Biotechnology

Single Protein Pores Containing Molecular Adapters at High Temperatures**

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Protein pores are being developed for use in biotechnology.[1] Many applications require that pores be stable at high temperatures. Herein we report single-molecule activity measurements on three proteinaceous membrane pores at temperatures close to 100 °C. We also show that one of the pores can bind a molecular adapter, β-cyclodextrin (βCD), at elevated temperatures. The complex retains the ability to recognize small molecules which permits stochastic sensing in aqueous solution under extreme conditions (Figure 1a). The structures of many integral membrane proteins remain intact at high temperatures,[2] and the existence of extremophiles implies that membrane proteins function at 100 °C and above.[3] 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.[4] The activity is compromised above 60 °C because the chromophore, a *trans*-retinal Schiff base, isomerizes to the 13-*cis* form. Transmembrane proton pumping was not measured directly in these experiments. Macroscopic current recordings with valinomycin, a macrocyclic antibiotic, have been carried out at up to 80 °C in bilayers comprising lipids from the hyperthermophile *Caldariella acidophila*.[5] 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.[6] Recently, single-channel recordings of the vanilloid receptor VR1 have been made at up to 55 °C.[7] The transmembrane domains of these proteins are presumed to be largely α-helical.

Measurements on the pore-forming toxin α-hemolysin (αHL), one of the proteins examined in the present work, have been made previously at up to 50 °C.[8–10] This protein is largely made up of β structure. Indeed, all three of the proteins examined herein contain β barrels, which are formed from either a single subunit (OmpG), seven subunits (αHL), or eight subunits (Luk). OmpG is a 280-residue polypeptide that most likely forms a β barrel of 16 antiparallel strands. Unlike most porins, which are trimeric, OmpG functions as a monomer.[11] The homoheptameric pore formed by αHL is a mushroom-shaped structure.[12] 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 subunit types, F and S, which are related in sequence and structure to αHL.[13,14] The pore is a heterooctamer containing four F and four S subunits.[15] 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.[16]

Previous work established the electrical stability of lipid bilayers at high temperatures. For example, planar bilayers made with bipolar lipids from the hyperthermophile *Caldariella acidophila* are stable at up to 80 °C.[5] Our experiments were carried out with planar bilayers made from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), which exist as a single phase at up to 120 °C as determined by NMR spectroscopy and X-ray diffraction.[17] In our hands, the bilayers remained stable at 98 °C as determined by capacitance measurements (Supporting Information). The saturated isoprenoid side chains of DPhPC resemble those found in certain thermophiles[18] and may contribute to the stability of the bilayers. Multichannel current recordings in response to a temperature ramp were carried out on wild-type αHL pores incorporated as preformed heptamers into DPhPC bilayers from the *cis* chamber. In all the experiments reported herein, both chambers contained 1 M NaCl with 10 mM sodium phosphate at pH 7.5, and unless otherwise noted, recordings were made at −40 mV. The αHL pores were stable at up to 94 °C (Supporting Information), which is surprising. Wild-type αHL pores had been shown by SDS-polyacrylamide gel electrophoresis to be stable in SDS at up to 65 °C.[19,20] The present experiments show that the pores are both stable and functional at much higher temperatures.

Single-channel current traces of wild-type αHL pores were obtained at up to 93 °C (Figure 1b). The single-channel current increased linearly from 26.4 pA at 22 °C to 91.9 pA at 93 °C. In a similar manner, single-channel recordings were obtained from the Luk and OmpG pores (Figure 1c). Again the currents increased 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 quoted are those that were reached before technical problems were encountered (such as the insertion of a second channel), or a reverse temperature ramp was intentionally initiated; the pores may well be stable at yet higher temperatures. The occurrence of numerous spikes toward zero current[21] dis-

