Stimuli-Responsive Polyguanidino-Oxanorbornene Membrane Transporters as Multicomponent Sensors in Complex Matrices

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1. Materials. All salts and buffers were of the best grade available from Sigma or Fluka and used as received. 5(6)-carboxyfluorescein (CF) was from Fluka. 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS), p-xylene-bis-pyridinium bromide (DPX), and Cascade Blue hydrazide trisodium salt (CB hydrazide) were from Invitrogen, Eugene, Oregon, USA. Egg yolk phosphatidylcholine (EYPC), egg yolk phosphatidyl glycerol (EYPG), dipalmitoyl phosphatidylcholine (DPPC) and a Mini-Extruder used for vesicle preparation were from Avanti Polar Lipids. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller (measurements at 25 °C unless otherwise noted). UV spectroscopy was carried out with a Jasco V-650 spectrophotometer.

2. Abbreviations. ADP: adenosine 5’-diphosphate; ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; ATP: adenosine 5’-triphosphate; CB hydrazide: Cascade Blue hydrazide trisodium salt; CF: 5(6)-carboxyfluorescein; DMSO: dimethylsulfoxide; DAN: dialkoxyanaphthalene; DPPC: dipalmitoyl phosphatidylcholine; DPX: p-xylene-bis-pyridinium bromide; EYPC: egg yolk phosphatidylcholine; EYPG: egg yolk phosphatidylglycerol, Hepes: N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid); LUVs: large unilamellar vesicles; PGON: polyguanidino-oxanorbornene; Tris: tris(hydroxymethyl)aminomethane.

3. Synthesis of Transporters

The synthesis of PGONs starts with the TFA salt of 1 from Scheme 1 in the text. To this salt (1.4 g, 4.34 mmol) N,N-Di-Boc-1H-pyrazole-1-carboxamidine (Aldrich, 4.0 g, 13.02 mmol) and triethylamine (Aldrich 4.1 g, 40 mmol) were added with 60 mL of solvent (90% CH₃CN, 10% H₂O). This solution was stirred at room temperature for 12 h. The solution was then diluted with ethyl acetate and washed twice each with H₂O and brine. The organic layer was evaporated and purified by silica column chromatography using a MeOH in CH₂Cl₂ gradient to afford the Boc-protected guanidinium oxanorbornene monomer, 2.
This monomer was polymerized by ring-opening metathesis polymerization (ROMP) using a derivative of the 2nd generation Grubbs’ catalyst, [(H2Imes)(3-Br-py)2-(Cl)2Ru=CHPh]. As a typical example, the polymerization of N14 entailed adding to a test tube monomer (100 mg) plus catalyst (13.7 mg). The test tube was capped with a septum and purged with N2 for 5 min, then 1 mL dry CH2Cl2 (Acros, packed under N2 and molecular sieves) was injected. The N2 line was removed and the clear, brown solution was stirred at 28 °C for 30 min after which 0.4 mL ethyl vinyl ether was injected to endcap the polymer. After stirring for 15 min the solution was added dropwise to 300 mL of stirring pentane to precipitate the polymer. The pentane solution was stirred an additional 30 min and left standing undisturbed for an hour. The precipitate was then collected by a fine sinter funnel. The polymer was then redissolved in 1 mL of CH2Cl2 and reprecipitated and collected then dried by vacuum for 8 h.

The Boc-protected polymers were deprotected by stirring 100 mg in 8 mL of 1:1 TFA:CH2Cl2 for 2 h. The solution was dried to an oil by rotary evaporator set at 40 °C and residual TFA was removed by sonicating the oil in more CH2Cl2 and evaporating the solvent again by rotary evaporator. The resulting solid was placed under vacuum for 2 h. Finally, the solid was fully dissolved in 4 mL H2O and filtered through a PES syringe filter (Whatman, 25 mm diameter, 0.45 µm pore) and freeze-dried for 48 h to give an eggshell colored soft solid. Final deprotected polymers were protected from moisture and stored at -20 °C.

