The present invention includes an apparatus, system and method for stochastic sensing of an analyte to a protein pore. The protein pore may be an engineered protein pore, such as an ion channel at temperatures above 55° C. and even as high as near 100° C. The analyte may be any reactive analyte, including chemical weapons, environmental toxins and pharmaceuticals. The analyte covalently bonds to the sensor element to produce a detectable electrical current signal. Possible signals include change in electrical current. Detection of the signal allows identification of the analyte and determination of its concentration in a sample solution. Multiple analytes present in the same solution may also be detected.
OTHER PUBLICATIONS


* cited by examiner
FIG. 1A

FIG. 1B
FIG. 1E

FIG. 2
FIG. 3

FIG. 9C
HIGH TEMPERATURE ION CHANNELS AND PORES

This application claims priority to U.S. Provisional Patent Application Ser. No. 60/637,919, filed Dec. 21, 2004, the entire contents of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT INTEREST

The present invention was conceived or reduced to practice using funds provided by DARPA, the DoD Tri-Service Technology Program, DOE, NASA, NIH and ONR. The US government has certain rights in the invention.

TECHNICAL FIELD OF THE INVENTION

The present invention relates in general to the field of ion channels, and more particularly, to protein pores and ion channels that are functional and stable at temperatures above 55° C.

BACKGROUND OF THE INVENTION

Without limiting the scope of the invention, its background is described in connection with stochastic sensing based on the detection of individual binding events between analyte molecules and a single sensor element. Upon binding, a property of the sensor element is altered. This property or the effects of the changed property are measured.

In a simple example, the sensor element is a protein that is altered when it binds another molecule. The binding molecule to be detected is referred to as the analyte. The alteration of the sensor element that occurs upon binding is measured either directly or indirectly. In simple systems the alteration produces a simple signal, such as a difference in electrical current, force or florescence. Measurements of the signal indicate whether the analyte is bound and how long it remains bound. The frequency of occurrence of binding events is determined by the concentration of the analyte. The nature of the binding event is determined by the binding properties of the analyte, which determine, for example, the magnitude and duration of the resulting signal. Thus, a single sensor element to which multiple analytes may bind either directly may be used to determine which of those analytes are in a solution and the concentration of each particular analyte.

Protein pores are being developed for use in stochastic sensing. For several applications, the pores must be stable at high temperatures. The structures of many integral membrane proteins remain intact at high temperatures and the existence of extremophiles implies that membrane proteins function at 100° C. and beyond. However, few measurements of membrane protein activity have been made at temperatures above ~55° C., and none have been made for ion channels and pores.

The photocycle of bacteriorhodopsin, for example, has been examined at temperatures up to 85° C. The activity is compromised above 60° C. because the chromophore, a transretinal Schiff’s base, isomerizes to the cis form. Transmembrane proton pumping was not measured directly in these experiments. Macroscopic current recordings with valinomycin (a macr cyclic antibiotic) have been carried out at up to 80° C. in bilayers comprising lipids from a hyperthermophile, Caldiviella acidophila.

In the case of proteinaceous channels and pores, activity has been measured at up to ~55° C. For example, multichannel recordings of vanilloid receptors (temperature-sensitive cation channels) have been made after expression in Xenopus oocytes, and several subtypes of the receptor remain active at 55° C. Recently, single channel recordings of the vanilloid receptor VR1 have been made at up to 55° C. The transmembrane domains of these proteins are presumably largely α-helical.

Stochastic sensing may be accomplished with various sensing elements, using various modes of detection. One simple model uses an ion channel protein pore embedded in a membrane between a cis chamber and a trans chamber. When the pore is fully open a large ion flux occurs (e.g., 10^8 ions/s) which constitutes an electrical current that may be monitored by single channel recording. When an analyte binds to the pore, ion flux is altered, usually by decreasing the flow of ions. This generates a current trace that shows conduction over time.

One particular pore that has been used in stochastic sensing is Staphylococcus alpha hemolysin (αHl), which is actually an exotoxin secreted by Staphylococcus aureus. The monomeric 293 amino acid polypeptide can self-assemble on lipid bilayers, such as membranes, to form a heptameric pore. Alternatively, pre-formed pores may be inserted into a lipid bilayer. The pore is a mushroom-shaped structure in which the lower half of the stem forms a transmembrane channel. The interior of the pore is referred to as the “lumen” and may be accessible from outside the pore. By convention, when the pore is situated in a membrane, the side of the membrane on which the top of the mushroom shape is located is designated as the “cis” side of the membrane. The side of the membrane to which the stem portion leads is designated the “trans” side of the membrane. The pore essentially forms a hole in the membrane through which ions will flow if an electric potential is generated between the two chambers.

Measurements on the pore-forming toxin, α-hemolysin, one of the proteins examined in the present application, have been made previously at up to 50° C. This protein largely includes β structures. Indeed, all three of the proteins examined here contain β barrels, which are formed from either a single subunit, OmpG; seven subunits, α-hemolysin (αHl); or eight subunits, leukocidin (Luk). OmpG is a 280-residue polypeptide that forms a β barrel of, most likely, 16 antiparallel strands. Unlike most porins, which are trimERIC, OmpG functions as a monomer.

The homoheptameric pore formed by αHl is a mushroom-shaped structure. The stem of the mushroom is a 14-stranded transmembrane β barrel with two strands contributed by each subunit. The stem is capped by a large hollow extracellular domain. The Luk pore contains two types of subunits, F and S, which are related in sequence and structure to αHl. The pore is a heterorotamer containing four F and four S subunits. Despite the presence of only one additional subunit, the unitary conductance of the Luk pore is more than three times that of the αHl pore.

