Solute Carrier Family 26 Member a2 (Slc26a2) Protein Functions as an Electroneutral SO$_4^{2-}$/OH$^-$/Cl$^-$ Exchanger Regulated by Extracellular Cl$^-$

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**Background:** Slc26a2 is an SO$_4^{2-}$ transporter, mutations in which cause diastrophic dysplasia. How Slc26a2 transports SO$_4^{2-}$ is unknown.  

**Results:** We found that Slc26a2 exchanges SO$_4^{2-}$ for 2OH$^-$ or 2Cl$^-$ and is regulated by a promiscuous extracellular anion site.  

**Conclusion:** Slc26a2 functions as SO$_4^{2-}$/2OH$^-$ or SO$_4^{2-}$/2Cl$^-$ exchanger, regulated by extracellular Cl$^-$.

**Significance:** The findings should help in understanding aberrant SLC26A2 function in diastrophic dysplasia.

Slc26a2 is a ubiquitously expressed SO$_4^{2-}$ transporter with high expression levels in cartilage and several epithelia. Mutations in Slc26a2 are associated with diastrophic dysplasia. The mechanism by which Slc26a2 transports SO$_4^{2-}$ and the ion gradients that mediate SO$_4^{2-}$ uptake are poorly understood. We report here that Slc26a2 functions as an SO$_4^{2-}$/2OH$^-$, SO$_4^{2-}$/2Cl$^-$, and SO$_4^{2-}$/OH$^-$/Cl$^-$ exchanger, depending on the Cl$^-$ and OH$^-$ gradients. At inward Cl$^-$ and outward pH gradients (high Cl$^-_{o}$ and low pH$_o$) Slc26a2 functions primarily as an SO$_4^{2-}$/2OH$^-$ exchanger. At low Cl$^-_{o}$ and high pH$_o$, Slc26a2 functions increasingly as an SO$_4^{2-}$/2Cl$^-$, Cl$^-$, and SO$_4^{2-}$/2OH$^-$ exchange. Slc26a2 also exchanges Cl$^-$ for I$^-$, Br$^-$, and NO$_3^-$ and Cl$^-_{o}$ competes with SO$_4^{2-}$ on the transport site. Interestingly, Slc26a2 is regulated by an extracellular anion site, required to activate SO$_4^{2-}$/2OH$^-$ exchange. Slc26a2 can transport oxalate in exchange for OH$^-$ and/or Cl$^-$ with properties similar to SO$_4^{2-}$/2OH$^-$ transport. Modeling of the Slc26a2 transmembrane domain (TMD) structure identified a conserved extracellular sequence and a putative transmembrane domain (TMD) structure. Mutation of Glu417 eliminated transport by Slc26a2, whereas mutation of Phe363 increased the affinity for SO$_4^{2-}$ 8-fold while reducing the affinity for Cl$^-_{o}$ 2 fold, but without affecting regulation by Cl$^-_{o}$. These findings clarify the mechanism of net SO$_4^{2-}$ transport and describe a novel regulation of Slc26a2 by an extracellular anion binding site and should help in further understanding aberrant SLC26A2 function in diastrophic dysplasia.

**Protein sulfation, and thus SO$_4^{2-}$, is essential for cellular and tissue survival.** Many proteins undergo post-translational modification by sulfation. Tyrosine sulfation of signaling molecules, like the G protein-coupled receptor chemokine receptors (1), modifies signaling pathways. Protein sulfation contributes to detoxification of endogenous compounds (2). A critical role of protein sulfation is sulfation of proteoglycans (3). Proteoglycans are constituents of the extracellular matrix that mediate the cell response to growth factors (4). Several disorders are caused by mutations in genes that affect proteoglycan synthesis or sulfation. The sulfate groups in proteoglycans are critical in formation of active domains, and the high polyanionic charge density of the proteoglycans is neutralized by SO$_4^{2-}$ (5). Sulfation of secretory proteins, like digestive enzymes and mucins, is essential for their synthesis, processing through the biosynthetic pathway and packaging in secretory granules (6). Hence, understanding SO$_4^{2-}$ homeostasis is essential for understanding cell development and function.

