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An Outer Membrane Protein undergoes Enthalpy- and Entropy-driven Transitions

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Abstract

β -barrel membrane proteins often fluctuate among various open sub-states, yet the nature of these transitions is not fully understood. Using temperature-dependent, single-molecule electrophysiology analysis, along with rational protein design, we show that OccK1, a member of the outer membrane carboxylate channel from *Pseudomonas aeruginosa*, features a discrete gating dynamics comprising of both enthalpy-driven and entropy-driven current transitions. OccK1 was chosen to analyze these transitions, because it is a monomeric transmembrane β -barrel of known high-resolution crystal structure and displays three distinguishable, time-resolvable open sub-states. Native and loop-deletion OccK1 proteins showed substantial changes in the activation enthalpies and entropies of the channel transitions, but modest alterations in the equilibrium free energies, confirming that the system never departs from equilibrium. Moreover, some current fluctuations of OccK1 indicated a counterintuitive, negative activation enthalpy, which was compensated by a significant decrease in the activation entropy. Temperature scanning of the single-channel properties of OccK1 exhibited a thermally-induced switch of the energetically most favorable open sub-state at the lowest examined temperature of 4°C. Therefore, such a semi-quantitative assessment of the current fluctuation dynamics not only demonstrates the complexity of channel gating, but also reveals distinct functional traits of a β -barrel outer membrane protein under different temperature circumstances.

Keywords

Gating; Kinetics; Single-channel electrical recording; OccK1; The kinetic rate theory

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Supporting Information Available: (i) physical features of the extracellular loops of the WT-OccK1 protein, (ii) titration experiments with potassium phosphate buffer, (iii) purification test of the OccK1 protein channel by SDS-PAGE assay, (iv) temperature-dependent single-channel electrical recordings at a negative applied transmembrane potential, (v) standard histograms of fitted current amplitudes of the single-channel events, (vi) typical all-points current amplitude histograms of the WT-OccK1, (vii) semilog Eyring plot of the four kinetic rate constants of the WT-OccK1 protein channel acquired at a negative applied transmembrane potential, (viii) properties of loop-deletion OccK1 mutants, (ix) graphic representations of the loop-deletion OccK1 mutants, (x) single-channel electrical recordings with loop-deletion OccK1 mutants at room temperature, (xi) enthalpic contributions to the single-channel fluctuations of the WT-OccK1 protein and loop-deletion OccK1 mutants, (xii) determination of the minimum transition entropies and maximum transition energies of the WT-OccK1 protein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

It is thought that a β -barrel structure is quite rigid, because of the network of backbone hydrogen bonds among the neighboring anti-parallel β strands (1). Spontaneous, thermally-induced current fluctuations are often observed in β -barrel outer membrane proteins (2;3). Two major mechanisms were proposed for the gating of β -barrel membrane protein channels: (i) the electrostatic mechanism, which involves local electrostatic forces within the constriction of the protein channel, thus precluding the ions from traversing the limiting barrier (4;5); (ii) the steric mechanism, which implies stochastic movements of long extracellular loops folded back into the channel lumen (6;7). Gating of β -barrel proteins has been studied extensively using various methodologies, such as single-channel electrical recordings (8;9), rational membrane protein design (6;7;10;11), atomic force microscopy (12), NMR spectroscopy (13), X-ray crystallography (14) and molecular dynamics simulations (15;16). Despite significant progress in identifying the mechanisms responsible for discrete fluctuation dynamics (12;17), we do not have a detailed quantitative understanding of the kinetics and energetics of the thermally-activated current fluctuations in a β -barrel protein channel.

Recently, we explored the gating of the OccK1 protein and found that it exhibits three well-defined open sub-states (18). OccK1, which was previously named OpdK (19), is an archetype of the outer membrane carboxylate channel K (OccK) subfamily from *Pseudomonas aeruginosa* (20–23). The X-ray crystal structure of OccK1 reveals a monomeric, 18-stranded β -barrel with a kidney-shaped central pore (Fig. 1) (22;24). The channel lumen includes long extracellular loops L3, L4 and L7, and features a 5 Å-diameter central constriction. Our hypothesis was that the presence of large extracellular loops L3, L4 and L7 within the channel lumen impacts the nature of the current fluctuations of the OccK1 protein (Supporting Information, Table S1). Thus, we relied upon recent high-resolution X-ray crystal structure of this outer membrane protein (Fig. 1) (22).

Here, the discrete gating dynamics of OccK1 reveals both enthalpy- and entropy-driven current fluctuations. We employed single-channel, temperature-dependent electrical recordings and loop-deletion protein design (Supplementary Information) to explore alterations in the kinetics and energetics of the discrete current fluctuations of the OccK1 protein. A semi-quantitative Arrhenius-based approach was implemented to obtain activation enthalpies as well as equilibrium free energies, enthalpies, and entropies for all current fluctuations. Loop-deletion OccK1 derivatives employing L3 and L4, each of which lacking an intramolecular salt bridge and several hydrogen bonds, showed significant modifications in the transition and equilibrium enthalpies, but modest changes in the equilibrium free energies.

Materials and Methods

Cloning, overexpression, and purification of the OccK1 protein and loop-deletion OccK1 mutants

The *occk1* gene lacking the part coding for the signal sequence was amplified from genomic DNA of *P. aeruginosa* and was then cloned into the pB22 vector (25) with the *E. coli* YtfM signal sequence at the N-terminus. We also introduced a 7-His tag at the N-terminus and a TEV protease cleavage site for the removal of the His tag. The Δ L3, Δ L4 and Δ L7 loop deletions of the OccK1 protein were made by PCR (Expand high fidelity PCR system, Roche) (Supporting Information, Table S2). The proteins were expressed in C43 (DE3) *E. coli* cells and purified by His tag chromatography. Other details of the standard protein chemistry protocols used in this work were published previously (18). The purity of the OccK1 protein samples was assessed by SDS-PAGE gel electrophoresis (Supplementary

Information, Fig. S2). Expression and purification of the loop-deletion OccK1 mutants were carried out in the same way as for the WT-OccK1 protein.

