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Published in:
Current Opinion in Biotechnology

DOI:
[10.1016/j.copbio.2018.11.005](https://doi.org/10.1016/j.copbio.2018.11.005)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Henderson, R. K., Fendler, K., & Poolman, B. (2019). Coupling efficiency of secondary active transporters. *Current Opinion in Biotechnology*, 58, 62-71. <https://doi.org/10.1016/j.copbio.2018.11.005>

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Coupling efficiency of secondary active transporters

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Secondary active transporters are fundamental to a myriad of biological processes. They use the electrochemical gradient of one solute to drive transport of another solute against its concentration gradient. Central to this mechanism is that the transport of one does not occur in the absence of the other. However, like in most of biology, imperfections in the coupling mechanism exist and we argue that these are innocuous and may even be beneficial for the cell. We discuss the energetics and kinetics of alternating-access in secondary transport and focus on the mechanistic aspects of imperfect coupling that give rise to leak pathways. Additionally, inspection of available transporter structures gives valuable insight into coupling mechanics, and we review literature where proteins have been altered to change their coupling efficiency.

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Current Opinion in Biotechnology 2019, **58**:62–71

This review comes from a themed issue on **Nanobiotechnology**

Edited by **Giovanni Maglia** and **Wesley R Browne**

<https://doi.org/10.1016/j.copbio.2018.11.005>

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Introduction

Biological membranes are selective barriers that enclose all cells and (most) organelles, separating the internal from the external. A membrane consists of a lipid bilayer and embedded proteins. Membrane bilayers are selectively permeable, with small, nonpolar molecules crossing most readily but ions and other polar molecules being essentially impermeable. This serves to trap biomolecules inside the cell or organelle and defend the cell against the environment. The inability of charged molecules to diffuse easily across the membrane supports asymmetries in the number of molecules, pH, and charge (ionic strength) and allows (electro)chemical gradients to be maintained. Membrane-embedded proteins have evolved transport processes to import and export a wide

variety of solutes across this barrier, including ions, carbohydrates, peptides, drugs, and even folded proteins.

Membrane transport proteins are generally classified as carriers (transporters) or channels. Transporters operate via a series of conformational changes to bind and release a substrate molecule on opposite sides of the membrane, in such a way that a defined substrate-binding site can only be accessed from one side of the membrane at a time. This is known as the ‘alternating-access mechanism’ [1] and is supported by substantial biochemical and structural evidence [2,3,4*]. On the other hand, the substrate of a channel may be solvent-accessible from both sides of the membrane simultaneously and therefore channels only permit the flow of molecules down their concentration gradients [5]. Transporters are sorted into uniporters (facilitators), primary active transporters, and secondary active transporters, each of which utilizes a different energy source to drive transport.

Facilitators transport a solute down its concentration gradient, but are distinct from channels due to their alternating-access mechanism. Active transporters catalyze the translocation of specific substrates across the membrane against their concentration gradients. Primary transporters are driven by metabolic energy source such as ATP. Secondary transporters couple the transport of a specific substrate to the energy stored in the transmembrane electrochemical potential of another solute, typically protons (H^+) or sodium ions (Na^+). Transmembrane proton (ΔpH) or sodium (ΔpNa) gradients are usually generated using primary transporters; ΔpH can also be formed via metabolic reactions. ΔpH and ΔpNa act together with the electrical potential ($\Delta \Psi$) to provide the energy for secondary transport. Coupling substrate translocation to an ion gradient results in uphill transport, which leads to concentration of the substrate on one side of the membrane. Traditionally, secondary transporters fall into three classes: symporters, which transport both the substrate and coupling molecules in the same direction, antiporters, which transport the two molecules in opposite directions, and uniporters, which transport the substrate down its concentration gradient; if the substrate of a transporter carries a charge, the transport will be influenced by the $\Delta \Psi$.