Gel permeation chromatography (GPC) was performed on the Boc-protected polymers with a Polymer Lab LC1120 pump equipped with a Waters differential refractometer detector. The mobile phase was tetrahydrofuran with a flow rate of 1.0 mL/min. Separations were performed with 105, 104, and 103 Å Polymer Lab columns and molecular weights were calibrated versus narrow molecular weight polystyrene standards. The Boc-protected PGON is THF soluble and GPC (polystyrene-calibrated) was used to approximate molecular weight (Mn) and polydispersity index (PDI). 1H-NMR and 13C-NMR spectra were obtained on a Bruker DPX-300 NMR spectrometer. Spectra were calibrated to the DMSO-d6 solvent signal.

**DiBoc-protected monomer 2.** 1H-NMR (300 MHz, DMSO-d6): δ 11.44 (1H, s), 8.35 (1H, t, J = 6.0 Hz), 6.56 (2H, s), 5.10 (2H, s), 3.52 (2H, m), 3.43 (2H, m), 2.85 (2H, s), 1.47 (9H, s), 1.36 (9H, s). 13C-NMR (75 MHz, DMSO-d6): δ = 176.3, 156.6, 153.0, 136.5, 83.3, 80.9, 47.6, 39.0, 38.4, 28.3, 28.1.
**Boc-protected N14.** $^1$H-NMR (300 MHz, DMSO-$d_6$): $\delta = 11.51$ (1H, br), 8.47 (1H, br), 5.90 (trans) and 5.70 (cis) (2H total, br), 4.89 (cis) and 4.39 (trans) (2H total, br), 1.45 (9H, s), 1.38 (9H, br), cis:trans ratio = 47:53.

**N14.** $^1$H-NMR (300 MHz, DMSO-$d_6$): $\delta = 7.88$ (1H, br), 7.36 (4H, br), 5.96 (1H, br), 5.74 (1H, br), 4.92 (1H, br), 4.43 (1H, br) 3.47 (4H, br).

4. **Synthesis of Inactivators**

*Cascade Blue Hydrazide (CB Hydrazide).* S3 Concentrations of CB hydrazide stock solutions were determined by UV spectroscopy ($\varepsilon_{377} = 22900$ M$^{-1}$cm$^{-1}$, $\varepsilon_{399} = 27500$ M$^{-1}$cm$^{-1}$). S4 The derivatizations were carried out in two variants. **Variant A:** Total reaction volume of derivatizations was 50 µl or 100 µl. A stock solution of CB hydrazide (50, 10, 2, 1, or 0.2 mM) in DMSO was diluted with DMSO to half of the desired total reaction volume. The other half was composed of either 10 or 2 mM of analyte (i.e. pyruvate, ketoglutarate, or oxaloacetate) in 0.4 M NaOAc/HOAc, pH 4.5. Subsequently, the mixtures were combined and incubated for 30 min at 50 °C. Aliquots from that solution were diluted with a 1:1 mixture of DMSO and 0.4 M NaOAc/HOAc (pH 4.5). These aliquots were subjected to fluorescence analysis as described below. **Variant B:** 50 µl CB hydrazide in DMSO (1 mM) was mixed with 10 µl analyte stock solution (pyruvate, ketoglutarate = 1 µM 100 mM), and 40 µl 0.4 M NaOAc/HOAc (pH 4.5). The resulting series of reaction mixtures were incubated for 30 min at 50 °C, and 20 µl aliquots of the mixtures were subjected to fluorescence analysis as described above (see below). Consistent $EC_{50}$s for pyruvate with both variants exclude possible influences from incomplete derivatization. In the case of ketoglutarate, the $EC_{50}$ with Variant A was significantly larger than with Variant B, which might limit practical applications. S3

