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# Structure of a slow CLC CI-/H+ antiporter from a cyanobacterium

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## **Abstract**

X-ray crystal structures have been previously determined for three CLC-type transporter homologues, but the absolute unitary transport rate is known for only one of these. The E. coli Cl<sup>-</sup>/H<sup>+</sup> antiporter (EC) moves ~2000 Cl<sup>-</sup> ions/s, an exceptionally high rate among membranetransport proteins. It is not known whether such rapid turnover is characteristic of ClCs in general or if the E. coli homologue represents a functional outlier. Here, we characterize a ClC Cl<sup>-</sup>/H<sup>+</sup> antiporter from the cyanobacterium Synechocystis sp PCC6803 (SY) and determine its crystal structure at 3.2 Å resolution. The structure of SY is nearly identical to that of EC, with all residues involved in Cl<sup>-</sup> binding and proton coupling structurally similar to their equivalents in EC. SY actively pumps protons into liposomes against a gradient and moves Cl<sup>-</sup> at ~20 s<sup>-1</sup>, 1% of the EC rate. Electrostatics calculations, used to identify residues contributing to ion binding energetics in SY and EC, highlight two residues flanking the external binding site that are destabilizing for Cl<sup>-</sup> binding in SY and stabilizing in EC. Mutation of these two residues in SY to their counterparts in EC accelerates transport to ~150 s<sup>-1</sup>, allowing measurement of Cl<sup>-</sup>/H<sup>+</sup> stoichiometry of 2/1. SY thus shares a similar structure and a common transport mechanism to EC, but it is by comparison slow, a result that refutes the idea that the transport mechanism of CLCs in general leads to intrinsically high rates.

> The CLC family of Cl<sup>-</sup> transport proteins is made up of two mechanistically distinct subclasses: Cl<sup>-</sup> channels and Cl<sup>-</sup>/H<sup>+</sup> exchangers 9also called antiporters). These proteins, which enable transmembrane movement of Cl<sup>-</sup> (or in plants, NO<sub>3</sub><sup>-</sup>) for many different physiological purposes throughout the biological world, have been extensively investigated by electrophysiological, biochemical, and crystallographic techniques (1, 2). Only CLC-ec1, a Cl<sup>-</sup>/H<sup>+</sup> exchanger from E. coli, has yielded to all three approaches. Accordingly, this homologue (henceforth denoted EC), has provided a unique paradigm for structure-function analysis of CLC proteins. Some of its basic properties, such as its homodimeric architecture, its anion binding and selectivity characteristics, and its 2-for-1 Cl<sup>-</sup>/H<sup>+</sup> exchange stoichiometry, also appear in all known CLC exchangerss. But other characteristics of EC do not apply generally to the CLC family; all eukaryotic and some prokaryotic CLC proteins, for example, carry a large cytoplasmic C-terminal regulatory domain (3) which is absent in EC and in many other bacterial homologues. A striking feature of EC is its absolute turnover rate,  $2000-3000 \text{ s}^{-1}$  (4, 5), which is atypically high among all varieties of transporters. Moreover, this rate can be increased ~20-fold further by "channelizing" the protein's ionbinding region through designed mutagenesis (6). Since EC is the only CLC transporter for which this fundamental kinetic property has been determined, it is impossible to judge whether this high rate is intrinsic to CLC mechanisms, or whether it is an outlier within this family.

For these reasons, we have been seeking additional CLC homologues appropriate for analysis of both structure and function. Here we report an X-ray crystal structure of CLC-sy1 (denoted SY herein), a cyanobacterial CLC of the exchange-transporter subclass. The structure of SY is very similar to that of EC, with which it shares 39% sequence identity (Fig. 1), and its fundamental transport characteristics are conserved as well. But SY displays a transport rate about 100-fold lower than that of EC. By comparing EC and SY sequences, we locate two residues which, when mutated to the equivalent in EC, substantially increase the SY turnover rate, while supporting the 2-to-1 Cl<sup>-</sup>/H<sup>+</sup> coupling stoichiometry typical of CLC exchangers. These results provide functional and structural information for an additional CLC exchanger, which enriches comparative studies of the mechanism of Cl<sup>-</sup> and H<sup>+</sup> transport in this family of proteins.