SUMMARY OF THE INVENTION

The present invention relates to protein pores and ion channels that are functional and sable at higher temperatures and may be used for stochastic sensing. In specific embodiments, the pores and ion channels are functional and stable for stochastic sensing at temperatures up to 55° C. The present invention includes the use of multiple pores and channels may be functional and stable for stochastic sensing at temperatures approaching 100° C. Pores and channels used for stochastic sensing in certain embodiments of the invention include αHl, Omp G and Luk. In a particular embodiment, the present invention includes βCD used in combination with αHl.
The apparatus, systems and methods of the present invention will find particular uses for the detection and characterization of single molecules as they affect a single protein pore. For example, the single-channel or pore for use at high temperatures of the present invention may be used for the study, characterization and manipulation of extremophiles and even for the study of single-molecule covalent chemistry.

The present invention may facilitate any stochastic sensing at temperatures above 55°C. One advantage of some embodiments as compared to antibiotic and other analyte sensing systems operable at higher temperatures lies in the ability to engineer easily alternative protein pores. Additionally, as compared to older protein pore stochastic sensing systems, embodiments of the present invention may be more useful for nanotechnology because of the capacity to operate at higher temperatures. Embodiments of the present invention may also be particularly useful in technologies in which DNA is measures, such as direct DNA sequencing. For a single-stranded DNA to enter a protein pore, it must be denatured, which requires a temperature above 55°C in almost all instances.

More particularly, the present invention includes a system for sensing one or more analytes in a solution by using a sensing device separated into first and second chambers separated by a divider having one or more protein pores operably disposed in or about the divider. A detection system operable to detect current between the first and second chambers is provided such that a solution with the one or more analytes that is capable of bonding to the protein pore, wherein bonding of the reactive analyte to the one or more protein pores produces a change in current between the first and second chambers detectable by the current detection system and is operable at a temperature at or above 55°C.

The protein pore may be an engineered protein pore, such as a αHL, Omp G and Luk protein pore, fusion proteins and combinations thereof. For example, the analyte may be disposed in an ionic solution that includes one or more species of chemical distinct analytes capable of bonding with the protein pore. The detection system may be any that is capable of detecting conductance or other ionic variations, e.g., those capable of stochastic sensing. When detecting stochastically, the change in current may be a change in the magnitude of the current.

The system may also be capable of detecting at least a second analyte, wherein the second analyte bonds to the protein pore and a detection system operable to detect a signal produced by covalent bonding of an analyte to the protein pore, wherein the detection system is capable of distinctly detecting the signals produced by bonding of at least the first and second analytes to the protein pore. Furthermore, the one or more different protein pores that have been functionalized to detect different analytes. In certain embodiments, the system is operable at a temperature at or near, 60, 70, 80, 90 or even 100°C.

The present invention also includes methods for sensing one or more analytes in a solution by disposing operably a protein pore in a divider between a first and a second chamber and detecting a current between the first and a second chamber with a current detector when the protein pores are exposed to a solution suspected of having the one or more analytes at a temperature at or above 55°C, wherein the one or more analytes produces a change in current between the first and a second chambers detectable by the current detector.

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

FIG. 1A presents a schematic representation of one of the pores used in embodiments of the present invention. Three states of the α-hemolysin (αHL) pore in a lipid bilayer are shown. At high temperatures, the unoccupied pore retains its ability to bind molecular adapters such as β-cyclodextrin (βCD, center), which can in turn bind guest molecules (right).

FIG. 1B are representative traces that illustrate single channel current tracess of unoccupied WT-αHL pores at different temperatures.

FIG. 1C is a graph that shows the variation of single channel currents with temperature for WT-αHL (○). Luk (Δ) and OmpG (□) pores. The values from four different experiments are compiled in each plot. The single channel currents depended linearly on the temperature: WT αHL, I(pA)=4.18+0.944T(°C) (R=0.999); Luk, I(pA)=26.4+2.041T(°C) (R=0.999); OmpG, I(pA)=3.73+1.057T(°C) (R=0.999).

FIG. 1D is a graph that shows that changes in single-channel currents are reversible: WT-αHL (○,●), Luk (Δ,▲) and OmpG (□,▼) pores. The data are from single representative experiments. The unfilled symbols are data points obtained as the temperature was increased, the filled symbols are data points obtained as the temperature was subsequently decreased.
FIG. 1 E is a graph that plots of the percentage change in g/k as a function temperature for WT-aHL (○), aHL (M113N) (●), and OmpG (□) and Luk (△) pores. The values at 23°C were set to 0%. Values of g from FIG. 2 were used, with additional data for aHL (M113N), and aHL (M113N), βCD obtained under the same conditions. Values for solution conductivity, k, were obtained from a linear fit to values of k measured at different temperatures.

FIG. 2 includes representative traces that illustrate the interaction of βCD with aHL-(M113N), pores obtained at various temperatures. Broken line, zero current; level 1, current through open (M113N), pore; level 2, current through (M113N),-βCD. The conditions were as follows: the buffer in both chambers was 10 mM Na phosphate, pH 7.5, containing 1 M NaCl and transmembrane potential was ~ -40 mV.

FIG. 3 includes traces that illustrate the interaction of the model analyte adamantane-1-carboxylic acid with βCD lodged in the aHL-(M113F/K147N)7 pore. Representative traces show the interaction of aHL-(M113F/K147N),-βCD with adamantane-1-carboxylic acid at various temperatures. βCD (40 µM) was applied to the cis side of the bilayer; adamantane-1-carboxylic acid (20 µM) was applied to the trans side. Otherwise, the conditions were as follows: the buffer in both chambers that was 10 mM Na phosphate, pH 7.5, containing 1 M NaCl and transmembrane potential was ~ -60 mV. Broken line, zero current; level 1, aHL-(M113F/K147N),-βCD; level 2, aHL-(M113F/K147N), βCD blocked with adamantane-1-carboxylic acid.

FIG. 4 illustrates one embodiment of the apparatus of the present invention.