Cells have two sources of SO$_4^{2-}$, a minor source from degradation of cysteine and methionine and active uptake of SO$_4^{2-}$ mediated largely by the SO$_4^{2-}$/2Cl$^-$ transporters Slc26a1 and Slc26a2 (7, 8). Slc26a1 and Slc26a2 belong to the family of the SL26 transporters, which includes 11 genes with Slc26a10 being a pseudogene (9). Members of the family transport remarkably diverse substrates, including Cl$^-$, HCO$_3^-$, I$^-$, SO$_4^{2-}$, formant, and oxalate, and can function as coupled electroneutral or electrogenic transporters or as ion channels (9, 10). Mutations in several members of the family are associated with human diseases, including autosomal recessively inherited chondrodysplasias (SLC26A2) (11, 12), congenital chloride...
diabetes (SLC26A3) (13), Pendred syndrome (SLC26A4) (14), deafness (SLC26A5) (15), and perhaps reduced fertility (SLC26A8) (16). In addition, deletion of Slc26a6 in mice resulted in nephrolithiasis due to aberrant oxalate transport (17) and in aberrant pancreatic and parotid ducts HCO3- transport (18, 19).

Although Slc26a1 has limited tissue distribution, Slc26a2 is ubiquitously expressed with particularly high levels in developing and mature cartilage as well as in epithelial tissues like pancreas, salivary glands, colon, bronchial glands, tracheal epithelium, and eccrine sweat glands (20, 21). The central role of Slc26a2 in supplying the bulk of cellular SO42- is evident from the lethality of deletion of the SLC26A2 gene in humans and mice (20, 22), mainly due to under-sulfation of proteoglycans leading to aberrant development (23). Indeed, measurement of SO42- uptake in fibroblast from patients with a severe form of the disease showed reduced or lack of SO42- uptake (20, 24). Most mutations causing diastrophic dysplasia are missense mutations that affect either trafficking to the plasma membrane or showed reduced SO42- transport (25, 26).

The phenotype of chondrodysplasias is highly variable, ranging from mild (27) to lethal before or shortly after birth (11). To better understand the disease and cellular SO42- homeostasis, it is necessary to understand transport and regulation of Slc26a2. To date, characterization of transport by Slc26a2 was based on measurement of isotopic fluxes (24, 25, 28) that are the sum of both net and exchange fluxes, with the exchange dominating the fluxes. These studies revealed that Slc26a2 can transport SO42-, Cl-, and oxalate (24, 25, 28), and a recent detailed characterization of the fluxes suggested that Slc26a2 functions as an electroneutral transporter when mediating isotopic fluxes. SO42- fluxes appeared to be sensitive to intracellular and extracellular pH (24). An unusual finding was that inhibition of SO42- and oxalate isotopic uptake by external Cl- exhibited simple saturation, whereas Slc26a2-mediated exchange of intracellular SO42-, oxalate, or Cl- for external Cl- was non-saturable (24), suggesting that the measured fluxes, at least isotopic efflux, is mostly exchange rather than net fluxes.

The available information is not sufficient to determine the mode of SO42- and other ions transport by Slc26a2 and the cellular ionic gradients that drive net transport. We used Xenopus oocytes expressing Slc26a2 to report that Slc26a2 functions as SO42-/2OH-, SO42-/2Cl-, and SO42-/OH-/Cl- exchanger, depending on the cellular Cl- and OH- (H+) gradients. Slc26a2 can also mediate Ox2-/2OH-/Cl- exchange and transport I-, Br-, and NO3-. Slc26a2 activity is regulated by an extracellular anion binding site, which is not involved in ion transport. Modelling of the Slc26a2 transmembrane sector identified an extracellular loop, which contains the conserved sequence R67G/E57P in the vicinity of the gating Glu417 as a potential part of the permeation pathway. These findings should help in further understanding ion transport by the SLC26 transporters and aberrant SLC26A2 function in diastrophic dysplasia.

**EXPERIMENTAL PROCEDURES**

**Solutions and Reagents**—For experiments in oocytes, the standard HEPES-buffered ND96 solution contained (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.5. Cl- free solutions were prepared by replacing chloride with gluconate in the presence of calcium cyclamate substituted for CaCl2. A 100 mM solution of disoethylenetriamine-2,2'-disulfonic acid (DIDS) (Invitrogen) dissolved in DMSO was prepared freshly and diluted to a final concentration of 10 or 50 µM in the relevant solutions. All other chemicals and reagents were purchased from Sigma.

**cRNA Preparation**—The pCMV-Sport6-Slc26a2 (GenBankTM/EMBL/DDBJ, accession no. BC028345) was purchased from Open Biosystems and was used as template for cRNA preparation. The plasmid was linearized with NotI and used to transcribe cRNA with an mMESSAGE mMACHINE Sp6 kit (Life Technologies, Applied Biosystems), respectively. Mutation in Slc26a2 were generated by a site-directed mutagenesis kit (Agilent Technologies) and verified by sequencing.