*Dialkoxynaphthalene Hydrazide (DAN Hydrazide).* S3 580 µg (2 µmol) DAN hydrazide were suspended in 200 µl DMSO and mixed with 160 µl 0.4 M NaOAc/HOAc (pH 4.5) and 40 µl 100 mM pyruvate in H$_2$O. As a control, the pyruvate was substituted with 0.4 M NaOAc/HOAc (pH 4.5). The reaction mixtures were incubated for 2 h at 50 °C and subsequently centrifuged for 1 min at 2000 rpm. Aliquots from that solution were diluted with a 1:1 mixture of DMSO and 0.4 M NaOAc/HOAc (pH 4.5) and subjected to fluorescence analysis as described below.
5. Activity, Activators and Inactivators (EYPC-LUVs ⊆ CF)

Preparation of EYPC-LUVs ⊆ CF. A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/CHCl₃ (1:1) on a rotary evaporator (40 °C) and then in vacuo overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N₂, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Tris, 107 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

PGON Activity in EYPC-LUVs ⊆ CF. 25 µl EYPC-LUVs ⊆ CF were added to 1975 µl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity Iₜ (λₑₓc = 492 nm, λₑₘ = 517 nm) was monitored during the addition of PGON (20 µl stock solution in DMSO, 1 nM - 100 µM final concentration) at t = 0 min, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Time courses of Iₜ were normalized to fractional intensities Iₗ using equation S1.

\[ Iₗ = (Iₜ - I₀) / (Iₜₖₐₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜ৯

where I₀ = Iₜ before pore addition and Iₖ = Iₜ after lysis. Iₜ at 200 s after the start of the experiment just before lysis was defined as transmembrane activity Y. For Hill analysis, Y was plotted against PGON concentration cₚГОN and fitted to the Hill equation S2 to give effective concentration EC₅₀ and the Hill coefficient n.

\[ Y = Yₖ + (Y₀ - Yₖ) / \{1 + cₚГОN / EC₅₀\}ⁿ \]

where Y₀ is Y in absence of PGON, Yₖ is Y with excess PGON.

PGON Inactivators. 20 µl of inactivator stock solution (in H₂O or derivatization buffer, see above) and 25 µl EYPC-LUVs ⊆ CF were added to 1955 µl gently stirred,
thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. When necessary, the pH was readjusted to 7.5 by addition of 1 M NaOH or 1 M HCl. Care was taken that volume changes were insignificant, i.e. below 1 %. The time-dependent change in fluorescence intensity $I_t$ ($\lambda_{\text{exc}} = 492$ nm, $\lambda_{\text{em}} = 517$ nm) was monitored during the addition of 20 µl PGON stock solution (50 µM PGON14 or 10 µM PGON41 in DMSO, final concentrations 0.5 µM and 0.1 µM, respectively) at $t = 0$ min, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Fluorescence time courses were normalized and converted into transmembrane activity $Y$ (equation S1) and further converted into fractional activity $Y_f$ according to equation S3.

$$Y_f = \frac{Y - Y_0}{Y_\infty - Y_0} \quad \text{(S3)},$$

where $Y_0$ is $Y$ without inactivator, and $Y_\infty$ is $Y$ with excess inactivator. For Hill analysis, $Y_t$ was plotted against inactivator concentration $c_i$ and fitted to the Hill equation S4 to give the concentration at which inactivator was 50 % ($IC_{50}$) and the Hill coefficient $n$.

$$Y_t = Y_{\infty,f} + \frac{(Y_{0,f} - Y_{\infty,f})}{\{1 + c_i / IC_{50}\}^n} \quad \text{(S4)},$$

where $Y_{0,f}$ is $Y_t$ without inactivator, and $Y_{\infty,f}$ is $Y_t$ with excess inactivator.

**PGON Activators.** 20 µl of activator stock solution (in H₂O or DMSO depending on solubility) and 25 µl EYPC-LUVs ⊆ CF were added to 1955 µl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity $I_t$ ($\lambda_{\text{exc}} = 492$ nm, $\lambda_{\text{em}} = 517$ nm) was monitored during the addition of 20 µl PGON stock solution (3 µM PGON14 or 30 µM PGON5 in DMSO, final concentrations 30 nM and 0.3 µM, respectively) at $t = 0$ min, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Fluorescence time courses were normalized and converted into fractional activity $Y_t$ as described for inactivation. The obtained Hill plots were then analyzed in analogy to equation S4, in which the inactivator concentration was replaced by the activator concentration, and the $IC_{50}$ by the $EC_{50}$ (as in equation S2 but for varied activator concentration at constant transporter concentration).