FIGS. 5A and 5B are graphs that illustrate bilayer capacitance and multichannel currents at elevated temperatures. FIG. 5A shows the variation of bilayer capacitance (C) with temperature (T). The capacitance was determined by applying a triangular wave of 100 mV_{pp} at 25 Hz and monitoring the current response. FIG. 5B presents a recording of the current passed by multiple aHL pores as a function of temperature. A representative current trace at ~ -40 mV is shown. The numbers on the trace are the numbers of channels present at a given time, estimated from the observed number of current steps. The buffer in both chambers was 10 mM Na phosphate, pH 7.5, containing 1 M NaCl.

FIG. 6 is a graph that shows the dependence of 1/τ_{on} (FIG. 6A) and 1/τ_{off} (FIG. 6B) on the concentration of βCD (trans) for the aHL-(M113N), pore at 78°C.

FIGS. 7A to 7C show the interaction of βCD with aHL-(M113N), pores at 23°C and OmpG (□) and Luk (△) pores. The conditions were as described in FIG. 2. Kinetic constants were derived as described herein. FIG. 7A shows ln k_{on} versus 1/T. FIG. 7B shows ln k_{off} versus 1/T. FIG. 7C shows ln K_{f} versus 1/T. FIGS. 7A, 7B and 7C each show the result from a typical experiment (from four total). k_{on}, k_{off}, and K_{f} are marked on the y-axes as dimensionless as explained for FIG. 7.

DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but whose usage does not delimit the invention, except as outlined in the claims.

The present invention relates to protein pores that are stable at high temperatures. The pores may be used to measure the properties of various analytes. In specific embodiments, the protein pores are active at temperature above 55°C. Pores may remain active up to temperatures near 100°C. The pore functions as a sensor element and may be an isolated or engineered protein pore, such as an ion channel.

Additionally, a variety of signals and bonding detection procedures may be used. Signals include conductance, fluorescence, force and any other effects of analyte binding to the chosen sensor element. These signals may be detected using electrical and optical measurements, force measurements, chemical techniques and any combination of the above.

As used herein, the term "analyte" refers to reactive chemicals or agents that covalently or non-covalently bond to the sensor element (e.g., the pore and divider) under stochastic sensing conditions. Covalent bonds may be reversible or irreversible. The analytes may be any type of chemical including toxins, environmental indicators, chemical process products or by-products and contaminants. Chemical warfare agents that may be detected using the present invention include, e.g., arsenicals, organophosphates, mustard gasses, etc. Other chemicals that may be detected include those in, e.g., foodstuffs, such as onion and garlic. Other chemicals that may be detected include those in, e.g., soil samples to detected, e.g., pesticides such as organophosphates. Analytes may also include naturally occurring environmental toxins or reactive pharmaceuticals. Many physiological molecules such as nitric oxide, endo and exo peroxides are extremely reactive messengers and may also be analytes that are detectable using the present invention.

Analytes may bond to the sensor element alone or in combination with a second molecule, such as a host or adaptor molecule. The analyte may bond to the second molecule, which then bonds to the sensor element. Alternatively, the second molecule may simply affect the reaction kinetics of
analyte bonding. If used to affect the reaction kinetics, the second molecule may be used to slow down or speed up rate of bonding or duration of the bound state to facilitate detection of the identity, concentration, or other properties of the analyte. The second molecule may also exert an effect on analyte bonding by reacting with the analyte while not bound to the sensor element.

Other chemicals that act upon the bound analyte or are otherwise affected by the analyte/sensor element combination may also be added to the sensing system to augment sensing. For instance, an analyte bound to an αHL pore that causes a change in ion flux, but is not easily distinguishable from another analyte by current trace may be distinguished by introduction of a chemical that cleaves one of the indistinguishable analytes, but not the other. Additionally, added chemicals may be used to further explore the chemical identity properties of the analyte using sensing methods other than the original sensor element.

Previous inventions have discussed the usefulness of protein pores and ion channels in analyte sensing. For example, U.S. Pat. No. 6,426,231, U.S. Published Application No. 2003/0215881 and U.S. application Ser. No. 10/180,792, all relate to stochastic sensing of one or more analytes using a protein pore or ion channel, such as the αHL pore, relevant portions of each of which are incorporated herein by reference. The assay systems disclosed in these references may be used to perform stochastic sensing or other assays.

The stochastic sensing methodologies of the present invention may use a variety of physical arrangements and be provided in numerous configurations. For example, systems using protein pore arrangements may have the pore embedded in a membrane, located between separate cis and trans chambers. It may be recommended to place the analyte in either the cis or the trans chamber exclusively, or the system may be readily functional regardless of the chamber in which the analyte is placed. The same is true for chamber placement of any adapter or chemical reagent for breaking of irreversible bonds. In most systems the chemical reagent will be placed in one chamber and the analyte in the other chamber.

It is also possible to prepare a sensing system in which portions of the system are introduced in a time-dependent manner or varied over time. In other embodiments the sample to be tested for analyte may be discharged and replaced over time. This may result from periodic introduction of new samples or by flowing a continuous stream of sample through the sensor. For example, a sensor system to detect the presence of a reactive chemical in industrial effluent may contain a chamber through which a small diverted stream of effluent flows. In such examples it may be necessary to regulate flow so that the sample remains in proximity with the sensor element for a sufficient amount of time to statistically allow detection of a particular analyte at or above a selected concentration.

The systems and methodologies of the present invention may be used in sensors for detection of various reactive molecules. These sensors may be fixed or portable and they may be designed for single-use applications or any number of multiple uses. Remote application sensors that may be placed or dropped in a target location and then transmit sensor information may also be used. Solutions or other chemicals for use in the sensors may be supplied with the sensors in a kit, or made from any of the materials outlined hereinabove, however, in certain applications the materials for the membrane may be a flexible membrane, e.g., silicone and may even be coated with a non-reactive material. The interaction of the analyte, such as an organoarsenic compound, with the αHL, blocks the flow of ions through the pore. This change in ion concentration in the cis and trans chambers modulates a current flow through a conductor connecting the two chambers. Measurement of this current to produce a current trace allows determination of when the analyte bonds with the αHL and when it becomes unbound.