Single-channel current recordings on planar lipid bilayers

Single-channel current measurements were performed by planar lipid membranes (26). The electrolyte in both chambers was 2000 mM KCl, 10 mM potassium phosphate, pH 7.4, unless otherwise stated. The bilayer was formed with 1,2-diphytanoyl-*sn*-glycerophosphocholine (Avanti Polar Lipids Inc., Alabaster, AL, USA). OccK1 was added to the *cis* chamber, which was at ground. Current flow shown as positive represents a positive charge moving from the *trans* to *cis* chamber. Currents were recorded by using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) connected to the chambers by Ag/AgCl electrodes (7). The single-channel electrical traces were filtered by an 8-pole low-pass Bessel filter (Model 900, Frequency Devices, Haverhill, MA) at a frequency of 10 kHz and sampled at 50 kHz. The rise time of the filter is $T_r = 339/f_c$, where f_c is the corner frequency of the low-pass Bessel filter (27;28). For a value of $f_c = 500$ Hz, we obtain $T_r = \sim 680$ μ s. This value would give us a dead time $T_d = 0.54 \times T_r = 365$ μ s. Employing single-channel analysis of the missed events (29–31), we estimated that the missed current blockades were not more than 8% of the total number of events in each recorded trace.

Temperature controller for single-channel electrical recordings with planar lipid bilayers

The temperature-control experiments were achieved using a Dagan HCC-100A controller (Dagan Corporation, Minneapolis, MN), which was adapted to planar bilayer recordings (32;33). Temperature was simultaneously monitored in the aluminum stage holding the bilayer chamber as well as in the bilayer chamber with thermocouple probes.

Molecular graphics

The OccK1 protein model was generated by the Chimera software package (34) using 2qtk.pdb (24).

Results

Exploring the optimal experimental conditions for temperature-dependent, single-channel analysis

Single-channel examination of the temperature dependence of the current fluctuations among the three open sub-states required a careful consideration of the experimental conditions (35;36). We decided to employ an ionic aqueous phase containing 2 M KCl for amplifying the signal-to-noise ratio, such as in the previous work with temperature-responsive, loop-containing α -hemolysin protein nanopores (32). Importantly, the number of the open sub-states remains preserved by altering the salt concentration of the aqueous phase from 1 to 4 M KCl (18). In addition, there is no substantial change in the kinetic rate constants when the salt concentration was varied from 1 to 2 M KCl.

A second issue is the choice of the buffer. Most buffers are unstable under different temperature regimes, meaning that there are substantial changes in pH accompanying changes in temperature. To cancel all the temperature-dependent factors that affect pH, we used a buffer with a low temperature coefficient (37;38). One excellent candidate, already tested in prior temperature-dependent single-channel experiments, is potassium phosphate (32;33;39). However, one possibility is that the presence of the potassium phosphate buffer alters the single-channel electrical signature of the OccK1 protein channel. To test this hypothesis, we pursued titration experiments with potassium phosphate buffer. Using various potassium phosphate concentrations, in the range 2–20 mM, we were not able to detect statistically significant changes in the dwell time of all open sub-states, O₁, O₂ and O₃.

(Supporting Information, Fig. S1), indicating no effect of the buffer on the single-channel kinetics.

All our temperature-dependent single-channel electrical recordings were performed at pH 7.4. However, how is the single-channel electrical signature impacted by pH? The potassium phosphate buffer operates well in the pH range 6–8 (37;40). Therefore, we executed single-channel electrical recordings with OccK1 at pH values of 6 and 8. Interestingly, we found no statistically significant alterations of the dwell times of the open sub-states O_1 , O_2 and O_3 . Representative single-channel electrical are shown in Fig. 2. In 1 M KCl, 10 mM potassium phosphate, pH 6, the fitted dwell times of the O_1 , O_2 and O_3 open sub-states were 1.8 ± 0.3 ms, 7.6 ± 0.1 ms and 3.9 ± 0.2 ms, respectively. Under similar experimental conditions, the values of these dwell times, determined at pH 8, were 2.1 ± 0.2 ms, 6.9 ± 0.2 ms and 3.6 ± 0.2 ms, respectively. These results indicate a lack of chargeable groups within the channel lumen under inspected experimental circumstances (pH 6–8). We examined the high-resolution X-ray crystal structure of the OccK1 protein and found that indeed histidine residues ($pK_a = 6$) are located outside the channel lumen (23).

Changing pH to either a more alkaline or a more acidic value requires a replacement of the potassium phosphate buffer. Therefore, we performed single-channel electrical recordings at pH 3 in 10 mM potassium acetate and at pH 9.0 in 10 mM Tris.HCl. The data were collected with an aqueous phase containing 1 M KCl. Typical single-channel traces are illustrated in Fig. 3. In both cases, we determined a large number of non-resolvable single-channel current blockade events. Moreover, we found that the open sub-state current levels were not well-defined at acidic pH. Therefore, a neutral pH obtained with a potassium phosphate buffer is a robust choice for temperature-dependent single-channel recordings.

