning of a polypeptide into the protein nanopore and its translocation through it; (C) The right-hand single-channel trace shows an "event-free" signal, which reflects an "unperturbed" ionic flow across the channel lumen; (D) The single-channel current blockade indicates an interaction of the translocating protein with the transmembrane pore.

Specific Aims and Research Design

A. Interaction of the polypeptides with large transmembrane β-barrel channels

A1. The nanopore

The proteins transported by the various protein translocation machineries found in cells may be hydrophobic, negatively charged or positively charged. Therefore, the translocation machineries must be quite promiscuous. There have been no systematic studies to evaluate the rates of protein translocation, and no quantitative work has been done to determine the means by which protein transport is driven. It is very important that the structure of the interior of this protein pore is known.

Primarily, I intend to pursue a simplified system in which polypeptides are transported through a model aqueous pore (Fig. 1 A-B) formed by a large transmembrane β-barrel protein, staphylococcal α-hemolysin (αHL). This system is consisted of a reconstituted bilayer lipid membrane with a single aqueous protein pore (Fig. 1A). The αHL pore is a heptameric protein of known crystal structure, which forms pores in lipid bilayers (Fig. 2)[13]. There are two distinct regions of the lumen of pore. On the cis side of the bilayer (Fig. 2C), the protein contains a large cavity, which measures ~46 Å in internal diameter and is entirely located outside the membrane. In the transmembrane domain, the channel lumen narrows to form a 14-stranded β-barrel with an average internal diameter of ~20 Å[13]. The two domains are separated by a constriction with a diameter of 15 Å (Fig. 2C).

The protein transport will be monitoring by single-channel recordings. The pore complex allow the energy-independent passage of solutes across a membrane. Concentration-, pH-, and voltage-gradients can be applied. Alternatively, translocation can be driven by refolding or protein-protein interactions on the trans side of the bilayer. In most of the examples we will be exploring, the diameter of the aqueous lumen of the pore will be about 20 Å, so that the passage of a protein can proceed only in its unfolded conformation (Fig. 3, panels (2) or (3)).

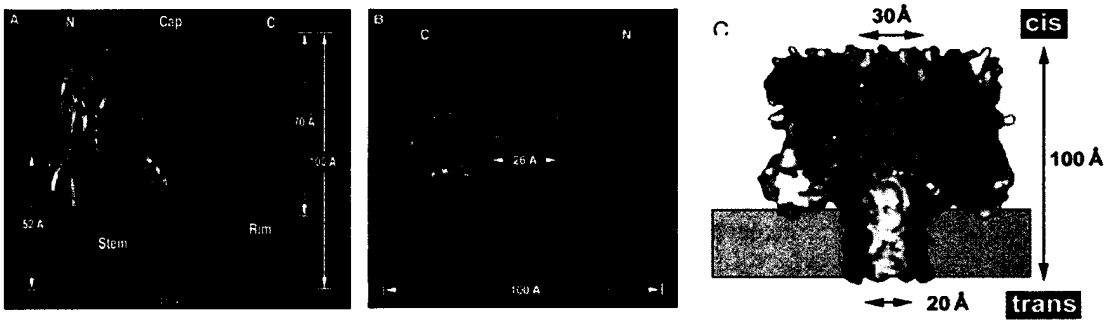

Figure 2 Structure of the staphylococcal αHL[13] that will be used for protein translocation studies: (A) Ribbon representation of the αHL heptamer with each protomer in a different color. View perpendicular to the sevenfold axis and approximately parallel to the putative membrane plane. (B) The same ribbon representation, but with a view from the top of the structure and parallel to the sevenfold axis. (C) The cross-sectional surface representation of (A). Irregularities of molecular surface are given by the contributions of bulky aminoacid side chains.

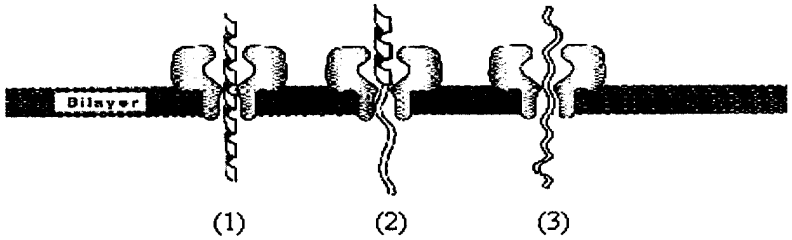

Figure 3 Various possibilities for the translocation of polypeptides through transmembrane nanopores: (1) the polypeptide remains in folded state; (2) the polypeptide denatures as it crosses the nanopore; (3) the polypeptide is denatured before its translocation across the nanopore.

