The structural basis of promiscuity in small multidrug resistance transporters

Ali A. Kermani1,7, Christian B. Macdonald2,7, Olive E. Burata3, B. Ben Koff1, Akiko Koide4,5, Eric Denbaum4,6, Shohei Koide4 & Randy B. Stockbridge1,2

By providing broad resistance to environmental biocides, transporters from the small multidrug resistance (SMR) family drive the spread of multidrug resistance cassettes among bacterial populations. A fundamental understanding of substrate selectivity by SMR transporters is needed to identify the types of selective pressures that contribute to this process. Using solid-supported membrane electrophysiology, we find that promiscuous transport of hydrophobic substituted cations is a general feature of SMR transporters. To understand the molecular basis for promiscuity, we solved X-ray crystal structures of a SMR transporter Gdx-Clo in complex with substrates to a maximum resolution of 2.3 Å. These structures confirm the family’s extremely rare dual topology architecture and reveal a cleft between two helices that provides accommodation in the membrane for the hydrophobic substituents of transported drug-like cations.

1 Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA. 2 Program in Biophysics, University of Michigan, Ann Arbor, MI 48109, USA. 3 Program in Chemical Biology, University of Michigan, Ann Arbor, MI 48109, USA. 4 Laura and Isaac Perlmutter Cancer Center, New York University Langone Medical Center, New York, NY 10016, USA. 5 Department of Medicine, New York University School of Medicine, New York, NY 10016, USA. 6 Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY 10016, USA. 7 These authors contributed equally: Ali A. Kermani, Christian B. Macdonald. ✉email: stockbr@umich.edu
Membrane proteins from the small multidrug resistance (SMR) family are a major driver of the spread of drug resistance genes among bacteria. Genes encoding SMR proteins (variously annotated *emrE, sugE, smr, qac, ebr*) are frequently found in mobile drug resistance gene arrays, and provide a broad selective advantage by conferring resistance to ubiquitous environmental pollutants with low-grade toxicity to microbes. The adaptive effects of the SMR proteins lead to co-selection of other genes in the arrays that confer resistance to the more potent drugs in the antimicrobial arsenal, including sulfonamides, β-lactams, and aminoglycosides, increasing the frequency of these genes in environmental reservoirs. Thus, the dispersal of drug resistance genes among bacteria, the transport capabilities of SMR proteins, and the distribution of SMR substrates in the biosphere are intimately linked. Despite their importance, functional experiments to test the chemical scope of transported compounds have been limited to a narrow range of SMR homologs and drugs, and although the overall fold has been determined, sidechain-resolution structural data have not been reported for any family member. In this study, we have two objectives: (1) determine the chemical characteristics of substrates transported by representative SMR family proteins; and (2) establish the structural basis of substrate binding and transport by SMR transporters.

The sequence diversity of bacterial SMR exporters can be visualized using a sequence-similarity network (Fig. 1a and Supplementary Fig. 1). The SMR family has two major subtypes that share high sequence identity (~40%) and similarity (Supplementary Fig. 2). Both are broadly distributed across bacterial taxa, and many bacteria possess both subtypes. One group contains proteins that provide resistance against quaternary ammonium cations, including structurally diverse polyaromatic cations such as ethidium and methyl viologen. This group, the Qac cluster, includes EmrE, an *Escherichia coli* homolog and the best-studied member of the SMR family. The other group was characterized more recently, and encompasses guanidinium (Gdm+) antiporters (Gdx proteins; *E. coli* gene name *sugE*). Gdm+ is an endogenously produced, nitrogen-rich metabolite that is transformed or exported by genes organized in Gdm+-related operons. These operons are often controlled by riboswitches that are selectively responsive to Gdm+ binding.

Initial experiments suggested that the Qac and Gdx subtypes fulfill discrete functional roles, since EmrE does not transport Gdm+, and the Gdx proteins do not transport canonical EmrE drugs. Of the two roles, export of quaternary ammonium ions is most readily associated with multidrug resistance, since these compounds have been used as antiseptics for almost a century. But genes from the Gdx cluster also commonly colocalize with horizontal gene transfer elements (Fig. 1a)10,11, and have been explicitly identified in mobile multidrug resistance gene arrays (Fig. 1). Is the functional dichotomy between the Qac and Gdx subtypes as strict as early experiments suggested? Or do proteins in the SMR family share transport capabilities that make them broadly adaptive in human-impacted environments?