Temperature-dependence of the single-channel electrical recordings with the OccK1 protein

The OccK1 protein was expressed and purified by standard protein chemistry techniques (Supporting Information, Fig. S2). We examined OccK1 in a temperature regime below room temperature, between 4 and 20°C. This strategy enabled a detailed single-channel analysis of all current fluctuations observed in a previous work performed at room temperature (18). This experimental design was motivated by the fact that the kinetic rate constants were expected to substantially increase at elevated temperatures, reaching values beyond the resolution of our instrument. In Fig. 4A, we show a typical single-channel electrical trace recorded with the wild-type OccK1 (WT-OccK1) protein at a temperature of 20°C and at an applied transmembrane potential of +40 mV. Clearly, this trace displays three major open sub-states (O_1 , O_2 and O_3) that were further explored using standard dwell-time histograms (Fig. 4B, Fig. 4C, Fig. 4D). A similar number of sub-states were found at an applied transmembrane potential of –40 mV (Supporting Information, Fig. S3), permitting direct meaningful comparisons among data recorded at both negative and positive voltage biases.

These three current sub-state levels were determined using both fitted current amplitude histograms (Supporting Information, Fig. S4) as well as all-points current amplitude histograms (Supporting Information, Fig. S5). The number of open sub-states was also conserved at 4°C (Fig. 4E, Fig. 4F and Fig. 4G). No O_1 to O_3 or O_3 to O_1 transition was observed in this work, indicating a linear kinetic scheme with three distinguishable open sub-states, $O_1 \leftrightarrow O_2 \leftrightarrow O_3$, and with the most probable O_2 open sub-state at 20°C, from which reversible transitions to the flanked open sub-state O_1 and O_3 levels occurred.

Temperature dependence of the unitary conductance of the OccK1 protein channel

We pursued the effect of temperature on the unitary conductance associated with each open sub-state recorded in single-channel traces with the OccK1 protein channel. These data sets are illustrated in Fig. 5. While the unitary conductance of the O₂ and O₃ open sub-states underwent a linear increase by elevating the temperature, with slopes of ~0.018 and ~0.022 nS/°C, respectively, the conductance value corresponding to the low-conductance O₁ open sub-state displayed a rectified enhancement (Fig. 5A). We normalized the unitary conductance (g_{channel} ; Fig. 5A) to the conductivity of the bulk aqueous phase ($g_{\text{bulk solution}}$; Fig. 5B). Again, the normalized conductance values of the O₂ and O₃ open sub-states followed a linear increase with temperature, whereas the normalized conductance value corresponding to the low-conductance O₁ open sub-state increased steeply at elevated temperatures (Fig. 5C). Therefore, for the three open sub-states, the slope of the unitary conductance by increasing temperature is greater than the slope of the conductivity of the bulk aqueous phase.

Determination of the kinetic rate constants

These kinetic rate constants were calculated using the event frequencies (f) and the average dwell times (τ) of the flanked O₁ and O₃ sub-states (18). Using standard chemical kinetics formalisms for single-molecule fluctuations of the OccK1 protein channel (41), we formulate the following system of partial differential equations (42–44):

$$\begin{aligned}\frac{dP_{O1}}{dt} &= -k_{O1 \rightarrow O2}P_{O1} + k_{O2 \rightarrow O1}P_{O2} \\ \frac{dP_{O2}}{dt} &= +k_{O1 \rightarrow O2}P_{O1} - k_{O2 \rightarrow O1}P_{O2} + k_{O3 \rightarrow O2}P_{O3} - k_{O2 \rightarrow O3}P_{O2} \\ \frac{dP_{O3}}{dt} &= -k_{O3 \rightarrow O2}P_{O3} + k_{O2 \rightarrow O3}P_{O2}\end{aligned}\quad (1)$$

where P_{O1} , P_{O2} and P_{O3} are the probabilities to occupy the O₁, O₂ and O₃ open sub-states, respectively. These probabilities are given by the following expressions (42–44):

$$\begin{aligned}P_{O1} &= \frac{T_{O1}}{T} = \frac{N_{O1}\tau_{O1}}{T} = f_{O1}\tau_{O1} \\ P_{O2} &= \frac{T_{O2}}{T} = \frac{N_{O2}\tau_{O2}}{T} = (f_{O1} + f_{O3})\tau_{O2} \\ P_{O3} &= \frac{T_{O3}}{T} = \frac{N_{O3}\tau_{O3}}{T} = f_{O3}\tau_{O3}\end{aligned}\quad (2)$$

T_{O1} , T_{O2} and T_{O3} are the total times occupied by the O₁, O₂ and O₃ open sub-states, respectively. N_{O1} , N_{O2} and N_{O3} are the total recorded events that correspond to the O₁, O₂ and O₃ sub-states, respectively. T indicates the total recording time. f and τ denote the event frequency and the average dwell time for an open sub-state, respectively. The equations (2) indicate two components for the O₂ open sub-state, corresponding to the transitions toward the O₁ and O₃ open sub-states. The well made by the O₂ open substate is flanked by two barriers for reaching the O₁ and O₃ open sub-states.

