Guanidinium export is the primal function of SMR family transporters

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The small multidrug resistance (SMR) family of membrane proteins is prominent because of its rare dual topology architecture, simplicity, and small size. Its best studied member, EmrE, is an important model system in several fields related to membrane protein biology, from evolution to mechanism. But despite decades of work on these multidrug transporters, the native function of the SMR family has remained a mystery, and many highly similar SMR homologs do not transport drugs at all. Here we establish that representative SMR proteins, selected from each of the major clades in the phylogeny, function as guanidinium ion exporters. Drug-exporting SMRs are all clustered in a single minority clade. Using membrane transport experiments, we show that these guanidinium exporters, which we term Gdx, are very selective for guanidinium and strictly and stoichiometrically couple its export with the import of two protons. These findings draw important mechanistic distinctions with the notably promiscuous and weakly coupled drug exporters like EmrE.

guanidinium | SMR | EmrE | SugE | dual topology

The small multidrug resistance (SMR) family comprises small four-transmembrane (TM) helical proteins that first became prominent because of their role in antibiotic and antiseptic resistance and their rare “dual topology” architecture (1–4). At ~100 residues, they are among the smallest known membrane transport proteins, and their small size makes them amenable to many biophysical analyses. The best studied member of the SMR family is the multidrug efflux pump EmrE, which protects bacteria from an array of hydrophobic, cationic antimicrobials including ethidium and methyl viologen (1). SMRs that are phenotypically similar to EmrE have been disseminated widely among bacteria through horizontal gene transfer (5). But many bacterial genomes harbor SMR proteins that are highly similar to the multidrug pumps but that do not transport drugs (variously annotated *qacE*, *sugE*, *ebrA*, *ebrB*, or *emrE*). For example, in *Escherichia coli*, EmrE and its non-drug-exporting SMR homolog share 33% sequence identity and 61% similarity, including a conspicuous midmembrane glutamate that is essential for function in EmrE (6).

The function of these “other” SMRs has remained enigmatic, from their initial erroneous assignment as chaperone-like molecules (7), stemming from a PCR error that interfered with an adjacent chaperone (8, 9) (to this day the genes are often annotated “*sugE*,” a misnomer that stands for “Suppressor of GroEL”), to the unsuccessful attempts to measure transport or conformational change in the presence of drugs (9–11). Moreover, no clear sequence-based criteria have been identified to distinguish SMR proteins that export drugs and those that do not. The determination of whether an SMR contributes to a pathogen’s drug resistance is typically determined using case-by-case bacterial fitness assays (5). And without understanding the native role of SMR proteins, the evolutionary history that spawned this particular variety of multidrug resistance in bacteria is indecipherable.

A solid lead on the native role of the SMRs was provided by the recent discovery of bacterial operons dedicated to guanidinium (Gdm⁺) metabolism (12–15). These operons are controlled by

Gdm⁺-binding riboswitches, which frequently up-regulate either enzymes that chemically modify Gdm⁺ or membrane proteins from the SMR family (15). These riboswitches are widespread in both Gram-negative and Gram-positive bacteria, including firmicutes, cyanobacteria, actinobacteria, proteobacteria, and others. There exist three structurally distinct Gdm⁺-responsive riboswitches, termed guanidine-I (or ykkC) (12, 16, 17), guanidine-II (or mini-ykkC) (14, 18, 19), and guanidine-III (or ykkC-III) (13). These riboswitches are mechanistically variable as well, with guanidine-I exerting transcriptional control (12), whereas guanidine-II and -III are translational regulators (13, 14). Their common feature is the genes whose expression they control, including, for all three riboswitch classes, genes that are annotated as SMRs (15).

That the nondrug transporting SMRs might instead transport Gdm⁺ is an appealing hypothesis. Despite the obvious difference in size between Gdm⁺ and the bulky substrates transported by EmrE, the substrates share a chemical essence that alludes to the familial relationship: cationic aromaticity. Gdm⁺ is a chemically unusual “Y-aromatic”—a planar molecule with electrons delocalized in pi orbitals over both faces (20, 21). It is easy to imagine how a Gdm⁺ exporter might share common traits with a promiscuous exporter of larger substrates with shared chemical attributes (Scheme 1). Indeed, experiments have shown that a representative riboswitch-controlled SMR binds Gdm⁺ (12), but there is no indication of whether Gdm⁺ is the transported substrate or merely an effector, whether drug- and Gdm⁺-related functions overlap, and how broadly Gdm⁺ exporters are distributed in the SMR family.