Here we show that SMR proteins from both the Qac and Gdx subtypes engage in promiscuous transport of hydrophobic substituted cations. Both subtypes transport a variety of hydrophobic guanidinyl compounds, and proteins belonging to the Qac subtype additionally transport substituted ammonium compounds and polyaromatic cations. X-ray crystal structures of Gdx-Clo in complex with substituted guanidinyl substrates reveal a cleft between two helices that provides accommodation in the membrane for the hydrophobic substituents of transported drug-like cations.

**Results**

**Overlapping, promiscuous substrate transport by Qac and Gdx subtypes.** In order to probe the chemical characteristics of transported substrates, we performed transport experiments using exemplars of both the Qac and Gdx subtypes (Fig. 1b): the polyaromatic cation exporter EmrE, and Gdx-Clo, a functionally characterized Gdm+ transporter from Clostridiales. Radioactive uptake assays confirm that EmrE transports methyl viologen, but not Gdm+; that Gdx-Clo transports Gdm+, but not methyl viologen; and that both proteins discriminate against a substituted guanidinyl metabolite, agmatine (Fig. 2a).

To expand the repertoire of substrates tested, we used solid-supported membrane (SSM) electrophysiology. These experiments are feasible because the transport cycle of SMR proteins is electrogenic: the Gdx proteins couple import of two H+ with export of one Gdm+ ion, and EmrE, though it has been shown to stray slightly from strict 2:1 stoichiometry, transports monovalent substrates in an electrogenic manner as well. In SSM electrophysiology, proteoliposomes are capacitively coupled to a gold electrode by adsorption to a lipid monolayer. When the liposomes containing SMR proteins are perfused with substrate, initiating electrogenic transport, transient capacitive currents are evoked (Fig. 2b). The peak currents are negative, consistent with a net-negative transport cycle expected for 2 H+ substrate exchange. (In contrast, translocation of a positively charged substrate, without concomitant proton antiport, would be expected to evoke a positive current.) The amplitudes of the currents are proportional to the initial rate of transport, but decay rapidly to baseline as a membrane potential builds up in the liposomes and the system achieves electrochemical equilibrium. Subsequent replacement of the substrate-containing solution with a substrate-free solution yields a transient current of the opposite polarity, reflecting efflux of the
accumulated substrate from the liposomes, and a return to the starting condition (Fig. 2b).

We tested substrates in the following categories: Gdm\(^+\), guanidinylated metabolites, hydrophobic substituted guanidinium ions, and hydrophobic substituted amines. For all of these, analogous experiments with protein-free liposomes exhibit no currents (Supplementary Fig. 3). (In contrast, polyaromatic molecules like ethidium and tetraphenylphosphonium produced currents due to nonspecific partition into the membrane, and were therefore not analyzed here; Supplementary Fig. 4.) Because an unexpected shift in stoichiometry to 1 H\(^+\):1 substrate antiport would be electrically silent, all negative results were validated using a second method, exchange with radiolabeled substrate (Supplementary Fig. 5 or ref. 6). We observed no discrepancies between the electrophysiological results and the radioactive uptake experiments.

Our electrophysiology experiments (Fig. 2c) recapitulate prior observations for metabolites: EmrE does not transport Gdm\(^+\), and both proteins are strongly selective against substituted guanidinium metabolites like arginine, agmatine, and creatine6. However, many of the non-natural compounds we tested were readily transported by both subtypes. Gdx-Clo transported guanidinyl compounds with hydrophobic single substitutions, including the bulky phenylGdm\(^+\). Currents decreased for doubly