The rates for reaching the O₁ and O₃ open sub-states are just the corresponding event frequencies, which are normalized to the P_{O2} probability:

$$\begin{aligned}k_{O2 \rightarrow O1} &= \frac{f_{O1}}{P_{O2}} = \frac{f_{O1}}{1 - f_{O1}\tau_{O1} - f_{O3}\tau_{O3}} \\ k_{O2 \rightarrow O3} &= \frac{f_{O3}}{P_{O2}} = \frac{f_{O3}}{1 - f_{O1}\tau_{O1} - f_{O3}\tau_{O3}}\end{aligned}\quad (3)$$

At equilibrium, the partial derivatives of the equations (1) are zero, since the event probabilities are constant. Therefore,

$$\begin{aligned} k_{O_1 \rightarrow O_2} &= \frac{1}{\tau_{O_1}} \\ k_{O_3 \rightarrow O_2} &= \frac{1}{\tau_{O_3}} \end{aligned} \quad (4)$$

The equations (3) and (4) indicate that the four kinetic rate constants, which describe the kinetic scheme with three open sub-states, can be calculated using the event frequencies and the average dwell times of the flanked O_1 and O_3 open sub-states. Moreover, the equations (3) and (4) confirm the general rule that the average dwell time of a certain open sub-state is given by the reciprocal of the sum of the kinetic rate constants for the transitions occurring away from that respective open sub-state (33;41;45;46):

$$\frac{1}{\tau_{O_2}} = k_{O_2 \rightarrow O_1} + k_{O_2 \rightarrow O_3} \quad (5)$$

The temperature dependence of the kinetic rate constants underwent a linear dependence in a semi-log Arrhenius representation (Fig. 6A; Supporting Information, Fig. S6). As expected, $k_{O_2 \rightarrow O_1}$, $k_{O_2 \rightarrow O_3}$ and $k_{O_3 \rightarrow O_2}$ increased upon increasing the temperature. Surprisingly, $k_{O_1 \rightarrow O_2}$ decreased at elevated temperatures.

Rational design of the loop-deletion OccK1 proteins

We asked whether deletions of major extracellular loops L3, L4 and L7 impact the temperature-dependent kinetics and energetics of the current fluctuations (Supplementary Information, Table S2, Figs. S6–S9). We made loop-deletion OccK1 proteins based upon inspection of the high-resolution structure of WT-OccK1 (22). These mutants were designed with the first requirement that the residues immediately before and after the deletion were close enough in space so that they could be replaced by a single glycine residue without introducing significant conformational perturbations of the altered loop. Single-channel data with a loop deletion in L7 (OccK1 Δ L7, S281–G287), which lacks an intramolecular R284–D116 salt bridge between loops L7 and L3, showed a noisy electrical signature, which was the reason not to pursue further temperature-dependent single-channel electrical recordings with this mutant (24). However, this mutant showed a greater most probable conductance than the unitary conductance of the WT-OccK1, confirming that L7 is indeed exposed to the channel lumen. We also constructed the deletion mutant OccK1 Δ L3 (D124–P129), which, in the high-resolution crystal structure (22), lacks an intramolecular D124–R16 salt bridge between loop L3 and the pore wall (Supplementary Information, Table S2, Fig. S7, Fig. S8). In addition, we examined the OccK1 Δ L4 deletion mutant (L166–K175), which does not contain any salt bridge. A second criterion for the selection of deletion-loop OccK1 mutants was that the single-channel conductance and the number of the open sub-states were similar to the WT-OccK1 protein, so a meaningful temperature-dependent comparison of kinetic and thermodynamic parameters among them was possible. While the OccK1 Δ L7 protein did not fulfill the second criterion, OccK1 Δ L3 and OccK1 Δ L4 exhibited a closely similar unitary conductance and an identical number of open sub-states to WT OccK1 (Supplementary Information, Fig. S9). It is worth mentioning that these loop-deletion OccK1 mutants lack several hydrogen bonds as well as Van der Waals interactions with other loops (L2, L3, L6, L7) and the B-barrel pore wall (Supplementary Information, Table S2).

Enthalpic and minimum entropic contributions to the gating transitions of the OccK1 proteins

Using linear semi-log Eyring plots of the kinetic rate constants versus the reciprocal of the absolute temperature, we determined the enthalpic ($\Delta H^\ddagger$) and entropic ($\Delta S^\ddagger$) contributions to the activation free energies ($\Delta G^\ddagger$) (39;47–49):

$$\ln k = \ln ff + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{R} \times \frac{1}{T} \quad (6)$$

where $ff = k_B T/h = 6 \times 10^{12} \text{ s}^{-1}$ is Eyring frequency factor for the gas phase. k_B , T and h are the Boltzmann constant, the absolute temperature and the Plank constant, respectively. The ff parameter is much smaller for conformational transitions of proteins in aqueous phase (50). Eyring transition state theory (TST) was first used for elementary chemical reactions in the gas phase. In this work, we did not use or assume a frequency factor for quantitative determinations of the activation free enthalpies as well as equilibrium free energies, enthalpies and entropies (see below). However, we employed TST formalism for the phenomenological exponential dependence of the kinetic rate constants on the activation free energies, which is routinely used in single-channel electrical data analysis (39;42;50–55). It is also true that the extension of TST to the condensed phase by Hänggi involved complicated expressions of the frequency factor (56). This result makes TST impractical for accurate quantitative determinations of the entropic contribution to the activation free energy in the single-channel kinetics and protein folding (53).

Determinations of $\Delta H^\ddagger$ were made directly using the slope ($-\Delta H^\ddagger/R$) of the semi-log k plots (Fig. 6A; Supplementary Information, Table S3). They were based on the assumption that the thermodynamic parameters $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are not temperature dependent in the temperature range examined in this work (57). The uncertainty of ff produces inaccuracy in calculating the exact values of $\Delta G^\ddagger$ and $\Delta S^\ddagger$. However, knowing that $ff < 6 \times 10^{12} \text{ s}^{-1}$ for protein transitions in aqueous phase, we can obtain an estimate of the maximum value of the activation free energy ($\Delta G_{\text{max}}^\ddagger = RT \times \ln(ff/k)$) and the lower limit of the activation entropy ($\Delta S_{\text{min}}^\ddagger = (\Delta H^\ddagger - \Delta G_{\text{max}}^\ddagger)/T$) (Supporting Information, Table S4) (54).