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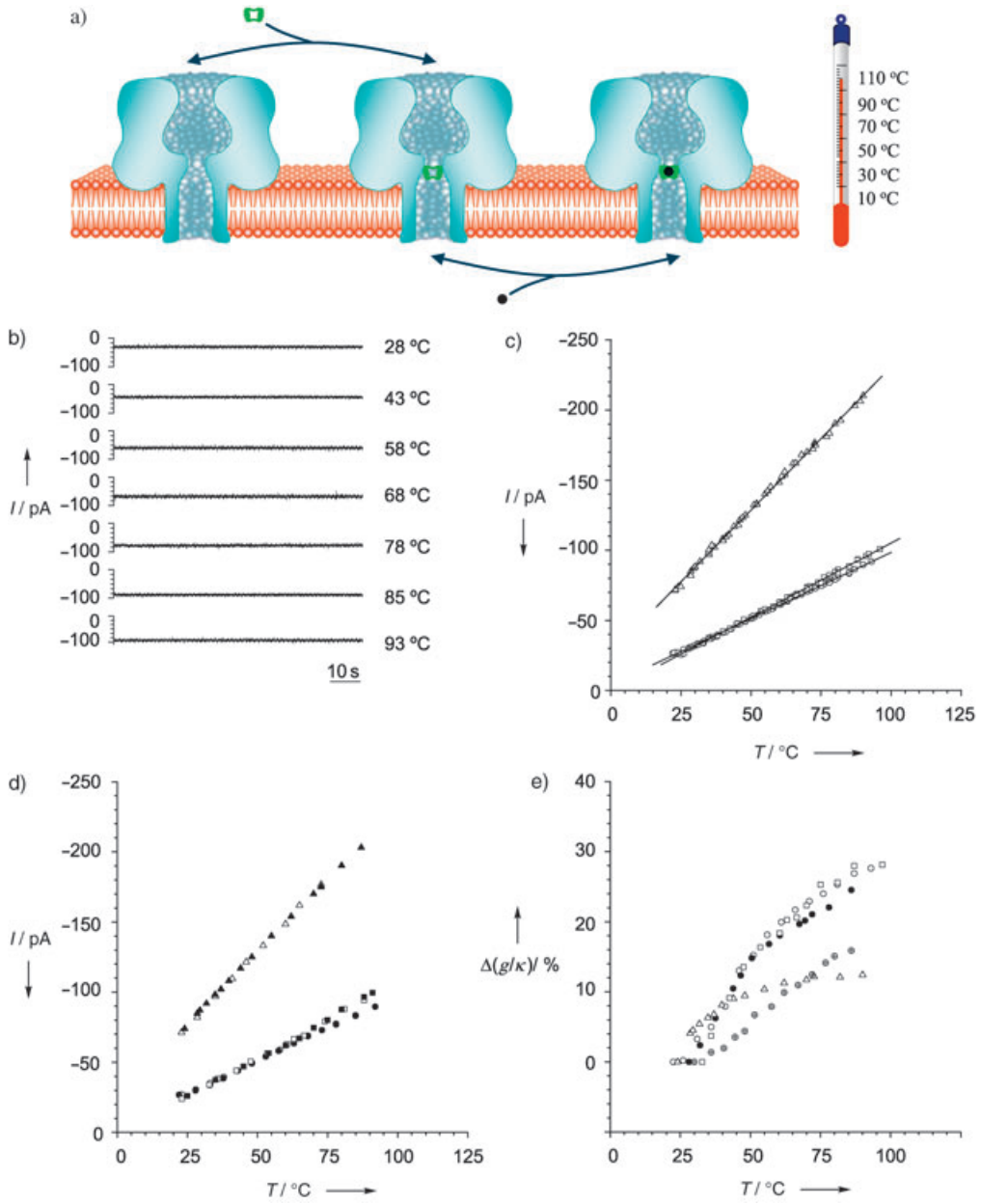