A2. Model peptides and specific experimental design

In the initial stage of this project, we will be using a collection of model peptides known to form α-helical structures of different thermodynamic stabilities in water. The aims are the following: (1) To monitor the conductance of the nanopore in the presence of a test peptide. The extent of the single-channel conductance blockade in the presence of a test peptide and the constants k_{on}, k_{off}, K_d will reveal (a) interactions between the peptide and the pore; (b) the energetics of partitioning of the test peptide into the lumen of the pore. (2) To derive the activation free energy required for complete translocation of a polypeptide chain through the protein pore based on the relationship between translocation and melting temperature (T_m).

We propose to use the polypeptides, which contain residues with different α-helix parts containing variable aminoacid with which the hydrophobicity and/or the melting temperature T_m of the polypeptide can be varied. The thermodynamic stabilities of the peptides have already been studied in detail[14]. The peptide sequence was based on a negatively charged "leader", spacer groups and a reactive cysteine side chain at the terminus. The reactive cysteine residue may be used for a variety of applications such as biotinylation of the peptide or attaching

different labels, capturing the terminus in one side of the membrane with a larger macromolecule (a second protein that can binds to a ligand that is already covalently attached to the translocating peptide), tethering this polypeptide within a defined site of the nanopore and so on. For a control experiment, we propose a peptide of the same length that does not form a α-helix. Spectroscopic and calorimetric data, such as circular dichroism and differential scanning calorimetry, will be used to obtain information about the unfolding transitions of the test peptides for correlation with the single-channel electrical recordings.

The fundamental goal here is to study the interaction of the peptides with the lumen of the αHL pore. The translocation rates of the polypeptides will be studied as a function of transmembrane potential, temperature and transmembrane gradient of pH. This is expected to provide a basic understanding on the protein diffusion across nanopores. We will study whether a driving force will assist the unfolding of the α-helix and, ultimately, a full translocation of a polypeptide through the nanopore.

To better understand the charge role to interaction peptide-protein pore, we will use polymers of the same length and similar propensities, but with different charges. For example, the highly charged peptides Ac-Y-$(AAKAA)_m$-NH_2 (Fig. 4) form very stable α-helical structures. I will compare the data collected with this series of peptides with those corresponding to Ac-Y-$(EAAAK)_m$-NH_2. The latter ones obey to a closely similar helical propensity, have the same extended length, but are neutral. In addition, the influence of polypeptide chain flexibility, persistence length and helical fraction content will be pursued. The single-channel data with helical peptides will also be compared with the results corresponding to peptides with the same length and charge, but with a random coiled structure. Finally, the effect of bulky versus short aminoacid sidechains on the polypeptide-pore interaction will be considered.

Since the protein pore - membrane system is very robust and stable for wide external conditions, it is advantageous to examine the interaction of the polypeptide chain - pore interior during an unfolding process. I expect that temperature-induced unfolding CD spectroscopy experiments, titration calorimetry and single-channel recordings in a wide temperature regime will add to our insight regarding the transient partitioning of peptides within protein pores. At this point of the project, other experiments with chemical denaturants such as urea and guanidine hydrochloride will be pursued as alternatives to get a detail of the interaction at the single molecule level when progressive polymer unfolding is induced. The electrical recordings will be compared with the analysis of solvent denaturation curves, thus permitting a new concept of conformational stability of a polypeptide chain confined within a nanopore at the single molecule level. Alternative design for folding-unfolding equilibrium of peptides includes the use

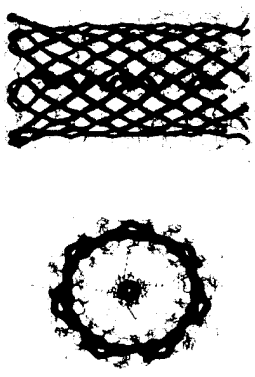

Figure 4 *Graphic model of an alanine-based α-helical peptide located in the β-barrel transmembrane protein pore of αHL: a backbone representation of a medium peptide P5 (26 aminoacids, the backbone is represented in dark blue) within a β-barrel protein pore, represented in green (the upper panel); cross-sectional view of in a backbone-bonds graphic representation. The α-helical peptide bonds are colored in blue. The αHL aminoacid bonds located within the constricted region of the pore (Met113 and Lys147) are colored in red, otherwise in black (lower panel).*

of polymers that have disulfide bonds. The experiments will be carried out in both unoxidized and oxidized states of the peptide sulfhydryl groups, thus revealing unprecedented details of the interaction that cannot be detected by other instrumentation. Disulfide-thiol exchange, in the

peptide chain, at dynamic equilibrium, will be carried out. Dithiothreitol (DTT) and triscarboxyethylphosphine (TCEP) will be used as reducing agents, whereas 5,5'-dithiobis(2-nitrobenzoic acid) will be a good candidate for thiolic active reagent. I expect that single-channel electrical signatures will discriminate between the reduced and unoxidized state of the peptide during the transit entry within the pore.