Fig. 6 illustrates the effect of the loop deletion on the transition enthalpy as well as the impact of the voltage sign at 40 mV. The large-amplitude $O_2 \rightarrow O_1$ current transitions showed a favorable enthalpic contribution (Fig. 6B; Supporting Information, Table S3), which was compensated by unfavorable entropic contributions. For WT-OccK1, at +40 mV and 20°C, minimum entropic contribution and maximum activation free energy were $-T\Delta S_{O_2 \rightarrow O_1\text{-min}} = -42 \pm 6 \text{ kJ/mol}$ and $\Delta G_{O_2 \rightarrow O_1\text{-max}}^\ddagger = 66 \pm 10 \text{ kJ/mol}$, respectively (Supporting Information, Table S4). Contrasting the $O_2 \rightarrow O_1$ transition, the large-amplitude $O_1 \rightarrow O_2$ current fluctuations were characterized by a negative activation enthalpy (Fig. 6C). This large-amplitude, $O_1 \rightarrow O_2$ current transition showed a substantially favorable entropic contribution, with $-T\Delta S_{O_1 \rightarrow O_2\text{-min}} = 119 \pm 15 \text{ kJ/mol}$, giving a maximum free energy of activation, $\Delta G_{O_1 \rightarrow O_2\text{-max}}^\ddagger = 60 \pm 10 \text{ kJ/mol}$ at +40 mV and 20°C. At a voltage of 40 mV, a distinctive feature of the low-amplitude $O_2 \rightarrow O_3$ current transition is that its $\Delta H^\ddagger$ was not impacted by either the sign of the voltage or the deletions operated in L3 or L4 (Fig. 6D). However, $\Delta H^\ddagger$ for the $O_2 \rightarrow O_3$ transition of the OccK1 protein channel was decreased at applied transmembrane potentials of +60 and –60 mV, revealing the complexity of the voltage dependence of this transition. The low-amplitude $O_3 \rightarrow O_2$ current transition exhibited a fairly large enthalpic contribution, in the range 86–149 kJ/mol (Fig. 6E), and was affected by the loop deletions in L3 and L4. For example, at +40 mV, the absolute magnitude of $\Delta H_{O_3 \rightarrow O_2}^\ddagger$ followed the sequence WTOccK1 < OccK1 Δ L3 < OccK1 Δ L4 (Fig. 6C; Supporting Information, Table S3).

Energetics of the current fluctuations

The equilibrium free energies (ΔG° s), enthalpies (ΔH° s) and entropies (ΔS° s) from open sub-states O_1 or O_3 to open sub-state O_2 were calculated using the fits of the ratio between corresponding kinetic rate constants, which is similar to a van't Hoff's equation (58;59):

$$\ln \left(\frac{k_{O_1 \rightarrow O_2}}{k_{O_2 \rightarrow O_1}} \right) = \left(-\frac{\Delta H_{O_1 \rightarrow O_2}^\circ}{R} \right) \frac{1}{T} + \frac{\Delta S_{O_1 \rightarrow O_2}^\circ}{R}$$

$$\ln \left(\frac{k_{O_3 \rightarrow O_2}}{k_{O_2 \rightarrow O_3}} \right) = \left(-\frac{\Delta H_{O_3 \rightarrow O_2}^\circ}{R} \right) \frac{1}{T} + \frac{\Delta S_{O_3 \rightarrow O_2}^\circ}{R} \quad (7)$$

The large-amplitude current transitions of the WT-OccK1 protein were characterized by a highly favorable enthalpic contribution (-161 ± 21 kJ/mol; **Table 1**). This value is in the order of 8 hydrogen bonds ($\Delta H_{HB} \sim 8-20$ kJ/mol), indicating that the channel transition is accompanied by large conformational alterations of the extracellular loops blocking the channel lumen. This highly favorable enthalpic component was balanced by a large entropic penalty (-533 ± 167 J/mol·K), giving a small equilibrium free energy at 25°C (-1.8 ± 0.2 kJ/mol, **Table 1**). OccK1 $\Delta L3$ featured closely similar thermodynamic parameters at +40 mV. In contrast, these findings for the $O_1 \leftrightarrow O_2$ transitions were reversed at -40 mV, namely $\Delta H_{O_1 \rightarrow O_2}$ of OccK1 $\Delta L4$ was greater than the values corresponding to WTOccK1 and OccK1 $\Delta L3$. The energetic barrier between the O_2 and O_3 open sub-states of the WT-OccK1 protein showed a favorable entropic component $\Delta S_{O_3 \rightarrow O_2}^\circ = 139 \pm 5$ J/mol·K at a transmembrane potential of +40 mV. This favorable entropic contribution was compensated by an unfavorable enthalpic contribution $\Delta H_{O_3 \rightarrow O_2}^\circ = 40 \pm 2$ kJ/mol, producing a small equilibrium free energy at 25°C ($\Delta G_{O_3 \rightarrow O_2}^\circ = -1.4 \pm 0.1$ kJ/mol, **Table 1**). OccK1 $\Delta L3$ and OccK1 $\Delta L4$ exhibited increased values of the unfavorable enthalpic contributions of 60 ± 4 and 52 ± 3 kJ/mol, but with more favorable and compensating entropic components of 215 ± 12 and 179 ± 8 J/mol·K, respectively. Similarly, at -40 mV, as compared to WTOccK1, OccK1 $\Delta L3$ and OccK1 $\Delta L4$ showed a greater favorable entropic contribution of 184 ± 5 and 116 ± 5 J/mol·K, which was compensated by an unfavorable enthalpic contribution of 52 ± 2 and 33 ± 1 kJ/mol, respectively (**Table 1**).