Figure 1. Single-channel currents at elevated temperatures. The buffer in both chambers was sodium phosphate (10 mM, pH 7.5), containing NaCl (1 M). Transmembrane potential was −40 mV. a) Schematic representation of one of the pores used in this work. Three states of the αHL pore in a lipid bilayer are shown. At high temperatures, the unoccupied pore (left) retains its ability to bind molecular adapters such as βCD (center, rendered in green), which can in turn bind guest molecules (right, shown in black). b) Representative single-channel current traces of unoccupied wild-type αHL pores at different temperatures. c) The variation of single-channel currents with temperature for wild-type αHL (○), Luk (△), and OmpG (□) pores. The experimental values from four different experiments are compiled in each plot. The single-channel currents depended linearly on the temperature: wild-type αHL, I(pA) = 4.18 + 0.944 T(°C) (R = 0.999); Luk, I(pA) = 26.4 + 2.04 T(°C) (R = 0.999); OmpG, I(pA) = 0.373 + 1.05 T(°C) (R = 0.999). d) The changes in single-channel currents are reversible: wild-type αHL (○,●), Luk (△,▲), and OmpG (□,■) pores. The data are from single representative experiments. Empty symbols represent data obtained as temperature increased, the filled symbols show data collected as temperature subsequently decreased. e) Plots of the percent change in g/κ as a function temperature for wild-type αHL (○), $(M113N)_7$ (●), $(M113N)_7$·βCD (⊕), OmpG (□) and Luk (△) pores. The values at 23 °C were set to 0%. Single-channel conductance values (g) were from Figure 1c, with additional data for $(M113N)_7$ and $(M113N)_7$·βCD obtained under the same conditions (Supporting Information). Values of solution conductivity (κ) were determined from a linear fit to experimental κ values measured at different temperatures. αHL = α-hemolysin; βCD = β-cyclodextrin; Luk = leukocidin; $(M113N)_7$ = Met113-to-Asn replacement on αHL, in which all seven subunits bear the mutation.

tinguished the OmpG traces from those of the αHL pore, which has a similar conductance, thereby ruling out sample contamination. Again, 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,[15b] and the OmpG protein unfolds in *n*-octyl-β-D-

glucopyranoside at 63 °C.[11] The changes in single-channel current associated with the temperature ramp were fully reversible (Figure 1 d). Three main factors contributed to the ability to record single-channel currents at high temperatures (Supporting Information). First was the use of an aperture with a diameter of ≈ 100 μm. (With a larger orifice, multiple channels were incorporated too readily and the bilayer tended to break.) Second, bilayers were formed with a large mass of DPhPC (200 μg lipid per chamber: electrolyte volume = 1.5 mL, surface area = 0.72 cm^2). Third, dilute protein samples were used (experimental details can be found in the Supporting Information).

The strong temperature dependence of the single-channel conductance values (g) of the three pores is largely a result of the variation of solution conductivity (κ) with temperature, which suggests that there is no appreciable molecular reorganization or subunit dissociation at elevated temperatures. The conductivity of the buffer was found to increase linearly with temperature from 20 to 90 °C ($\kappa = 3.74 + 0.19\,T\,S\,m^{-1}$ (T = temperature in °C; R = 0.998 for all data points plotted from three experiments)), which is similar to literature values for 1 M NaCl.[22] When g/κ is plotted as a function of temperature, the value increases slightly with temperature for all the pores examined (Figure 1 e). For the Luk pore, g/κ increases by ≈ 13 % over the 70 °C range. For wild-type αHL, the αHL mutant form $(M113N)_7$, and OmpG, the changes in g/κ are larger at 26 %, 25 %, and 24 %, respectively. Because these relatively wide pores allow the passage of hydrated ions, the dominant effect of κ in determining g is reasonable. The small change in g/κ with temperature did not result from a change in the pH value of the solution nor from the development of a small electrical potential in the apparatus (Supporting Information). Therefore, the most likely explanation derives from the mechanism of ion transport through the pores. While the pores we have examined transport hydrated ions, they are weakly ion-selective. The selectivity derives from the interactions of the ions with the walls of the pore lumen. As the temperature increases, these interactions are weakened and the conductance of the pore increases to a greater extent than would be predicted from bulk conductivity measurements. By comparison with the β barrels, it is notable that the gating kinetics of several channels, including the temperature-gated vanilloid receptors, are characterized by dramatic responses to temperature (at < 55 °C).[23]