### **Methods**

Special reagents were obtained from Avanti (lipids), Anatrace (detergents), and Roche (Lys-C protease). An open reading frame (GI 1652876) coding for a 451-residue CLC protein was cloned from the cyanobacterium Synechocystis sp PCC6803. This sequence was inserted into the pASK vector with a C-terminal hexahistidine tag (7), as diagramed in Supplementary Fig 1. Protein was expressed in E. coli with growth conditions described for EC (8). Cells were lysed by sonication and extracted in 40 mM decylmaltoside (DM) for 1-2 hr at room temperature. After clarifying the extract by centrifugation (25,000×g, 45 min) the supernatant was passed through cobalt-affinity beads (1 mL resin/L bacterial culture), washed with 100 mM NaCl, 20 mM imidazole and eluted with 400 mM imidazole. The protein was further purified on a Superde×200 gel filtration column in 100 mM NaCl, 20 mM tris-HCl pH 7.5, 5 mM DM. For crystallization experiments, the preparation was treated with lysine-C endoproteinase (1 hr, 0.01U/mg, 25°C) before the Superde×200 step to remove the first 14 N-terminal residues. Crystals were grown by vapor diffusion for 3–12 days in sitting drops at 18–25 mg/mL protein, with ~40 mM DM. The drops were set with 2 μL of protein mixed with 1–2 μL of reservoir solution containing 30–32% PEG 400, 200 mM CaCl<sub>2</sub>, 100 mM Tris-HCl, pH 8.0-8.2; additives were then introduced to the indicated final concentrations: C-Hega-11 (14 mM), SrCl<sub>2</sub> (12 mM), PEG 400 (6.2%), and in some cases Hega-10 (9 mM) and decanoyl sucrose (3 mM). Only crystals larger than ~200 µm diffracted well enough for data-collection. The structure was solved by molecular replacement with PHASER using the EC homodimer (PDB #1OTS) as search model and prime-and-switch density modification to suppress model bias. The model was refined using Refmac5 and Phenix (9, 10).

For functional analysis, purified SY protein was added to reconstitution buffer at 5–10 µg protein/mg lipid containing 20 mg/mL E. coli polar lipid and 35 mM CHAPS. Detergent was removed by dialysis against the desired intraliposomal solution over 36 hours. For concentrative uptake experiments, liposome samples (100 µL) containing 450 mM KCl, 25 mM citrate-phosphate pH 4.5 were centrifuged through 1.5-mL G50 columns equilibrated in uptake buffer (400 mM sorbitol, 25 mM K<sub>2</sub>HPO<sub>4</sub>, 33 mM K<sub>3</sub>-citrate, 17 mM citric acid, pH 4.0–7.0). <sup>36</sup>Cl<sup>-</sup> (1 mM) was added to the extra-liposomal solution to start the uptake, and liposomes were collected at various time points by passage through a 1.5 mL Dowex-1X4glutamate column (7). Proton pumping experiments were carried out with 300 mM KCl, 40 mM citrate/pH 4.8 for the internal solution; liposomes were spun through Sephadex G50 columns equilibrated in external buffer (300 mM K<sup>+</sup>-isethionate, 1 mM KCl, 2 mM citrate-KOH pH 4.8), then diluted tenfold into a stirred cell containing 1.8-mL this solution. Valinomycin (Vln, 0.5 μM) was added to set the membrane potential to zero and initiate transport. The resulting proton uptake was followed by recording external pH with a glass electrode, and after reaching a steady level the pH gradient was dissipated by addition of 0.5 μM proton ionophore FCCP. Initial rate of Cl<sup>-</sup> efflux uncoupled from H<sup>+</sup> uptake (4) was

measured under similar conditions, except that 25 mM citrate was included in internal and external solutions and efflux was started by addition of Vln and FCCP together; Cl $^-$  appearance in the external solution was recorded electrochemically with a Ag/AgCl electrode. After several minutes, liposomes were disrupted by addition of 50 mM  $\beta$ - octylglucoside to measure total trapped Cl $^-$ . Cl $^-/H^+$  stoichiometry is reported as the ratio of initial rates (measured from the first 5 seconds of uptake) under proton-pumping conditions, with calibration by 150 nmol HCl. For isothermal calorimetry (ITC) measurements, protein was purified as described above except that the final gel-filtration step was carried out in Cl $^-$  free buffer: 100 mM Na $^+/K^+$  tartrate, 20 mM Na $^2$ HPO $_4/$ pH 7.5, 5 mM DM. Protein was concentrated to 200  $\mu$ M, and ITC was performed in a 1.8-mL cell, using 5  $\mu$ L injections of 10 mM NaCl in the gel filtration buffer.