Discussion

Because we were not able to detect direct $O_1 \rightarrow O_3$ and $O_3 \rightarrow O_1$ transitions, we judge that the large-amplitude current fluctuations and low-amplitude current fluctuations were made by distinct parts of the WT-OccK1 protein within the channel lumen. This characteristic of the WT-OccK1 protein is advantageous, because the temperature-dependent, single-channel analysis can be easily pursued for individual transitions independently from each other. Interestingly, we determined that the temperature dependence of the single-channel conductance of the OccK1 protein is different from that of the effective conductance given by the bulk aqueous phase. For example, increasing temperature produced an amplification of the ratio between the single-channel conductance and the bulk conductivity. Remarkably, similar results were obtained in prior temperature-dependent, single-channel studies with the outer membrane proteins C (OmpC) and F (OmpF) of *E. coli* (60;61). Chimere and co-workers argued that two possible mechanisms are responsible for this observed phenomenon: the dynamics of the surface charges and the lifetime of the ion pairs within the channel lumen (60). For example, the confined nature of the channel lumen catalyzes the formation of more ion pairs inside the channel lumen than its outside. Elevating the temperature drastically decreases the lifetime of the ion pairs, which favors an increase in the single-channel conductance in a steeper way than that expected from bulk aqueous phase.

The most probable open sub-state at 20°C was O₂, from which the system departed to either the O₁ open sub-state crossing an enthalpic barrier or to the O₃ open sub-state crossing an entropic barrier. The former fluctuation is a large-amplitude current transition that is characterized by a large activation enthalpy ($\Delta H_{O_2 \rightarrow O_1} = 108 \pm 16$ kJ/mol), whereas the latter fluctuation is a low-amplitude current transition featured by a smaller activation enthalpy ($\Delta H_{O_2 \rightarrow O_3} = 56 \pm 9$ kJ/mol). The free energy landscape model corresponding to a temperature of 4°C is illustrated in Fig. 7A (the left panel, dashed line). In this case, the most probable open sub-state is no longer O₂, so there is a switch in the gating profile of the WT-OccK1 protein between the O₂ and O₃ open sub-states. The gradual shift of the O₃ open sub-state at more negative free energies at lower temperatures was accompanied by a progressive drift of the O₁ sub-state at less negative free energies. Therefore, the O₁ and O₃ open sub-states displayed reversal behavior with respect to temperature alterations in the chamber.

Based upon the single-channel data acquired at low-temperature, we predicted that at elevated temperatures all activation free energies, except $\Delta G_{O_1 \rightarrow O_2}$, will decrease by increasing temperature (Fig. 7B, the left-hand panel, dashed line). Indeed, the event frequency and duration of the O₁ current blockades increased at 50°C (Fig. 7B, the right-hand panel). Moreover, we were not able to detect the low-amplitude O₂ ↔ O₃ current fluctuations, again confirming that these events occurred with a much faster duration than the time resolution of our instrument. It is also conceivable that most of these transitions will be missed at elevated temperatures, owing to their low current amplitude. The lack of the low-amplitude current transitions corroborates the model prediction for substantially decreased activation free energy $\Delta G_{O_3 \rightarrow O_2}$ (Fig. 7B, the left-hand panel, dashed line).

Another interesting aspect of these results is the counterintuitive negative value of $\Delta H_{O_1 \rightarrow O_2}$, which resulted from an increase of the microscopic $k_{O_1 \rightarrow O_2}$ transition rate constant at lower temperatures. We judge that this finding is counterintuitive, because it used to be thought that the rates of chemical reactions become faster by increasing temperature. What is the reason of this anomaly? The O₁ → O₂ gating transition is accompanied by strongly negative $\Delta S_{O_1 \rightarrow O_2}$ ($\Delta S_{O_1 \rightarrow O_2-\text{min}} = -405 \pm 52$ J/mol·K). This drastic decrease in the activation entropy might be produced by a local formation of intramolecular interactions during the conformational alterations accompanying the transition process. Here, we report for the first time that the gating fluctuations of a β-barrel protein channel undergo a negative activation enthalpy. It was long believed that negative activation energies of reactions are governed by barrierless transitions, in which the reactive molecule is captured in a potential well. In our system, we show the presence of a barrier between O₁ and O₂, $\Delta G_{O_1 \rightarrow O_2}$, whose energetic value decreases at lower temperatures. Oliveberg and colleagues explained that large-scale conformational transitions of proteins represent highly dimensional reactions between many residues and solvent molecules, which under some experimental circumstances may feature negative activation enthalpies (57). Recently, other groups determined negative activation enthalpies accompanying the kinetics of protein folding (62;63). Noronha and colleagues, using a combination of rapid-mixing temperature jump and laser temperature-jump with fluorescence detection, determined that the activation enthalpy of folding required for a 76-residue ubiquitin-based polypeptide is ~ -45 kJ/mol (63).