**Pyrene Excimers.** Aqueous solutions of pyrenebutyrate were prepared by addition of 1 equivalent of NaOH to pyrenebutyric acid in water. Solutions of pyrenebutyrate (120 µM)
in buffer (2 ml, 10 mM Na\textsubscript{m}H\textsubscript{n}PO\textsubscript{4}, pH 7.4) with \textbf{N14}, \textbf{N41} or polyarginine (0 - 50 µl of concentrated stock solutions) were gently mixed, and the fluorescence emission spectra were measured with $\lambda_{\text{ex}}$ 340 nm. The obtained spectra were normalized at 375 nm, and fractional excimer emission intensities at 470 nm ($I_{470} / I_{375}$) were plotted as a function of polymer concentration and subjected to Hill analysis (Figure S1).

\textbf{Figure S1.} (Top) Fluorescence emission spectra of pyrenebutyrate (120 µM) in water (10 mM Na\textsubscript{m}H\textsubscript{n}PO\textsubscript{4}, pH 7.4) in the presence of \textbf{N14} (left) and \textbf{N41} (right); excitation at 340 nm. (Bottom) Relative excimer emission intensity ($I_{470} / I_{375}$) as a function of \textbf{N14} (left) and \textbf{N41} (right) concentration.
6. Dependence on Membrane Fluidity (DPPC-LUVs ⊘ CF)

Preparation of DPPC-LUVs ⊘ CF,55 A thin lipid film was prepared by evaporating a solution of 25 mg DPPC in 1 ml MeOH/CHCl₃ (1:1) on a rotary evaporator (40 °C) and then in vacuo overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5) at 60 °C (which is well above the phase transition temperature),56 the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N₂, 60 °C water bath). The extruder was assembled and placed on top of a hot plate to warm up to 60 °C, which was controlled by measuring the temperature inside the heating block. The vesicle suspension was >15 times extruded through a polycarbonate membrane (pore size 100 nm) and extravesicular components were subsequently removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Tris, 10 mM NaCl, pH 7.5. Final conditions: ~2.5 mM DPPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

PGON Activity in DPPC-LUVs ⊘ CF,53,55 25 or 50 µl DPPC-LUVs ⊘ CF were added to 1975 or 1950 µl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The mixture was equilibrated for >10 min and temperatures were assumed to be the same as that of the circulating water bath. Fluorescence intensity was monitored during addition of 20 µl PGON14 stock solution (50 µM in DMSO, 0.5 µM final concentration) at t = 0 min, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Normalization of fluorescence time traces and conversion into transmembrane activity Y was performed as described above for EYPC-LUVs ⊘ CF.

7. Dependence on Surface Potentials (EYPC/EYPG-LUVs ⊘ CF)

Preparation of EYPC/EYPG-LUVs ⊘ CF. Thin lipid film were prepared by evaporating a 1 ml MeOH/CHCl₃ (1:1) solution of 25 mg lipids in molar ratios 10:0, 9:1, 7:3, and 1:1 of EYPC and EYPG on a rotary evaporator (40 °C) and then in vacuo overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N₂, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-
Aldrich) with 10 mM Tris, 107 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

**PGON Activity in EYPC/EYPG-LUVs ⊆ CF.** 25 µl of each lipid preparation were added to 1975 µl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity $I_t$ ($\lambda_{exc} = 492$ nm, $\lambda_{em} = 517$ nm) was monitored during the addition of PGON14 (20 µl stock solution in DMSO, 1 nM - 100 µM final concentration) at $t = 0$ min, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Time courses of $I_t$ were normalized to fractional intensities $I_f$ and analyzed using equations S1 and S2 as described above.