**Biotinylation and Western Blot Analysis**—To monitor surface expression of Slc26a2 WT, E417A, and E417K, HEK cells transfected with vector alone or Myc-tagged Slc26a2 constructs were incubated with EZ link Sulfo-NHS-LC-Biotin (0.5 mg/ml, Thermo Fisher Scientific) for 30 min at room temperature. Subsequent steps were as previously described (29) with the following modifications: 50 µl of 1:1 slurry of immobilized avidin beads (Thermo Fisher Scientific) was added to 300 µg of protein in 300 µl of cell extract, and the mixture was incubated overnight. To monitor protein expression the PVDF membranes were incubated overnight with anti-Myc antibodies diluted 1:1,000 (Cell Signaling) and for 1 h with HRP-conjugated goat anti-mouse (Invitrogen) diluted 1:2,000. For β-actin detection membranes were incubated for 1 h with monoclonal anti-β-actin peroxidase (Sigma-Aldrich) diluted 1:20,000.

**Xenopus laevis Oocyte Preparation**—All experiments in this study were conducted under the National Institutes of Health guidelines for research on animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee. Oocytes were isolated by partial ovariectomy of anesthetized female X. laevis (Xenopus Express, Brooksville, FL) and treated by collagenase B (Roche Applied Science), as described previously (30). Stage V–VI oocytes were injected with 10 ng of cRNA using glass micropipettes and a microinjection device (Nanoliter 2000; World Precision Instruments) in a final volume of 27.6 nl. Control oocytes were injected with equal volumes of H2O. Oocytes were incubated at 18 °C in ND96 supplemented with 2.5 mM pyruvate and antibiotics and were studied 72–144 h after injection.

**Voltage, pH, and Cl- Measurement in Oocytes**—Voltage recordings were performed at room temperature with two-electrode voltage clamp, exactly as described previously (29, 30). Voltage, pH, and Cl- concentrations were measured as detailed previously (31, 32). In the present study, the Cl- sensitive electrode was also used to record intracellular Br-, I-, and NO3- with the resin and the procedure used to measure Cl- (see “Results”).

**Measurement of Buffer Capacity**—To determine OH- (H+) fluxes by Slc26a2 we determined the buffer capacity of oocytes bathed in HEPES-buffered medium. Because we can measure both Cl- and pH, we determined the buffer capacity directly rather than relying on pH changes induced by weak acids.
Slc26a2 Transport Properties and Regulation

Supplemental Fig. 1A shows that two consecutive injections of the oocytes with 13.8 nl of 100 mM HCl reduced pHi and increased Cli. Similar determination in five experiments and using the pHi and Cli changes of the first injection resulted in a buffer capacity of 17.1 ± 2.2/pH unit, which is similar to that reported by others (33).

Modeling and Prediction of the Slc26a2 Transmembrane Domains Structure—The transmembrane sector of the mouse Slc26a2 model was generated using the Deepview Swiss-PDB viewer by raw sequence fit of the Slc26a2 sequence (NCBI accession no. NP_031911) onto the putative Slc26a6 model previously generated by us based on structural similarity to the bacterial ClC-ec protein (29). The predicted binding site of DIDS on the Slc26a2 model was performed with the AutoDockVina software (34), according to software tutorial instructions. Briefly, the box grid determining the Slc26a2 region of binding was set using the AutoDockTools software with the following coordinates (center: x = 0.472, y = 1.222, z = 0.472) (size: x = 30, y = 26, z = 24). Exhaustiveness level was set to default. AutoDockTools was further used to select all rotatable bonds of the DIDS molecule. The AutoDockVina software generated nine different models, and herein we present the best model as ranked by the software with a predicted affinity of −8.9 kcal/mol for the binding of Slc26a2 and DIDS. The final model (cartoon and surface representations) was generated using PyMOL (Schrödinger, LLC).

RESULTS AND DISCUSSION

Slc26a2 Functions as an SO4$^{2-}$/OH$^-$/H$^+$/Cl$^-$ Exchanger—Slc26a2-mediated net fluxes were assayed in Xenopus oocytes by measuring intracellular pH (pHi) and Cli (Cl$^-$i), and the membrane potential in the same oocytes. Fig. 1A shows that removal of extracellular Cli (Cl$^-$o) had no effect on pHi, and the membrane potential with a slow rate of reduction in Cl$^-$i. Exposing Slc26a2-expressing oocytes bathed in Cl$^-$-free solution to 0.2 mM SO4$^{2-}$ resulted in a precipitous reduction in pHi and Cl$^-$i. Removal of SO4$^{2-}$ with the concomitant addition of Cl$^-$ resulted in increased pHi and Cl$^-$i. Fig. 1C shows almost no change in Cl$^-$i and pHi in water-injected oocytes under the same conditions.

