Sided Functions of an Arginine−Agmatine Antiporter Oriented in Liposomes

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S *Supporting Information*

ABSTRACT: The arginine-dependent extreme acid resistance system helps enteric bacteria survive the harsh gastric environment. At the center of this multiprotein system is an arginine−agmatine antiporter, AdiC. To maintain cytoplasmic pH, AdiC imports arginine and exports its decarboxylated product, agmatine, resulting in a net extrusion of one "virtual proton" in each turnover. The random orientation of AdiC in reconstituted liposomes throws up an obstacle to quantifying its transport mechanism. To overcome this problem, we introduced a mutation, S26C, near the substrate-binding site. This mutant exhibits substrate recognition and pH-dependent activity similar to those of the wild-type protein but loses function completely upon reaction with thiol reagents. The membrane-impermeant MTSES reagent can then be used as a cleanly sided inhibitor to silence those S26C-AdiC proteins whose extracellular portion projects from the external side of the liposome. Alternatively, the membrane-permeant MTSEA and membrane-impermeant reducing reagent, TCEP, can be used together to inhibit proteins in the opposite orientation. This approach allows steady-state kinetic analysis of AdiC in a sided fashion. Arginine and agmatine have similar Michaelis−Menten parameters for both sides of the protein, while the extracellular side selects arginine over argininamide, a mimic of the carboxylate-protonated form of arginine, more effectively than does the cytoplasmic side. Moreover, the two sides of AdiC have different pH sensitivities. AdiC activity increases to a plateau at pH 4 as the extracellular side is acidified, while the cytoplasmic side shows an optimal pH of 5.5, with further acidification inhibiting transport. This oriented system allows more precise analysis of AdiC-mediated substrate transport than has been previously available and permits comparison to the situation experienced by the bacterial membrane under acid stress.
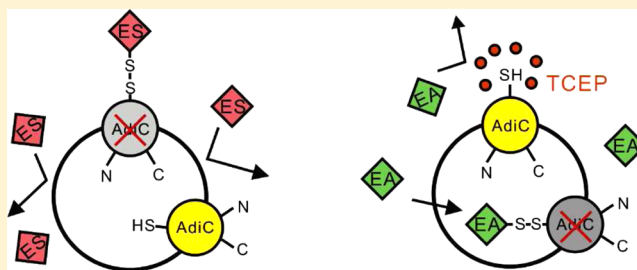
From the viewpoint of enteric bacteria, the mammalian stomach, in which the pH can be as low as 1.5, is a potentially lethal barrier that must be surmounted to colonize the intestinal tract. Many bacteria breach this host defense by activating multiple extreme acid resistance (XAR) systems.[1,2] This study concerns one of these, the arginine-dependent XAR system, which is composed of an arginine−agmatine antiporter, AdiC, and an acid-activated arginine decarboxylase, AdiA.[3,4] To counter acidification of the cytosol during exposure to low pH, AdiC imports arginine, which is then decarboxylated by AdiA; this reaction consumes an aqueous proton, which replaces the α-carboxyl of arginine to form agmatine (1-amino-4-guanidino-n-butane). Agmatine is then exported by the same transporter, resulting in net extrusion of one "virtual proton"[5] from the cytosol in each AdiC transport cycle (Figure 1A).

Previous studies have revealed basic biochemical, functional, and structural characteristics of AdiC.[6−9] The protein is a homodimer in which each subunit acts as a self-contained antiporter that mediates strict one-to-one exchange of arginine for agmatine and is activated by low pH. AdiC's detailed transport mechanism, however, remains unclear. Several central questions are yet to be answered, including how AdiC senses pH changes and how it avoids transporting the protonated carboxylate form of the substrate, Arg²⁺, the predominant form of arginine at pH <2.3, as it must do to achieve acid resistance.

Recent high-resolution structures of AdiC in multiple states[7−9] provide opportunities to tackle issues like these.

But crystal structures alone are not enough. Close analysis of transport mechanism requires reconstituting purified protein into liposomes for flux measurements. However, AdiC, like many membrane proteins, inserts into liposome membranes randomly, with two populations of protein oriented in opposite directions. This heterogeneous orientation is a severe impediment to examining a transport mechanism, because it precludes assignment of specific functions to defined sides of the protein and makes it impossible to obtain the protein's kinetic parameters in a quantitatively meaningful way. A method for orienting AdiC in liposomes is therefore necessary for detailed mechanistic analysis. In some cases, oriented reconstituted systems may be achieved by the application of sided inhibitors, which exclusively silence one of the two protein populations present. However, no sided inhibitor of AdiC has yet been found.

Here, guided by crystal structures, we introduce an innocuous serine-to-cysteine substitution at a position that
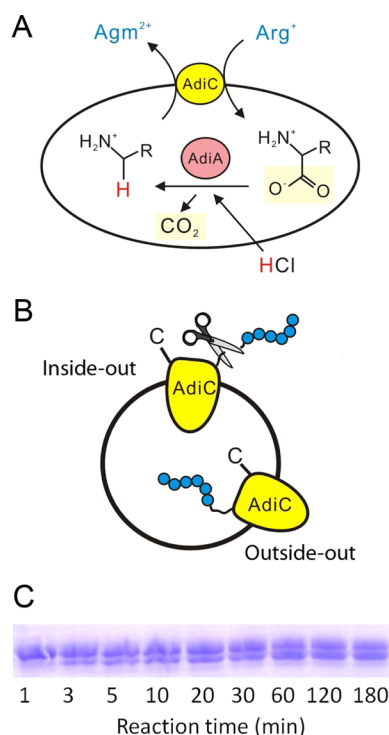
Figure 1. Orientation of AdiC in liposome membranes. (A) Mechanism of the arginine-dependent XAR system. (B) AdiC in inside-out and outside-out orientations. The N-terminal H6 tag (blue circles) of the inside-out protein, exposed to the outside of the liposome, is susceptible to thrombin cleavage (scissors). (C) Determination of the AdiC orientation. Thrombin (0.01 unit) was added to 100 μL liposome aliquots (10 μg of AdiC/mg of lipid). At the indicated time points, proteolysis was stopped by 100 μM PMSF, and samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

contributes to AdiC's arginine-binding site. This maneuver permits the use of thiol-directed methanethiosulfonate (MTS) reagents to inhibit AdiC in a strictly sided fashion, to yield liposomes with protein reconstituted in either of two orientations, outside-out or inside-out. An initial kinetic analysis reveals the sidedness of the protein's basic transport parameters, as well as the distinct pH sensitivities of the cytoplasmic and extracellular sides. This work paves the way for future detailed examination of AdiC's transport mechanism and structural correlates thereof.