8. **Dependence on Membrane Potentials (EYPC-LUVs ⊆ ANTS/DPX)**

**Preparation of EYPC-LUVs ⊆ ANTS/DPX.** A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/CHCl$_3$ (1:1) on a rotary evaporator and then in vacuo overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Hepes, 20 mM KCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N$_2$, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Hepes, 100 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Hepes, 20 mM KCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5; outside: 10 mM Hepes, 100 mM NaCl, pH 7.5.

**Membrane Polarization.** x µl K$^+$-buffer (10 mM Hepes, 100 mM KCl, pH 7.5), 20 µl safranin O (6 µM in H$_2$O) and 50 µl EYPC-LUVs ⊆ ANTS/DPX were added to 1950-x µl gently stirred, thermostated Na$^+$-buffer (10 mM Hepes, 100 mM KCl, pH 7.5) in a disposable plastic cuvette. By assuming no K$^+$ in the Na$^+$-buffer, the resulting membrane potential $\psi$ can be calculated according to the Nernst equation S5

$$\psi [\text{mV}] = 59 \times \log([K_{out}]/[K_{in}]) \quad (S5),$$

where $[K_{out}]$ and $[K_{in}]$ are the potassium concentrations inside and outside of the vesicle. During the addition of 20 µl valinomycin stock solution (60 µM in DMSO), the emission of safranin O ($\lambda_{exc} = 522$ nm, $\lambda_{em} = 581$ nm) was monitored, which gradually increased according
to the expected built-up of membrane potential until a plateau was reached. Lysis was initiated by addition of 20 µl melittin stock solution (200 µM in H₂O). The calibration curve was obtained by plotting the averaged safranin O fluorescence emission intensity of three measurements versus the calculated membrane potential. A linear correlation was found between −60 mV and −140 mV.

\[ \text{PGON Activity in EYPC/EYPG-LUVs} \supseteq CF \times \mu l \ K^+-\text{buffer} \ (10 \ mM \ Hepes, 100 mM KCl, pH 7.5), \ 20 \ µl \ safranin \ O \ (6 \ µM \ in H_2O) \ and \ 50 \ µl \ EYPC-LUVs \supseteq ANTS/DPX were added to 1950-x µl gently stirred, thermostated Na^+-buffer (10 mM Hepes, 100 mM KCl, pH 7.5) in a disposable plastic cuvette. Simultaneously recording the emission of safranin O \ (\lambda_{exc} = 522 \ nm, \lambda_{em} = 581 \ nm) \ and \ ANTS \ (\lambda_{exc} = 353 \ nm, \lambda_{em} = 510 \ nm) \ gave \ the \ applied \ membrane \ potential \ after \ addition \ of \ 20 \ µl \ valinomycin \ stock \ solution \ (60 \ µM \ in \ DMSO) \ by \ comparing \ the \ safranin \ O \ emission \ intensity \ with \ the \ calibration \ curve \ and \ the \ transmembrane \ activity \ of \ PGON14 \ after \ addition \ of \ 20 \ µl \ PGON14 \ stock \ solution \ (0.1 \ mM, 0.3 \ mM, \ and \ 1 \ mM \ in DMSO, i.e. 1, 3, and 10 µM final concentration) and 20 µl melittin stock solution (200 µM in H₂O) by normalizing and analyzing the ANTS emission intensity using equations S1 and S2 as described above.

9. Lactate Sensing

Detection of Enzyme Activity. 20 µl of 50 mM (L)-lactate in H₂O (adjusted to pH 6.5 with NaOH), 10 µl of 22000 U/ml catalase in H₂O, and 10 µl lactate oxidase (0, 10, and 50 U/ml in H₂O) were added to 160 µl 50 mM Na₂HPO₄ (pH 6.5) at 37 °C. Final concentrations: 5 mM (L)-lactate; 1100 U/ml catalase; 0, 0.5, and 2.5 U/ml lactate oxidase. Aliquots (5 µl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 µl 0.4 M NaOAc/HOAc, pH 4.5. 25 µl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 µl aliquots were subjected to fluorescence analysis as described above (cf. paragraph 5, equation S3).