We discovered earlier that host molecules such as cyclodextrins can become lodged within the αHL pore, where they can in turn bind guest molecules (Figure 1 a).[24] In the work described herein, we examined the interaction of βCD with the $(M113N)_7$ pore at high temperatures. At room temperature, $(M113N)_7$ binds βCD > 10000-fold more tightly than the wild-type protein.[25] At 31 °C, βCD binding events with a mean duration (τ_{off}) of 14 s are observed (Figure 2). As the temperature increases, several phenomena are observed at a fixed βCD concentration:

1. The conductance values of both the unoccupied and occupied states of the pore increase.
2. Short additional blockades from the $(M113N)_7$·βCD level (substates) are observed. The frequency of occurrence of these events was independent of βCD concentration, so they do not arise from the binding of a second βCD molecule. Instead, they are assigned as a second conformation of the occupied state, $(M113N)_7$·βCD. Perhaps they represent the rotation of βCD at the binding site or dewetting transitions[26] within the narrow cyclodextrin ring.
3. The dwell time of βCD (τ_{off}) and the intervals between the binding events (τ_{on}) both decrease.

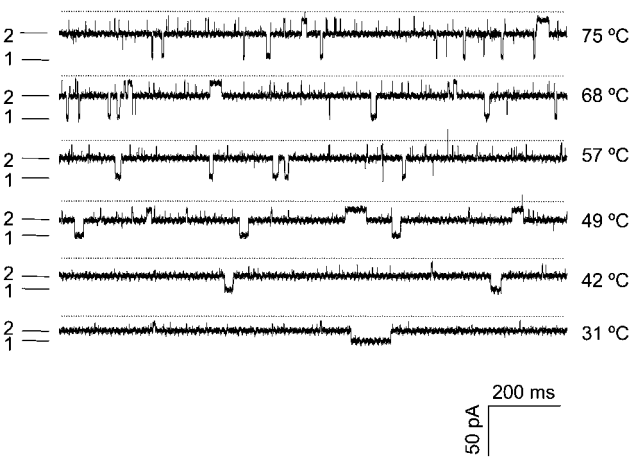

Figure 2. Interaction of βCD with $(M113N)_7$ pores. Representative traces obtained at various temperatures. Broken line, zero current; level 1, current through open $(M113N)_7$ pores; level 2, current through $(M113N)_7$·βCD. The conditions were as described in Figure 1 caption.

In earlier work at room temperature, we showed that βCD takes part in a simple binary interaction with $(M113N)_7$.[25] By a kinetic analysis, we confirmed that this was also the case at 78 °C (Supporting Information). Measurements of the mean dwell time (τ_{off}) and the mean inter-event interval (τ_{on}) were used to derive association (k_{on}) and dissociation (k_{off}) rate constants for βCD. At 25 °C, the values were $4.5 \pm 0.6 \times 10^5\,M^{-1}s^{-1}$ and $0.031 \pm 0.01\ s^{-1}$ ($n = 3$) respectively, yielding $(k_{on}/k_{off}) = K_f = 1.5 \times 10^7\,M^{-1}$. (The literature reports a K_f value of $7.7 \times 10^6\,M^{-1}$, which was determined in a different sequence background for αHL, "RL2", which might explain the small difference).[25] This value is over 10000-fold greater than the value for the interaction of wild-type αHL with βCD ($K_f = 290\,M^{-1}$) reported previously.[25] As the temperature increased, k_{on} and k_{off} increased. For example, at 85 °C, the highest temperature reached in these experiments, k_{on} increased $\approx$15-fold to $6.5 \pm 0.8 \times 10^6\,M^{-1}s^{-1}$ and k_{off} increased $\approx$800-fold to $25.0 \pm 0.3\ s^{-1}$ ($n = 3$) over the values at 25 °C. Because there is a larger increase in k_{off} than with k_{on} as temperature increases, the formation constant K_f decreased to $2.6 \times 10^5\,M^{-1}$ at 85 °C.