■ MATERIALS AND METHODS

Chemical Reagents. Lipids, including *Escherichia coli* polar lipid (EPL), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), were purchased from Avanti Polar Lipids. MTS reagents, 2-sulfonatoethyl methanethiosulfonate (MTSES)/2-aminoethyl methanothiosulfonate (MTSEA), and detergents, including *n*-decyl maltoside (DM) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), were obtained from Anatrace. Tris(2-carboxyethyl)-phosphine (TCEP) was purchased from Soltec Ventures, fluorescein 5-maleimide from Sigma-Aldrich, and [14C]arginine was from PerkinElmer. All other chemicals were of the highest grade available.

Protein Expression and Purification. The coding sequence of *Salmonella enterica* AdiC was inserted into the

pASK-IBA2 vector between the XbaI and HindIII restriction sites.[7] A thrombin-cleavable hexahistidine tag (HHHHHHSGGLVPRGSGT) was placed immediately after the initiator methionine to yield our working "wild-type" (WT) construct. Standard two-step polymerase chain reaction was used for site-directed mutagenesis, and full coding sequences were confirmed for all mutants. A cysteine-free AdiC, henceforth denoted "C-less", was constructed by substituting serine for the eight native cysteines (positions 80, 98, 129, 238, 281, 286, 369, and 398).

Detailed protein expression and purification procedures have been described previously.[6] In brief, AdiC constructs were expressed in BL21(DE3) cells, extracted in 40 mM DM, 100 mM NaCl, and 50 mM Tris-HCl (pH 8.0), and purified on cobalt affinity (Talon) and size-exclusion columns (Superdex 200). For proteins with the S26C mutation, 2 mM TCEP was added after the extraction step to keep the introduced cysteine residue reduced. The final protein product was collected in 5 mM DM, 100 mM NaCl, and 25 mM Tris-HCl (pH 7.5) and concentrated to 10−20 mg/mL. The final yield was typically 10−15 mg for WT-AdiC or 5−10 mg for C-less mutants per liter of bacterial culture.

Isothermal Titration Calorimetry (ITC). AdiC, concentrated to 100−300 μM in 10−20 mM DM, was loaded into the 170 μL sample cell (Nano-ITC, TA Instruments). After equilibration at 25 °C, arginine at 2−10 mM in the same solvent as the protein solution was titrated into the sample cell by 20−30 successive 1 μL injections at 2−3 min intervals. Data were fit to single-site isotherms using software provided by the manufacturer of the isothermal titration calorimeter (Nano-Analyze version 2.1.9). For experiments with MTSEA-modified protein, AdiC was treated with 5 mM MTSEA for 30 min and then repurified by size-exclusion chromatography.

Reconstitution of AdiC. Lipid (EPL or 3:1 (w/w) POPE/POPG, stored in chloroform at −80 °C) was dried under N_2, washed twice with pentane, and suspended to a final concentration of 20 mg/mL in reconstitution buffer (RB) [150 mM KCl, 25 mM citrate, and KOH (pH 5.5)] with 35 mM CHAPS included. The suspension was sonicated to clarity, and AdiC was added to the desired concentration (5−10 μg of protein/mg of lipid). The protein/detergent/lipid mixture was dialyzed overnight at 4 °C against RB supplemented with 1 mM TCEP, followed by two more 24 h dialyses against a fresh RB solution without TCEP. The resulting proteoliposomes, frozen in aliquots at −80 °C, remain fully functional for at least 6 months.

Arginine Uptake Assay. Proteoliposomes were prepared for arginine fluxes by adding 5 mM arginine to the liposome suspension, which was then subjected to two or three freeze–thaw cycles. After the final thawing step, liposomes were sonicated briefly, and extraliposomal arginine was removed by spinning 100 μL aliquots through 1.5 mL Sephadex G-50 columns equilibrated with RB and dried by prespinning. Transport was initiated by adding 50 μM [14C]arginine (1 μCi/mL). At desired time points, 50 μL samples were loaded onto 2.2 mL Sephadex G-50 columns equilibrated with RB to terminate the uptake reaction. Liposomes were collected into scintillation vials by flushing the column with 1.6 mL of RB. We frequently use "fractional uptake" as a transport metric, defined as the fraction of total radioactivity that is trapped within the liposomes. This procedure was slightly modified to measure initial rates of arginine uptake. Instead of G-50 columns, a 2.2 mL Dowex 50-X4 column, *N*-methyl glucamine (NMG) form,

equilibrated with RB was used to stop arginine uptake, a maneuver that shortens the system's dead time to ~5 s. After the 30 s uptake reaction, columns were flushed with 2 mL of RB to collect liposomes into scintillation vials.

To gauge the pH dependence of arginine transport with symmetrical pH 4 or 7, EPL proteoliposomes were prepared by dialyzing an AdiC/lipid/detergent solution against 150 mM KCl buffered with either 25 mM succinate (pH 4) or 25 mM MOPS (pH 7). Arginine uptake was assessed as described above, except that these succinate or MOPS buffers were used rather than RB. For experiments with asymmetrical pH values (5.5 inside and 3−8 outside), it was necessary to use POPE/POPG liposomes, as these, unlike EPL liposomes, are much less permeable to H^+ on the uptake time scale (Figure S1 of the Supporting Information). After the liposomes had been loaded with 5 mM arginine, the external solution was exchanged, as described above, on G-50 spin columns equilibrated with 150 mM KCl and 1 mM citrate (pH 5.5). Immediately before the transport reaction was started, the extraliposomal pH was altered by diluting the liposomes into 2.5 volumes of 150 mM KCl, along with the appropriate buffer at 25 mM: citrate for pH 3, 3.5, 5, and 5.5; succinate for pH 4 and 4.5; MES for pH 6 and 6.5; MOPS for pH 7; and HEPES for pH 8. The uptake reaction was stopped with Dowex-NMG columns, and liposomes were eluted and counted as described above.

