Mechanosensitive Channel Activation by Diffusio-Osmotic Force

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▶ Abstract. For ion channel gating, the appearance of two distinct conformational states and the discrete transitions between them is essential, and therefore of crucial importance to all living organisms. We show that the physical interplay between two structural elements that are commonly present in bacterial mechanosensitive channels – namely a charged vestibule and a hydrophobic constriction – creates two distinct conformational states, open and closed, as well as the gating between them. We solve the nonequilibrium Stokes-Poisson-Nernst-Planck equations, extended to include a molecular potential of mean force, and show that a first order transition between the closed and open states arises naturally from the diffusio-osmotic stress caused by the ions and water inside the channel and the elastic restoring force from the membrane.

Introduction. Osmotic shock presents a fatal risk to unicellular organisms. A sudden increase of the environmental solute concentration, known as hypertonic shock, leads to water loss and cell volume decline, whereas a sudden decrease, referred to as hypotonic shock, causes water to enter the cell rapidly, inducing cytolysis. As a final resort in case of severe hypotonic shock, many bacteria, archaea and fungi avert cell lysis by activating non-selective membrane channels to release solutes from the cytoplasm [1]. In E. coli bacteria, two wellstudied membrane protein channels are responsible for the release of solutes: the mechanosensitive channel of large conductance (MscL) [2] and the mechanosensitive channel of small conductance (Mscs) [3]. Based on the observation that mechanosensitive channels are activated *in vitro* by an applied hydrostatic pressure, the prevalent model for the gating mechanism invokes a conformational change in the protein triggered by tension applied to the cell membrane [2, 3, 4]. A free energy landscape for channel activation can be constructed by considering an elastic force proportional to the applied pressure, assuming predetermined open and closed states [5]. In E. coli MSCL mutants, however, added charge in the pore region activates the channels also in the absence of a hydrostatic pressure difference [6, 7, 8, 9], highlighting the importance of electrostatic interactions in the activation process. Indeed, the transmembrane domains of both MSCL and MscS carry a substantial net charge: Each of the ten transmembrane helices of the pentameric MSCL protein carries a net charge of -1e [10], and the heptameric Mscs protein carries an arginine residue with a charge of +1e on each of its monomers [11]. Charge-induced activation is a robust feature of MSCL channels and has been used for drug delivery into mammalian cells [12]. Despite its significance, however, the electrostatic contribution to the activation energy, and in particular the diffusio-osmotic force originating in the dynamic overlap-



Figure 1: (A) Crystal structure cross sections of the mechanosensitive channels Mscs (2OAU) and MscL (2OAR). (B) Pore outlines of the closed and open conformations [2, 3]. (C) Sketch of the channel embedded in a section of the membrane. (D) Computational domain in cylindrical coordinates. See the Appendix for details.

ping double layer at the channel's charged surface, has not been considered up to now.

The permeation pathways of both MscL and MscS are funnel-shaped, with the conical vestibule opening to the periplasmic side [2, 3], and the stem of the funnel lined with uncharged hydrophobic residues (Fig. 1A). Upon activation the pore walls move radially outward (Fig. 1B). In weakly polar channels, water can fill constrictions down to the size of a single water molecule [13], but even strongly hydrophobic channels are intermittently filled with water [14, 13, 15]. Ions, on the other hand, are subject to a strong repulsive potential of mean force (PMF) up to channel radii much larger than the ionic radius, caused by their hydration shells [16, 17, 18], steric and van der Waals interactions and self energy [19]. Using molecular dynamics (MD) simulations, the energy barrier for ion permeation through MscS has been estimated to be $17-34 k_{\rm B}T$ [20], explaining the lack of electric conductivity of MscS in the closed state despite its relatively wide permeation pathway. A similar hydrophobic lock mechanism has been found in MscL [2] and many different membrane channels [21, 22, 23]. Using mutation analysis, it has been established that the hydrophobic constriction in MscL provides a threshold for channel activation [24].