Reduction in pHi can be due to H$^+$ influx or OH$^-$ efflux. Because some of the SO4$^{2-}$ transport is coupled to Cl$^-$, and the Cl$^-$ coupling is affected by pHi (see below), we will refer to the transported ion as OH$^-$, although we cannot distinguish between the transport of OH$^-$ and H$^-$. The average Slc26a2-mediated SO4$^{2-}$-coupled net Cl$^-$ and OH$^-$ transports are shown in Fig. 1B and indicate that under the conditions of Fig. 1A ~40% of SO4$^{2-}$ is transported in exchange for Cl$^-$ and ~60% in exchange with OH$^-$. SO4$^{2-}$ transport is electroneutral, because it is not associated with a change in membrane potential (Fig. 1), and SO4$^{2-}$-coupled OH$^-$ (Fig. 1D) and Cl$^-$ (not shown) fluxes are the same at membrane potentials of +40 and −100 mV. This indicates that the coupling stoichiometry of SO4$^{2-}$ exchange with Cl$^-$ and OH$^-$ is likely 1:2 with Slc26a2 functioning as SO4$^{2-}$/2OH$^-$, SO4$^{2-}$/2Cl$^-$ and possible SO4$^{2-}$/OH$^-$/Cl$^-$ exchanger.

To further determine the relationship between SO4$^{2-}$ and Cl$^-$, we measured the effect of Cl$^-$o on the apparent affinity for SO4$^{2-}$. Fig. 2A shows an example of the protocol used for these experiments. Oocytes expressing Slc26a2 were exposed to solutions containing the desired Cl$^-$o (0, 5, 20 or 50 mM) and SO4$^{2-}$ concentration for 5 min to obtain the rate of OH$^-$/Cl$^-$ efflux. Then the oocytes were incubated in Cl$^-$-containing solution without SO4$^{2-}$ to extrude the SO4$^{2-}$ and recover pHi before exposure to the subsequent SO4$^{2-}$ concentration. The plots in Fig. 2B...
obtained from these experiments were used to calculate the apparent $K_m$ for $SO_4^{2-}$ that were then plotted as a function of $Cl^-$ (Fig. 2C). The linear relationship in Fig. 2C indicates that $Cl^-$ competes with $SO_4^{2-}$ for interaction with the external substrate site. This competition is different from the non-saturating $Cl^-$ dependence reported for the isotopic exchange of $Cl^-$ with intracellular $Cl^-$ ($Cl^{-}_i$) or intracellular oxalate (24). This may reflect the different dependence of $Cl^{-}_i$ of the half (exchange) and full turnover cycle (net) of transport by Slc26a2.

Many of the SLC26 transporters can transport $HCO_3^-$ in exchange for $Cl^-$ (9). However, Fig. 2D shows that Slc26a2 does not function as a $Cl^{-}$/$HCO_3^-$ exchanger. The capacity of Slc26a2 to transport other anions, such as $I^-$, $Br^-$ and $NO_3^-$, in addition to $SO_4^{2-}$, $OH^-$ and $Cl^-$ was further tested by measuring their intracellular concentration. Supplemental Fig. 1B shows that the resin used to detect $Cl^-$ can also detect $Br^-$ and $NO_3^-$ $\sim$10 times better than $Cl^-$ and $\sim$100 times better than $Cl^-$ (see also (29)). The left panel of Fig. 2D shows that exposing Slc26a2-expressing oocytes to $Cl^-$-free solution containing 2 mM $I^-$ resulted in a rapid influx of $I^-$. Removal of $I^-$ in the absence of $Cl^-$ stopped the influx. To initiate $I^-$ influx it was necessary to add $Cl^-$ with as little as 1 mM $Cl^-$, resulting in nearly maximal rate of $I^-$ influx. Similar behavior was observed with $Br^-$ and $NO_3^-$ (Fig. 2D, right panel) and no $I^-$ (Fig. 2D, gray trace, left panel), $Br^-$ or $NO_3^-$ (not shown) fluxes were observed in water-injected oocytes. These findings indicate that the Slc26a2 permeation pathway is not very selective and can accommodate $I^-$, $Br^-$ and $NO_3^-$ to mediate $I^-/Cl^-$, $Br^-/Cl^-$ and $NO_3^-/Cl^-$ exchange.