When using the αHL pore, most analytes may be added to either the cis or the trans side, although analytes can more readily reach the pore from the trans side. The same is true for many adapters or chemical reagents for reversal of irreversible bonds. In certain embodiments of the present invention, single-molecule activity measurements have been performed using three proteinaceous membrane pores at temperatures close to 100°C. Specifically, one of the pores can bind a molecular adapter, β-cyclodextrin (βCD), at elevated temperatures. The complex retains the ability to recognize small molecules, permitting stochastic sensing in aqueous solution under extreme conditions (see FIG. 1A).

Lipid bilayers are electrically stable at high temperatures. For example, planar bilayers made with bipolar lipids from a hyperthermophile, *Caldariella acidophila*, are stable at up to 80°C. The studies described herein were carried out with planar bilayers made from 1,2-diphtanoyl-sn-glycero-3-phosphocholine (DPhPC), which exist as a single phase at up to 120°C as determined by NMR and X-ray diffraction. In the studies described herein, the bilayers remained stable at 98°C, as determined by capacitance measurements (FIG. 5A). The saturated isoprenoid side-chains of DPhPC resemble those found in certain thermophiles which contribute to the stability of the bilayers. Multichannel current recordings in response to a temperature ramp were carried out on WT (wild-type) αHL pores incorporated as preformed heptamers into DPhPC bilayers from the cis chamber.

In all the studies conducted herein, both chambers of the measurement device (FIG. 4) contained 1 M NaCl with 10 mM Na phosphate, pH 7.5, and unless otherwise noted recordings were made at −40 mV. The αHL pores were stable up to 94°C (FIG. 5B). WT-αHL pores had been shown to be stable in SDS at up to −68°C, as judged by SDS polyacrylamide gel electrophoresis. This stability and functionality at higher temperatures has not previously been demonstrated, nor was it expected based upon earlier studies. It is
well known that most proteins experience increasing instability at higher temperatures. This decrease in stability correlates with a decrease in function that normally abrogates functionality at higher temperatures, or decreases it so severely that one would not expect the protein to be useful in any assay relying upon its function.

Single-channel current traces of WT-øHL pores were measured at up to 93°C (FIG. 1B). The single-channel current increased roughly linearly from 26.4 pA at 22°C to 91.9 pA at 93°C. In a similar manner, single-channel recordings were measured using the Luk and OmpG pores (FIG. 1C). Again, the currents increased roughly linearly with temperature: Luk, 72.0 pA at 23°C to 210 pA at 90°C; OmpG, 25.5 pA at 25°C to 101 pA at 97°C. The highest temperatures reached are those that were reached before technical problems (such as the insertion of a second channel, which can occur at any temperature) were encountered or a reverse temperature ramp was intentionally initiated in the experiments being conducted at that time. However, the significance of the occurrence of these events on the functionality of the pores is not understood and in no way limits the present invention. Thus, the pores may well be stable and functional at yet higher temperatures. The occurrence of numerous spikes towards zero current distinguished the OmpG traces from those arising from the øHL pore, which has a similar conductance, thereby ruling out sample contamination. The stabilities of the Luk and OmpG pores were surprising based on their established properties in detergent solutions; Luk pores dissociate in SDS at ~78°C. (L. Jayasinghe, personal communication) and the OmpG protein unfolds in n-octyl-β-D-glucopyranoside at 65°C. (11) The changes in single-channel current associated with the temperature ramp were fully reversible (FIG. 1D). Three main factors may contribute to the ability to record single channel currents at high temperatures (FIG. 4): (1) the use of an aperture with a diameter of ~100 nm (with a larger orifice, multiple channels were incorporated too readily and the bilayer tended to break); (2) the formation of a bilayer by using a large mass of DPhPC (200 μg lipid per chamber-electrolyte volume=1.5 mL, surface area=0.72 cm²); and (3) the use of dilute protein samples.

The strong temperature dependence of the single-channel conductance values (g) of the three pores is largely due to the variation of solution conductivity (κ) with temperature, which suggests that there is a measurable molecular reorganization or subunit dissociation at elevated temperatures. The conductivity of the buffer was found to increase linearly with temperature (FIG. 1E). When g/κ was plotted as a function of temperature, this decrease in stability correlated with a decrease in function that normally abrogates functionality at higher temperatures, or decreases it so severely that one would not expect the protein to be useful in any assay relying upon its function.

By comparison with the β barrels, the gating kinetics of several channels, including the temperature-gated vanilloid receptors, are characterized by dramatic responses to temperature (at below 55°C). (23) Host molecules such as cyclodextrins can become lodged within the øHL pore, where they can in turn bind guest molecules (FIG. 1A). (24) Therefore, the interaction of β-cyclodextrin (βCD) with the (M113N) pore at high temperatures was examined.

At room temperature, the M113N mutant of øHL binds βCD-10,000-fold more tightly than the WT protein. (25) At 31°C, βCD binding events with a mean duration, τ₁, of 14 s were seen (FIG. 2A). As the temperature increased, several phenomena were observed at a fixed βCD concentration: (1) the conductance values of both the unoccupied and occupied states of the pore increased; and (2) short additional blockades from the (M113N)-βCD level (sub states) were observed. The frequency of occurrence of these events was independent of βCD concentration, so they did not arise from the binding of a second βCD. Instead, they most likely represented a second conformation of the occupied state, (M113N)-βCD. Specifically, they likely represented the rotation of βCD at the binding site or dewetting transitions (25) within the narrow cyclodextrin ring. Finally, the dwell time of βCD (τ₈,₉) and the intervals between the binding events (τ₈,₃) both decreased.