The refined SY model was used for electrostatics analysis. The static field contribution of each residue was calculated at the central Cl<sup>-</sup> binding site by solution of the Poisson equation as described (11). As this computation was used merely for screening residue contributions, the calculations were carried out with the protein alone, without a membrane dielectric implementation. The protein dielectric constant was set to 4 and solvent to 80, and all residues were in native protonation states, with histidines set neutral. All calculations were carried out using the PBEQ module of CHARMM (12).

#### Results

#### Crystal structure of SY

Initial poorly diffracting, needle-like crystals appearing in PEG400 screens were improved by using three extra detergents and  $SrCl_2$  as additives. Even with these optimized conditions, diffraction was typically anisotropic with high mosaicity, but a few rare crystals were suitable for structure determination at 3.2 Å resolution. The EC homodimer served as a molecular replacement search model, locating a single SY homodimer in the asymmetric unit (Supplementary Fig. 2). Model bias was suppressed by refinement using prime-and-switch density modification (13), which was particularly powerful because of the high solvent content (80%) of these crystals. The resulting electron density maps allowed unambiguous tracing of the entire transmembrane region, residues 26–450 (crystallographic statistics reported in Supplementary Table 1).

The SY structure (Fig. 2a) closely matches that of its E. coli homologue ( $C_{\alpha}$  rmsd 1.3 Å). The protein is a homodimer in which each subunit, constructed from 18  $\alpha$ -helices, is mostly membrane-embedded. The transmembrane dimer interface ( $\sim$  1200 Ų) in SY is composed mainly of interdigitating alkyl sidechains projecting off helices  $\alpha$ H,  $\alpha$ I,  $\alpha$ P, and  $\alpha$ Q, as in EC (14). The key ion-coupling residues of EC all overlay their equivalents in the SY structure (Fig 2b): the external and internal proton-transfer glutamates (E144 and E198, respectively), and the central serine-tyrosine pair (S103-Y437), which forms the intracellular gate or kinetic barrier of the Cl $^-$  pathway (3, 6). The central Cl $^-$  ion is visible in Fourier difference maps, as is confirmed in anomalous difference maps from crystals grown in Br $^-$ . As in EC, this buried, dehydrated anion is coordinated by the central Ser and Tyr sidechains, as well as by several backbone amides.

The SY structure shows a few minor departures from that of EC. The backbone trajectories of outer helices  $\alpha A$ - $\alpha C$ ,  $\alpha J$ , and  $\alpha K$  are slightly displaced from those in the molecular replacement model (Fig 2c), but no significant changes in regions known to mediate Cl<sup>-</sup>/H<sup>+</sup> transport arise from these differences. In addition, the bound central Cl<sup>-</sup> ion density in SY is less prominent than in EC (15, 16). Equilibrium Cl<sup>-</sup> binding to SY, determined by ITC (Fig 3), yields  $K_D \sim 1.4$  mM, about two-fold higher than the value previously reported for EC (5); since crystals were grown at Cl<sup>-</sup> concentrations far higher than this, we assign the weaker

Cl<sup>-</sup> density to the poorer quality of SY vs EC crystals, rather than to lower ion occupancy. Another difference in the SY structure is the absence of a second Cl<sup>-</sup> ion, the weakly bound "internal" Cl<sup>-</sup> seen in EC at the intracellular end of the Cl<sup>-</sup> pathway (15–17), as is corroborated by our failure to observe anomalous difference density in this region with crystals grown in Br<sup>-</sup>. In contrast to the mechanistically pivotal central Cl<sup>-</sup>, the internal Cl<sup>-</sup> ion is thought not to participate directly in transport (5, 18), and so its absence here is not alarming.