Fig. 2 Substrate transport by Gdx-Clo and EmrE. a Radioactive exchange assays. For Gdx-Clo and EmrE, uptake of \(^{14}\text{C}-\text{Gdm}^+\) or \(^{14}\text{C}-\text{methyl viologen}, respectively, monitored in exchange for the indicated substrate. Points represent individual replicates; error bars represent the mean ± SEM from three independent experiments. b Typical SSM electrical recording of Gdx-Clo proteoliposomes perfused with indicated solutions. The area in the dashed box is used to determine initial rate kinetics. c Initial rate of substrate transport (peak currents) by Gdx-Clo (normalized to Gdm\(^+\) currents, left panel) and EmrE (normalized to phenylGdm\(^+\) currents, right panel). Singly substituted guanidinyl compounds are shown as maroon bars, doubly substituted guanidinyl compounds are shown as blue bars, with all other compounds shown as green bars. Data were collected from 3 to 4 independent sensor preparations, which were in turn prepared from 2 to 4 independent protein preparations. Individual measurements are shown as points, and error bars represent ±SEM.
substituted guanidinyl compounds, and were absent for tetramethylGdm\(^+\). Compared with Gdx-Clo, EmrE required additional hydropobicity and bulk in its substrates. In agreement with the radiolabeled Gdm\(^+\) uptake experiments, Gdm\(^+\) was not transported by EmrE. However, methyl-, ethyl-, and phenylGdm\(^+\) evoked increasingly larger currents. In contrast to Gdx-Clo, EmrE also accommodated substrates with reduced or no H-bonding capacity, tetramethylGdm\(^+\) and tetramethylammonium, respectively. These experiments show that polya-romaticity is not a requirement for transport by EmrE. Moreover, these experiments make clear that functional promiscuity is a general trait of the SMR family. The relative transport specificities are summarized in Supplementary Fig. 6.

Crystal structure of Gdx-Clo. What molecular features of SMR proteins enable these promiscuous transport functions, while simultaneously prohibiting export of endogenous substituted guanidinium metabolites? Even though this family has proved endlessly intriguing to biochemists, as one of just a few idiosyncratic examples of primitive dual topology antiparallel dimers, the only structural models available include a 7 Å electron microscopy structure of EmrE\(^5\), and an X-ray crystal structure of anisotropic datasets and addition of substituted Gdm\(^+\) substrates further improved resolution, ultimately to 2.3 Å. The asymmetric unit contains one Gdx-Clo dimer and two Clo-L10 monobodies, one bound to each subunit. The monobodies primarily use residues diversified in the library to bind to residues 24–32 from loop 1 of each Gdx-Clo subunit in slightly different orientations, each forming a \(~400 \text{ Å}^2\) interface (Fig. 3a and Supplementary Fig. 8). In electrophysiology experiments, Gdm\(^+\) currents mediated by Gdx-Clo decreased upon addition of Clo-L10, but fractional inhibition saturated at \(~40\%,\) suggesting that monobody complexation is not incompatible with the transport cycle (Supplementary Fig. 9).

The structural basis for conformational exchange. The two 4-TM helix subunits of Gdx-Clo are arranged antiparallel with respect to each other in non-equivalent “A” and “B” conformations. The overall fold agrees with previous low-resolution structural models of EmrE\(^5\), and our designation of A and B subunits matches that used for EmrE. A large aqueous chamber is open to one side of the membrane, with the strictly conserved substrate- and proton-binding glutamates, E13\(_A\) and E13\(_B\), accessible at the bottom. Positive density is visible between the E13 sidechains, but cannot be definitively assigned as Gdm\(^+\) at this resolution (Supplementary Fig. 10). Transport by the antiparallel SMR proteins involves a conformational swap between the two structurally distinct monomers, which seals the substrate binding site on one side of the membrane while opening an identical site on the opposite side (Fig. 3b, c). As a consequence of the antiparallel homodimeric architecture, there is no structural difference between the inward-open and outward-open conformations: they are structurally identical and related by twofold symmetry about an axis parallel to the plane of the membrane. To visualize conformational exchange, we have rendered this structure in both the inward- and outward-facing directions (Fig. 3c).