In earlier work at room temperature, it has been shown that βCD takes part in a simple binary interaction with (M113N). (25) By a kinetic analysis, it was confirmed that this was also the case at 78°C (FIG. 3). Measurements of the mean dwell time (τ₁) and the mean inter-event interval (τ₈,₃) were used to derive association (kₐ) and dissociation (k₈,₉) rate constants for βCD. At 25°C, the values were 4.5±0.6x10⁶ M⁻¹s⁻¹ and 0.03±0.1 s⁻¹ (n=3) respectively, yielding Kₐ=1.5x10⁷ M⁻¹ (literature 7x10⁶ M⁻¹), the latter value was determined in a different sequence background for øHL, “RL2”, which might explain the small difference. (25) This value is >10⁴-fold greater than the value for the interaction of WT-øHL with βCD (250 M⁻¹) reported previously. (25) As the temperature increased, Kₐ and kₐ increased. For example at 85°C, the highest temperature reached in these experiments, Kₐ and kₐ increased by ~15-fold and ~800-fold to 6.5±0.8x10⁷ M⁻¹ s⁻¹ and 25.0±0.3 s⁻¹ (n=3), respectively, over the values at 25°C. Because there is a larger increase in k₈,₉ with temperature, compared with k₈,₉, the formation constant Kₙ was reduced to 2.6x10⁻¹, M⁻¹ at 85°C.

From the slope of a linear fit to ln Kₐ versus 1/T (FIG. 7C), ΔH° and ΔS° values were found to be, respectively, ~90±4 kJ mol⁻¹ and ~62±5 J mol⁻¹ K⁻¹, yielding a value of ΔG°=~41±3 kJ mol⁻¹ at 25°C (n=4). The value for ΔH° is close to that for the binding of βCD to glucoamylase, while the value for ΔS° for glucoamylase is a less favorable -90±7 kJ mol⁻¹. (27-29) Suggesting a more favorable reorganization of the binding site in (M113N), which would be augmented by the matching C₇ symmetry of the øHL pore and βCD. By comparison, for the formation of an 8-nucleotide DNA duplex under similar conditions (10): ΔH°=~144 kJ mol⁻¹, ΔS°=~359 J mol⁻¹ K⁻¹, ΔG°=~37 kJ mol⁻¹. In this case, a far larger entropic penalty is compensated for by a highly favorable enthalpic contribution. Binding experiments with βCD and the heteromeric (30,31) pore WT₁(M113N), yielded ΔH°=~51±2 kJ mol⁻¹, ΔS°=~33±2 kJ mol⁻¹ K⁻¹, and ΔG°=~33±2 kJ mol⁻¹ at 25°C (FIG. 8). The decrease in affinity brought about by the loss of one Asn-113 residue is almost entirely derived from a change in ΔH°, again suggesting a reorganized binding site.
be examined at temperatures approaching 100°C. at the
dissociation by PCD, FIG. 2A), which are dependent on both temperature
interactions.

Accordingly, a homoheptameric pore made from the double
mutant M113F/K147N was used, which has the following
described previously (S. Conlan, Y. Zhang, S. Miles, S. Cheley, O. Braha, H. Bayley, Biochemistry 40, 636-
640 (2001)). The mutant aHL M113N, which was made in a WT-aHL background (L.-Q. Gu, S. Cheley, H. Bayley, Science 291, 636-
640 (2001)), was synthesized in vitro by coupled transcription and translation (IVTT) and assembled into
heptamers by the inclusion of rabbit red cell membranes (rRBCM) during synthesis (S. Cheley, O. Braha, X. Lu, S.
Conlan, H. Bayley, Protein Sci. 8, 1257-1267 (1999). The heptamers were purified by SDS-polyacrylamide gel electrophoresis and stored in aliquots at ~80°C. Leukocidin pores were prepared by co-translating of the F and S subunits in the presence of rRBCM and purified by electrophoresis (G. Miles, S. Cheley, O. Braha, H. Bayley, Biochemistry 40, 8514-8522 (2001)). Stock solutions of the aHL and Luk pores were ~200 ng mL^-1. They were diluted 10- to 20-fold with 10 mM Na phosphate, pH 7.4, containing 1 M NaCl, and 0.5 to 1.0 µL was added to the cis chamber of the bilayer apparatus (1.5 mL). The porin Omp G was prepared and purified as described previously (S. Conlan, Y. Zhang, S. Cheley, H. Bayley, Biochemistry 39, 11845-11854 (2000)). The stock solution (0.56 mg mL^-1) in 10 mM Tris.HCl, pH 8.0, containing 150 mM NaCl and 1% (w/v) Genapol X-080

The approach developed herein may be useful in studies of the fundamental functional properties of ion channels, and of how they fold and assemble. All three pores examined herein contain transmembrane β barrels, but the approaches described herein may also be applied to channels that are predominantly a helix. The pores examined are functional at high temperatures, although they originate in mesophilic bacteria. Pores from thermophiles may also be examined. From the point of view of biotechnology, the ability to observe channels and pores at high temperatures aids in the ability to engineer stable membrane proteins as components of devices such as sensors or DNA sequencers. Accordingly, aspects of the invention relate to an aHL pore containing a molecular adapter that retains its ability to bind a model analyte at elevated temperatures. Recently, the aHL pore has been used as a nanoreactor for the examination of single-molecule chemistry. The ability to record at high temperatures greatly extends the power of this methodology.

It will be apparent to one skilled in the art that various alternative embodiments of the invention exist. For example, one may change the lipid used, incorporate alternative pores, vary the composition of the electrolyte, and change the material employed, nature of the electrode, dimensions, or other physical attributes of the testing apparatus.