The Ratio of $SO_4^{2-}/2OH^-$ and $SO_4^{2-}/2Cl^-$ Exchange Is Determined by $pH_o$—Coupling of $SO_4^{2-}$ transport to $OH^-$ and $Cl^-$ raised the question of how the availability of substrate would affect the coupling. We addressed this question by examining the effect of $pH_o$ and $pH_i$ on $SO_4^{2-}$ transport. Fig. 3A shows example traces of the changes in $pH_o$ (left panel) and of the $Cl^-$ (right panel) as a result of $SO_4^{2-}$ transport at $pH_o$ of 6.5 (black traces) and 8.2 (gray traces). The rates of $OH^-$ and $Cl^-$ influx and efflux under both conditions are summarized in Fig. 3B. The models in Fig. 3B show the direction of ion fluxes during $SO_4^{2-}$ influx (left) and $SO_4^{2-}$ efflux (right) and the columns show the associated $OH^-$ ($H^+$) and $Cl^-$ fluxes at $pH_o$ of 6.5 and 8.2. $SO_4^{2-}$ influx is coupled to $Cl^-$ and $OH^-$ efflux, while $SO_4^{2-}$ efflux initiated by removal of $SO_4^{2-}$ and addition of $Cl^-$ is coupled to $Cl^-$ and $OH^-$ influx. During $SO_4^{2-}$ influx acidic $pH_o$ increases $OH^-$ efflux with low $Cl^-$ efflux while alkaline $pH_o$ has the opposite effect. On the other hand, during $SO_4^{2-}$ efflux acidic $pH_o$ inhibits $OH^-$ efflux and increases $Cl^-$ efflux while alkaline $pH_o$ has the opposite effect.

Fig. 3C further illustrates the reciprocal effect of $pH_o$ on $OH^-$ and $Cl^-$ fluxes. Exposing Slc26a2-expressing oocytes to a solu-
tion buffered to pH 7.5 and containing 110 mM Cl\(^-\) and 2 mM SO\(_4^{2-}\) resulted in a reduction in pH\(_i\), at a rate of \(~0.18 \pm 0.03\) mM/min (\(n = 8\)), with no change in Cl\(^-\). Removal of SO\(_4^{2-}\) resulted in recovery of pH\(_i\). H\(_2\)O injected oocytes showed no response to SO\(_4^{2-}\). Hence, at high Cl\(^-\) and pH\(_o\), of 7.4 all the Slc26a2-mediated SO\(_4^{2-}\) flux is mediated by SO\(_4^{2-}/2\text{OH}^-\) exchange (or SO\(_4^{2-}/2\text{H}^+\) cotransport). When the same oocytes were exposed to the same solution containing 110 mM Cl\(^-\) and 2 mM SO\(_4^{2-}\), but now buffered to pH of 6.5, SO\(_4^{2-}\) uptake resulted in a large reduction in pH\(_i\) with no change in Cl\(^-\) while SO\(_4^{2-}\) efflux initiated by removal of SO\(_4^{2-}\) responded in a small increase in pH\(_i\) and a large Cl\(^-\) influx (Fig. 3C). Thus, at low pH\(_i\), SO\(_4^{2-}\) uptake is predominantly mediated by SO\(_4^{2-}/2\text{H}^+\) exchange, while SO\(_4^{2-}\) efflux is dominated by SO\(_4^{2-}/\text{Cl}^-\) exchange.

The sulfate transported species can be SO\(_4^{2-}\) or HSO\(_3^-\). Although we did not examine this in great detail, the results in Figs. 1–3 favor SO\(_4^{2-}\). Thus, if the transported species is HSO\(_3^-\), then acidic pH\(_i\) should markedly enhance Sulfate influx. Fig. 3B indicates that is not the case. Second, SO\(_4^{2-}\) efflux after removal of SO\(_4^{2-}\) should be independent of pH\(_i\) since pH\(_i\) should have no effect of the transported SO\(_4^{2-}\) species. Again, this is not the case. Third, changes on pH\(_o\) have the same effect on SO\(_4^{2-}\) and O\(_x^{2-}\) transport (see below), suggesting that the transport rate follows the pH gradient rather than substrate species.

Coupling of SO\(_4^{2-}\) transport to both Cl\(^-\) and OH\(^-\) may function to ensure SO\(_4^{2-}\) uptake under acidic and alkaline conditions. Slc26a2 is expressed in the luminal membrane of polarized cells (31, 35) that can be exposed to acidic and alkaline pH. In the stomach and synovial fluid pH is acidic (36, 37) and SO\(_4^{2-}/2\text{OH}^-\) exchange mediates most SO\(_4^{2-}\) uptake. On the other hand, in secretory glands, like the pancreas (38) and salivary glands (39), luminal pH is alkaline, which inhibits SO\(_4^{2-}/\text{OH}^-\) exchange (Fig. 3B) and most SO\(_4^{2-}\) uptake is by SO\(_4^{2-}/2\text{Cl}^-\) exchange.