Here we demonstrate that Gdm⁺ export is the main functional role of the SMR family, whereas the experimentally validated

Significance

The small multidrug resistance (SMR family) was named for its founding member, a promiscuous exporter of antibiotics and antiseptics, and has become an important model system in membrane protein mechanism and evolution. But most members of this family do not share drug export activity, and their function has been enigmatic. Here we show that the SMR family is mostly comprised of proteins that export guanidinium ion, a small, cationic metabolic byproduct. In addition to opening up an unusual bacterial physiology for further exploration, this work provides a comprehensive view of the SMR family and provides critical context to understand the evolution and mechanism of the multidrug transporters.

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Scheme 1. Structural comparison of Gdm^+ and some canonical EmrE substrates.

multidrug exporters (5) are all confined to a single clade of the phylogenetic tree. We characterize several proteins dispersed throughout the phylogeny, showing that they couple Gdm^+ export to the proton motive force, exchanging one Gdm^+ for two protons. These Gdm^+ -exporting SMRs, which we call Gdx (guanidinium exporter), are mechanistically distinct from the multidrug exporters in two important ways: They are highly selective for their substrate, and they couple its export tightly with the import of two protons.

Results

Most SMR Proteins Are Associated with Guanidine Riboswitches. Over half of guanidine-riboswitch controlled genes are annotated as members of the SMR family, but of the SMRs with validated drug-export functions, none are associated with a riboswitch. [The *E. coli* riboswitch-controlled SMR, also called SugE, has been shown not to transport drugs (10).] To examine the evolutionary relationships between the riboswitch-controlled proteins and multidrug exporters from the SMR family, we constructed sequence alignments and a phylogeny from a representative sample of all known SMRs.

The SMR family comprises predicted homo-oligomers, encoded by a single gene, and predicted heterodimers, encoded by adjacent genes in the genome that arose from a gene duplication event. Analysis of the sequences shows that, in the single gene versions, the solvent-accessible loops possess few charged residues, suggesting dual topology, the propensity for subunits to insert into the membrane in both inward- and outward-facing orientations, setting up the possibility for antiparallel homodimers to occur (3, 22). In contrast, the subunits of putative heterodimers possess oppositely directed charge biases (Fig. 1A), circumstantial evidence that the subunits oligomerize with antiparallel orientation. In dual topology proteins, duplication events are typically followed by the relatively rapid evolution of positive charge bias (lysines and arginines) on the cytoplasmic

loops, fixing the topology of the heterodimer subunits (3, 23, 24). Our preliminary analyses suggested that several different duplication events have occurred in different SMR lineages, each yielding pairs with oppositely biased charge distributions. This example of convergent evolution, the evolution of similarly biased loops among several independent duplication events, is in conflict with the assumption of divergent evolution necessary for phylogenetic reconstruction. Therefore, we estimated solvent-exposed sequences based on structural homology to EmrE and trimmed these sequences from the alignment, a necessary maneuver to avoid bias by convergent evolution.

Our maximum likelihood phylogeny (Fig. 1B) provides strong support for five major distinct protein clades: one each comprised of proteins controlled by the guanidine-II and guanidine-III riboswitches, two clades of proteins associated with the guanidine-I riboswitches, and a fifth branch in which experimentally validated multidrug exporters (reviewed in ref. 5) cluster. Notably, most of the proteins from Gdx branches are associated with riboswitches, whereas many of the proteins from the multidrug resistance branch are associated with transposons or plasmids (5).

The SMRs controlled by guanidine-I riboswitches are distributed across two deeply diverged clades. Closer inspection of these SMRs reveals that most of them likely form heterodimeric pairs, with one partner from each clade. The genes encoding these pairs are almost always located adjacent to each other in genomes, and the paired proteins themselves possess opposite charge biases, implying that their topologies became fixed after an ancient gene duplication event. Other more recent duplication events yielding probable heterodimers, notably in the guanidine-III clade, are also evident.