Once these studies are completed, we will pursue single-channel recording experiments with larger proteins that exhibit more than one transition state. In these experiments, the two half-chambers of the planar bilayer set-ups will have distinct compositions: one, in which the protein is completely unfolded or follows a multi-state unfolding process; the other one, in which the protein is fully translocated and reaches a folded state.

The peptide translocation rate will be modulated by additional peptide design and by the use of sequence leaders. For example, if a net positive charge is required for translocation, and the peptide enters the pore with the N terminus first, then groups of Lys and Arg residues may be attached at the N terminus. Similarly, sequence leaders of Glu and Asp might be attached, if a net negative charge is required for translocation. The experiments will be carried out below and above the melting temperature of the peptides (T_m).

A critical issue in the experimental design is the detection of complete translocation of the peptide through the pore. One possibility is to engineer a cysteine residue at the opposite entrance to the one where the polypeptide enters the channel. A sulfhydryl reactive group will be attached to the test polypeptide. If the polypeptide is translocated through the pore, it will react with the engineered cysteine residue at the entrance through which it exits. The polypeptide will remain tethered to the pore and a distinct current event will therefore be recorded. Based on a similar approach, I recently showed that large neutral and flexible polymers are completely translocated across the αHL transmembrane protein pore[15]. Other procedures will be evaluated, such as radiolabeling the polypeptide in the exit chamber with high specific activity ^{125}I, ^{131}I or ^{32}P.

Once this phase of the project is achieved, I can move ahead to a more demanding protocol design. As an example, I am interested whether I can detect subtle differences between the helix propensities of two peptides containing multiple Glu and Lys pairs, with identical compositions, but with distinct sequence distributions. Examples are:

Ac-Y-S-EEEE-KKKK-EEEE-KKKK-NH_2

Ac-Y-S-EEKK-EEKK-EEKK-EEKK-NH_2

The results will be very instructive, as I will be able to relate the interactions between the polypeptide and the pore with the difference in helix content between the two sequences. Spectroscopic and calorimetric data, such as circular dichroism and differential scanning calorimetry, will be required to obtain information about the unfolding transitions of the test peptides for correlation with the single-channel recordings.

A3. Peptides involved in mitochondrial transport

As an application of the techniques developed in the previous part of the project, I will investigate peptides known to be taken up by mitochondria. Very recently, Kinnally and colleagues[16] showed that small cationic signal peptides, such as yCOX-IV_{1-13} (MLSLRQSIRFFKY), a peptide corresponding to the first 12 amino acids of the presequence of cytochrome c oxidase subunit IV, are translocated into mitochondria. This peptide blocks both the translocase of the inner membrane channel (TIM) and the translocase of the outer membrane channel (TOM). These channels, located in the mitochondrial membranes, are slightly cation-selective and high conductance channels responsible for protein translocation. The goal here is to study the interaction of the sequence peptides with the lumen of the αHL pore by single-channel recordings and compare the results (binding affinities, flickering rates) with previous electrophysiological data on TIM and TOM channels[16]. One of the issues addressed here is to understand whether cation-selectivity is a main determinant for the interaction with the channel,

as αHL pore, which has a closely similar internal diameter and conductance, is slightly anion-selective. The translocation rate of the mitochondrial peptide will be studied as a function of transmembrane potential and ΔpH that mimic conditions in mitochondria.

B. Pore diameter and the amino acid side chains facing the lumen are envisioned as structural determinants of the interactions with the translocated polypeptides

B1. Various handles for the engineered protein pores

Pore diameter and the net charges of the amino acid side chains facing the inner part of the protein pore are structural determinants of the interactions with the translocated polypeptide. Importantly, engineering binding sites inside the αHL transmembrane protein pore is possible. As mentioned above, the use of a large and robust protein channel of known crystal structure and model peptides is a model system for understanding the basic principles by which polypeptides interact with the channel interior during translocation events. It is likely that the interactions between the translocated polypeptides and the nanopore are strongly dependent on the net electric charges located in or near the constriction (in the transmembrane β-barrel domain). This barrier is determined by bulky amino acid side chains, particularly Met-113 and Lys-147. I plan to study interactions between translocated polypeptides and the channel for a variety of αHL pores by engineering of the pore via site-directed scanning mutagenesis. Different aminoacids will be substituted in the constriction zone, and the effects on the kinetics of the interactions between test peptides and mutated protein pores will be measured.