#### **Functional characteristics of SY**

The structural similarities above suggest that SY will hold few functional surprises. Indeed, this is the case. The protein's transport characteristics were assessed by reconstitution into liposomes. As with EC (8), SY supports acid-activated Cl<sup>-</sup> flux (Fig 4a), with a somewhat stronger pH-dependence than for EC (8). As with EC, competition of <sup>36</sup>Cl<sup>-</sup> uptake by nonradioactive anions (Fig 4b) reveals little selectivity among Cl<sup>-</sup>, Br<sup>-</sup>, and NO<sub>3</sub><sup>-</sup>, while SCN<sup>-</sup> competes strongly and SO<sub>4</sub><sup>2+</sup> weakly, the divalent anion requiring 10-fold higher concentration for effects comparable to Cl<sup>-</sup>. The central mechanistic feature of CLC exchangers - Cl<sup>-</sup>/H<sup>+</sup> antiport - is also observed in SY, as shown by uphill H<sup>+</sup> pumping at the expense of an oppositely directed Cl<sup>-</sup> gradient (Fig 5). Here, liposomes are loaded with high Cl<sup>-</sup> (300 mM) and suspended in low Cl<sup>-</sup> (1 mM), with internal pH slightly lower than external. Upon addition of Vln to counteract the charge imbalance set up by electrogenic Cl<sup>-</sup>/H<sup>+</sup> exchange, the suspension alkalinizes as H<sup>+</sup> is pumped inward, an effect reversed by a proton ionophore. While these ion movements qualitatively mimic those in EC, SY gives much smaller signals in our assays. We therefore suspected that the absolute turnover rate of SY might be much lower than the high value to which EC has accustomed us (4). This expectation is confirmed in a "Cl" dump" experiment (4), wherein Cl"-loaded liposomes passively release their contents into low-Cl<sup>-</sup> medium with no pH gradient present. The unitary turnover rate of SY is 20 s<sup>-1</sup>, (Table 1) a conventional value for transporters but 100-fold lower than the EC rate. This low turnover rate made it impossible to quantify the Cl<sup>-</sup>/H<sup>+</sup> exchange stoichiometry for wild-type SY or to obtain satisfactory electrical recordings of SY in planar lipid bilayers, as is routinely accomplished with EC (8, 19).

To seek residues responsible for the slow transport rate in SY, we carried out a comparative analysis of SY and EC sequences informed by their crystal structures. In particular, we focused on residues differing between SY and EC in electrostatics in the transport pathway, a factor that may account for the slightly weaker Cl<sup>-</sup> binding affinity for SY. The static field contribution of each residue in the protein was evaluated at the central Cl<sup>-</sup> binding site by solution of the continuum electrostatics Poisson equation. A residue-by-residue comparison of the two proteins shows several positions potentially capable of modulating the electrostatics along the transport pathway (Fig. 6). Mutations at these positions to the corresponding EC residues are expected to be stabilizing (F143R, H200K, H211R, E238K, D309F, E429Q) or destabilizing (K50D, Q107E) for Cl<sup>-</sup>. Most of these residues are located near the water-exposed surfaces and are thus likely to exert relatively weak effects. However, three residues, K50, F143 and D309, are partly buried and close to the Cl binding pathway. Residues F143 and D309 cap the N-termini of the αF and αL helices respectively, which point their positive dipoles towards the external Cl<sup>-</sup> binding site, and F143 is also adjacent, in sequence and structure, to the external glutamate gate E144. Furthermore, analysis of 2625 CLC homologues in the PFAM database shows that either Arg or Lys is found at the F143 equivalent position in over 70% of the entries, with zero occurrence of negatively charged residues (Supplementary Table 2). Remarkably, Phe appears at this position only once - in the entry for SY. Position 309 is more evenly distributed among residue-types, showing a significant preference for Tyr.

Each of the eight single substitutions listed above was reconstituted into liposomes, and unitary Cl<sup>-</sup> transport rates were measured by the "Cl<sup>-</sup> dump" assay (Fig 7a). The rates are similar to wild-type SY for most constructs (Table 2), except for the two external site mutants F143R and D309F, each of which showed a ~5-fold increase in Cl<sup>-</sup> transport to (130, 105 s<sup>-1</sup>, respectively). The increase in rate accompanying these mutations made it possible to measure the Cl<sup>-</sup>/H<sup>+</sup> transport stoichiometry. The F143R mutant (Fig. 7b) maintains H<sup>+</sup> pumping, with Cl<sup>-</sup>/H<sup>+</sup> exchange stoichiometry close to the 2:1 value for expected for CLC transporters (2), further corroborating the mechanistic similarity between SY and EC. We also examined the double substitution, F143R/D309F, which further increased the rate only slightly to  $\sim 150 \text{ s}^{-1}$  (Fig 7A). We noticed the possibility of a functional triad formed by these two residues and K50 (Fig 6), since these three sites in EC (Asp+ Arg+Phe) and SY (Lys+ Phe+Asp) conserve charge. However, the SY triple mutant (K50D, F143R, D309F), which recapitulates the EC triplet, fails to produce a fast transporter (85 s<sup>-1</sup>). At first glance, it appears that Cl<sup>-</sup> transport rates in SY correlate with net charge of this external region, but this conclusion is undermined by further experiments. For instance, in contrast to F143R, Lys or His substitutions here have no effect on transport (Table 2). Likewise the converse mutation in EC, R147F, shows only a modest (~30%) lowering of transport rate. For these reasons, we cannot invoke a general electrostatic mechanism to account for the large differences in SY and EC rates. There is a certain irony in this conclusion since F143 and D309 were originally identified as influential via an electrostatic computation.