Use of Thiol Reagents. MTSES or MTSEA was freshly prepared by dissolving the reagent in ice-cold water just before use. S26C-AdiC was treated for 30 min at room temperature in detergent solution [5 mM DM, 100 mM NaCl, and 25 mM Tris-HCl (pH 7.5)] with 0.5−1 mM reagent or with 1−5 mM reagent when in liposomes suspended in RB (pH 5.5) (Figure S2 of the Supporting Information). (For reasons we do not understand, arginine did not lower rate of MTS reaction with the substituted cysteine residue.) Stock aqueous solutions of TCEP (0.6 M, pH 5.5) were kept at 4 °C for up to 1 month. Stock solutions (20 mM) of fluorescein maleimide in DMSO were kept at −20 °C in the dark for no more than 2 weeks.

For labeling of S26C-AdiC in liposomes with fluorescein, EPL liposomes were prepared as usual, and the exposed His tags were removed by thrombin (0.01 unit for a 50 μL liposome sample, 90 min, room temperature). After 100 μM phenylmethanesulfonyl fluoride (PMSF) had been added to stop proteolysis, 5 mM MTSES or 1 mM MTSEA with 20 mM TCEP was added, and after the desired time, samples were spun through G-50 columns equilibrated with 150 mM KCl and 25 mM MOPS (pH 7) to remove thiol reagents. (Samples containing TCEP were spun through G-50 columns twice to ensure complete removal of the reductant.) Fluorescein maleimide (2 mM) was then added to the liposomes to label remaining free cysteine, and samples were run on 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels.

Synthesis of [14C]Agmatine. [14C]Agmatine was produced by enzymatic decarboxylation of [14C]arginine. A pT7 expression vector carrying the gene encoding speA, a pyridoxal phosphate (PLP)-dependent arginine decarboxylase, was generously provided by Y. Ding.[10] The plasmid was transformed into BL21(DE3) cells, which were grown in Terrific Broth at 37 °C to an A_{550} of ~1 and induced with 1 mM isopropyl α-D-1-thiogalactopyranoside (IPTG) at 25 °C for 4 h. Cells from a 1 L culture were disrupted in 100 mL of 100 mM NaCl and 50 mM Tris-HCl (pH 8) in the presence of protease inhibitors (1 mM PMSF or 1 μg/mL leupeptin/pepstatin) and

centrifuged at 30000g for 25 min. The supernatant was loaded at a rate of 1 mL/min onto an 8 mL Ni affinity column equilibrated with wash buffer (WB) [100 mM NaCl and 25 mM Tris-HCl (pH 7.5)]. The column was washed successively with 40 and 400 mM imidazole in WB to elute nonspecifically bound protein and speA decarboxylase, respectively. The protein was concentrated to 20 mg/mL and stored at −80 °C with 3 mM DTT and 5% glycerol supplemented. The final yield of speA was ~200 mg/L of culture.

To synthesize [14C]agmatine, 100 μCi of [14C]arginine dissolved in 1 mL of 2% ethanol was mixed with 20 μg of speA and 1 mL of 100 mM NaCl, 50 μM PLP, 2 mM $MgCl_2$, and 2.5 mM MES (pH 6). After 2 h at room temperature, the mixture was boiled for 20 min, dried under N_2, and resuspended in 50% ethanol twice to evaporate $^{14}CO_2$. The final preparation of [14C]agmatine was stored in 2% ethanol at −20 °C. The purity of the preparation was confirmed by high-pressure liquid chromatography on a C18 column after derivatization, as described previously.[4]

■ RESULTS

The orientation of membrane proteins incorporated into biochemically defined lipid membranes can be affected by the method used for reconstitution as well as molecular features specific to the protein. Denoting protein whose extracellular side faces outside of the liposome as "outside-out" and proteins in the opposite orientation "inside-out", we sought to assess these two possible orientations in AdiC-reconstituted liposomes (Figure 1B). To this end, proteoliposomes carrying WT AdiC with an N-terminal hexahistidine (H6) tag were incubated with thrombin, which cleaves off the H6 tag exclusively from the inside-out population, since the N-terminus of this protein is cytoplasmic (Figure 1B). Proteolysis was run to completion, and the sample was analyzed via SDS−PAGE. As illustrated in Figure 1C, thrombin cleavage produces a lower band representing the lighter, H6-removed AdiC protein; over an extended duration of proteolysis, this inside-out AdiC band reaches an intensity roughly equal to that of the uncleaved, outside-out protein. This assay demonstrates that AdiC is randomly oriented in the liposome membranes.

A Strategy To Orient AdiC in Liposome Membranes. A fully oriented reconstituted system for AdiC would be achieved if we could inhibit the protein in one orientation completely while leaving the other orientation unaffected. Although no AdiC inhibitor is known, thiol-specific reagents might serve this purpose in a properly cysteine-substituted transporter. An appropriate design for cysteine substitution should satisfy three conditions. First, the mutation should have limited functional impact, so that it faithfully models wild-type behavior. Second, cysteine modification should lead to complete elimination of functional activity, so that the modified protein would not generate any contaminating signal for transport or binding measurements. Third, the substituted cysteine should be accessible to thiol reagents from only one side of the protein to ensure that only one of the two orientations is subject to modification from a particular side of the membrane.

Guided by crystal structures of arginine-occupied AdiC, we chose Ser26 for cysteine mutation (S26C). This residue, located at the arginine binding site (Figure 2), interacts with arginine in both "extracellular-open" and "extracellular-occluded" conformations of the transporter;[8,9] a bulky thiol adduct here would be expected to block substrate binding and abolish transport function. Because S26 lies close to the
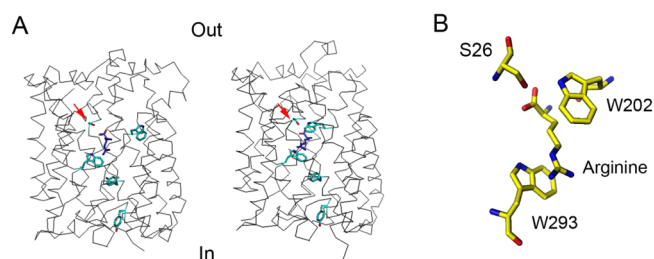
Figure 2. Ser26 in crystal structures of AdiC. (A) Extracellular-open (left, PDB entry 3OB6) and extracellular-occluded (right, PDB entry 3L1L) states of AdiC. Red arrows indicate the position of Ser26, which interacts directly with arginine (purple) and is separated from the cytoplasmic side by protein-containing layers of aromatic residues. (B) Arginine binding site of the extracellular-occluded state. The guanidinium side chain is sandwiched between Trp202 and Trp293, and the α-carboxylate forms a hydrogen bond with the hydroxyl of Ser26.