Regulation of Slc26a2 by Cl\(^-\)—While measuring net SO\(_4^{2-}\) efflux we noticed that removal of SO\(_4^{2-}\) in the continuous absence of Cl\(^-\) never resulted in SO\(_4^{2-}\) efflux, as would be expected from SO\(_4^{2-}/2\text{OH}^-\) exchange. This is illustrated in the period bordered by the dashed box of Fig. 4A. However, addition of as little as 1 mM Cl\(^-\) triggered a robust SO\(_4^{2-}/2\text{OH}^-\) exchange and a small Cl\(^-\) influx (Fig. 4A, period marked by gray box). The dependence of the SO\(_4^{2-}/2\text{OH}^-\) exchange rate of Cl\(^-\) followed simple saturation curve with apparent \(K_m\) of 3.7 ± 0.9 mM (Fig. 4B). Activation of the exchange was not specific for Cl\(^-\). Fig. 4C shows that 1 mM external Cl\(^-\), Br\(^-\), I\(^-\), NO\(_3^-\) and SCN\(^-\) similarly activated SO\(_4^{2-}/2\text{OH}^-\) exchange. Only 1 mM F\(^-\) did not activate the exchange (Fig. 4C), but actually inhibited the exchange initiated by the other anions (not shown).

The findings in Fig. 4 suggest that SO\(_4^{2-}\) transport by Slc26a2 is regulated by interaction of an anion with a regulatory site. The regulatory site is not selective for Cl\(^-\), but because Cl\(^-\) is the major extracellular anion, Slc26a2 is likely regulated by Cl\(^-\) interaction with the regulatory site. The Cl\(^-\) regulatory site is likely different from the transport site since increased Cl\(^-\) should increase SO\(_4^{2-}/2\text{Cl}^-\) exchange while reducing SO\(_4^{2-}/2\text{OH}^-\) exchange. However, the opposite is observed. Activation of Slc26a2-mediated SO\(_4^{2-}/2\text{OH}^-\) by Cl\(^-\) may be by stabilization of an active Slc26a2 conformation. However, the exact mechanism remains to be elucidated. The physiological significance of regulation of Slc26a2 activity by Cl\(^-\) is not known at present. The Cl\(^-\) content in the GI tract is high in the range of 100–150 mM and is determined largely by acid secretion (40). On the other hand, urine Cl\(^-\) can be below 4 mM when prerenal azotemia occurs with metabolic alkalosis (41) and regulation of Slc26a2 by Cl\(^-\) can become significant. In addition, the luminal membrane-localized Slc26a2 is exposed to variable Cl\(^-\) concentrations, as low Cl\(^-\) in ducts that absorb the Cl\(^-\), such as the pancreatic (38) and salivary (32) ducts, the
The rate measured at 110 mM Cl suggested based on the predicted similarity of Slc26a6 to the low resolution structure of a bacterial Slc26 of Slc26 transporters (48). This structure showed a surprising Slc26a5 STAS domain to assemble a detailed putative structure predicted Slc26a6 model and the crystal structure of the SO₄⁻/HCO₃⁻ exchange. The results are mean ± S.E. of 3–5 experiments and fitted to the Hill equation. The sequence GFXXP is predicted to be in the extracellular loop between transmembrane domains (TMDs) 7 and 8, with Phe368 predicted to be in the entrance of the permeation pathway. Interestingly, Fig. 7A shows that the F368A mutation had multiple effects. Fig. 7B shows that DIDS inhibited SO₄²⁻ efflux when added after SO₄²⁻ uptake. Also in this case DIDS completely inhibited OH⁻ (H⁺) influx, but with a residual Cl⁻ influx. Similar results were obtained with 10 and 50 μM DIDS, indicating that the DIDS sensitivity of Slc26a2 is in the same range of that reported for Slc26a6 (52). Fig. 6C summarizes the rates of OH⁻ and Cl⁻ fluxes in the absence and presence of SO₄²⁻ and DIDS, indicating that at pH o of 7.5 and the absence of Cl⁻ a ~60% of SO₄²⁻ uptake is coupled to OH⁻ efflux and 40% to Cl⁻ efflux. Fig. 6D test another prediction of the model in Fig. 5 by neutralizing (Slc26a2/E417K) the charge of the conserved Glu417. Both mutations eliminated SO₄²⁻ uptake. However the F368A mutation had multiple effects. Fig. 7C shows that Slc26a2(F368A) is ~50% less active than wild-type Slc26a2 in exchanging SO₄²⁻ for OH⁻ (left traces) and Cl⁻ (right traces). Most notably, the F368A mutation increased the apparent affinity of Slc26a2 for SO₄²⁻ by ~8-fold to reduce the apparent Kₘ for SO₄²⁻ from 79 ± 7 to 9.7 ± 0.7 μM. Unexpectedly from competition between SO₄²⁻ and Cl⁻ (Fig. 2), the F368A mutation increased the apparent Kₘ for inhibition of SO₄²⁻ uptake by Cl⁻ o from 26 to 50 μM (Fig. 7C). Hence, Phe368 appears to control the access of SO₄²⁻ and Cl⁻ to the permeation pathway. Interestingly, Fig. 7D shows that the F368A mutation had no effect of the apparent affinity for the Cl⁻ o regulatory site that activates SO₄²⁻/OH⁻ exchange. This finding provides the strongest evidence that inhibition of SO₄²⁻ uptake by Cl⁻ o (Figs. 4B and 7C) and activation of SO₄²⁻/OH⁻ exchange by Cl⁻ o probably involves interaction of Cl⁻ o with two separate sites. The findings in Fig. 7A–C provide additional evidence for the importance of the GSGIP or the GFXXP motifs in the function of the Cl⁻ transporters, in addition to the two additional
GXXXP motifs that participate in Cl⁻ transport in the bacterial ClCs (44). The bacterial ClC-ec1 crystal structure shows that the permeation pathway has three Cl⁻ interacting sites (44–46, 49). Ser107 and Gly108 in the GSGIP motif coordinate the Cl⁻ ion in the internal substrate site, and the side chain of Ser107 participates in binding of the middle Cl⁻ (45, 49). In Slc26a2 Phe368 appears to control the affinity for the substrate (SO₄²⁻), suggesting that Phe368 may participate in the access of SO₄²⁻ to the permeation pathway or in shaping the external SO₄²⁻ binding site. The increased apparent affinity for SO₄²⁻ and reduced apparent affinity for Cl⁻ by the F368A mutation suggests that Phe368 may hinder access of SO₄²⁻ and facilitate access of Cl⁻ to the permeation pathway or reduces the time SO₄²⁻ spends in the external binding site on its way across the plasma membrane. Perhaps this is necessary to allow SO₄²⁻/Cl⁻ exchange at high SO₄²⁻, when SO₄²⁻ efflux is required. Irrespective of the exact role of Phe368, the present findings further support the notion of similarities between the CIC and SLC26 transporters permeation pathways and that the opening of the permeation pathway is situated in the region of TMDs 7 and 8.