The crux of the conformational exchange is helix 3 (G56xxxAxxTG64IGxxxxXxG), which possesses two GxxxxXxGG helical packing motifs offset from each other by two amino acids, or just over 180°. The G64IG sequence at the helical midpoint is the fulcrum between an N-terminal domain (TM1, TM2, and the first half of TM3) and a C-terminal domain (the second half of TM3 and TM4). Comparing subunit A and subunit B, the domains possess near structural identity (RMSD 0.5 Å for Ca, 1–62), but are offset by a rigid body rotation of about 30° (Fig. 3d).

In agreement with our observations, the analogous G64VG sequence in EmrE has been identified in EPR studies as a “kink” about which the conformational change occurs\(^21\). Inspection of the regions that change in accessibility during the transport cycle shows that, for each TM3, only one of the two GxxxxXxG packing motifs is buried at one time, and that burial alternates with conformational exchange (Fig. 3c and Supplementary Fig. 11). We posit that competition between the two halves of TM3 to pack against structurally complementary regions of the protein contributes to the structural frustration and conformational exchange in the Gdx transporters. In addition, T63, which immediately precedes the GIG sequence, is in a position to backbond to the mainchain and further perturb the helical geometry. Mutation of the analogous serine at this position in EmrE interferes with the dynamics of the conformational exchange\(^22\).

The well-ordered extramembrane loops also exhibit major differences in packing on the open and closed sides of the transporter (Fig. 3e). On the open side of the transporter, several charged amino acids, K21\(_A\) from loop 1A and E80\(_A\) and R86\(_A\) from loop 3\(_A\), are solvent-exposed in the aqueous chamber. Upon conformational exchange, K21\(_A\), E80\(_A\), and R86\(_A\) converge on loop 2B and the N-terminal end of helix 3B, forming cross-subunit H-bond interactions with the backbone and sidechain atoms of L53\(_B\)-T57\(_B\). The hydrophobic loop 1A also contributes to sealing the binding pocket on the closed side of the transporter, where it is wedged between the antiparallel helices 2A and 2B. Thus, the extramembrane loops, which are the least well-resolved features of previous structural models of SMR proteins, likely play an important role in the energetics of subunit packing. The involvement of loop 3 in conformational exchange has also been proposed for EmrE based on spectroscopic studies\(^23,24\).

The substrate binding site. In order to visualize substrate coordination, we solved a structure of the Gdx-Clo/L10 monobody complex together with a non-natural transported substrate, phenylGdm\(^+\), since this compound’s bulky phenyl group would aid modeling of the substrate. Fortuitously, this also improved the resolution to 2.5 Å. We observed conspicuous density near the glutamates, to which we fit one phenylGdm\(^+\) molecule (Fig. 4a).

Neutralization of these glutamates has previously been shown to abolish substrate transport in Gdx-Clo\(^6\). The substrate’s guanidinyl group is coordinated by E13\(_B\), whose position is in turn stabilized by a stack of conserved H-bond donors and acceptors, including W62\(_B\), S42\(_B\), and W16\(_B\). W62 and S42 are highly conserved among SMRs, and have been previously implicated in substrate specificity and transport\(^25,26\). In Gdx-Clo, mutations that remove H-bond potential, S42A and W62F, reduced or
eliminated Gdm$^+$ exchange, respectively (Fig. 4b). Conspicuously, W16 is conserved among Gdx proteins, but conserved as a glycine or alanine among the Qac subtype. In Gdx-Clo, the W16G mutant reduces, but does not eliminate Gdm$^+$ exchange (Fig. 4b).