Here, we present an alternative view of the gating process, based on a model of a mechanosensitive channel consisting of the essential structural features found in MSCS and MSCL: a funnel-shaped pore with an uncharged hydrophobic stem and a vestibule carrying a fixed surface charge density, embedded in an impermeable membrane separating two solutions with salt concentrations c_1 and c_2 , respectively (Fig. 1C–D). This model is based directly on the experimentally determined protein crystal structure, and aims to explain experimental work showing, first, that added charge in the vestibule activates the channel [6, 7, 8, 9, 12], and second, that the hydrophobicity of the constriction provides a barrier for channel activation [24]. As an experimental benchmark, we consider measurements showing that MSCL and MSCS are activated at a hypotonic shock of at least $c_2 - c_1 = -0.3$ M [25]. We first summarize the main result in our paper, and then explain in detail how we have obtained it and examine various technical aspects and implication of our findings.

Emergence of two-state behavior. We find that the tension on the channel wall results from a competition between contractile forces due to the ionic PMF and the elastic membrane, and expansile forces due to the charged vestibule. The striking result of this competition is that the nonequilibrium free energy landscape G(R) [defined in Eq. (5)] exhibits two minima, corresponding to the closed and open states (Fig. 2A). Under isotonic conditions, exclusion of ions from the hydrophobic stem at small radii (inset 1 of Fig. 2A), which is known to reduce the pressure between like-charged parallel plates [26], gives rise to an energy barrier between the two states of ~ 3 $k_{\rm B}T$. Remarkably, the energy barrier arises naturally from only electrostatic and hydrodvnamic forces. The second energy minimum is caused by the expansile electrostatic force, which increases upon hypotonic shock. Whereas for R < 1.2 nm the increased electrostatic force is partly compensated for by the reduced pressure due to the ionic PMF (inset 2 of Fig. 2A), the electrostatic force dominates when the height of the repulsive potential in the stem of the funnel is negligible for R > 1.2 nm, and the channel activates (inset 3 of Fig.



Figure 2: (A) Nonequilibrium free energy [Eq. (5)] as a function of the channel radius R for different salt concentrations: isotonic ($c_1 = c_2 = 0.5$ M) and hypotonic ($c_2 - c_1 = -0.2$ M, -0.3 M and -0.4 M). For small radii, ions are excluded from the stem area by the ionic PMF, leading to a contractile net force under isotonic conditions at R = 1.0 nm (inset 1). After hypotonic shock, the increased electrostatic pressure diminishes the net force (inset 2). At R = 1.5 nm, the ionic PMF has vanished, ions enter the hydrophobic constriction and the channel activates under influence of the expansile electrostatic pressure (inset 3). (B) Electrical conductance of the channel at a salt concentration of $c_1 = c_2 = 0.3$ M.

2A). For large R the elastic term overcomes the electrostatic repulsion. The first order transition between closed and open states occurs at a hypotonic shock of approximately $c_2 - c_1 = -0.3$ M, in quantitative agreement with experimental results [25]. The channel activation is evident from the electrical conductance, which is shown in Fig. 2B.

▶ Governing equations. The Poisson equation relates the non-dimensional electrostatic potential $\psi(\mathbf{x}) = e\phi(\mathbf{x})/(k_{\rm B}T)$, with $\phi(\mathbf{x})$ being the potential in volts and $\boldsymbol{x} = (r, z)$ being the position in cylindrical coordinates, to the ion densities $c_{\pm}(\boldsymbol{x})$,

$$\nabla^2 \psi \left(\boldsymbol{x} \right) = -4\pi b \left[c_+ \left(\boldsymbol{x} \right) - c_- \left(\boldsymbol{x} \right) \right], \qquad (1)$$

with $b = e^2/(4\pi\varepsilon\varepsilon_0 k_{\rm B}T)$ being the Bjerrum length. At low Reynolds number, the solvent velocity $\boldsymbol{u}(\boldsymbol{x})$ is governed by the Stokes equations, which for an incompressible fluid in steady state read

$$\nabla \cdot [\mathsf{P}(\boldsymbol{x}) + \mathsf{T}(\boldsymbol{x})] + \boldsymbol{f}(\boldsymbol{x}) = 0 \text{ and } \nabla \cdot \boldsymbol{u}(\boldsymbol{x}) = 0.$$
 (2)