Riboswitch-Controlled SMRs Are Proton-Coupled Gdm^+ Transporters. To identify the molecular function of riboswitch-controlled SMRs, we identified, heterologously expressed, and purified representatives controlled by each riboswitch subtype, including both a homodimer and a heterodimer from the guanidine-I family (Table 1 and Fig. S1). Proteins were reconstituted into liposomes to assess Gdm^+ transport. Proteoliposomes were initially loaded with 10 mM unlabeled Gdm^+ and then diluted into an external solution containing 20 μM ^{14}C -labeled Gdm^+ . In the presence of a Gdm^+ transport pathway, the outward-directed Gdm^+ gradient drives uptake of radiolabeled Gdm^+ into the liposomes. All four proteins catalyzed robust uptake of ^{14}C - Gdm^+ . Gdm^+ exchange was abolished by mutation of a midmembrane glutamate, E13 (Fig. 2A), which is completely conserved among SMRs and known to be essential for substrate binding and transport in EmrE (6). Gdm^+ exchange is limited to riboswitch-controlled SMRs; despite its promiscuity among drugs, EmrE fails to bind or transport Gdm^+ (Fig. 2A and Fig. S2).

Fig. 1. Phylogeny and sequence alignment of the SMR family. (A) Sequence alignment of representative Gdx proteins in this study (Table 1) and multidrug exporter EmrE. (B) Phylogeny of representative SMR proteins constructed using maximum likelihood methods. Major nodes were statistically significant (support > 0.9), indicated with a black circle. Tree is annotated by colored branches: red, experimentally implicated in multidrug resistance; yellow, associated with guanidine-I riboswitch (12); green, associated with guanidine-II riboswitch (14); blue, associated with guanidine-III riboswitch (13). Duplication events leading to probable heterodimers are indicated by gray shading. Functionally validated proteins are labeled with the protein name. EbrA/EbrB is a *Bacillus subtilis* drug exporter that functions as an obligate heterodimer (45).

Table 1. Gdx proteins examined in this study

Protein name	Source	Sequence ID, NCBI	Riboswitch
Gdx-Eco	<i>E. coli</i>	WP_032226439.1	Guanidine-I
Gdx-Clo	<i>Clostridiales</i> bacterium oral taxon 876	WP_021653285.1	Guanidine-I
Gdx-Mic	<i>Micromonospora</i>	WP_013284696.1	Guanidine-II
Gdx-Sle 1	<i>Staphylococcus lentus</i> (subunit 1)	WP_016999238.1	Guanidine-I
Gdx-Sle 2	<i>S. lentus</i> (subunit 2)	WP_016999239.1	Guanidine-I

Based on the conservation of the TM1 glutamate, we speculated that Gdx, like EmrE, might couple substrate exchange to a proton gradient. To test this, we examined whether an H^+ gradient could drive ^{14}C -Gdm $^+$ accumulation in liposomes reconstituted with each of the three Gdx homodimers. Proteoliposomes were prepared with an internal pH of 6.5 and diluted into an otherwise identical buffer with a pH of 8.5. This outward-directed H^+ gradient induced ^{14}C -Gdm $^+$ uptake into the proteoliposomes (Fig. 2B). Radioactive uptake was not observed in the absence of a pH gradient, at either pH value. We also monitored the ability of Gdm $^+$ to drive H^+ translocation. Using a pH-sensitive fluorophore, pyranine, we monitored pH changes in the liposome interior upon addition of external Gdm $^+$. The fluorescence intensity increased when Gdm $^+$ was added to the external solution, indicating H^+ efflux from the liposomes (Fig. 2C). The steady-state magnitude of H^+ efflux increased in tandem with added Gdm $^+$. H^+ transport was not observed in liposomes without protein, and addition of urea (an uncharged structural analog of Gdm $^+$) or arginine to Gdx-Clo proteoliposomes also failed to reproduce transport (Fig. S3).