From the slope of a linear fit to $\ln K_f$ versus $1/T$ (Supporting Information), $\Delta H°$ and $\Delta S°$ values were found to be $-60 \pm 4\ kJ\,mol^{-1}$ and $-62 \pm 5\ J\,mol^{-1}K^{-1}$ respectively, yielding a value of $\Delta G° = -41 \pm 3\ kJ\,mol^{-1}$ at 25 °C ($n = 4$). The value of $\Delta H°$ is close to that for the binding of βCD to glucoamylase, whereas the value of $\Delta S°$ for glucoamylase is a less favorable $-90\ J\,mol^{-1}K^{-1}$,[27–29] suggesting a more favor-

able preorganization of the binding site in $(M113N)_7$, which would be augmented by the matching C_7 symmetry of the αHL pore and βCD. By comparison, for the formation of an eight-nucleotide DNA duplex under similar conditions,[10] $\Delta H° = -144\ kJ\,mol^{-1}$, $\Delta S° = -359\ J\,mol^{-1}\,K^{-1}$, and $\Delta G° = -37\ kJ\,mol^{-1}$. In this case, a highly favorable enthalpic contribution compensates for a far larger entropic penalty. Interestingly, binding experiments with βCD and the heteromeric[30,31] pore containing one wild-type (WT) subunit ($WT_1(M113N)_6$) yielded $\Delta H° = -51 \pm 2\ kJ\,mol^{-1}$, $\Delta S° = -59 \pm 3\ J\,mol^{-1}\,K^{-1}$, and $\Delta G° = -33 \pm 2\ kJ\,mol^{-1}$ at 25 °C (Supporting Information). The decrease in affinity brought about by the loss of one asparagine residue is almost entirely derived from a change in $\Delta H°$, again suggesting a preorganized binding site.

Values of $\Delta G^{\neq}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for βCD and $(M113N)_7$ were determined by using $\ln k/\phi = -(\Delta H^{\neq}/R)\cdot 1/T + \Delta S^{\neq}/R$; ϕ is a frequency factor in a simplified transition-state theory that is useful for comparisons with related systems (Supporting Information).[32,33] For the dissociation of βCD, $\Delta H^{\neq} = 99\ kJ\,mol^{-1}$ and $\Delta S^{\neq} = 130\ J\,mol^{-1}\,K^{-1}$, with ϕ at $1\ ns^{-1}$.[33] At 25 °C, $\Delta G^{\neq} = 60\ kJ\,mol^{-1} = 24\,RT$; at 85 °C, $\Delta G^{\neq} = 53\ kJ\,mol^{-1} = 21\,RT$. For the association of βCD, $\Delta H^{\neq} = 39\ kJ\,mol^{-1}$ and $\Delta S^{\neq} = 66\ J\,mol^{-1}\,K^{-1}$ when $\phi = 1\ ns^{-1}$. Association at 25 °C gives $\Delta G^{\neq} = 19\ kJ\,mol^{-1} = 7.7\,RT$; at 85 °C $\Delta G^{\neq} = 15\ kJ\,mol^{-1} = 6.1\,RT$. The value of $\Delta S^{\neq} = 130\ J\,mol^{-1}\,K^{-1}$ for dissociation can be compared with the value of $310\ J\,mol^{-1}\,K^{-1}$ ($\phi = 1\ ns^{-1}$) for the dissociation of the duplex formed by two complementary eight-base DNA strands.[10] In the latter case, the approach to the transition state must reflect a relatively large increase in disorder by comparison with that in βCD dissociation.