In a final, desperate attempt to increase the transport rate of SY, we resorted to radical surgery by removing both external and internal "gates" of the transporter's  $Cl^-$  pathway. In EC, removal of these gates by the double mutation E148A/Y445A produces a continuous pore connecting internal and external solutions, abolishes  $H^+$  transport, and increases  $Cl^-$  transport to ~20,000 s<sup>-1</sup> (6). Since the structure of SY is so similar to EC, we expect that removal of the gates should also greatly speed transport. However, the corresponding mutation in SY, E144A/Y437A, produces no change whatsoever over the slow  $Cl^-$  transport rate of wildtype SY (Fig 7A). This result is extremely surprising, but without a crystal structure of the double mutant, which we have still not achieved, we cannot fathom its meaning.

#### **Discussion**

Up until now, X-ray crystal structures of three CLC Cl<sup>-</sup>/H<sup>+</sup> exchange-transporters have been determined: of prokaryotic homologues from *Escherichia coli* and *Salmonella enterica typhimurium* (17, 20), and of a eukaryotic CLC from *Cyanidioschyzon merolae* (3). Of these, only EC has been subjected to detailed analysis of transport function, including quantitative measurements of the unitary turnover rate (4, 5). Moving Cl<sup>-</sup> ions at 2000–3000 s<sup>-1</sup> at 25 °C, EC is an unusually fast transporter. In light of the unusual structural architecture of ClC proteins and the absence of large conformational changes during the transport cycle, which appears to involve only subtle sidechain rearrangements (3, 18, 21), we have naturally suspected that atypically high transport rates may be a feature of all CLC transporters. This suspicion is buttressed by the similarities between SY and EC. All key structural elements - the transmembrane helices, Cl<sup>-</sup> binding residues and gates, and residues involved in H<sup>+</sup> transport - are found similarly disposed in the two homologues, and the mechanistic hallmark of ClC transporters - 2:1 Cl<sup>-</sup>/H<sup>+</sup> antiport stoichiometry - is also present in SY.

But our results refute this expectation without ambiguity. At 20 s<sup>-1</sup>, Cl<sup>-</sup> transport in SY is two orders of magnitude slower than in EC, with a rate conventional for membrane transporter mechanisms. This striking difference in rate between the two homologues must

lie in subtle structural variation and sequence differences. We have identified two residues in SY that increase the transport rate when mutated to their EC counterparts: F143R and D309F, substitutions that increase the local positive electrostatic character of the protein's ion-transport region. Positioned at the N-terminal ends of the two helices pointed at the external Cl<sup>-</sup> site and adjacent to the external glutamate gate, these residues appear to be in position to stabilize anions in the transport region. However, these mutations enhance transport only modestly, the double mutant F143R/D309F reaching only 8% of the EC rate. Nevertheless, SY now provides a structural model for comparison and a target for dissecting out new molecular contributors to transport function.

## **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

#### **Abbreviations**

**FCCP** carbonylcyanide-p-trifluoromethoxyphenylhydrazone

DM n-decylmaltosidePEG polyethylene glycol

## **Acknowledgments**

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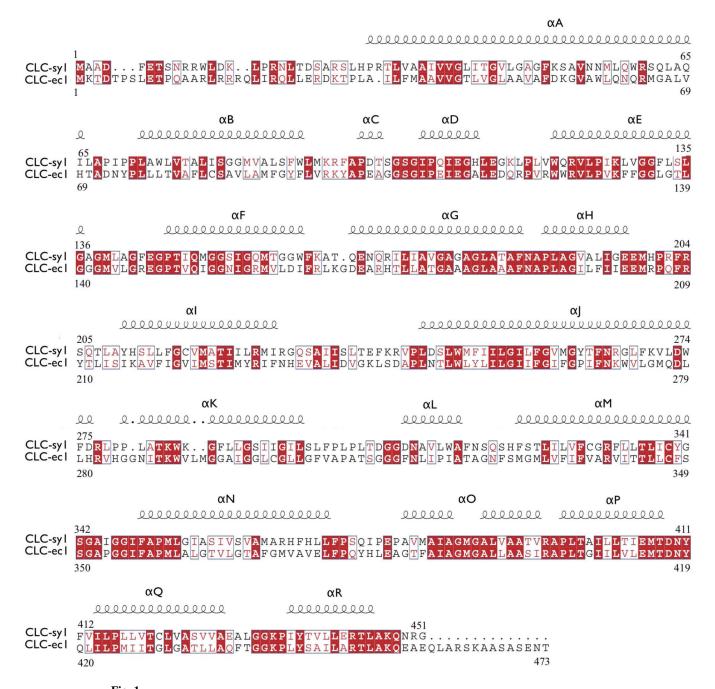