B2. Comparative study on leukocidin, a different β-barrel pore-forming toxin

With this in mind, I will extend the experiments proposed above to other β-barrel pore-forming channels. Leukocidin, one of the candidates for investigation, is a binary toxin, thereby differing from αHL (Fig. 5)[17,18]. Staphylococcal leukocidin pores are formed by the obligatory interaction of two distinct polypeptide chains. The single-channel conductance of the leukocidin pore is 3-fold larger that that of αHL, suggesting that it has a considerably wider lumen. The larger conductance is also explained from the lack of bulky side chains equivalent with those in the central constriction of the αHL nanopore. I will examine the interaction of polypeptides with the leukocidin pore. The results from section A1 will be compared with those obtained from section B2. Additional site-directed mutagenesis may be required to achieve a better understanding of the effect of pore dimensions on translocation.

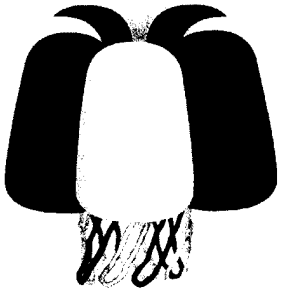

Figure 5 *Model of a staphylococcal leukocidin pore. Very recent experiments suggested that the transmembrane domain of the leukocidin pore is a 16-stranded β-barrel.*

C. Single-molecule dynamics of a single peptide tethered to a protein pore

Short oligopeptides will be tethered within the lumen the αHL transmembrane protein pore and their dynamics investigated at the single molecule level by planar bilayer recording. I will compare the results with previous recent measurements of tethered flexible (neutral or

charged) polymers within the lumen of the αHL pore[2,19]. One tethering site will be in the large cavity of the αHL protein pore at position Ser-106. Several issues will be addressed at this point: (1) How frequently does the polypeptide enter the transmembrane barrel domain? (2) What is the relative channel block, when the polypeptide is in the cavity? (3) Is the untethered end of the polypeptide able to translocate across the lumen under an applied potential? The answers to these questions will contribute to an understanding of the more complex situation that obtains when the peptides are free to move under a driving force. A longer-term goal is to follow the translocation of polypeptides through large protein channels by a combination of chemical modification of the peptides and single-channel electrical recording, using the latter to follow the kinetics of the interaction between the translocated polypeptide and the pore in real time. The experiments with tethered peptides may have applications in the field of biosensors. A tethered peptide responding to external analytes (structural changes following the presence of divalent cations/change in pH, temperature etc.) may act as a detection element in a particular biosensor that uses the principle of stochastic sensing. The small size of the detectors will allow their incorporation into microfluidic lab-on-a-chip devices.

It is important to design an experimental protocol for sensing a complete translocation, rather than a partial entrapping within the nanopore. Strictly, the single-channel current measurements will only tell us that a peptide or polypeptide interacts with the pore or partitions into it. A technique for the simultaneous recording of electrical currents and optical signals by fluorescence would be extremely useful. One candidate for the characterization of molecules at bilayer surfaces at the single molecule level is the total internal reflection fluorescence microscopy (TIRFM)[20]. The strategy involves distinct fluorescent labels for the translocated polypeptide and the protein-conducting channel. Although, the goal is challenging, recent efforts in this direction show promise[21,22] (Fig. 6).

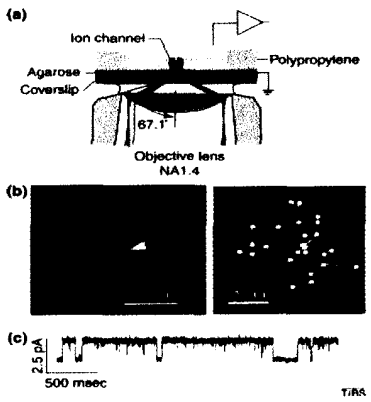

***Figure 6** Single-channel current and optical signal are measured simultaneously[21]. (a) Schematic picture of the objective-type total internal reflection fluorescence microscope with a bilayer membrane reconstituted on a agarose support. (b) Image of ion channel (left) and its Brownian walk in a lipid bilayer. (c) Electrical recording for single-channel in (b)[23]*

Potential outcomes and concluding thoughts

The program of research proposed above will be achieved by a combination of techniques including membrane protein engineering, electrophysiology and single-molecule biophysics. A long-term goal is to follow the translocation of polypeptides through large protein channels by a combination of single-molecule fluorescence microscopy and single-channel electrical recording, using the latter to follow the kinetics of the interaction between the translocated polypeptide and the pore in real time. The focus will be on understanding the ability of polypeptides to enter large transmembrane β-barrel pores as a result of driving forces that include transmembrane differences in pH, membrane potential, or other electrochemical gradients. In addition, we will address other important factors such as the geometry of the pore interior and the net charges of the aminoacid side chains that project into it.

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