Gdx Proteins Are Selective for Gdm⁺. A hallmark of multidrug exporting SMRs, like EmrE, is their ability to export diverse cationic substrates that range in size and in valence (25, 26). To determine whether Gdx is similarly nonselective, we monitored ¹⁴C-Gdm⁺ uptake in the presence of a number of different substrates, including urea, which is a neutral, isosteric analog of Gdm⁺; common biological compounds with guanidinyll or quaternary amine moieties (arginine, agmatine, creatine, betaine, choline, and spermidine); EmrE substrates (ethidium and TPP⁺); and

N-substituted guanidinyll analogs (methyl- and aminoguanidinium) (Fig. 3). Among these, only aminoguanidinium and methylguanidinium were comparable to cold Gdm⁺ in competition with ¹⁴C-Gdm⁺ accumulation for all three homologs (Fig. 3A). To distinguish between block and permeation, counterflow assays, in which liposomes were preloaded with amino- or methyl-Gdm⁺, rather than Gdm⁺, were performed (Fig. 3B). Both *N*-substituted analogs were exchanged for radiolabeled Gdm⁺, albeit more slowly. Gdm⁺ uptake in Gdx-Eco was also competitively inhibited by TPP⁺, in accord with previous reports that it binds this substrate (10), but TPP⁺ was not transported in our experiments (Fig. 3B).

H⁺/Gdm⁺ Antiport Occurs with 2:1 Stoichiometry. Recent experiments with EmrE have fashioned a model of a transporter in which substrate promiscuity and transport stoichiometry are intimately linked. Depending on the substrate, EmrE undergoes some combination of 2:1 and 1:1 proton:drug exchange (27, 28). Given the selective nature of the Gdxs, we sought to determine whether they behave more like classical transporters, with tightly coupled exchange, or whether they share flexible stoichiometry with their multidrug-transporting family members. To this end, we loaded Gdx-Eco proteoliposomes with pyranine and diluted these into an otherwise identical buffer. A membrane potential was set using the potassium ionophore valinomycin in the presence of a K⁺ gradient, which effectively “clamps” the voltage across the membrane at the Nernst potential for K⁺. When the K⁺ concentration is equal on both sides of the membrane, no substrate movement occurs because all ions in the system are at chemical and electrical equilibrium (Fig. 4A, middle trace). When we apply an inwardly

Fig. 2. Gdx-mediated Gdm⁺ transport. For A–C, liposome cartoons illustrate experimental conditions. (A) Time course of ¹⁴C-Gdm⁺ uptake into liposomes reconstituted with different SMR proteins. Uptake is reported as a fraction of total ¹⁴C counts added at the outset of the experiment. Individual data points are the mean and SEM of at least three independent experiments from at least two independent protein preps. Where not visible, error bars are smaller than the diameter of the point. (B) Time course of ¹⁴C-Gdm⁺ uptake into Gdx-containing liposomes holding the indicated internal/external pH gradients. Values are the mean and SEM of at least three independent experiments. (C) Fluorescence of pyranine trapped inside liposomes. Addition of guanidinium to the external buffer is indicated by solid triangle. After reaction reached steady state, FCCP was added to collapse the pH gradient (open triangle).

Fig. 5. Architecture of Gdx proteins. (A) Glutaraldehyde cross-linking of Gdx-Mic. Cartoon indicates the distribution of primary amines in Gdx-Mic and Gdx-Mic mutants, with blue stars indicating native primary amines and red stars representing introduced lysine residues. (B) ^{14}C -Gdm $^{+}$ uptake into liposomes by antiparallel Gdx-Eco concatamer.

described by Schuldiner and coworkers (30) and later used to correctly predict the topology of Fluc family fluoride channels (32). We constructed a fused homodimeric construct linked by a nondimerizable glycophorin A TM helix to allow the subunits to adopt an antiparallel topology (32). This construct expressed robustly in *E. coli* (Fig. S5B), and activity assays showed that the antiparallel concatamer transports Gdm $^{+}$ at low protein:lipid ratios (Fig. 5B). We also attempted to design a concatamer with short, charged linker to force a parallel construction. However, we were unable to express and purify this protein.

Discussion

These experiments establish that diverse SMR proteins are Gdm $^{+}$ /H $^{+}$ antiporters, exporting Gdm $^{+}$ from the cytoplasm by coupling its translocation to the proton motive force. These results resolve a decades-long mystery about the function of the non-drug-exporting SMRs. The diverse homologs selected here are all notable for their selectivity for Gdm $^{+}$ over other common biological molecules with guanidinyll or primary amine moieties, and we expect that these traits are general among SMR proteins in riboswitch-associated clades. This draws a sharp contrast with the promiscuous multidrug exporters within the SMR family, including EmrE.