Fig. 1. Alignment of CLC-sy1 (SY) and CLC-ec1 (EC) sequences. Solid red block represents identical residues, comprising 39% of the total sequence. The boxed segments highlight both identical and similar parts of the alignment. The 18  $\alpha$ -helices forming the transmembrane structure are labeled  $\alpha A$ - $\alpha R$ .

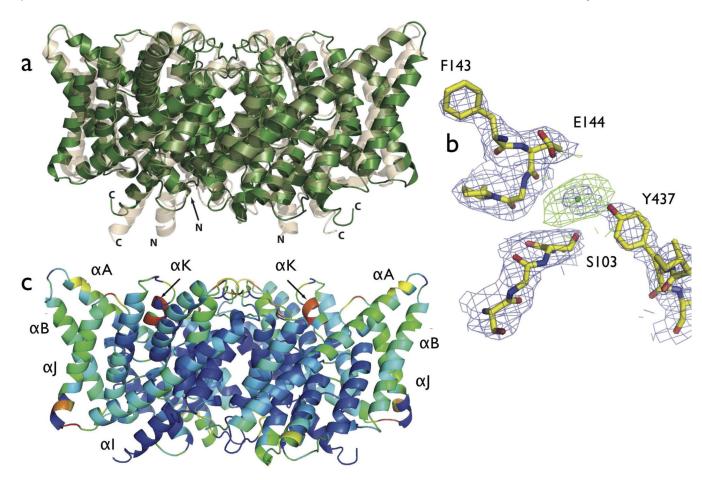


Fig. 2. Structure of SY. a. SY (green) and EC (tan) structure alignment of backbone atoms (1.4 Å rmsd). b. The central Cl<sup>-</sup> binding site in SY, formed by residues S103 and Y437. The external H<sup>+</sup>-transfer residue, E144, and its adjacent residue, F143 are also shown, with  $2F_{O}$ - $F_{C}$  map (blue) and anomalous difference density from a Br<sup>-</sup> dataset (green) contoured at 1.5 and 8  $\sigma$ , respectively. c. Regions of structural agreement of SY and EC mapped onto SY structure. Low rmsd regions are shown in blue, while increasing values of rmsd are in green, yellow, and red. Helices  $\alpha A$ ,  $\alpha B$ ,  $\alpha J$  and  $\alpha K$  are labeled as shown. Structure factors and coordinates of SY are deposited in the PDB (#3NCO for the Cl<sup>-</sup> structure and #3Q17 for the Br<sup>-</sup> structure)

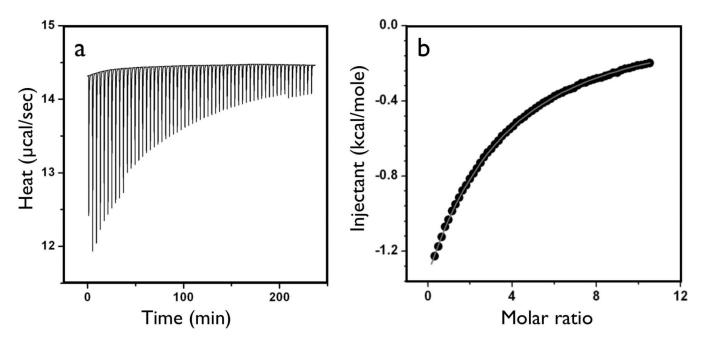
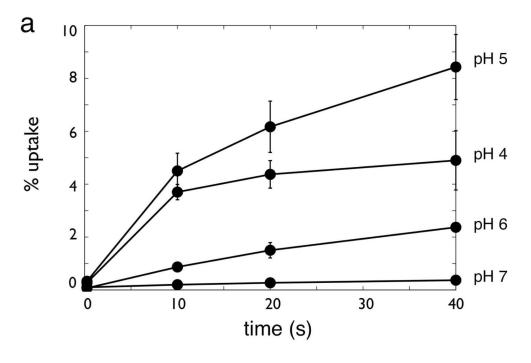
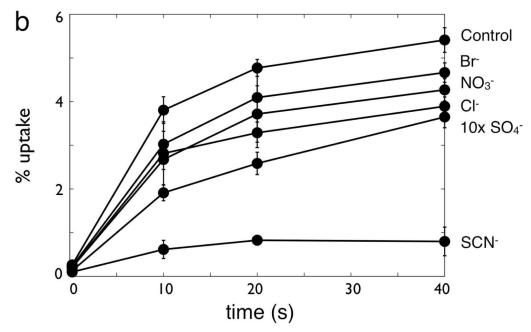