The components of the viscous and electrostatic stress tensors P(x) and T(x) and the force density f(x) due to the ionic PMF $\mu(x)$ are given by

$$P_{ij}(\boldsymbol{x}) = -p(\boldsymbol{x}) \,\delta_{ij} + \eta \left[\nabla_{i} u_{j}(\boldsymbol{x}) + \nabla_{j} u_{i}(\boldsymbol{x})\right]$$

$$T_{ij}(\boldsymbol{x}) = \frac{k_{\rm B}T}{8\pi b} \left[2\nabla_{i} \psi(\boldsymbol{x}) \,\nabla_{j} \psi(\boldsymbol{x}) - \delta_{ij} \left(\nabla \psi(\boldsymbol{x})\right)^{2}\right] \quad (3)$$

$$\boldsymbol{f}(\boldsymbol{x}) = -k_{\rm B}T \left[c_{+}(\boldsymbol{x}) + c_{-}(\boldsymbol{x})\right] \nabla \mu(\boldsymbol{x}),$$

with $p(\mathbf{x})$ being the hydrostatic pressure, η being the viscosity and i, j being r, z. The local concentrations $c_{\pm}(\mathbf{x})$ of positive and negative ions are determined by conservation of species. In steady state:

$$\boldsymbol{u}\left(\boldsymbol{x}\right)\cdot\nabla c_{\pm}\left(\boldsymbol{x}\right)=-\nabla\cdot\boldsymbol{J}_{\pm}\left(\boldsymbol{x}\right),\tag{4}$$

with $J_{\pm}(\boldsymbol{x}) = -D_{\pm} [\nabla c_{\pm}(\boldsymbol{x}) + c_{\pm}(\boldsymbol{x}) (\nabla \mu(\boldsymbol{x}) \pm \nabla \psi(\boldsymbol{x}))]$ being the ion fluxes and $D_{\pm} = 1 \text{ nm}^2/\text{ns}$ being the corresponding diffusion constants. We numerically solve Eqs. (1)–(4) in the domain shown in Fig. 1D, which allows us to analyze the diffusio-osmotic force exerted on the channel wall for the first time.

Boundary conditions. We employ a fixed surface charge density in the conical vestibule of σ = $-0.5 \ e \ nm^{-2}$ and uncharged boundaries everywhere else. Guided by experimental design, we set $c_1 = 0.5$ M The hydrodynamic equations obey the no-slip [25].boundary condition on the surface of the membrane and the vestibule, as is appropriate for hydrophilic surfaces [27, 28]. Inside the hydrophobic stem, on the other hand, perfect slip is assumed, consistent with the plug-like flow found in hydrophobic nanotubes [29]. Note that assuming no slip inside the stem instead leads to very similar results, implying that the model is robust regarding the characteristics of the hydrodynamic flow inside the constriction. Nevertheless, the hydrophobic stem and its associated water structure crucially affect the ionic PMF. In the stem area, we approximate the PMF by a one-dimensional function $\mu(z, R)$, which is a good approximation for narrow channels [18], as we verify by detailed calculations. See the Appendix for details. MD simulations show that the one dimensional ionic PMF $\mu(z, R)$ in narrow channels exhibits a peak, reaching a maximum $\mu_0(R)$ in the center of the channel, which decreases with increasing R [30, 18, 19, 17, 20]. At a radius of R = 1 nm, $\mu_0(R)$ is still several $k_{\rm B}T$'s in short

nanopores [30]. Therefore, we model the ionic PMF by a repulsive potential in the stem of the funnel of height $\mu_0(R)$, that decreases linearly from $\mu_0(R) = 18 \ k_{\rm B}T$ at R = 0.3 nm to zero at R = 1.2 nm. This potential comprises all interactions between the ions, the water and the pore, including changes in the hydration state of the pore [16]. The force on the surface S of the pore, consisting of the stem and the vestibule, is calculated from the normal stress, $\mathbf{F}(R) = -\int_{S} (\mathsf{P}(\mathbf{x}) + \mathsf{T}(\mathbf{x})) \cdot \hat{n} \ d\mathbf{x}$. We calculate the nonequilibrium free energy landscape as the sum of two terms: the integral over the radial force $F_r(R)$ due to the electrolyte, and an elastic term due to the protein and the membrane,

$$G(R) = -\int_{R_0}^{R} F_r(R') \, \mathrm{d}R' + \pi K \left(R^2 - R_0^2\right), \quad (5)$$

with $R_0 = 0.3$ nm being the minimum channel radius. For the elasticity coefficient of the protein and the membrane we assume $K = 0.5 k_{\rm B} T \,{\rm nm}^{-2}$, which is well within the range of values quoted in literature [31]. See the Appendix for details of the calculation, the PMF and the effect of the elastic restoring force.