The guanidinyl group of phenylGdm$^+$ is also in close proximity to the opposite E13A sidechain. However, E13A is deflected downward by a cross-subunit interaction with Y59B, so that the angle between the nitrogen, hydrogen (coplanar with the guanidinyl group), and oxygen atoms is not optimal for H-bond formation. Y59 is absolutely conserved among SMR proteins and the capacity to hydrogen bond has been identified as mechanistically essential at this position$^{18,27}$. Based on our Gdx-Clo structure, we propose that Y59B and the guanidinium group compete for E13A, and that displacement of Y59B by the guanidinyl group initiates the transport motion (Fig. 4c). Of all the amino acids, Y59 undergoes one of the largest changes in conformation, swinging out away from the binding site and into the aqueous pocket when the subunits swap conformations. Y59F, which cannot form a hydrogen bond with the E13 carboxylate, is not competent for substrate exchange (Fig. 4b), in accord with the requirement for an H-bond at this position. It is also notable that E13 only forms a single hydrogen bond with the Gdm$^+$ ion. This contrasts with the lowest energy coplanar, bidentate coordination of the guanidinium/glutamate complex in solution$^{28}$, and also draws a contrast to Gdm$^+$ coordination by the guanidine riboswitches, which provide hydrogen bond partners for most or all of the substrate’s five hydrogen bond donors$^{29–32}$. The more minimal coordination by the transporter explains its permissiveness towards guanidinium ions with methyl substitutions in one or two positions.

Fig. 3 Gdx-Clo structure and conformational exchange. a Structure of Gdx-Clo/monobody complex. Clo-L10 monobodies are shown in green. Transporter shown with subunit A in light blue and subunit B in tan. The N- and C-termini for each subunit are shown as blue and red spheres, respectively. Transporter residues that comprise the monobody binding interface are shown in magenta. b Cartoon schematic showing transport cycle for an antiparallel homodimer. The dashed box indicates the conformational exchange step highlighted in panel (c). Changes in accessibility during conformational exchange. For both the upper diagram, and the lower three-dimensional structure, regions that alternate in solvent accessibility are shown in magenta (TM2, loop 2, and the first GxxxAxxxG motif of TM3) and dark blue (TM1, loop 1, and second GxxxAxxxG motif of TM3). The N- and C-termini are shown as blue and red spheres. In the three-dimensional structure, E13 sidechains shown as sticks and solvent-accessible vestibule indicated with dots. d Overlay of Gdx-Clo A and B subunits aligned over C$\alpha$1–61. The sequence of TM3 is shown with GxxxAxxxG packing motifs colored in magenta and dark blue in structures and sequence. An arrow indicates I55. Three views are shown (counterclockwise from top): top-down view, view through the plane of the membrane (with GI65G indicated with an arrow), and rotated 90°. e Conformational exchange viewed from top down. E13 sidechains shown in red as surface representation and indicated with arrows. Sidechains that make polar contacts on the closed side of the transporter are shown as sticks.
A membrane portal accommodates hydrophobic substrate substituents. In the case of phenylGdm\(^+\), the substituent is packed between TM2\(_A\) and TM2\(_B\). At this point, the antiparallel TM2 helices splay apart, delimiting a portal from the membrane to the substrate binding site (Fig. 4d). In order to interrogate this feature, we solved a structure of Gdx-Clo in complex with octylGdm\(^+\), a cationic detergent with a Gdm\(^+\) head group and an eight-carbon tail. The guanidinium group sits in the same binding pocket as phenylGdm\(^+\), near E13\(_B\), and the aliphatic tail protrudes from the protein and into the detergent micelle (Fig. 4d, e). The tail is accommodated by rotameric rearrangements of the hydrophobic amino acids lining TM2 including M39 and F43 (Fig. 4e). Similar portals have been observed in other drug-binding membrane proteins, and are thought to provide binding site access for hydrophobic substrates that partition into the membrane33–35. Spectroscopic studies and molecular modeling have provided evidence for a similar portal between the TM2 helices of EmrE18,23.

It is clear that this membrane portal could be exploited by hydrophobic compounds to gain access to the binding site. We propose that this portal is also advantageous in the transporter's physiological context. Although this portal allows hydrophobic substituent accommodation by the membrane, metabolites like arginine, creatine, and agmatine all have polar groups on the tails for which insertion into the hydrophobic membrane environment would introduce a high energetic penalty, making the portal a convenient means for selecting against major guanidinylated metabolites, and rationalizing the conservation of this feature. The SLC35 solute transporters36–38 provide a notable point of contrast. SLC35 proteins are assembled as two-domain inverted repeat transporters in which each domain is homologous to the SMR fold, but have an additional two-helix insertion that seals off the portal so that the binding site is only accessible from aqueous solution (Supplementary Fig. 12).