Fig. 3. Equilibrium binding of  $Cl^-$  to SY. a. Traces of heat evolution in ITC run. SY at  $216~\mu M$  in DM micellar solution was challenged with NaCl injections. b. Binding isotherm. Integrated heats (filled points) were fit to single-site  $Cl^-$  binding isotherm (solid curve), with  $K_D=1.5$  mM. Thermodynamic parameters for binding are  $\Delta H^o=-10~kcal/mol,~\Delta S^o=-21~cal/mol-K$ . A duplicate experiment gave essentially identical results.





**Fig. 4.**  $^{36}\text{Cl}^-$  transport by SY. a. Acid-activated concentrative uptake. Proteoliposomes were preequilibrated and  $^{36}\text{Cl}^-$  uptake timecourses were performed at the indicated pH values. Uptake is reported as % of total  $^{36}\text{Cl}^-$  counts that become trapped inside the liposomes. b. Anion selectivity of transport. Selectivity was measured by inhibition of  $^{36}\text{Cl}^-$  uptake by addition of non-radioactive anions (1 mM for monovalent anions and 10 mM for  $\mathrm{SO_4}^=$ ). The control curve represents no addition of competing anions. Measurements were carried out at pH 4.5.

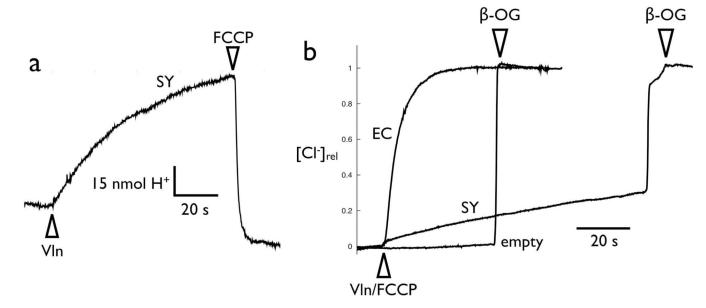


Fig. 5.  $H^+$  and  $Cl^-$  transport by SY. a.  $H^+$  pumping driven by a  $Cl^-$  gradient. Raw recordings of external solution pH are shown following addition of Vln at arrow to  $Cl^-$ -loaded liposomes to start the  $Cl^-$ - $H^+$  exchange; upward deflection represents alkanization. Proton buildup was dissipated at end of the uptake by addition of proton ionophore FCCP as indicated. b. Passive  $Cl^-$  efflux. Recordings of  $Cl^-$  appearance under similar conditions, except that FCCP was present throughout to prevent buildup of  $H^+$  gradient. Liposomes were disrupted with 50 mM  $\beta$ -octylglucoside ( $\beta$ -OG) to determine total trapped  $Cl^-$  which was used for normalization in calculating relative  $Cl^-$ . Efflux traces are indicated for EC, SY, and protein-free liposomes.