Detailed comparison analysis of the four current transitions observed with WT-OccK1, OccK1 ΔL3 and OccK1 ΔL3 revealed statistically significant distinctions in the transition enthalpies as well as equilibrium free energies, enthalpies and entropies of these proteins. One immediate question is why do these single-channel current fluctuations display large activation enthalpies? A transmembrane protein channel, such as many other proteins, exhibits remarkable temperature dependence. As a matter of fact, any protein is a great

temperature sensor. The Q_{10} temperature coefficient of the OccK1 protein channel, which is defined as the kinetic rate constant measured at temperature $T+10$ divided by the kinetic rate constant measured at temperature T (k_{T+10}/k_T), covered a range between 2 and 6 for all inspected transitions. The current gating of a protein channel has typical values of Q_{10} in the range 2–4 (53;64–66). If Q_{10} is 4, this value corresponds to an activation enthalpy of ~ 43 $k_B T$. Then, how is it possible to have thermally-induced current fluctuations characterized by an enormous activation enthalpy, which is much greater than $k_B T$? The probability of such a transition would be very small, so the transition would be barely detectable. However, it happens that such thermally-induced current transitions feature compensatory entropic contributions to the activation free energy. Large enthalpic modifications (ΔH s) to the transition energies are accompanied by large, compensating entropic alterations ($-T\Delta S$ s), which result in smaller ΔG s. Enthalpy-entropy compensation is a general property in many chemical reactions, protein folding and all processes dominated by weak interactions (67). The OccK1 protein channel comprises large extracellular loops L3, L4 and L7 folded back into the channel lumen (Supporting Information, Table S1). These heavy loops are likely responsible for the low-amplitude and large-amplitude current fluctuations.

The loop deletions in L3 and L4 increased $\Delta S^{\circ}_{O_3 \rightarrow O_2}$ (**Table 1**), indicating an increase in the disorder of the local environment at the expense of the loss of intramolecular contacts in OccK1 $\Delta L3$ and OccK1 $\Delta L4$, but with no substantial changes in equilibrium energies. On the other hand, the variability of the effect of these deletions on transition and equilibrium enthalpies (Fig. 6; **Table 1**) suggests that alterations in one long extracellular loop (L3 or L4) not only remove native intramolecular contacts, but also produce new interactions between respective loop and other loops or among the other loops (68). Since both deletions in L3 and L4 impacted the single-channel current transitions $O_1 \rightarrow O_2$, $O_2 \rightarrow O_1$ and $O_3 \rightarrow O_2$, these findings confirm the high-resolution X-ray crystal structure of WT-OccK1 that reveals exposure of these large loops into the channel lumen (22).

In summary, we examined the temperature dependence of the three-state discrete dynamics of the current gating fluctuations of OccK1, an outer membrane protein channel from *P. aeruginosa*. OccK1 exhibited enthalpy-driven, large-amplitude and infrequent current transitions between the O_1 and O_2 open sub-states. The reversible $O_1 - O_2$ fluctuations are accompanied by a drastic decrease of equilibrium entropy, rendering the channel system more ordered in the energetically most favorable O_2 open sub-state than in the O_1 open sub-state. Moreover, OccK1 displayed entropy-driven, low-amplitude and highly frequent current transitions between the O_3 and O_2 open sub-states. The small equilibrium free energies demonstrate that these transitions were never far from the equilibrium, whereas the large enthalpic and entropic contributions suggest significant structural alterations within the channel lumen upon small changes in temperature. We also revealed a change in the energetically most favorable open sub-state at the lowest examined temperature of 4°C. This finding has significant implications for other membrane protein channels that operate differently under various temperature regimes. For example, reversal of the most probable sub-state was also observed with the vanilloid receptor (VR) channel (64). At temperatures $< 42^\circ\text{C}$, the VR channel showed a mostly closed sub-state, which was decorated by rare and short-lived openings. However, at elevated temperatures above 42°C , VR exhibited a drastic increase in overall activity. Based on these findings, it may be feasible, with further experimentation and rational molecular design, to construct β -barrel protein nanopore-based devices, whose functional features display thermally-induced switches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of the wild-type OccK1 protein (WT-OccK1) (24)

(A) A cross-sectional view of the WT-OccK1 protein from the extracellular side. The location of the pore constriction is shown by an asterisk; (B) A cross-sectional view along the axis of the pore lumen of the WT-OccK1 protein inserted into a planar lipid bilayer. Regions marked in green, magenta and blue indicate the positions of the major extracellular loops L3, L4 and L7, respectively.

Figure 2. Representative single-channel electrical recordings acquired with the OccK1 protein at different pH values

(A) Typical single-channel electrical trace recorded at pH 6.0; (B) Dwell-time histogram of the O₁ events from (A); (C) Dwell-time histogram of the O₂ events from (A); (D) Dwell-time histogram of the O₃ events from (A); (E) Typical single-channel electrical trace recorded at pH 8.0; (F) Dwell-time histogram of the O₁ events from (E); (G) Dwell-time histogram of the O₂ events from (E); (H) Dwell-time histogram of the O₃ events from (E). The fits were based upon log likelihood ratio (LLR) tests with a given confidence level of 0.95 (46;69). The results of the fits were the following: (B) $\tau_{O1} = 1.8 \pm 0.3$ ms; (C) $\tau_{O2} = 7.6 \pm 0.1$ ms; (D) $\tau_{O3} = 3.9 \pm 0.2$ ms; (F) $\tau_{O1} = 2.1 \pm 0.2$ ms; (G) $\tau_{O2} = 6.9 \pm 0.2$ ms; (H) $\tau_{O3} = 3.6 \pm 0.2$ ms. For the sake of clarity, the single-channel electrical traces were low-pass Bessel filtered at 500 Hz. The buffer solution contained 1 M KCl, 10 mM potassium phosphate at room temperature. The applied transmembrane potential was +40 mV.

Figure 3. Typical single-channel electrical recordings with the OccK1 protein channel at acidic and alkaline pH values

(**A**) Representative trace acquired at pH 3.0 in 10 mM potassium acetate buffer; (**B**) Representative trace recorded at pH 9.0 in 10 mM Tris.HCl buffer. The aqueous phase contained 1 M KCl. The applied transmembrane potential was +40 mV. For the sake of clarity, the single-channel electrical traces were low-pass Bessel filtered at 500 Hz.