Several factors point to Gdm $^{+}$ export as the primal role of the SMR family. In both number and diversity, SMRs associated with guanidine riboswitches exceed those that are highly similar to multidrug exporters. Put another way, multidrug exporters are a minority clade in a family of Gdm $^{+}$ exporters. Gdx proteins are in well-structured sequences within the genomic DNA of both Gram-negative and Gram-positive bacteria, typically associated with regulatory RNA elements and often adjacent to additional genes involved in Gdm $^{+}$ remediation, suggesting that Gdm $^{+}$ export function has been useful to microbes over evolutionary time. In contrast, SMR proteins associated with multidrug resistance are typically found on plasmids or associated with horizontal gene transfer elements, suggesting that these genes have spread among microbes in response to relatively more recent stressors (5, 12). Our phylogeny shows that proteins in Gdx clades are genetically diverse, despite performing highly similar functions, and that there are examples of gene duplications that lead to deeply diverged heterodimer subunits that nonetheless assemble to perform Gdm $^{+}$ export function. The multidrug resistance clade, in contrast, is less deeply diverged, even though its functions are more variable (25, 27). The most parsimonious interpretation of our data is that multidrug export evolved from an ancestral Gdm $^{+}$ exporter, which is also the forebear of homodimeric and heterodimeric Gdx proteins.

The evolutionary relationships among SMR family members are especially noteworthy in light of the structural similarities and mechanistic differences between EmrE and the Gdx proteins. In

terms of architecture, Gdx and EmrE are overwhelmingly similar. They possess ~60% sequence similarity; antiparallel, four-TM helical construction; similarities in hydropathy; and a mechanistically essential glutamate residue in TM1. What, then, is the origin of the differences in both substrate specificity and mechanism? EmrE is mechanistically sloppy, exchanging substrates with different stoichiometries and undergoing uncoupled transport and substrate-free conformational exchange (27, 28). This mechanism has been proposed to facilitate its handling of a broad array of substrates (27). In contrast, the Gdx proteins hew closely to 2:1 coupled stoichiometry, and we do not observe 1:1 transport cycles or uncoupled H $^{+}$ movement by Gdx proteins in our experiments. These two SMR subtypes provide an excellent platform to probe the structural factors that contribute to promiscuity versus specificity and coupling efficiency. Gdx might even realize the original hope for EmrE: serving as a stripped-down model system to understand nature's minimalist solution to coupled, alternating access transport.

A final mystery of these proteins, and the other recently established components of Gdm $^{+}$ -responsive operons, is what role Gdm $^{+}$ plays in bacteria. These proteins are widespread, conserved, and evidently fulfilling an ancient function. Gdm $^{+}$ has been recognized as a byproduct of bacterial creatine metabolism since the late 1800s, when high concentrations of Gdm $^{+}$ were found in spoiled meats (33), but there is scant reference to Gdm $^{+}$ in the microbial literature before the discovery of the guanidine riboswitches. Recent results show that Gdm $^{+}$ is produced by bacteria when nutrients are scarce (12), but neither the metabolic pathway that leads to accumulation nor the motivation for export is known. It is certainly possible that Gdm $^{+}$ is simply a broad-spectrum toxin, to be recognized and removed, like fluoride ion (32, 34). Since a number of metabolites, including arginine, agmatine, and creatine, possess guanidinyll groups, it seems likely that Gdm $^{+}$ has interfered with chemical biology since early evolution. While biological levels of Gdm $^{+}$ are probably much less than the ~molar concentrations required to unfold proteins, Gdm $^{+}$ has been shown to inhibit a number of cellular processes at low-millimolar concentrations. Gdm $^{+}$ competitively inhibits arginine-binding metabolic enzymes like arginase (35), is a pore blocker of potassium channels (36), and electrostatically inhibits an ATPase involved in prion formation in yeast (37, 38). Further research will be required to determine whether Gdm $^{+}$ is a general toxin or whether it has some heretofore unknown dedicated role in bacterial life.