βCD is a host for a wide variety of guest molecules.[34] Therefore, the αHL pore equipped with βCD as a molecular adapter can act as a sensor element for the stochastic detection of small organic compounds.[24] Herein we demonstrate this approach at elevated temperatures with adamantane-1-carboxylic acid as a model analyte.[24] Although the $(M113N)_7$ pore binds βCD at high temperatures, the appearance of substates (partial closures during occupancy by βCD, Figure 2), which are dependent on both temperature and the applied potential, limits its use in stochastic detection. We therefore used a homoheptameric pore made from the double mutant M113F/K147N, which has the following characteristics (unpublished work): 1. there are no substates during occupancy by βCD; 2. the binding affinity for βCD is high, with K_f (*trans*) $= 1.3 \pm 0.2 \times 10^5\ M^{-1}$ at −40 mV ($n = 4$), in comparison with wild-type αHL,[25] K_f (*trans*) $= 3.0 \times 10^2\ M^{-1}$; and interestingly, 3. βCD binds from both the *cis* and *trans* sides of the bilayer. In wild-type αHL and most mutant forms, βCD binds only from the *trans* side. βCD (*cis*) bound to $(M113F/K147N)_7$ for extended periods ($\tau_{off} = 7.0 \pm 0.3$ s at −60 mV ($n = 3$)), during which an interaction with adamantane-1-carboxylic acid (*trans*) could be observed (Figure 3). At 22 and 65 °C, we ascertained that the mean residence time (τ_{off}) of the analyte was independent of analyte concentration and that $1/\tau_{on}$ (τ_{on} denotes the inter-event interval) was linearly dependent on the analyte concentration; this is diagnostic of a bimolecular interaction between the analyte and βCD.[25,35] Kinetic constants were determined from τ_{on} and τ_{off} values. Over the temperature range from 22 to 78 °C, k_{on} for adamantane-1-carboxylic acid increased by about sixfold from $2.5 \pm 0.2 \times 10^6\ M^{-1}\,s^{-1}$ ($n = 3$) to $1.4 \pm 0.1 \times 10^7\ M^{-1}\,s^{-1}$ ($n = 3$), and k_{off} increased by ≈43-fold from $2.1 \pm 0.2 \times 10^2\ s^{-1}$ ($n = 3$) to $9.3 \pm 0.6 \times 10^3\ s^{-1}$ ($n = 3$) (Supporting Information). The corresponding K_f values at 22 and 78 °C, were $1.2 \pm 0.1 \times 10^4\ M^{-1}$ and $1.5 \pm 0.1 \times 10^3\ M^{-1}$, respectively. From a plot of $\ln K_f$ versus $1/T$ (Supporting Information), we obtained $\Delta H°$ and $\Delta S°$ values of $-31 \pm 2\ kJ\,mol^{-1}$ and $-28 \pm 1\ J\,mol^{-1}\,K^{-1}$, respectively, yielding $\Delta G° = -23 \pm 2\ kJ\,mol^{-1}$ at 25 °C ($n = 3$). At least seven values for the standard thermodynamic constants are available in the literature for adamantane-1-carboxylic acid as the carboxylate.[34] They are in rough agreement with each other: $\Delta H° = -22\ kJ\,mol^{-1}$, $\Delta S° = +10\ J\,mol^{-1}\,K^{-1}$, $\Delta G° = -25\ kJ\,mol^{-1}$. Although the value of $\Delta G°$ is close to that obtained in our work, $\Delta H°$ and $\Delta S°$ differ. Within the αHL pore, the enthalpy change for the interaction is more favorable, but a less favorable $T\Delta S°$ compensates. Perhaps the βCD presents only one face to the guest presented from the *trans* side or the βCD is in a different conformation when lodged inside the pore than it is in solution. Pore-bound βCD may form a binding site that offers more favorable noncovalent bonding interactions, but which is associated with, for example, less favorable solvent reorganization.