Properties of Slc26a2-mediated Oxalate Transport—Slc26a2 was reported to transport Oxalate (Ox²⁻) by mediate Ox²⁻/SO₄²⁻ exchange (8, 24–26, 28) and that Ox²⁻/SO₄²⁻ exchange is 10 times slower than SO₄²⁻/Cl⁻ exchange (24). However, the properties and mode of Ox²⁻ transport and the capacity of net Ox²⁻ transport by Slc26a2 are not known. We set to estimate net Ox²⁻ transport by measuring Ox²⁻-mediated OH⁻ and Cl⁻ fluxes. Fig. 8A shows that Slc26a2 mediates net Ox²⁻/OH⁻ and Ox²⁻/Cl⁻ exchange in oocytes bathed in Cl⁻-free solution containing 1 mM Ox²⁻, pH 7.5. Removal of Ox²⁻ was not followed by Ox²⁻ efflux until the addition of 1 mM Cl⁻ to activate the efflux. Importantly, addition of 1 mM Cl⁻ resulted in minimal Ox²⁻/Cl⁻ exchange but near maximal Ox²⁻/OH⁻ exchange. Increasing Cl⁻ to 110 mM caused a small additional increase in Ox²⁻/OH⁻ exchange and modest Ox²⁻/Cl⁻ exchange. As expected, Fig. 8B shows that
reducing pH

\[ \text{Ox}^2^- / \text{OH}^- \] exchange and increasing pH

\[ \text{Ox}^2^- / \text{OH}^- \] exchange. Finally, the F368A mutation increased
the apparent affinity for \( \text{Ox}^2^- \) and reduced the apparent \( K_m \) for
\( \text{Ox}^2^- \) from 90 ± 12 to 50 ± 8 µM. Although this was not as

![Figure 6](link)

**Figure 6. Inhibition of Slc26a2 by DIDS and by mutations of Glu417.** Example traces depicting inhibition of \( \text{SO}_4^{2-} \) flux (A) and \( \text{SO}_4^{2-} \) efflux (B) by 50 µM DIDS. Note the complete inhibition of the coupled \( \text{OH}^- \) but not of \( \text{Cl}^- \) fluxes. C, the average rates (mean ± S.E. of the indicated number of experiments) of \( \text{OH}^- \) and \( \text{Cl}^- \) fluxes. In D shown are examples of oocytes expressing either wild-type Slc26a2, Slc26a2(E417A), or Slc26a2(E417K) that were used to measure the \( \text{SO}_4^{2-} \)-associated \( \text{OH}^- \) and \( \text{Cl}^- \) fluxes. E, shows the surface expression of Slc26a2 and mutants with actin used as a control for the biotinylation.