Discussion

In summary, our transport experiments show that a representative of the SMR family’s Gdx subtype, like the better characterized Qac SMRs, promiscuously transports a series of hydrophobic non-natural compounds, and that functional promiscuity is thus a general feature of the SMR family. Although Gdx-Clo’s physiological role is transport of the metabolite Gdm\(^+\), it is not exquisitely selective for Gdm\(^+\), and whereas there is a biological imperative to prevent export of valuable guanidinylated

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Fig. 4 Substrate binding by Gdx-Clo. a PhenylGdm\(^+\) binding site. Subunits colored in light blue and tan as in Fig. 3. The aqueous accessible vestibule is shown as a gray surface rendering. Sidechains that coordinate substrate or E13 shown as sticks, and interactions with appropriate distance and geometry for hydrogen bonds are shown as dashed lines. The electron density assigned to phenylGdm\(^+\) (2Fo-Fc map contoured at 1.3\(\sigma\)) is shown as blue mesh. b Uptake of 14C-Gdm\(^+\) into proteoliposomes mediated by the indicated mutant. Total uptake is normalized relative to uptake by WT at 10 min. Error bars represent the SEM for three independent replicates. c Illustration of the proposed conformational transition around the transported Gdm\(^+\). Colored sidechain sticks are in the positions observed in the structure; white sidechain sticks and arrows show proposed conformational change. d Membrane portal. The structure from Fig. 3 is shown at left, and the octylGdm\(^+\) bound structure is shown at right. Cartoon is shown with helices 3 and 4 removed for clarity. Sidechains lining the portal, and E13 sidechains, are shown as sticks. OctylGdm\(^+\) is shown as stick representation, with octyl tail extending toward the viewer. Dashed line indicates the level at which the protein is sliced in panel (e). e Top-down view of Gdx-Clo surface and helices with octylGdm\(^+\), sliced at approximately the midpoint of the membrane. E13 sidechains are shown as sticks. Experimental 2Fo-Fc density for the ligand, contoured at 1.8\(\sigma\), is shown as gray mesh. The arrow indicates C5 of the octyl substituent. Agmatine and arginine bear amino groups at this position.
metabolites like arginine or agmatine, there is no selective pressure to be discerning towards non-native compounds. Promiscuous functions, those that are not under direct selection, provide a rich source of cryptic variation that can be harnessed to provide evolutionary novelty—perhaps rationalizing the broad distribution of both the Qac and Gdx subtypes with a conserved selectivity mechanism against biguanides have also been identified with antiseptic use and substituted guanidinium ions and contamination by hydrophobic quaternary amines is associated made these latent functions adaptive. Indeed, environmental could include various human-introduced biocides, may have provided evolutionary novelty that impact the human microbiome and that are excreted in waste water.

Structural analysis of Gdx-Clo reveals numerous features that correspond to biochemical or spectroscopic observations made for EmrE, indicating a high degree of mechanistic conservation between the Qac and Gdx subtypes. The Qac and Gdx subtypes also share multiple binding pockets, which have also been implicated in polyspecificity in other systems, including QacR transcriptional regulators and P-glycoprotein. The structure also identifies other features that contribute to promiscuous substrate transport in the SMR family, including minimal coordination of the substrate and direct access from the membrane to the binding site. We conjecture that SMR proteins have enjoyed such evolutionary success in the modern world because this portal, a conserved selectivity mechanism against major physiological metabolites, proved to be extremely adaptive for the binding and export of hydrophobic, human-introduced chemicals.