extracellular surface and is well-separated from the intracellular side by layers of aromatic residues (Figure 2A), we also anticipate that thiol-modifying reagents would reach this position only from the protein's extracellular side. A major concern is the functional impact that the mutation might cause, as in the crystal structure the hydroxyl of S26 forms a hydrogen bond with the carboxylate of bound arginine (Figure 2B), and conventional wisdom suggests that a Ser-to-Cys mutation would significantly reduce the arginine affinity of the protein because of diminished binding energy. However, the fact that AdiC also effectively transports agmatine, which lacks a carboxylate, implies that S26 may not be as crucial as inferred from the crystal structure. We were therefore motivated to examine the suitability of the S26C mutation for functional studies.

Function of S26C Mutant and Its Inhibition by MTS Reagents. The basic functional characteristics of WT AdiC and the S26C mutant were compared as the first step in gauging whether the three criteria above are satisfied. We note that the S26C mutant to which we henceforth refer is constructed on a C-less protein that has all eight native cysteines mutated to preclude background reaction with thiol reagents. Isothermal calorimetry was used to extract equilibrium substrate binding parameters (Figure 3A). Titration of arginine in detergent solution produces endothermic signals with both WT and S26C. The binding data are described well by single-site isotherms with similar values ($\sim$30 μM) of K_D for both. Thus, equilibrium binding of arginine to AdiC is not compromised by the S26C substitution.

Transport function for these AdiC variants was assessed by arginine–arginine exchange in liposomes.[6] In a typical experiment, $[^{14}\text{C}]$arginine enters the liposomes and a steady level is achieved in 5–10 min (Figure 3B). The S26C mutant is virtually identical in arginine uptake to the C-less control (which itself is approximately 10-fold slower than the full-cysteine WT transporter). Moreover, the acid activation of AdiC is preserved in the S26C mutant (Figure 3C). Taken together, these results show that the S26 mutant on its C-less background functionally mirrors the WT protein.

According to the second requirement for an oriented system, the S26C protein must lose function entirely upon modification by thiol-directed reagents. We find that arginine binding, as followed by ITC, is completely lost by treating the mutant with MTSEA in a detergent solution (Figure 4A). Transport activity of S26C is also abolished by MTS modification (Figure 4B), while the C-less control is unaffected (Figure S3 of the Supporting Information). Moreover, addition of TCEP during the transport reaction reactivates the inhibited protein by
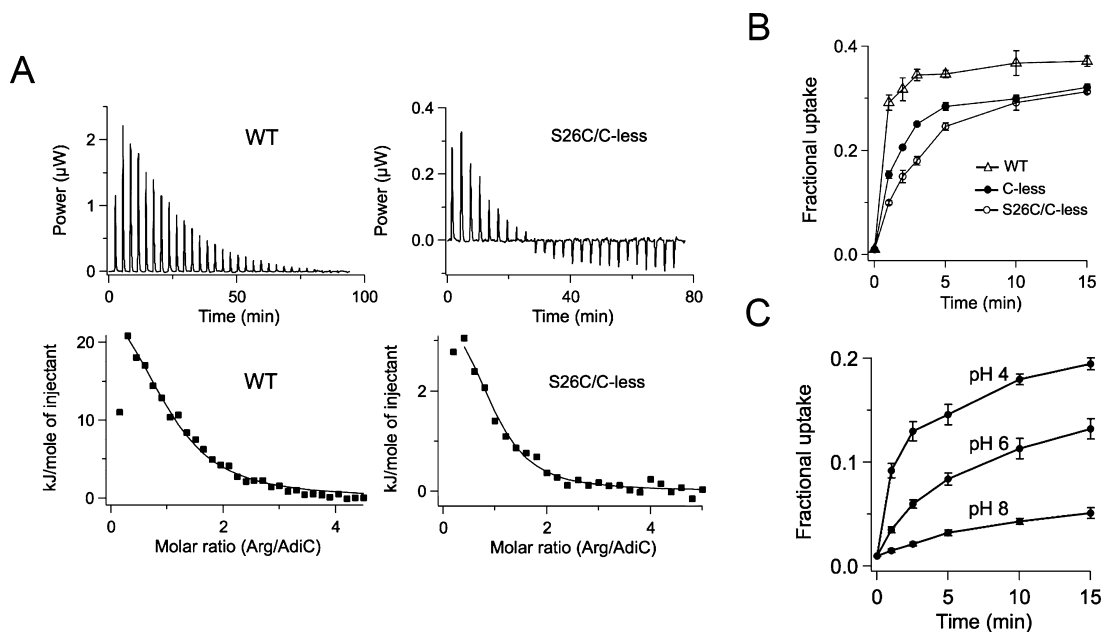

Figure 3. Functional impact of the S26C mutation. (A) Equilibrium binding of arginine to WT and the S26C mutant. ITC data (top) were fit with single-site isotherms (bottom), yielding the following parameters. WT ($n = 4$): $K_D = 32 \pm 2$ μM, $\Delta H° = 6.4 \pm 0.3$ kcal/mol, $\Delta G° = -6.2 \pm 0.1$ kcal/mol, and $\Delta S° = 42 \pm 1$ cal mol^{-1} K^{-1}. S26C ($n = 3$): $K_D = 22 \pm 2$ μM, $\Delta H° = 3.1$ 0.4 kcal/mol, $\Delta G° = -6.3$ 0.1 kcal/mol, and $\Delta S° = 32 \pm 1$ cal mol^{-1} K^{-1}. (B) Transport of arginine by WT and mutant AdiC. The uptake time course of $[^{14}\text{C}]$arginine was determined with indicated AdiC constructs reconstituted at 10 μg/mg of lipid. (C) pH-dependent transport activity of S26C. Uptake time courses were performed at the indicated symmetrical pH with protein reconstituted at 5 μg/mg.
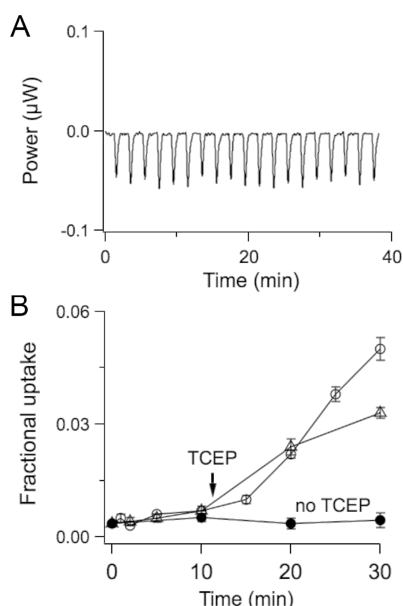
Figure 4. Inhibition of S26C-AdiC by MTS reagents. (A) An illustrative ITC experiment with arginine titrated into a sample containing 150 μM MTSEA-modified S26C protein showing no binding signal. (B) Effects of MTS reagents on transport activity of S26C-AdiC. S26C protein treated with 5 mM MTSEA (O or ●) or MTSES (△) in detergent was reconstituted into liposomes at 10 μg/mg of lipid, and arginine uptake was measured over a 30 min period. Eleven minutes into the time ourse, 20 mM TCEP was added (arrow), except to the control samples (●).