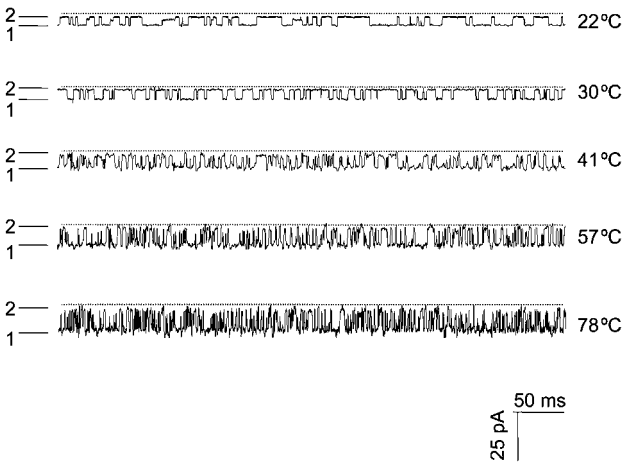

Figure 3. Interaction of the model analyte adamantane-1-carboxylic acid with βCD lodged in the $(M113F/K147N)_7$ pore. Representative traces showing the interaction of $(M113F/K147N)_7$·βCD with adamantane-1-carboxylic acid at various temperatures. βCD (40 μM) was applied to the *cis* side of the bilayer; adamantane-1-carboxylic acid (20 μM) was applied to the *trans* side. All other conditions were as described in Figure 1 caption, except the applied potential was −60 mV. Broken line, zero current; level 1, $(M113F/K147N)_7$·βCD; level 2, $(M113F/K147N)_7$·βCD blocked with adamantane-1-carboxylic acid. $(M113N/K147N)_7$ = mutant form of αHL, in which all seven subunits bear the double mutation Met113- and Lys147-to-Asn.

Our experiments show that the properties of protein pores can be examined at temperatures approaching 100 °C at the single-molecule level by planar bilayer recording. The approach we have developed will be useful in studies of the fundamental functional properties of ion channels, and how they fold and assemble. All three pores that we examined

contain transmembrane β barrels, and it will be interesting to apply this approach to channels that are predominantly α-helical. The pores we examined are functional at high temperature, although they originate in mesophilic bacteria. It will also be worth examining channels and pores from thermophiles to understand how they contribute to the physiology of these organisms.[3] From the biotechnological point of view, the ability to observe channels and pores at high temperatures will aid our ability to engineer stable membrane proteins[1] to act as components of devices such as sensors[36] or DNA sequencers.[37] In that respect, we have shown herein that an αHL pore containing a molecular adapter retains its ability to bind a model analyte at elevated temperatures. Recently, we have used the αHL pore as a nanoreactor for the examination of single-molecule chemistry.[38–40] The ability to record at high temperatures will greatly extend the power of this methodology.