▶ lonic conductivity and fluid flow. We calculate the electrical conductance (presented in Fig. 2B) from $C(R) = \frac{dI(R)}{d\Delta\psi}$, with $\Delta\psi = \psi_2 - \psi_1$ being an applied potential difference across the channel and I(R) = $e \int J_+(x) - J_-(x) + u(x) [c_+(x) - c_-(x)] dx$ being the resulting electric current, where the integration can be carried out over any plane spanning the pore¹. The conductance is minute up to a radius of R = 1 nm (Fig. 2B), owing to the repulsive PMF. Between 1.0 < R < 1.2 nm, the conductance increases dramatically, before adopting linear growth with R. The conductance of the open channel at $c_1 = c_2 = 0.3$ M agrees well with the experimental values of 2.5 - 3.7 nS measured for MscL [32, 4].

To examine the functionality of the channel, we monitor the ion concentrations and water flux throughout the activation process. In the closed state (R = 0.5 nm), the ionic PMF excludes both ion types from the stem of the funnel, as revealed by the concentrations $c_{\pm}(\boldsymbol{x})$ (Figs. 3A–B). In the open state (R = 1.5 nm), on the other hand, ions flow through the channel uninhibited (Figs. 3C–D). In response to a hypotonic shock, water rushes into the cell, driven by the osmotic pressure (arrows in Fig. 3B). When the channel activates, ions flowing outward through the channel drag the fluid along, and the water flow reverses (arrows in Fig. 3D), thus reproducing the experimentally observed behavior.

▶ Hydrostatic pressure. In vitro, also an applied hydrostatic pressure difference induces gating in mechanosensitive channels. Applied pressure affects the gating dynamics in two ways. First, the pressure exerts tension directly on the channel wall. Even very large

¹The asymmetry in the conductance with respect to the direction of the applied potential difference, which is due to the asymmetric geometry of the channel, is negligible.



Figure 3: Ion concentrations $c_+(\mathbf{x})$ (left panels) and $c_-(\mathbf{x})$ (right panels) and fluid velocity (arrows) for channels in the closed (R = 0.5 nm) and open (R = 1.5 nm) states. Under isotonic conditions, the fluid velocity is zero, and ions are repelled from the stem region in the closed state (A), but not in the open state (C). Under hypotonic conditions, the fluid velocity is directed cell inward in the closed state (B) and outward in the open state (D). The scale of the arrows in D has been increased by a factor 40 relative to those in B.

pressure differences of 2 bar have only a minor influence on the nonequilibrium free energy profile (Fig. 4A). Second, the pressure reduces the effective elastic constant Kin Eq. (5) [5]. For changes in K close to the values used in experiments (see the Appendix), this mechanism has a much more profound effect, tilting the energy landscape toward the open state while preserving the twostate character (Fig. 4B).

Discussion. The use of continuum hydrodynamics in nanometer-sized tubes has been shown to be justified for radii in the nanometer range [29]; a noteworthy result, which can be rationalized by analytic arguments [27] and has been used recently to calculate the hydrodynamic resistance of aquaporin channels [33]. Similarly, the Nernst-Planck equation for ion transport is applicable down to a radius of R = 0.3 nm, provided that the ion concentrations are estimated accurately [34]. Ion concentrations at solid surfaces and lipid bilayers can be accurately calculated from mean-field theory when the ionic PMF, estimated using MD simulations, is included as a non-electrostatic contribution to the potential [35, 28]. Combined, extended mean-field theory and continuum



Figure 4: Nonequilibrium free energy G(R) (Eq. 5) in case of an applied hydrostatic pressure difference. (A) Different pressure boundary conditions affect the tension exerted on the channel wall. (B) Effect of the hydrostatic pressure induced reduction of the effective elastic constant K.

hydrodynamics reproduce the electrokinetic properties found in experiments and atomistic simulations of both hydrophilic and hydrophobic surfaces [36, 37]. A complementary view on the effect of solvent structure may be obtained from kinetic theory [37, 38, 39]. Applicability of the current approach is inherently limited to the class of aqueous pores. Note, furthermore, that the gating process may not only involve the ion density, but also collective variables, such as the water density inside the channel and internal degrees of freedom of the protein. Capturing dewetting transitions, a collective effect which may become important near the lower end of the range of radii considered here, would require molecular [40, 41, 16] or coarse-grained [42, 43] modeling. However, of primary interest here are the mesoscopic electrokinetic properties of the channel, which we show to be insensitive to the hydrodynamic characteristics of the hydrophobic constriction. Although we will not be able to predict the electrolyte dynamics inside the stem area in atomic detail, this theoretical framework provides a reliable description of the electrokinetic properties at the mesoscopic scale of the protein channel. Nevertheless, solving the coupled Stokes-Poisson-Nernst-Planck equations in complex geometries has proven to be a challenging endeavor [44].