Figure 5. Sided inhibition of S26C-AdiC by MTSES. (A) Time course of inhibition by MTSES. Liposomes reconstituted with S26C-AdiC (10 μg/mg of lipid) were exposed to 5 mM MTSES. At the indicated times, a 100 μL sample was spun through G-50 columns to remove MTSES, and a 1 min arginine uptake assay was performed. For samples with >15 min MTSES exposures, an extra dose of 5 mM MTSES (arrow) was added to compensate for the loss of MTSES due to hydrolysis. (B) Calibration of MTSES inhibition. Arginine uptake time courses were determined using liposomes reconstituted with S26C-AdiC at 2.5, 5, or 10 μg of protein/mg of lipid, as indicated: (●) liposome pretreated with 5 mM MTSES for 30 min and (O) untreated control liposomes.

reducing the MTS-modified side chain back to cysteine. These results establish that MTS reagents fully inhibit S26C.

MTSES Silences Outside-Out S26C-AdiC. A crucial condition for achieving our purpose here is that during all stages of the transport cycle, the cysteine substitution site must be accessible from only one side of the membrane-embedded protein. To test this possibility, we challenged reconstituted S26C with the membrane-impermeant reagent MTSES, which can act only from the extraliposomal solution.[11] Arginine uptake activity was assayed after incubating liposomes with MTSES for various times. The treatment inhibits arginine import, but not fully (Figure 5A); substantial transport remains resistant to the inhibitor, even after prolonged exposure. Calibration of transport kinetics as a function of protein concentration in the liposomes shows that the MTSES-treated liposomes have ~50% of the activity of untreated liposomes (Figure 5B), as if MTSES targets only half of the reconstituted protein population. These experiments strongly refute the idea that C26 may be accessed from both sides of the protein during transport; instead, MTSES acts to fully inhibit AdiC in a sided fashion.

We are uncertain, however, about the absolute orientation of proteins remaining functional after MTSES inhibition. Crystal structures[7−9] suggest that C26 would be accessible only from the extracellular side, but this conjecture requires direct scrutiny. Accordingly, we designed an experiment (Figure 6A) to identify those proteins that remain unmodified after MTSES treatment of the liposomes. After MTSES had been removed from the liposome sample, a membrane-permeant fluorescent maleimide is added to label all AdiC proteins that retain a free thiol. Thrombin is also added to remove the H6 tag exclusively from the inside-out protein fraction, which are
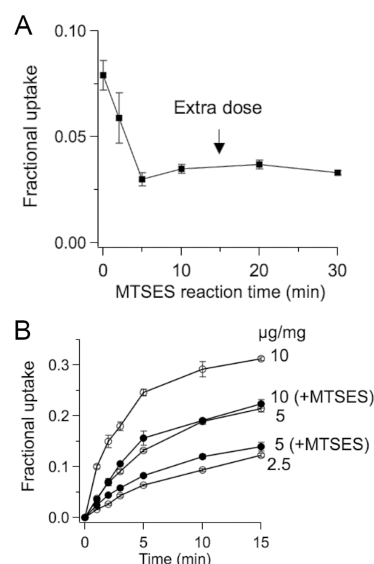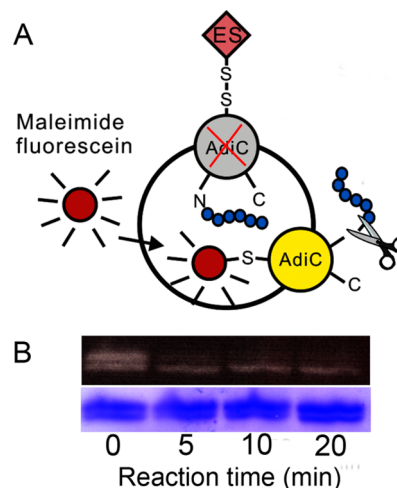

Figure 6. Inside-out orientation of AdiC in MTSES-treated liposomes. (A) Strategy for creating an inside-out system. (B) Fluorescein labeling of AdiC in MTSES-treated liposomes. MTSES (5 mM) was added to 100 μL liposome samples (10 μg of protein/mg of lipid) and subsequently removed by spinning the samples through G-50 columns at the indicated times. Unmodified proteins were then labeled to completion with 2 mM fluorescein maleimide and cleaved with thrombin. Samples were analyzed by SDS−PAGE and visualized by fluorescence (top) or Coomassie staining (bottom).

then easily distinguished from the outside-out proteins by SDS−PAGE. This strategy demonstrates (Figure 6B) that the inside-out proteins are spared from MTSES modification, as MTSES prevents fluorescent labeling of only the H6-tagged upper band of AdiC. The results establish that MTSES can be

used as a sided inhibitor to create a reconstituted system with functional S26C-AdiC proteins all in the inside-out orientation.