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[1] H. Bayley, L. Jayasinghe, *Mol. Membr. Biol.* **2004**, *21*, 209 – 220.
[2] T. Haltia, E. Freire, *Biochim. Biophys. Acta* **1995**, *1241*, 295 – 322.
[3] L. J. Rothschild, R. L. Mancinelli, *Nature* **2001**, *409*, 1092 – 1101.
[4] J. Wang, M. A. El-Sayed, *Biophys. J.* **2000**, *78*, 2031 – 2036.
[5] A. Gliozza, R. Rolandi, M. De Rosa, A. Gambacorta, *J. Membr. Biol.* **1983**, *75*, 45 – 56.
[6] M. J. Caterina, T. A. Rosen, M. Tominaga, A. J. Brake, D. Julius, *Nature* **1999**, *398*, 436 – 441.
[7] B. Liu, K. Hui, F. Qin, *Biophys. J.* **2003**, *85*, 2988 – 3006.
[8] G. Belmonte, L. Cescatti, B. Ferrari, T. Nicolussi, M. Ropele, G. Menestrina, *Eur. Biophys. J.* **1987**, *14*, 349 – 358.
[9] A. Meller, L. Nivon, E. Brandin, J. Golovchenko, D. Branton, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1079 – 1084.
[10] S. Howorka, L. Movileanu, O. Braha, H. Bayley, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12996 – 13001.
[11] S. Conlan, H. Bayley, *Biochemistry* **2003**, *42*, 9453 – 9465.
[12] L. Song, M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, J. E. Gouaux, *Science* **1996**, *274*, 1859 – 1865.
[13] R. Olson, H. Nariya, K. Yokota, Y. Kamio, E. Gouaux, *Nat. Struct. Biol.* **1999**, *6*, 134 – 140.
[14] J.-D. Pédelacq, L. Maveyraud, G. Prévost, L. Baba-Moussa, A. González, E. Courcelle, W. Shepard, H. Monteil, J.-P. Samama, L. Mourey, *Structure* **1999**, *7*, 277 – 288.
[15] a) G. Miles, L. Movileanu, H. Bayley, *Protein Sci.* **2002**, *11*, 894 – 902; b) L. Jayasinghe, personal communication.
[16] G. Miles, S. Cheley, O. Braha, H. Bayley, *Biochemistry* **2001**, *40*, 8514 – 8522.
[17] H. Lindsey, N. O. Petersen, S. I. Chan, *Biochim. Biophys. Acta* **1979**, *555*, 147 – 167.
[18] V. Luzzati, A. Gambacorta, M. DeRosa, A. Gulik, *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16*, 25 – 47.
[19] A. C. McNiven, P. Owen, J. P. Arbuthnott, *J. Med. Microbiol.* **1972**, *5*, 113 – 122.
[20] B. Walker, H. Bayley, *Protein Eng.* **1995**, *8*, 491 – 495.
[21] S. Conlan, Y. Zhang, S. Cheley, H. Bayley, *Biochemistry* **2000**, *39*, 11845 – 11854.
[22] V. V. M. Lobo, J. L. Quaresma, *Handbook of Electrolyte Solutions.* Part B, Elsevier, New York, **1989**.
[23] M. Pusch, U. Ludewig, T. J. Jentsch, *J. Gen. Physiol.* **1997**, *109*, 105 – 116.
[24] L.-Q. Gu, O. Braha, S. Conlan, S. Cheley, H. Bayley, *Nature* **1999**, *398*, 686 – 690.
[25] L.-Q. Gu, S. Cheley, H. Bayley, *J. Gen. Physiol.* **2001**, *118*, 481 – 494.
[26] O. Beckstein, M. S. P. Sansom, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7063 – 7068.
[27] B. W. Sigurskjold, B. Svensson, G. Williamson, H. Driguez, *Eur. J. Biochem.* **1994**, *225*, 133 – 141.
[28] B. W. Sigurskjold, T. Christensen, N. Payre, S. Cottaz, H. Driguez, B. Svensson, *Biochemistry* **1998**, *37*, 10446 – 10452.
[29] J. Sauer, T. Christensen, T. P. Frandsen, E. Mirgorodskaya, K. A. McGuire, H. Driguez, P. Roepstorff, B. W. Sigurskjold, B. Svensson, *Biochemistry* **2001**, *40*, 9336 – 9346.
[30] O. Braha, B. Walker, S. Cheley, J. J. Kasianowicz, L. Song, J. E. Gouaux, H. Bayley, *Chem. Biol.* **1997**, *4*, 497 – 505.
[31] S. Howorka, S. Cheley, H. Bayley, *Nat. Biotechnol.* **2001**, *19*, 636 – 639.
[32] H. Gutfreund, *Kinetics for the Life Sciences*, Cambridge University Press, Cambridge, **1995**.
[33] O. S. Andersen, *J. Gen. Physiol.* **1999**, *114*, 589 – 590.
[34] M. V. Rekharsky, Y. Inoue, *Chem. Rev.* **1998**, *98*, 1875 – 1917.
[35] E. Moczydlowski in *Ion Channel Reconstitution* (Ed.: C. Miller), Plenum, New York, **1986**, pp. 75 – 113.
[36] H. Bayley, P. S. Cremer, *Nature* **2001**, *413*, 226 – 230.
[37] D. W. Deamer, D. Branton, *Acc. Chem. Res.* **2002**, *35*, 817 – 825.
[38] S.-H. Shin, T. Luchian, S. Cheley, O. Braha, H. Bayley, *Angew. Chem.* **2002**, *114*, 3859 – 3861; *Angew. Chem. Int. Ed.* **2002**, *41*, 3707 – 3709.
[39] T. Luchian, S.-H. Shin, H. Bayley, *Angew. Chem.* **2003**, *115*, 1970 – 1973; *Angew. Chem. Int. Ed.* **2003**, *42*, 1926 – 1929.
[40] T. Luchian, S.-H. Shin, H. Bayley, *Angew. Chem.* **2003**, *115*, 3896 – 3901; *Angew. Chem. Int. Ed.* **2003**, *42*, 3766 – 3771.