![Figure 7](link)

**Figure 7. Phe368 in Slc26a2 permeation pathway.** A, example traces for pH, and Cl− measurement in oocytes expressing either wild-type (gray traces) or Slc26a2(F368A) (black traces). This protocol was used to monitor Slc26a2-mediated the \( \text{SO}_4^{2-} \) dependence of \( \text{SO}_4^{2-} / \text{OH}^- \) exchange (B) and inhibition of the exchange by \( \text{Cl}^- \) (C). \( \text{SO}_4^{2-} / \text{OH}^- \) exchange was used to monitor activation of the reverse exchange by \( \text{Cl}^- \) (D). All plots (B–D) were fitted to the Hill equation, and \( K_m \) values are given as mean ± S.E.
prominent as the increased apparent affinity for SO$_4^{2-}$ (Fig. 7), it was in the same direction. The results in Fig. 8, A–D indicate that the properties of Ox$_2^{-}$ transport closely resemble those of SO$_4^{2-}$ transport, although at the same conditions the Ox$_2^{-}$ transport rate was ~50% slower than the SO$_4^{2-}$ transport rate.

In summary, the present study reports the mechanism of SO$_4^{2-}$ and Ox$_2^{-}$ transport by Slc26a2. Both anions are transported in exchange for Cl$^-$ and OH$^-$ or by cotransport with H$^+$. Based on the rate of the coupled OH$^-$(H$^+$) fluxes in the absence of Cl$^-$, and at substrate concentration of 1 mM, net SO$_4^{2-}$ transport by Slc26a2 is about twice faster than net Ox$_2^{-}$ transport. Under normal conditions plasma oxalate is in the micromolar range and even in patients with primary hyperoxaluria plasma oxalate is around 40 µM (53). Moreover, although Slc26a2 is expressed at high level in the luminal membrane of colonic crypts (21), SO$_4^{2-}$ in the colon can be in the millimolar range both in human (54) and animals (55) that will favor SO$_4^{2-}$ uptake by Slc26a2. Indeed, the colon is a major site of SO$_4^{2-}$ absorption (54, 56) that is likely mediated by Slc26a2. Similarly, although Slc26a2 is expressed in the proximal tubule luminal membrane (31), SO$_4^{2-}$ concentration in the proximal tubule is in the millimolar range, and although the role of Slc26a2 in the kidney is not known, if any it is likely to function mainly as an SO$_4^{2-}$ transporter (8). The only possible scenario where Slc26a2 can affect Ox$_2^{-}$ homeostasis is by mediating Ox$_2^{-}$ secretion in exchange for external SO$_4^{2-}$ when external Cl$^-$ is low and pH is high. Even then, this process will be inhibited by the high cytoplasmic Cl$^-$ typical of epithelia and by intracellular SO$_4^{2-}$. Thus Slc26a2 is not likely to play a major role in oxalate metabolism in the colon or the kidney.

The permeation pathway includes the conserved SLC26 transporter Glu$^-$ and may lay between TMD7 and TMD8, where a phenylalanine conserved in the loop predicted to connect the TMDs may control SO$_4^{2-}$ and Cl$^-$ access to the permeation pathway. As yet, mutations of these residues, or even in the vicinity of these residues, have not been found in patients with diastrophic dysplasia (57). This is most likely because Slc26a2 is an essential gene and the mutations that markedly affect Slc26a2 activity may not be compatible with life. Indeed, analysis of several disease causing Slc26a2 mutations showed retention of some SO$_4^{2-}$ transport capacity by the mutants and a good correlation between loss of SO$_4^{2-}$ transport and disease severity (25, 26). The coupling of SO$_4^{2-}$ transport to both OH$^-$ and Cl$^-$ likely serves to ensure transport at both acidic pH when most SO$_4^{2-}$ uptake is mediated by SO$_4^{2-}$/2OH$^-$ exchange and alkaline pH when most SO$_4^{2-}$ uptake is mediated by SO$_4^{2-}$/2Cl$^-$ exchange. Slc26a2 is also regulated by an extracellular anion binding site different from the transport site, the physiological function of which remains to be determined, although it may control SO$_4^{2-}$ uptake when Cl$^-$ is very low.

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