Reconstituted AdiC in an Outside-Out Orientation. Kinetic analysis of membrane transport in liposomes is usually conducted by varying substrates, inhibitors, pH, etc., in the extraliposomal solution. It would therefore be desirable to supplement the inside-out AdiC system described above with proteins reconstituted in the alternative outside-out orientation. This goal may be accomplished by inhibiting all transporters with a membrane-permeant MTS reagent, MTSEA,[11] and then rescuing the outside-out proteins with a membrane-impermeant disulfide reductant, TCEP (Figure 7A).
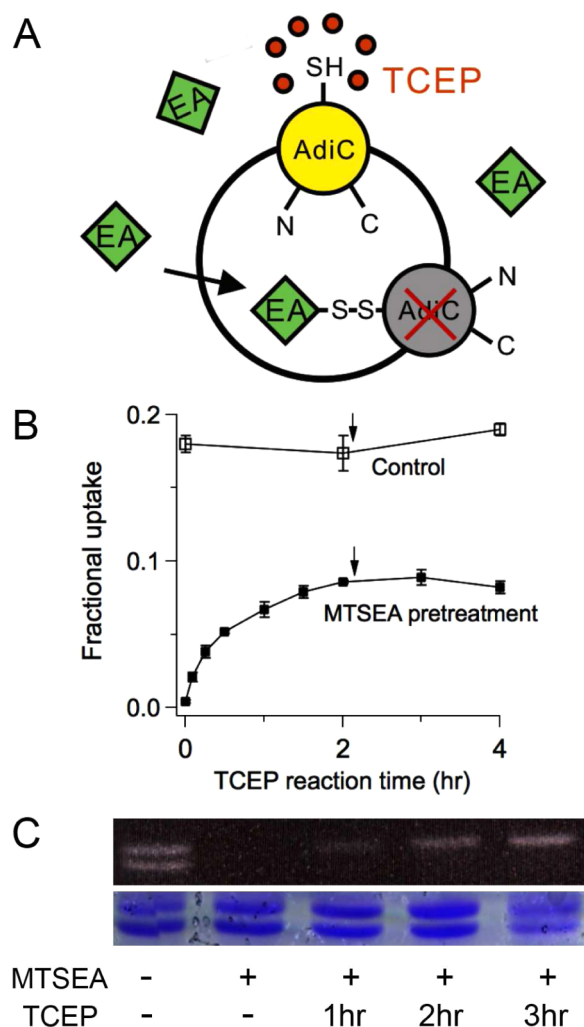

Figure 7. Outside-out orientation of AdiC in MTSEA- and TCEP-treated liposomes. (A) Strategy for creating an outside-out system. (B) Arginine transport rescued by TCEP. S26C-reconstituted liposomes (10 μg/mg) with (■) or without (□) a 30 min exposure to 1 mM MTSEA were treated with 20 mM TCEP for various times, with an extra dose (20 mM) added at 2 h (arrow). After TCEP had been removed, a 3 min arginine uptake was measured to ascertain the recovery of transport function. (C) Fluorescein maleimide labeling after indicated MTSEA/TCEP treatment. After being labeled, samples were analyzed by SDS–PAGE as in Figure 6.

To test the feasibility of this strategy, we inhibited all AdiC in the liposomes with MTSEA and subsequently examined arginine transport activity. No arginine import is detected after MTSEA treatment, but addition of 20 mM TCEP restores

transport to ~50% of that of the control sample, as expected if the reductant acts exclusively upon the outward-facing C26 adduct (Figure 7B). To confirm this picture, we further treated the liposomes with fluorescein maleimide and thrombin as described above. Because only the upper band becomes fluorescent following TCEP reduction (Figure 7C), we conclude that a combination of MTSEA and TCEP can produce fully oriented outside-out AdiC transporters.

Kinetic Analysis of Substrate Transport. Now with sided reconstituted systems available, the stage is set for exploring the substrate specificity for the two sides of AdiC. Arginine in the external solution was varied, keeping the intraliposomal arginine level fixed at a saturating concentration, and initial rates of uptake were measured in outside-out and inside-out orientations (Figure 8). Arginine uptake follows Michaelis–Menten kinetics in both cases, with K_m values of ~100 and ~50 μM for the cytoplasmic and extracellular sides, respectively, and similar V_{max} values for both orientations, on the order of 0.1 s^{-1}. Similar experiments with [^{14}C]agmatine uptake were also conducted (Figure 8C), yielding K_m and V_{max} values similar to those for arginine, as summarized in Table 1.

The physiological role of AdiC to counter acidification of cytosol demands that AdiC transports only the deprotonated carboxylate form of arginine (Arg$^+$), a minor species at pH <2.3.[6] We have shown previously that argininamide (ArgNH$_2$), an analogue of protonated Arg^{2+}, is a poor transport substrate compared to Arg$^+$.[6] Here, we revisit this issue in an oriented system, using ArgNH$_2$ as a competitor for [^{14}C]arginine–arginine exchange. Arginine uptake is slowed by ArgNH$_2$ in either orientation according to simple competitive inhibition (Figure 9), but the competitor is 5-fold less effective on the extracellular versus intracellular side of AdiC (Table 1). This result conforms to the biological context, as it is the low-pH extracellular side of the transporter that must distinguish Arg$^+$ from Arg^{2+}; this selectivity is unimportant in the cytoplasm, where Arg^{2+} is negligible because intracellular pH never falls below 4.5.[12,13]

Sided pH Sensitivity of AdiC. AdiC-mediated transport is stimulated by low pH (Figure 3C), as is unsurprising for a protein whose biological task is performed in a strongly acidic environment. To determine the side of AdiC where pH is sensed, we varied pH for one side of the protein while fixing the other side at pH 5.5. For extracellular variation, the extent of arginine transport is low around neutrality and increases ~30-fold to a plateau as the pH is lowered from 8 to 3 (Figure 10A). This finding makes physiological sense, as it is the extracellular side of AdiC that directly experiences strong acidification in the stomach. The cytoplasmic pH also drops during extreme acid challenge, to as low as pH 4.5 but not lower.[12,13] This biological response is reprised in the bell-shaped dependence of transport on cytoplasmic pH (Figure 10B), which shows a 3-fold increase in activity from neutrality to a maximum near pH 5, followed by a decrease as the pH is further lowered, a condition that the acid-resisting cytoplasm never experiences. We therefore suppose that each side of AdiC carries its own pH sensor, each tuned to the conditions prevailing during the arginine-dependent XAR response.