Materials and Methods

Phylogenetic Tree. A representative set of ~400 genes from the Multi_Drug_Res PFAM family (PF00893) were aligned together with a set of genes associated with multidrug resistance in the literature using MAFFT (39). Based on a structural alignment to EmrE, loop regions were removed from the alignment. A maximum-likelihood phylogenetic tree was constructed using IQ-TREE with evolutionary model JTT+G4, selected by ModelFinder on the basis of the Bayesian information criterion (40, 41). Branch support was calculated using UFBoot to provide rapid bootstrap approximation (42).

Protein Purification and Liposome Reconstitution. Protein was expressed in *E. coli* C41 (DE3) cells from pET21c plasmids with synthetic Gdx genes (GenScript) with a C-terminal protease recognition site and hexahistidine tag. Expression was induced with 0.2 mM IPTG at an OD $_{600}$ of 1.0, for 3 h at 37 °C. Cell membranes were extracted with 2% (wt/vol) decyl- β -D-maltoside (DM; Anatrace), and the detergent-solubilized fraction was purified with cobalt affinity beads, washed with 100 mM NaCl and 20 mM imidazole, and eluted with 400 mM imidazole. After histidine tag removal, protein was further purified on a Superdex200 gel-filtration column in 10 mM Hepes, pH 8.1, 200 mM NaCl, and 5 mM DM. Proteoliposomes were prepared from a 3:1 mixture of POPE (1-palmitoyl, 2-oleoylphosphatidylethanolamine) and POPG (1-palmitoyl, 2-oleoylphosphatidylglycerol), with 0.2 μ g protein per milligram of lipid (0–1 Gdx dimers per liposome) by dialysis against the desired intraliposomal solution, typically 200 mM NaCl and 20 mM Hepes, pH 7.5. Proteoliposomes were stored in aliquots at –80 °C until use and extruded 21 times through a 400-nm membrane filter before functional

assays. Heterodimeric and concatameric Gdx proteins were prepared with slight alterations to this protocol. EmrE was expressed and purified as described previously (43).

Radiolabeled Guanidinium Transport Assays. Liposomes preloaded with 1–10 mM test ion (typically Gdm^+) were passed over a Sephadex G-50 column pre-equilibrated with reaction buffer. Proteoliposomes were diluted two-fold into transport buffer (25 mM Hepes, pH 7.5, and 400 mM sorbitol). Transport was initiated by addition of 20 μM ^{14}C radiolabeled Gdm^+ (American Radiolabeled Chemicals, Inc.). At time intervals, 100 μL of reaction mix was passed over a 1.6-mL Dowex ion exchange resin column (*N*-methyl D -glucamine form) to remove external ^{14}C - Gdm^+ and resuspended in liquid scintillation mixture (Ultima Gold; Perkin-Elmer) for scintillation counting. For competition assays, 1–10-mM test substrate was added immediately before initiation with ^{14}C - Gdm^+ .

H^+ Transport and Stoichiometry Assay. Liposomes were preloaded with 1 mM pyranine (trisodium 8-hydroxypyrene-1, 3, 6-trisulfonate; Sigma-Aldrich) and external buffer exchanged using Sephadex G-50, as described above. Proteoliposomes were diluted 200-fold into buffer (25 mM Hepes, pH 7.4, and 400 mM sorbitol). Transport was initiated by addition of 10 mM Gdm^+ , and fluorescence was monitored (excitation, 460 nm; emission, 510 nm). At the end of the assay, the proton gradient was collapsed by addition of 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone). For assays

monitoring the effect of membrane potential, liposomes were prepared with 3–100 mM KCl, Gdm-Cl (10 mM for electrogenicity experiments and 0.4 mM for stoichiometry experiments), 25 mM Hepes 7.5, and 1 mM pyranine. NaCl was added to maintain ionic strength at 200 mM. External buffer contained 25 mM Hepes, pH 7.5, and 3–100 mM KCl to establish a potassium gradient (as indicated in the text/figures), NaCl to maintain 200 mM ionic strength, 10 mM Gdm^+ (for experiments to determine the electrogenicity) or 4 mM Gdm^+ (for experiments to determine stoichiometry), and valinomycin (20 $\mu\text{g/mL}$) to set the membrane potential. Membrane potential was calculated from the K^+ gradient according to the Nernst potential. The stoichiometry was determined using the method described by Mindell and coworkers (29, 44), from the experimentally determined reversal potential, E_{rev} . Pyranine fluorescence was monitored as a proxy for proton transport as before.

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