In conclusion, two-state mechanosensitive channel gating emerges naturally from the electrokinetic transport equations and an elastic restoring force in a geometry based on the crystal structure. Our proposed gating mechanism is fully supported by mutation experiments, showing a strong influence of protein surface charge and hydrophobicity on the gating kinetics, as well as hydrostatic pressure induced gating. Moreover, it agrees quantitatively with experiments regarding hypotonic shock threshold and electrical conductivity. The activation mechanism can be verified further using mutation experiments, substituting charged residues for neutral ones. Because hydrophobic constrictions and charged vestibules are shared features of many different ion channels, our analysis is likely to be important for a broad range of ion channels. Moreover, this new insight into the gating mechanism constitutes an essential step toward the design of artificial mechanosensitive channels and ion channel-targeting therapeutics.

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Appendix

▶ Vorticity formulation. Inserting Eq. 3 into Eq. 2 and taking the curl results in the following equations for the vorticity $\omega(\mathbf{x}) = \nabla \times \mathbf{u}(\mathbf{x}) = \nabla_z u_r(\mathbf{x}) - \nabla_r u_z(\mathbf{x})$,

$$\eta[r^{-3} \nabla_r^2 \xi(\boldsymbol{x}) - \nabla^2 \omega(\boldsymbol{x})] = \nabla \times [\nabla \cdot \mathsf{T}(\boldsymbol{x}) + \boldsymbol{f}(\boldsymbol{x})]$$

$$\omega(\boldsymbol{x}) = r^{-1} \left(\nabla_z^2 + \tilde{\nabla}_r^2\right) \xi(\boldsymbol{x}), \qquad (6)$$

with $\tilde{\nabla}_r^2$ being given by $r \nabla_r r^{-1} \nabla_r$. From the latter definition of $\xi(\boldsymbol{x})$ it follows $u_r(\boldsymbol{x}) = r^{-1} \nabla_z \xi(\boldsymbol{x})$ and $u_z(\boldsymbol{x}) = -r^{-1} \nabla_r \xi(\boldsymbol{x})$, which guarantees that the incompressibility condition is satisfied.

Boundary conditions. At each boundary, the governing equations are complemented by one boundary condition for the electrostatic potential, one boundary condition for the ion flow and two boundary conditions for the fluid flow velocity. The boundaries of the domain are sketched in Fig. 1D, and the boundary conditions used are summarized in Table A1. We denote the unit normal vector on the surface, pointing outward from the fluid, by \hat{n} . At the solid surface and at the symmetry boundary $\xi(\mathbf{x})$ is constant and $\mathbf{J}_{\pm}(\mathbf{x}) \cdot \hat{n} = 0$, which means there is no flow of water and ions perpendicular to the surface. The no-slip boundary condition is set by $\nabla \xi(\mathbf{x}) \cdot \hat{n} = 0$, and perfect slip is characterized by the absence of vorticity, $\omega(\mathbf{x}) = 0$. Setting the electric field proportional to the surface charge density σ at the charged surface ensures overall charge neutrality, and vanishing electric field and vorticity in the center of the channel is a necessary condition for rotational symmetry. The vorticity at the open boundary must vanish, and $\nabla \xi(\mathbf{x}) \cdot \hat{n} = 0$ because of translational invariance. The fluid flux out of the domain is given by

$$\phi = \int_{\mathcal{S}} \boldsymbol{u} \left(\boldsymbol{x} \right) \cdot \hat{n} \, \mathrm{d} \boldsymbol{x}, \tag{7}$$

integrated along the open boundary of the domain. Since $\xi(\mathbf{x}) = 0$ at the membrane, integration of Eq. 7 leads to $\phi = 2\pi\xi_c$. A fixed pressure difference is achieved by adjusting ξ_c every iteration.