Sensor Calibration. x (0.1 to 30) µl of 100 mM (L)-lactate in H₂O (adjusted to pH 6.5 with NaOH), 5 µl of 22000 U/ml catalase in H₂O, and 5 µl lactate oxidase (50 U/ml in H₂O) were mixed with 90-x µl 50 mM Na₂HPO₄ (pH 6.5) at 37 °C for 60 min (concentration of lactate in the reaction mixture \( c_{rx} = 0.1 - 30 \) mM). Aliquots (5 µl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 µl 0.4 M NaOAc/HOAc, pH
4.5. 25 µl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 µl aliquots were withdrawn, subjected to fluorescence analysis and converted into fractional activity $Y_f$ as described above (cf. paragraph 4, equation S3). Hill analysis was carried out in analogy to equation S4 with equation S6 to give the concentration in the enzymatic reaction mixture which finally leads to 50% inactivation of membrane transport ($IC_{50,rx}$).

$$Y_f = Y_{\infty,f} + \left( Y_{0,f} - Y_{\infty,f} \right) / \left\{ 1 + c_{rx} / IC_{50,rx} \right\}^n$$  \hspace{1cm} (S6),

The obtained $c_{rx} = 1.8 \pm 0.3$ mM can be related to the $IC_{50}$ by considering 10x dilution during derivatization and 100x dilution during transduction. This gives an $IC_{50} = 1.8 \pm 0.3$ µM which is in excellent agreement with the $IC_{50} = 1.5 \pm 0.1$ µM of pyruvate.

**Determination of Lactate Content in Sour Milk.** Sour milk (Valflora M-Dessert, Migros, CH) was centrifuged twice for 2 min at 14500 rpm. The upper, white phase was removed, and the lower, clear phase (milk serum) was filtered through glass wool. The filtered serum (source phase) was dialyzed against 1 ml 50 mM KH$_2$PO$_4$, pH 6.5 (receiving phase) for 3 h (cut off = 5 kDa), followed by the replacement of the source phase with fresh serum, and further dialysis for 16 h (total: 1.33fold dilution). The pH of the receiving phase was carefully controlled and remained constant during dialysis. Subsequently, $x$ µl (initial volume $V_i$) of the receiving phase, 5 µl of 22000 U/ml catalase in H$_2$O, and 5 µl lactate oxidase (50 U/ml in H$_2$O) were mixed with 90-$x$ µl 50 mM Na$_2$HPO$_4$ (pH 6.5) at 37 °C for 60 min (total volume $V_0 = 100$ µl). Aliquots (5 µl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 µl 0.4 M NaOAc/HOAc, pH 4.5. 25 µl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 µl aliquots were withdrawn, subjected to fluorescence analysis and converted into fractional activity $Y_f$ as described above (cf. paragraph 4, equation S3). Hill analysis was carried out in analogy to equation S4 and S6 with equation S7 to give the effective volume of milk serum which finally leads to 50% inactivation of membrane transport ($V_{50}$).

$$Y_f = Y_{\infty,f} + \left( Y_{0,f} - Y_{\infty,f} \right) / \left\{ 1 + V_i / V_{50} \right\}^n$$  \hspace{1cm} (S7),
The obtained $V_{50} = 3.3 \pm 0.3 \, \mu l$ can be converted into the lactate concentration in the receiving phase $c_{rec}$ using equation S8

$$c_{rec} = \frac{(V_0 \times IC_{50,rx})}{V_{50}} \quad \text{(S8),}$$

which gives $c_{rec} = 54.5 \, \text{mM}$. Considering the 1.33fold dilution during dialysis, one obtains a concentration of 72.7 mM (L)-lactate in the milk serum, which is in excellent agreement with expectations from the literature.\textsuperscript{S3,S8} Blockage of membrane transport caused by the receiving phase treated in the same manner, but without exposing it to lactate oxidase, was not observed (cf. Supporting Online Material of ref. S3).

10. References

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