■ DISCUSSION

AdiC belongs to the amino acid-polyamine-organocation (APC) transporter superfamily, whose members appear throughout the biological world. APC transporters mediate a panoply of physiological tasks, such as supplying amino acids
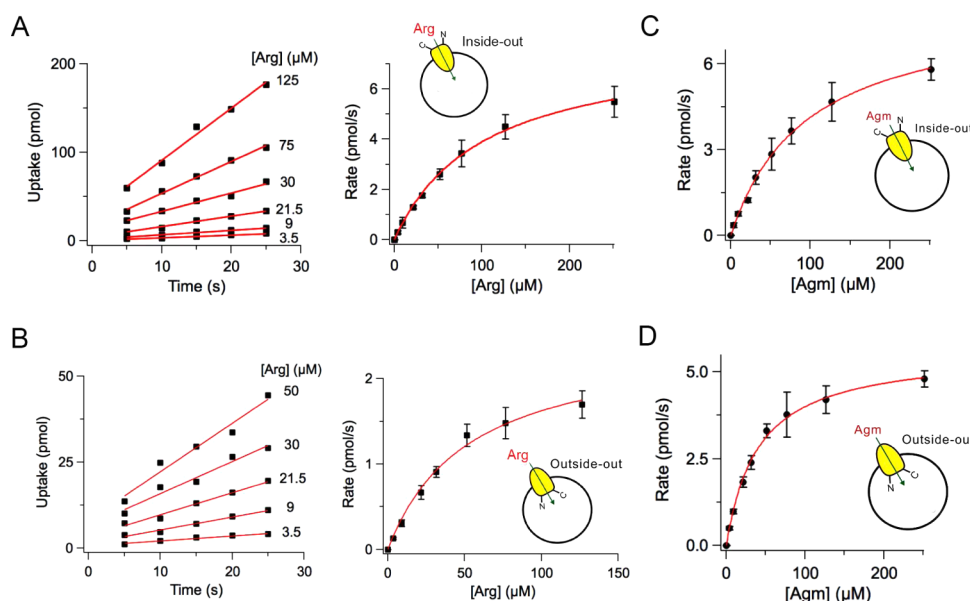
Figure 8. Sided kinetic analysis of substrate transport. (A and B) Kinetic analysis of arginine uptake. Uptake of [14C]arginine into liposomes loaded with 5 mM arginine and carrying inside-out (A) or outside-out (B) AdiC (10 μg/mg of lipid) was examined as a function of the indicated external arginine concentration. The left panels show illustrative initial rate data over a 25 s time course in a single run in which each sample contains 6.5 μg of AdiC. In the right panels, rates plotted vs arginine concentration were fit to the Michaelis−Menten model (—), with the parameters summarized in Table 1; each point represents the mean ± the standard error from three to five separate runs. (C and D) Kinetic analysis of [14C]agmatine uptake. Experimental conditions were as described for panels A and B, except that uptake of agmatine was followed in inside-out (C) or outside-out (D) AdiC liposomes.

Table 1. Michaelis−Menten Parameters for Arginine, Agmatine, and Argininamide[a]

	arginine			agmatine			ArgNH2
AdiC orientation	K_m (μM)	V_{max} (pmol/s)	k_{cat} (s⁻¹)	K_m (μM)	V_{max} (pmol/s)	k_{cat} (s⁻¹)	K_i (mM)
inside-out	101	7.8	0.12	96	8.1	0.12	1.1
outside-out	52	2.5	0.04	40	5.6	0.08	5.2

[a]Parameters reported are from the least-squares fits shown in Figures 8 and 9.
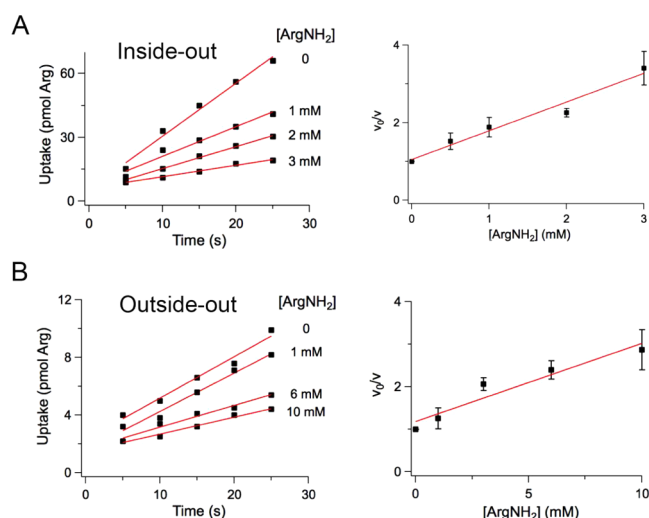

Figure 9. ArgNH2 inhibition of arginine uptake. Inside-out (A) or outside-out (B) liposomes were prepared as described in the legend of Figure 8, and [14C]arginine uptake was measured in the presence of indicated external concentrations of ArgNH2. Left panels show initial rate time courses, and right panels show reciprocal plots of rate relative to its value in the absence of ArgNH2 (v_o/v). Lines are fits to Michaelis−Menten kinetics with parameters reported in Table 1.

for metabolism, arginine for nitric oxide synthesis, and headgroups such as choline, ethanolamine, and inositol for phospholipid synthesis, as well as energizing membranes of certain anaerobic bacteria in the absence of electron transport. In bacterial XAR, AdiC specifically imports arginine from gastric fluid and exports agmatine along with a "virtual proton"[5,6] out of the cytosol during each turnover. Specific recognition of extracellular arginine can be understood from X-ray crystal structures of AdiC determined in two arginine-bound states: extracellular-open[9] and extracellular-occluded.[8] In these structures, the aromatic side chain of Trp293 appears to stabilize arginine binding by a cation−π interaction with the substrate's guanidinium group. Mutagenesis of a variety of APC transporters has identified aromatic interactions as being crucial for substrate binding, and indeed, mutations of Trp293 in AdiC itself lead to a complete loss of transport activity.[6]