▶ **Potential of mean force.** The one dimensional channel radius-dependent ionic PMF is modeled by a

Table A1: The boundary conditions for Eqs. 1–4. The unit normal vector \hat{n} on the surface points outward from the fluid.

Boundary	Stokes	Poisson	Nernst-Planck
Charged surface	$\xi = 0$	$\nabla \psi \cdot \hat{n} = 4\pi b\sigma$	$\boldsymbol{J}_{\pm}\cdot\hat{n}=0$
	$\nabla \boldsymbol{\xi} \cdot \hat{\boldsymbol{n}} = \boldsymbol{0}$		
Uncharged surface	$\xi = 0$	$\nabla \psi \cdot \hat{n} = 0$	$\boldsymbol{J}_{\pm}\cdot\hat{n}=0$
– no slip	$\nabla \boldsymbol{\xi} \cdot \hat{\boldsymbol{n}} = \boldsymbol{0}$		
– perfect slip	$\omega = 0$		
Open boundary	$\nabla \boldsymbol{\xi} \cdot \hat{\boldsymbol{n}} = \boldsymbol{0}$	$\psi = \psi_1$	$c_{\pm} = c_1$
	$\omega = 0$	$\psi = \psi_2$	$c_{\pm} = c_2$
Symmetry	$\xi = \xi_c$	$\nabla \psi \cdot \hat{n} = 0$	$\boldsymbol{J}_{\pm}\cdot\hat{n}=0$
	$\omega = 0$		

block function of height $\mu_0(R)$, convoluted with a sphere of radius a,

$$\mu(z,R) = \frac{\mu_0(R)}{4a^3} \begin{cases} \frac{(2a-z+z_b)^2}{(1-a-z+z_b)^{-1}} & \text{if } z_b < z < z_b + 2a \\ 4a^3 & \text{if } z_b + 2a < z < z_t - 2a \\ \frac{(z-z_t)^2}{(3a+z-z_t)^{-1}} & \text{if } z_t - 2a < z < z_t \\ 0 & \text{otherwise,} \end{cases}$$

$$(8)$$

with z_b and z_t being the bottom and top positions of the hydrophobic constriction, respectively, and a = 0.3 nm being the radius of a hydrated ion. The PMF of Eq. 8 is shown in Fig. A1A, together with a sketch of the computational domain on the same scale (Fig. A1B). The height of the PMF in the center of the channel $\mu_0(R)$ as a function of the channel radius R is estimated based on atomistic molecular dynamics simulations [20, 17, 30], analytical results for an infinitely long channel in a lowdielectric continuum [19], and a hybrid method [45]. For short channels, the PMF vanishes for sufficiently large R. The radius where $\mu_0(R)$ reaches zero is based on the range of the short-range non-electrostatic interaction in water, which typically extends up to approximately 1 nm away from the interface [28],

$$\mu_0(R) = \begin{cases} 18 - 20(R - 0.3) & \text{if } d < 1.2 \text{ nm} \\ 0 & \text{otherwise.} \end{cases}$$
(9)

The function of Eq. 9 is shown in Fig. A1C together with the literature estimates mentioned above.

▶ **Two-dimensional potential of mean force.** The approximation of the PMF by a one-dimensional function $\mu(z, R)$ is a good approximation for narrow channels [18]. For wider channels, the PMF may depend on the radial coordinate as well. Although no accurate PMFs are available for mechanosensitive channels, we can estimate the



Figure A1: (A) The ionic potential of mean force (PMF) $\mu(z)$ in units of $k_{\rm B}T$ (Eq. 8). (B) The computational domain. The parameters a, z_b and z_t of Eq. 8 are indicated. (C) The height μ_0 of the PMF in the center of a channel of radius R, together with estimates based on continuum theory by Parsegian [19], molecular dynamics simulations by Anishkin *et al.* [20], Richards *et al.* [17] and Beckstein *et al.* [30], and a hybrid method by Zwolak *et al.* [45].

effect of a PMF perpendicular to the channel wall by calculating the free energy of two parallel plates separated by a distance d, for which detailed PMFs have been calculated using molecular dynamics simulations [28]. We calculate the free energy per unit area as described in Ref. [46], using two contributions to the PMF: one repulsive component due to the confinement in the nanochannel, and one component perpendicular to the channel's surface. The first component is given by the function used for $\mu_0(R)$ in cylindrical geometry, with the distance between the plates d = 2R,

$$\mu_0(d) = \begin{cases} 18 - 10(d - 0.6) & \text{if } d < 2.4 \text{ nm} \\ 0 & \text{otherwise.} \end{cases}$$
(10)