The following examples are included to demonstrate specific embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples below represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Pore formation. Wild-type aHL pores were formed by treating monomeric aHL, purified from Staphylococcus aureus, with deoxycholate (S. Bhakdi, R. Fülsö, J. Traum-Jensen, Proc. Natl. Acad. Sci. USA, 78, 5475-5479 (1981)). Heptamers were isolated from SDS-polyacrylamide gels (O. Braha, B. Walker, S. Cheley, J. K. Kasianowicz, L. Song, J. E. Gouaux, H. Bayley, Chem. Biol. 4, 497-505 (1997). The stock solution (0.56 mg mL^-1) in 10 mM Tris.HCl, pH 8.0, containing 150 mM NaCl and 1% (w/v) Genapol X-080

The value of $\Delta S^0$ is close to that obtained herein, $\Delta H^0$ and $\Delta S^0$ differ. Within the aHL pore, the enthalpy change for the interaction is less favorable, but a more favorable TAS compensation. Perhaps the βCD presents only one face to the guest presented from the trans side, or the βCD is in a different conformation when in solution, but offer less favorable non-covalent bonding interactions.

These studies show that the properties of protein pores may be examined at temperatures approaching 100°C. at the

single-molecule level by planar bilayer recording.
was diluted 5-fold with 10 mM Na phosphate, pH 7.4, containing 1 M NaCl, and 1.0 µL was added to the cis chamber (1.5 mL).

Example 2

Apparatus. One example of the detector 10 used in conjunction with the studies described herein includes a specially designed Teflon block 12, a stainless steel stand 14, and a Peltier device 16 (FIG. 4, side, top and bottom views). The block 12 includes two chambers (18A, 18B), designated cis and trans (or alternatively, first and second chambers). The planar lipid bilayer is formed across a 100-150 µm-diameter orifice in a 25 µm thick Teflon film 20 that separates the two chambers 18A, 18B. The bigger cylindrical holes (22A, 22B) (diameter=5 mm), which are connected to the main chambers 18A, 18B, are used for holding the electrodes (not depicted). The smaller holes (24A-D) (diameter=1.5 mm) are used for holding a thermocouple (not depicted), the tip of which is exposed to an electrolyte and therefore accurately monitors the temperature in the main chamber. The two chambers 18A, 18B are clamped tightly together in a stand 14 made of, e.g., stainless steel. The bottoms of the chambers 18A, 18B were covered with a single thin sheet of borosilicate glass 26 (0.16 mm thick) for efficient heat transfer between the solution in the chambers 18A, 18B and the surface of the Peltier device 16. The Peltier device 16 and the chambers 18A, 18B are mounted on a stainless steel stand 14, which provides efficient heat dissipation during cooling. The temperature in the chamber 12 was controlled by varying the current through the Peltier device 16 with a DC power supply (not depicted). The apparatus 10 may also include one or more stirring motors (28A, 28B).

Example 3

Temperature Control. The temperature in the chamber 12 (FIG. 4) was controlled by using a Peltier device 16 (Ferro-Tec, T221084-01). The top part of the chamber was built from Teflon, but the bottom was formed from a thin piece of borosilicate glass 26 (0.16 mm thick; Lab-Tek, Part No. 155361), so that heat transfer between the solution in the chamber 12 and the surface of the Peltier device 16 element was efficient. The temperature in the chamber 12 was controlled by varying the current through the Peltier device 16 with a DC power supply (TENMA, 72-6153). To reverse the temperature ramp, the polarity of the DC power was switched. Cooling was aided by the steel stand 14 underlying the glass bottom. The temperature of the electrolyte in the chamber 12 was recorded with a calibrated thermocouple. The system rapidly and accurately controls the solution temperature: heating rate 35°C min⁻¹; cooling rate 10°C min⁻¹; temperature ±0.2°C. It can be used over a broad temperature range, from room temperature to 100°C. The electrical noise is low (≈1.4 pA rms), similar to that found in room temperature recordings. In practice, steps of 5 to 10°C were made (see e.g. FIG. 5); larger steps produced distortions in the current recordings caused by bubble formation in the chambers. The temperature in the chambers was adjusted by manually varying the voltage of the DC power supply (this might readily be automated). Except for multichannel recordings (FIG. 5), continuous heating and recording was not used because the absence of stirring led to interference by bubbles. During heating, and before recording, the solution was stirred. When the temperature of the solution stabilized at the desired value, the stirring was stopped and recording initiated.

Example 4

Planar Bilayer Recording. The buffer used for bilayer recording was 10 mM Na₂HPO₄/10 mM NaH₂PO₄, pH 7.5, containing 1 M NaCl. Planar lipid bilayer membranes of 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPhPC) (Avanti Polar Lipids, Alabaster, Ala.) were formed by the method of Montal and Mueller (M. Montal, P. Mueller, Proc. Natl. Acad. Sci. USA 69, 3561-3566 (1972)) on a 100- to 150-µm-diameter orifice in a 25-µm thick Teflon film (Goodfellow Corp., Malvern, Pa.) separating the cis and trans compartments (each 1.5 mL) of a Teflon apparatus. Prior to formation of the bilayer, the orifice was pretreated with a 1:10 hexadecane/ pentane mixture. The level of the buffer in each chamber was set just below the aperture and 1% (w/v) DPhPC in pentane (20 µL) was transferred to each chamber and allowed to spread on the surface of the buffer. After 3 min, during which the pentane evaporated, the buffer level in each chamber was raised above the aperture. The formation of a bilayer was monitored by observing the increase in membrane capacitance to a value of approximately 8-10 µF µm⁻². Larger orifices and the application of less lipid tended to give less stable bilayers at elevated temperatures.