Figure 4. Representative single-channel electrical recordings acquired with the WT-OccK1 protein at different temperatures

(A) Typical single-channel electrical trace recorded at 20 °C; (B) Dwell-time histogram of the O₁ events from (A); (C) Dwell-time histogram of the O₂ events from (A); (D) Dwell-time histogram of the O₃ events from (A); (E) Typical single-channel electrical trace recorded at 4 °C; (F) Dwell-time histogram of the O₂ events from (E); (G) Dwell-time histogram of the O₃ events from (E). The fits were based upon a log likelihood ratio (LLR) test with a given confidence level of 0.95. The results of the fits were the following: (B) $\tau_{O_1} = 6.3 \pm 1.1$ ms; (C) $\tau_{O_2} = 17.5 \pm 0.1$ ms; (D) $\tau_{O_3} = 7.4 \pm 0.1$ ms; (F) $\tau_{O_2} = 28.3 \pm 6.1$ ms; (G) $\tau_{O_3} = 32.17 \pm 8.4$ ms. At 4 °C, the number of the O₁ events was not satisfactory for the construction of a standard dwell time histogram, so a different protocol for the single-channel analysis of individual O₁ events was employed. The single-channel electrical traces were low-pass Bessel filtered at 500 Hz. The transmembrane potential was +40 mV. The buffer solution contained 2 M KCl, 10 mM potassium phosphate, pH 7.4.

Figure 5. Temperature dependence of the single-channel conductance of the OccK1 protein channel

(A) Single-channel conductance of the three open sub-states of the OccK1 protein channel; (B) The conductivity of 2 M KCl solution as a function of temperature, in 10 mM potassium phosphate, pH 7.4; (C) Single-channel conductance of the three open sub-states of the OccK1 protein channel normalized to the conductivity of the bulk aqueous phase from (B). The applied transmembrane potential was +40 mV.

Figure 6. Determination of thermodynamic parameters using semi-log Eyring plots

(A) Dependence of the four kinetic rate constants of the WT-OccK1 protein versus the reciprocal of the absolute temperature at an applied transmembrane potential of +40 mV. All experimental conditions were similar to those shown in Fig. 5. Panels (B), (C), (D) and (E) show activation free enthalpies of all single-channel current transitions. In panels (B), (C), (D) and (E), vertical bars marked by *WT-OccK1 and **WT-OccK1, indicate the activation free enthalpies inferred from single-channel data acquired at an applied transmembrane potential of -60 and +60 mV, respectively.

Figure 7. Alterations in the free energy landscape of the WT-OccK1 protein due to modifications of temperature

(A) The continuous line is the free energy landscape at 20°C, whereas the dashed line is the free energy landscape at 4°C; (B) The continuous line is the free energy landscape at 20°C, whereas the dashed line corresponds to the free energy landscape at 50°C. The single-channel electrical traces on the right-hand side show a comparison between the electrical signatures acquired at 20°C (A) and 50°C (B). In (B), the O_3 events, if present, were much shorter than the time resolution of the experiment, so they were not detectable. The other experimental conditions were the same as in Fig. 5. The reaction coordinate is the position of the fluctuating part of the OccK1 protein with respect to the *trans* opening.

Table 1

The equilibrium free energies, enthalpies and entropies of WT-OccK1, OccK1 Δ L3 and OccK1 Δ L4.

Voltage (mV)	Protein	$\Delta H^{\circ}_{01 \rightarrow 02}$ (kJ/mol)	$\Delta S^{\circ}_{01 \rightarrow 02}$ (J/mol.K)	$\Delta G^{\circ}_{01 \rightarrow 02}$ (kJ/mol)	$\Delta H^{\circ}_{03 \rightarrow 02}$ (kJ/mol)	$\Delta S^{\circ}_{03 \rightarrow 02}$ (J/mol.K)	$\Delta G^{\circ}_{03 \rightarrow 02}$ (kJ/mol)	$\Delta G^{\circ}_{03 \rightarrow 02}$ (kJ/mol)
+40	WT-OccK1	-161 $\pm$ 21	-533 $\pm$ 167	-15.1 $\pm$ 2.3	-1.8 $\pm$ 0.2	40 $\pm$ 2	139 $\pm$ 5	2.1 $\pm$ 0.2
+40	OccK1 Δ L3	-163 $\pm$ 28	-541 $\pm$ 99	-14.9 $\pm$ 1.3	-1.5 $\pm$ 0.6	60 $\pm$ 4	215 $\pm$ 12	1.1 $\pm$ 0.8
+40	OccK1 Δ L4	-110 $\pm$ 9	-357 $\pm$ 32	-12.9 $\pm$ 0.6	-4.0 $\pm$ 0.1	52 $\pm$ 3	179 $\pm$ 8	1.6 $\pm$ 0.7
-40	WT-OccK1	-133 $\pm$ 9	-431 $\pm$ 32	-15.2 $\pm$ 0.5	-4.4 $\pm$ 0.3	25 $\pm$ 1	91 $\pm$ 12	1.7 $\pm$ 0.2
-40	OccK1 Δ L3	-109 $\pm$ 14	-365 $\pm$ 14	-13.4 $\pm$ 2.5	-3.2 $\pm$ 0.4	52 $\pm$ 2	184 $\pm$ 5	2.0 $\pm$ 0.3
-40	OccK1 Δ L4	-185 $\pm$ 15	-613 $\pm$ 54	-18.0 $\pm$ 0.1	-2.6 $\pm$ 0.3	33 $\pm$ 1	116 $\pm$ 5	1.5 $\pm$ 0.7
								-1.4 $\pm$ 0.8

^aData were calculated at 0°C.

^bData were calculated at 25°C.