For the component perpendicular to the surface we use the PMF for NaCl at hydrophilic and hydrophobic self assembled monolayers, which have a similar structure as



Figure A2: Free energy per unit area of two parallel plates at distance d between (A) hydrophobic uncharged surfaces, using $\mu(x, d) = \mu_0 (d) + \mu_{\text{NaCl}}(x, d)$, and (B) hydrophilic surfaces with $\sigma = -0.5 \ e/\text{nm}^2$, using $\mu(x, d) = \mu_{\text{NaCl}}(x, d)$.

the protein surface [28]. To account for the effect of both plates, the PMF at a single plate $\tilde{\mu}_{\text{NaCl}}(x)$, with x the distance from the plate, is modified for the parallel plate geometry using $\mu_{\text{NaCl}}(x, d) = \tilde{\mu}_{\text{NaCl}}(x) + \tilde{\mu}_{\text{NaCl}}(d-x).$ Mimicking the conditions in the hydrophobic constriction, the free energy is shown in Fig. A2A for two uncharged hydrophobic parallel plates using $\mu_0(d)$ from Eq. 10 with and without the perpendicular component $\mu_{\text{NaCl}}(x, d)$. Mimicking the charged vestibule, the free energy of charged hydrophilic surfaces ($\sigma = -0.5 \ e/\mathrm{nm}^2$) is shown in Fig. A2B, using $\mu_0(d) = 0$ with and without the perpendicular component of the PMF. Although taking the perpendicular component of the PMF into account gives rise to clear quantitative differences, the important qualitative features of the energy profiles are not affected. In particular, the contractile force between hydrophobic plates at short distances and the expansile force between the charged hydrophilic plates are captured well when using only $\mu_0(d)$. At the same time, the graphs show that in future work, a more detailed two-dimensional PMF for mechanosensitive channels could be used to refine the current calculations.

▶ Effect of the ion diffusion coefficient. The ion diffusion coefficient inside a narrow channel is different from the bulk value. Calculations based on atomistic molecular dynamics simulations indicate that the diffusion constant inside a Gramicidin channel is reduced to 70% of its bulk value [47]. To estimate the effect of a varying ion diffusion coefficient on our calculations, we calculate the nonequilibrium free energy profile for half and twice the original value of $D_{\pm} = 1 \text{ nm}^2/\text{ns}$, keeping D_{\pm} constant in space. The effect of varying D_{\pm} by these amounts is negligible, from which we infer that also the small spatial variation of D_{\pm} found in atomistic simulations is unlikely to affect the nonequilibrium free energy landscape.

The effective elastic restoring force. The prefactor K of the elastic term in Eq. 5 parameterizes the combined response of the protein and the membrane to radial displacements. This constant is likely to be different for different proteins, depending on whether the transmembrane domain consists of alpha helices, a beta barrel, or

any other architecture. In addition, the effective force is reduced by an applied hydrostatic pressure difference [5], as explained in the main text. For a given experiment, where a pressure difference across the bilayer of a giant vesicle is applied using a pipette, the relation between the applied pressure difference Δp and the change in bilayer tension ΔK is given by [48]

$$\Delta K = \frac{R_p \,\Delta p}{2 \left(1 - R_p / R_v\right)},\tag{11}$$

with R_p and R_v being the radius of the pipette and the vesicle, respectively. Early experiments on *E. coli* mechanosensitive channels found gating at applied pressures of tens of mm Hg and almost full activation at $\Delta p =$ 40 mm Hg, using a pipette of $R_p = 0.5 \ \mu m$ on a vesicle of $R_v = 6 \ \mu m$ [49], corresponding to $\Delta K = 0.35 \ k_{\rm B}T/{\rm nm}^2$. In Fig. 4B, we show the nonequilibrium free energy profile, calculated using Eq. 5, for different values of the effective elastic constant, all within the literature range of $10^{-4} - 1 \ k_{\rm B}T/{\rm nm}^2$ [31]. Whereas for low *K* (flexible protein or strong hydrostatic pressure difference) the energy landscape is tilted toward the open state, the twostate character vanishes for high *K* (rigid protein).

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