All proteins were added to the cis chamber, which was held at, e.g., ground. A positive potential indicates a higher potential in the trans chamber, and a positive current is one in which cations flow from the trans to the cis side. With respect to the oLHL pore, this convention means that the cap domain (E. Song, M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, J. E. Gouaux, Science 274, 1859-1865 (1996)) is exposed to the cis chamber, while the entrance to the transmembrane β barrel at the tip of the stem domain is exposed to the trans chamber. A potential difference was applied across the bilayer with two freshly prepared Ag/AgCl electrodes in 1.5% agarose (Bi-Rad) saturated with 3 M KCl. Before each experiment, the two electrode potentials were carefully checked against a standard calomel electrode to ensure that the potential difference between them was <1.5 mV. Currents were recorded at a holding potential of ~40 mV by using a patch clamp amplifier (Axopatch 200B, Axon Instruments). The signal was low-pass filtered with a built-in four-pole Bessel filter set at 5 kHz and sampled at 20 kHz by computer with a Digidata 1200 A/D converter (Axon Instruments). β-Cyclodextrin (Aldrich) was added to the trans chamber, except in the case of the oLHL- (M113F/K147N)₂ pore when it was added to the cis chamber.

Example 5

Data analysis. Data were analyzed and presented by using the software pClamp 7.0 (Axon Instruments) and Origin 5.0 (Microcal, Northampton, Mass.). Conductance values were obtained from the peaks of all-point histograms. To determine kinetic constants for the binding of PCD to the oLHL pore, τᵦᵢ (the interevent interval) and τᵦᵣ (the lifetime of the oLHL-PCD complex) were obtained from dwell-time histograms fitted to single exponentials by the Levenberg-Marquardt procedure. To determine each set of kinetic constants, three or more experiments were performed. In each case, data were analyzed that had been acquired for at least 2 min. In all cases, the coefficient of determination of the fits was R²≈0.85. Separate segments of the data yielded similar τ values, demonstrating that stationary kinetics prevailed. Kinetic constants were calculated by using kᵦᵢ=1/τᵦᵢ, kᵦᵣ=1/τᵦᵣ, [PCD], and Kᵦᵣ/kᵦᵢ=Kᵦᵣ=Kᵦᵣ/[PCD], where [PCD] is the concentration of PCD. Standard thermodynamic values, ΔG°, ΔH° and ΔS°, were determined by using ΔG°=ΔH°-TΔS°=-RT ln Kᵦᵣ and ln Kᵦᵣ=(ΔH°/R)/1/T+ ΔS°/R. ΔG°, ΔH° and ΔS° were determined by using ln kᵦᵣ=(ΔH°/R)/1/T+ΔS°/R, where ψ is a frequency factor in transition state theory. The kinetics of the interaction of ada-
Example 6

Bilayer stability and macroscopic currents. Bilayer stability was determined by measuring the dependence of capacitance on temperature (FIG. 5A). Before single channel recordings were performed, preliminary experiments were conducted with multichannel currents at elevated temperatures (e.g., FIG. 5B). The displayed example of multichannel recording is typical. Additional pores became incorporated into the bilayer as the temperature increased. In the study shown, excess protein in the cis chamber was not washed out before the recording. However, washing did not prevent the incorporation of additional pores, which probably originated from protein that had adhered to the bilayer surface. Eventually, the bilayer shown contained about eight channels. At 94° C., the current dropped sharply to zero, indicating that either all eight channels closed simultaneously or that the entire bilayer had become occluded. At the same time the bilayer capacitance decreased from 120 pF to 50 pF, indicating an increase in thickness of unknown origin. All such recordings ended in this way.

Example 7

The effect of temperature on solution pH values. All measurements were carried out in 10 mM Na phosphate at pH 7.5 (titrated at 25° C.) containing 1 M NaCl. Phosphate buffer was chosen because of its low temperature coefficient: d(pH)/dT=+0.0013 pH deg⁻¹ in the range 25° C. to 50° C. and (pH)/dT=+0.0013 pH deg⁻¹ in the range 50° C. to 90° C. [R. G. Bates, J. Res. Natn. Bur. Stand., Phys. Chem. 66A (2), 179 (1962); R. P. Buck, S. Rondinini, A. K. Covington, F. G. K. Baecke, et al., Measurement of pH. Definition, standards and procedures. Pure Appl. Chem. 74, 2169-2200 (2002)]. In addition, the temperature coefficient was determined experimentally in the presence of 1 M NaCl by using a Ross® glass electrode (TABLE 1). The pH meter was calibrated at each temperature by using standard buffers. At or below 50° C., two-point calibration with standard buffers at pH 7.0 and pH 10.0 was used. Above 50° C., single-point calibration was used. The change in the glass electrode slope with temperature was automatically compensated with an ATC probe. Small changes in pH with temperature were observed and were in agreement with the values in the literature.

Example 8

The Effect of Temperature on the Electrode Potential. The cell in this example may be described as follows:

Ag/AgCl, NaCl (3.0 M) | electrolyte solution NaCl (1.0 M) | NaCl (3.0 M) | AgCl/Ag

Theoretical Calculations

The half-cell potential for a Ag/AgCl electrode can be written:

For the reaction, Ag⁺+Cl⁻=AgCl(s), Ksp=αAg⁺αCl⁻. Therefore, for the first electrode:

\[ E_i = E^\circ_{Ag^+Cl^-} + RT/nF \ln(\alpha_{Ag^-}) \]

Similarly, for the second electrode:

\[ E_i = E^\circ_{Ag^+Cl^-} + RT/nF \ln(\alpha_{Ag^-}) + RT/nF \ln(\alpha_{Cl^-}) \]

In the chamber, the potential difference between the two electrodes is:

\[ V = E_2 - E_1 + RT/nF \ln(\alpha_{Ag^-}/\alpha_{Cl^-}) \]

where: I, current; R, resistance; V, applied potential. For two matched Ag/AgCl electrodes at the same temperature and bathed in the same concentration of chloride ions:

\[ E_i = E^\circ_{Ag^+Cl^-} + RT/nF \ln(\alpha_{Ag^-}/\alpha_{Cl^-}) \]

However, it is worth considering the effect of a small temperature differential. If it is assumed that there is a temperature difference ΔT=T₂-T₁ between the two electrodes:

\[ ΔE = ΔE_1 - E_2 = RT/nF \ln(\alpha_{Ag^-}/\alpha_{Cl^-}) \]

where: R, Ksp₁, Ksp₂, and F are the gas constant (8.314 J mol⁻¹ K⁻¹), the solubility product of AgCl at T₁, the solubility product of AgCl at T₂, and the Faraday constant (96,500 C mol⁻¹), respectively.