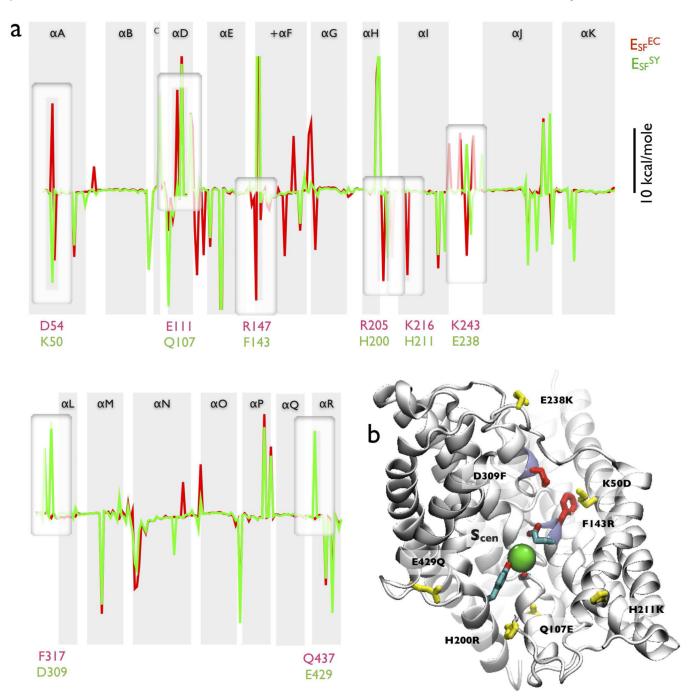
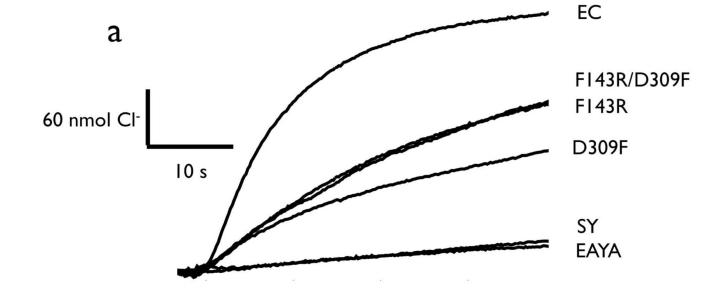


Fig. 6.
Electrostatic analysis of residues affecting Cl<sup>-</sup> binding in SY and EC. a Static field contribution for each residue in SY (green) and EC (red) for a Cl<sup>-</sup> ion bound to the central site. Deflections represent stabilizing (downward) or destabilizing (upward) energies for Cl<sup>-</sup> binding. Residues showing significant differences between SY and EC are highlighted. b. Single subunit of SY viewed from the dimer interface, with helices H, I, P and Q removed for visualization of the Cl<sup>-</sup> binding pathway. Residues near the central site (Y437, S103) and the external gate (E144) are shown. Residues with significant differences in electrostatics between SY and EC are highlighted in yellow, and the two residues (F143, D309) adjacent to the external site (blue ends of helices) are highlighted in red.



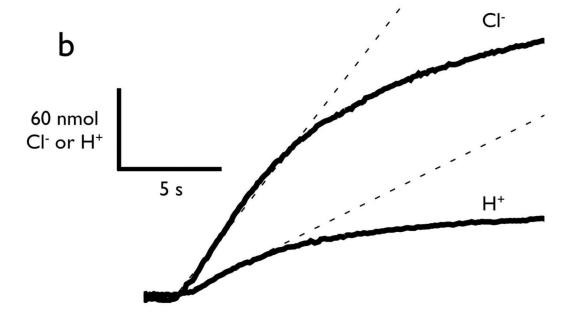


Fig. 7. SY turnover rates and antiport stoichiometry. a. Passive Cl $^-$  efflux. Raw recordings of Cl $^-$  release are shown for SY and indicated mutants, and rates are reported in Table 1. Protein was reconstituted at 5  $\mu g$  protein/mg lipid, except E144A/Y437A (EAYA) which was at 10  $\mu g/mg$ . b. Stoichiometry of Cl $^-/H^+$  transport in F143R mutant. Stoichiometry is measured by comparing initial rates of transport (dashed lines); Cl $^-/H^+$  stoichiometry was determined to be 2.3  $\pm$  0.4 (s.e., N=3). Cl $^-$  and H $^+$  signals were calibrated by addition of 150 nmol HCl.

Table 1

Cl<sup>-</sup> turnover rate for SY and specific mutants

Mutant	unitary Cl <sup>-</sup> rate (s <sup>-1</sup> per subunit)
protein-free	4 ± 2
WT	20 ± 3
K50D	$14 \pm 1$
QI07E	$26\pm7$
FI43R	$130\pm 5$
FI43K	$14 \pm 1$
FI43H	$22\pm3$
E238K	$15\pm6$
D309F	$105 \pm 7$
E429Q	$14 \pm 2$
FI43R/D309F	$149 \pm 21$
K50D/FI43R/D309F	$84 \pm 14$
EI44A/Y437A	19 ± 2

Cl $^-$  turnover rates were measured electrochemically as in Fig. 5b for the indicated SY variants (and control protein-free liposomes). Each value represents mean  $\pm$  s.e. of 3–4 separate determinations (or range of duplicates for F143R/D309F).