Methods

Sequence-similarity network. A sequence-similarity network was generated using the EBI GMT program (Protein Data Bank Int. Res.), with an alignment score of 20, and visualized with 50% similarity in Cytoscape using the preface force-directed layout. A genome neighborhood network was then generated with the EBI-GNT tool, using a neighborhood size of 10. The coordinates of the Guanidine-I, Guanidine-II, and Guanidine-III riboswitches were retrieved from RFAM and used to annotate PMF members if they were to be found in the genome neighborhood. The coordinates of the Guanidine-I, Guanidine-II, and Guanidine-III riboswitches were then generated with the EFI-GNT tool, using a neighborhood size of 10. The genome neighborhood network was then generated with the EBI-GNT tool, using a neighborhood size of 10. The sequence-similarity network was generated using the prefuse force-directed layout. A genome neighborhood network was then generated with the EBI-GNT tool, using a neighborhood size of 10.

Transporter expression, purification, and proteoliposome reconstitution. Lipids were from Avanti, detergents from Anatrace. Proteins were expressed and purified as previously described. Briefly, Gdx-Clo bore a C-terminal hexahistidine affinity tag and a LysC recognition site, and were cloned into a PET-21c expression vector, and transformed into C41 (DE3). When cultures reached an OD600 of 1.0, protein expression was induced with 0.2 mM Isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Cell lysate was extracted with 2% (w/v) decayl-β-D-maltoside (DM), and the soluble fraction was purified over cobalt affinity column, washed with 100 mM NaCl, 20 mM imidazole, and then eluted with 400 mM imidazole. The affinity tag was cleaved by incubation with LysC (200 ng per mg protein, 2 h at room temperature), before a final size exclusion purifica- tion step using a Superdex 200 gel-filtration column equilibrated in 100 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES)-NaOH, 5 mM DTT, pH 8.1. EmrE was expressed and purified similarly, but the construct bore an N-terminal histidine affinity tag with a thrombin recognition site. When cultures reached an OD600 of 0.5, protein expression was induced with 0.2 mM Isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Cell lysate was extracted with 2% (w/v) decayl-β-D-maltoside (DM), and the soluble fraction was purified over cobalt affinity column, washed with 100 mM NaCl, 20 mM imidazole, and then eluted with 400 mM imidazole. The affinity tag was cleaved by incubation with LysC (200 ng per mg protein, 2 h at room temperature), before a final size exclusion purifica- tion step using a Superdex 200 gel-filtration column equilibrated in 100 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES)-NaOH, 5 mM DTT, pH 8.1. EmrE was expressed and purified similarly, but the construct bore an N-terminal histidine affinity tag with a thrombin recognition site. After induction with IPTG, protein was expressed overnight at 16 °C. E. coli polar lipids dissolved in chloroform were dried under a nitrogen stream and residual chloroform was removed by washing and drying three times with ethanol and three times with water and dried by taping on a paper towel. 1.5 µl of lipid solution (7.5 µg/µl 1,2-diphtanyloyl-sn-glycerol-3-phosphocholine in n-decane) was painted on the gold electrode surface using a pipette tip, followed by Postscript, and 500 µM g DNase, 2 mM MgCl2, 1 mM PMSF, 1 mg/ml lysozyme, then incubated with TEV protease (0.03 mg/ml cobalt affinity resin) overnight to cleave Postscript, and lysis, and lysed by sonication for 5 min. Monobody expression and puri- fication. Monobody proteins were expressed in E. coli (BL21-DE3) grown in Studier’s autoinduction medium at 37 °C. All refolding reactions were performed in 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 100 mM KCl, 100 mM KPO4, pH 7.5. For measurements in the presence of monobody, recording buffers contained 50 mM MgCl2, 4 mM DM, 100 mM KCl, 100 mM KPO4, pH 7.5. For measurements in the presence of monobody, recording buffers contained 50 mM MgCl2, 4 mM DM, 100 mM KCl, 100 mM KPO4, pH 7.5. Measurements were performed at an angle of 15° to the gold electrode surface using a pipette tip, followed immediately by addition of 50 µl of nonactivating buffer (100 mM KCl, 100 mM KPO4, pH 7.5). Proteoliposomes were diluted 25-fold in buffer and sonicated 30–60 s before addition to the protein sensor and centrifugation at 29,000 × g for 30 min.