A less understood molecular interaction in AdiC's crystal structures is a hydrogen bond between the α-carboxylate of arginine and the hydroxyl group of Ser26, a residue conserved among APC virtual proton pump exchangers.[7] Although it is natural to imagine that this interaction would provide significant binding energy for extracellular amino acids, we find with some surprise that Cys (Figure 3) or Ala (Figure S4 of the Supporting Information) substitutions at Ser26 do not undermine arginine transport. Viewed from an evolutionary perspective, however, these results appear less puzzling, because
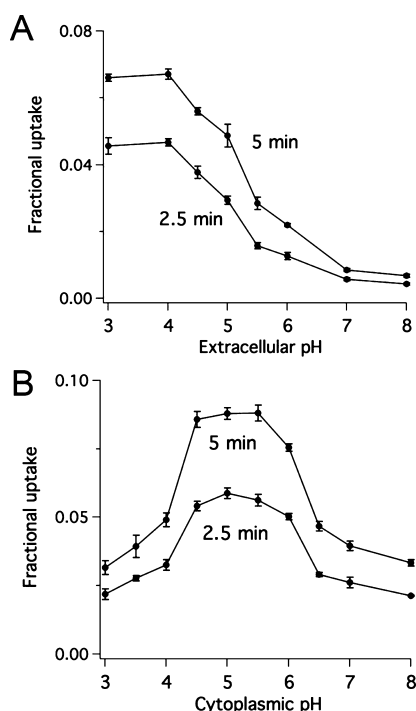
Figure 10. Distinct pH sensitivities of the two sides of AdiC. Liposomes with AdiC in outside-out (A) or inside-out (B) orientations were prepared with an internal pH of 5.5. Arginine uptake was performed at the indicated external pH, for either 2.5 or 5 min.

in the stomach, where amino acids are available, the outward open conformation of AdiC faces no evolutionary pressure to differentiate amino acids from substrates with other chemical groups attached to the α-carbon. A vivid example seen here is that agmatine, which lacks the α-carboxylate, has an extracellular transport K_m ($\sim$50 μM) similar to that of arginine.

No AdiC structure has yet been determined in the cytoplasmic-open conformation. With the sided system developed here, we may begin to gain insight into ligand binding and specificity for AdiC's intracellular side. Kinetic analysis reveals that arginine and agmatine have similar K_m values ($\sim$100 μM) for this side of the protein, a result that appears surprising at first glance, as transport of arginine from the cytoplasm would produce a futile cycle useless for acid resistance, and one might therefore expect intracellular selectivity for agmatine. We should remember, however, that the cytoplasmic level of arginine is kept low by robust decarboxylation and thus presents no problem as a cytoplasmic competitor for agmatine.

In the gastric fluid, the two sides of AdiC operate under very different conditions. The extracellular pH can fall as low as 1.5, while the cytoplasmic pH is maintained in the range of 4.5−5.5 by the XAR response.[13] Therefore, the two sides of AdiC must deal with distinct environmental challenges. With a pK_a of 2.3, arginine's carboxylate presents a problem of molecular recognition, because the outward-facing conformation must select the minor, deprotonated α-carboxylate form, Arg$^+$, over the preponderant protonated form, Arg^{2+}, to achieve acid resistance.[6] Previous work showed that AdiC transports ArgNH$_2$, a mimic of Arg^{2+}, with substantially lower efficiency than Arg$^+$, but the sidedness of this selectivity could not be determined because of the random protein orientation in the liposomes.[6] Now with the oriented system, we find that

extracellular ArgNH$_2$ is substantially less effective than intracellular ArgNH$_2$ as a competitive inhibitor of Arg$^+$ transport. In other words (assuming that ArgNH$_2$ serves as a valid analogue of Arg^{2+}), the outward-facing conformation is more sensitive than the inward-facing conformation to the protonation state of arginine, a circumstance resonant with the biological imperatives that AdiC must satisfy.

Previous work[6] established that AdiC transport activity is stimulated by acidification, but the random protein orientation in that work made it impossible to evaluate the sidedness of this effect. Now with the oriented system, we find that both sides of the protein sense pH, each with its own characteristic pH profile. The major, extracellular pH sensor leads to $\sim$30-fold stimulation upon acidification from neutrality, while intracellular acidification produces a biphasic response with 3-fold stimulation followed by inhibition at pH <4. We do not currently understand the molecular basis for this pH-dependent transport activity, but the differing pH profiles for the two sides of the protein imply that at least two pH sensors are involved, one for each side of the membrane. This situation makes biological sense. Under nonstressed conditions, near neutrality on both sides of the bacterial inner membrane, AdiC transport activity is low, and only upon extracellular acidification does the transporter spring into action. We estimate that the two sensors working together in response to typical acid stress conditions, extracellular and cytoplasmic pH values of 3 and 5.5, respectively, accelerate the rate of virtual proton pumping $\sim$50-fold (Figure S5 of the Supporting Information). It is also notable that the bell-shaped cytoplasmic pH profile for transport is similar in shape to the pH dependence of the acid-activated arginine decarboxylase AdiA,[14,15] a satisfying example of a sophisticated, multiprotein system in which all components are poised to respond to a stress condition in a coordinated way.

Our results also offer a cautionary note regarding the logic sometimes mustered to argue for H$^+$-coupled mechanisms in transport proteins. For instance, in a recent description of the structure and mechanism of ApcT, a wide-spectrum APC amino acid transporter, it was shown[16] that the level of substrate uptake is increased $\sim$3-fold in the presence of a pH gradient (pH 4 outside to pH 7 inside), as compared with symmetrical pH 4 conditions. This result was taken to mean that a proton is cotransported with the amino acid, i.e., that ApcT is a H$^+$-coupled amino acid symporter. However, as shown here, AdiC, which does not cotransport protons,[6] is also stimulated by a similar pH gradient (pH 3 outside to pH 5.5 inside, vs a symmetrical pH of 5.5). Thus, the type of evidence adduced to identify ApcT as a H$^+$-coupled symporter neither supports nor refutes that conclusion.

In this study, we developed an oriented reconstituted system for AdiC and used it to extract sided kinetic parameters for substrate transport and other functional properties of AdiC. Many unresolved problems and ambiguities concerning this protein remain for future examination. For example, the precise role of Ser26 in substrate selectivity is still in question, despite the implication from crystal structures alone, of a strong H-bond donated to substrate by this side chain. In addition, the specific influences of pH on substrate transport have not yet been dissected mechanistically, nor have the protein's pH sensors been identified. The sided system described here will be a useful tool for future approaches to these problems.

■ ASSOCIATED CONTENT

S Supporting Information

Proton leakage through proteoliposomes (Figure S1), dose response of MTS reagents (Figure S2), effects of MTSEA on the C-less mutant (Figure S3), arginine binding and transport of S26A-AdiC (Figure S4), and maximal activation of AdiC by acid stress (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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