When the temperatures are 26° C. at electrode 2 and 25° C. at electrode 1, ΔT=1° C., Ksp₁=1.85x10⁻⁹ M² at 25° C., Ksp₂=2.02x10⁻¹⁰ M² at 26° C. [Ksp₁ was obtained from “Solubilities of inorganic and organic compounds. Vol. 1: Binary systems, Part 1”, H. Stephen, ed., Pergamon Press, Oxford; Ksp₂ was calculated by using ln(Ksp₁/Ksp₂)=ΔHf°/R(1/T₁−1/T₂), where ΔHf° (65.6 kJ mol⁻¹) is the enthalpy of dissolution of AgCl [Y. Sun and Y. Xia, Anal. Chem. 74, 5279-5305 (2002)]. In 3 M NaCl solutions, there is:

\[ ΔE = ΔE_1 - E_2 = −0.24 \text{ mV} \]

In an applied potential of −40 mV, the current flowing through a single WT αHL pore at 25° C. in 10 mM Na phosphate at pH 7.5, containing 1 M NaCl, is −28 pA. So, the current shift ΔI caused by a potential change of −0.24 mV is −0.17 pA or 0.6% of the total current.

TABLE 1

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>25° C.</td>
<td>7.50</td>
</tr>
<tr>
<td>30° C.</td>
<td>7.49</td>
</tr>
<tr>
<td>40° C.</td>
<td>7.47</td>
</tr>
<tr>
<td>50° C.</td>
<td>7.47</td>
</tr>
<tr>
<td>60° C.</td>
<td>7.47</td>
</tr>
<tr>
<td>70° C.</td>
<td>7.48</td>
</tr>
<tr>
<td>80° C.</td>
<td>7.49</td>
</tr>
<tr>
<td>90° C.</td>
<td>7.51</td>
</tr>
</tbody>
</table>

TABLE 2

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>ΔT (T-25, °C)</th>
<th>ΔE (mV)</th>
<th>ΔI (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° C.</td>
<td>−0.24</td>
<td>−0.17</td>
<td></td>
</tr>
<tr>
<td>2° C.</td>
<td>−0.54</td>
<td>−0.38</td>
<td></td>
</tr>
<tr>
<td>3° C.</td>
<td>−0.78</td>
<td>−0.55</td>
<td></td>
</tr>
<tr>
<td>4° C.</td>
<td>−1.05</td>
<td>−0.74</td>
<td></td>
</tr>
<tr>
<td>5° C.</td>
<td>−1.24</td>
<td>−0.87</td>
<td></td>
</tr>
</tbody>
</table>

Measurement of the potential difference between two Ag/AgCl electrodes at different temperatures. Measurements were carried out in 10 mM Na phosphate at pH 7.5 (titrated at
Binary interaction of βCD with the αHL (M113N)₆ pore at an elevated temperature. The existence of a binary interaction between βCD and the αHL (M113N)₆ pore was confirmed by examining the temperature dependence of 1/κₑ and 1/κₒ on the concentration of βCD at 78°C. (Fig. 6).

Interaction of βCD with a heteromeric αHL pore. The nature of the interaction of βCD with the αHL (M113N)₆ pore was further explored by examining the WT₁₆ (M113N)₆ heteromer (Fig. 8).

Temperature dependence of the interaction of the model analyte adamantane-1 carboxylic acid with βCD lodged in the αHL-(M113F/K147N)₆ pore. Kinetic constants were derived as described above and used to derive ΔG, ΔH and ΔS values from ln k (or K) versus 1/T plots (Fig. 9).

It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention.

More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references are indicated by numeral throughout the specification.

All references above and otherwise cited herein are incorporated by reference.

What is claimed is:

1. A method for sensing one or more analytes in a solution comprising:
   disposing operably one or more protein pores in a divider between a first and a second chamber exposing said one or more protein pores to a solution suspected of comprising one or more analytes at a temperature at or above 55° C.; and detecting a current between the first and a second chambers with a current detector when the one or more protein pores are exposed to a solution suspected of comprising the one or more analytes at a temperature at or above 55° C., wherein each of the one or more protein pores comprise at least one high temperature stabilization molecular adapter that stabilizes the one or more protein pores at a temperature at or above 55° C. and the one or more analytes produces a change in current between the first and a second chambers detectable by the current detector.

2. The method of claim 1, wherein the one or more protein pores are engineered protein pores.

3. The method of claim 1, wherein one or more of the one or more protein pores is an aHL pore.

4. The method of claim 1, wherein the solution is a pH buffered KCl solution.

5. The method of claim 1, wherein the one or more analytes comprise an environmental toxin.

6. The method of claim 1, wherein the one or more analytes comprise a chemical weapon.

7. The method of claim 1, wherein the one or more analytes comprise a pharmaceutical.

8. The method of claim 1, wherein the one or more analytes comprise an arsenical.

9. The method of claim 1, wherein the solution comprises an anionic solution suspected of comprising one or more chemically distinct analytes capable of bonding with the one or more protein pores.

10. The method of claim 1, wherein the current detector is capable of stochastic sensing.

11. The method of claim 1, wherein the change in current comprises a change in the magnitude of the current.

12. The method of claim 1, wherein the at least one high temperature stabilization molecular adaptor comprises β-cyclodextrin (βCD).