Before experiments, sensors were checked for conductance and capacitance using SURFER software protocols. Sensors for which capacitance and conductance measurements were outside an acceptable range (10–40 nF capacitance, 1–5 nS conductance) were not used for experiments. Sensors were periodically rechecked for quality during the course of an experiment. Each substrate was tested for transport at a concentration of 1 mM in buffer containing 100 mM KCl, 100 mM KPO4, pH 7.5. For measurements in the presence of monobody, recording buffers contained 50 mM bovine serum albumin/ml. To compare measurements recorded on different sensors, currents were normalized relative to a reference sensor, as described in the text. Currents elicited by the reference compound were measured both at the outset of the experiment and after collecting data on test compounds. If currents for the first and last perfusions of reference compound differed by more than 10%, this indicated that the amount of reconstituted protein had not remained stable over the course of the experiment, and data collected in this series were not used for further analysis. Data were collected from 3 to 4 independent sensor preparations, which were in turn prepared from 2 to 4 independent protein preparations. Reported data are for peak currents, which represent the initial rate of substrate transport before a membrane potential builds up and inhibits further electrogenic transport.

Monobody development. Monobody selection was performed following previously published methods. Four rounds of phage selection with target concentrations of 100, 100, 50 and 20 mM was performed in 10 mM Hepes pH 7.5, 200 mM NaCl, 20 mM GdmCl, 4 mM DM, then sorted pools were subcloned into a yeast display library following recombination of 5’ and 3’ fragments to increase library diversity. Three rounds of yeast library sorting were performed: the first round for clones binding to 50 nM target, second round for clones exhibiting no binding to 10 μM streptavidin (negative sorting), and the third round for binding with 5 nM target. Isolated clones were validated for target binding using a yeast-display binding assay, as described in detail.

Monobody expression and purification. Monobody proteins were expressed in E. coli (BL21-DE3) grown in Studier’s autoinduction medium at 15–18 °C at 37 °C. After harvest, cells were solubilized with recombinant lysozyme culture (Takara; 3–5 mg/ml for 3 ml culture) for 15 min on ice. The column with bound monobody was washed with 10 CV of denaturing buffer, 10 CV of breaking buffer. The resin containing the L10 inclusion was resuspended in denaturing buffer (20 mM Tris–Cl pH 8.0, 6 M GdmCl) and incubated at room temperature with rotation for 1 h. Debris were removed by centrifugation (17,500 × g for 45 min), and the supernatant was loaded onto a cobalt affinity column (100 ml for 3 ml culture). After washing with 10 CV of denaturing buffer, 10 CV of breaking buffer. The resin, with bound, refolded monobody, was incubated with TEV protease (0.03 mg/ml cobalt affinity resin) overnight to cleave the His tag, and digested monobody was eluted with breaking buffer. A final size
exclusion purification step was performed using a Superdex 75 gel-filtration column equilibrated in 10 mM HEPES pH 7.5, 10 mM NaCl.

Crystal preparation. For X-ray crystallography, Gdx-Clo and monobody Clo-L10 were purified as described above. For the Clo purification, the size exclusion buffer contained 200 mM NaCl, 10 mM HEPES pH 8.1, and 10 mM Gdm-8N. Proteins were concentrated to 10 mg/ml, Clo-L10 was supplemented with 4 mM DM, and monobody and Gdx-Clo dimer were mixed in a 2:1:1 ratio. The protein solution was then mixed with an equal volume of crystallization solution (0.3 μl in 96-well plates). Initial hits grew in 200 mM CaCl2, 0.1 M Tris/HCl pH 8.0 and 32.5% PEG 600. Crystals were subsequently improved by addition of charged detergents lauryldimethylamine-N-Oxide (LDAO; final concentration 6.6 mM), dimethylcyclohexylamine oxide (ApO12; final concentration 2 mM), or octylGdm+ (final concentration 3.3 mM) to the protein solution prior to ad来看看吧.
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Author contributions

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Competing interests

A.K. and S.K. are listed as inventors for patents (US9512199 B2 and related patents and 1436514371 (2004).)

Additional information

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Correspondence and requests for materials should be addressed to R.B.S.

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