Conformational changes and coupling mechanisms

Alternating-access requires that transporters complete a series of conformational changes between outward-facing and inward-facing states via one or more fully occluded states (Figure 1) [2,3]. Facilitating this cycle are gates that

Figure 1

Conformational changes in the transport cycle of a solute-ion symporter and antiporter. The hallmark of transport by symporters and antiporters is the conformational cycling via an occluded intermediate such that access to the substrate binding site alternates between the two sides of the membrane. These illustrations show a simple rocker-switch mechanism of alternating-access, exemplifying the lactose- H^+ symporter (LacY) and the multidrug/ H^+ antiporter (EmrE), *vide infra*. For symport (left), the occluded intermediate is possible for the apo transporter or when both substrate (pink hexagons) and coupling ion (yellow circles) are bound. Antiport (right), on the other hand, passes through the occluded intermediate when only one of the two substrates is bound.

are distinct in transporters from the concept of ‘gating’ in channels, in which a gate opens or closes the channel pore in response to a stimulus. Instead, transporter gates are structural components of the protein that separate the substrate-binding site from the surroundings at some point in the catalytic cycle [3]. The occluded intermediate is a key property of transporters, whereby the substrate-binding cavity is fully shielded from the aqueous surroundings by protein mass. Structural studies have revealed distinct types of alternating-access mechanisms in secondary transporters (reviewed in Ref. [4*]), and various conformational states are available for proteins exhibiting the MFS fold [6,7*], LeuT fold [8–12], and the SLC1 family [13–19], among others.

In a canonical secondary transporter, conformational changes are tightly regulated to prevent significant uncoupled transport. In symport, the substrate and co-substrate are always transported together, so the protein may only alternate access in either the presence or absence of both substrates. A large energetic barrier should exist when only one of the two is bound. Proton-coupled antiporters such as the Na^+/H^+ antiporter NhaA operate differently in that the sodium ion and protons share a single binding site but move in opposite directions [20]. In the case of metabolite antiporters such as the mitochondrial carriers [21] or the microbial arginine/ornithine antiporters only one of the two substrates is transported at a time; for example arginine in the

inward half reaction and ornithine in the outward half reaction of ArcD [22,23]. Hence, the binary complexes are conformationally mobile and there must be an energetic barrier for isomerization of the carrier in the absence of bound solute. The exact mechanism of coupling may vary from protein to protein, but it is founded in the solute binding and/or translocation steps [24]. For instance, the crystal structures of the Na^+ or H^+ -coupled melibiose transporter MelB from *Salmonella typhimurium* suggest interdependent formation of the discrete sugar-binding and cation-binding sites [25]. On the other hand, sugar binding to the *Escherichia coli* proton-xylose symporter XylE appears to close part of the extracellular gate, but the protein cannot undergo the transition from outward-facing to inward-facing without protonation of a conserved acidic residue (Box 1) [6,26–28]. Alternative proton-coupling mechanisms can exist within single families of transporters, as has been suggested for proton-coupled symporters from the Proton-dependent Oligopeptide Transporter (POT) family [29*] or Sugar Porter (SP) family [30*].

Energetics and kinetics of secondary transport

The two common coupling ions are H^+ and Na^+ , and cells maintain gradients of each across most biological membranes (Box 2). Proton-coupled symport of a neutral solute is driven by the electrochemical proton gradient ($\Delta \sim \mu_{\text{H}^+}$), which is composed of a transmembrane pH

Box 1 The Sugar Porter family: a case study in (un)coupling.

In some proton-coupled symporters, mutation of a single acidic residue to a neutral variant leads intuitively to an ES-leak mutant in which proton-binding can no longer occur in the catalytically-relevant position, thereby eliminating flux through the ternary complex (ESH) and effectively converting a solute-proton symporter into a solute uniporter (Cycle A, Figure 2b). However, such a mutation often leads to transport deficiencies beyond only coupling. For example, in the Sugar Porter (SP) family, which counts both symporters and uniporters among its members, there is a conserved aspartate in TM1 of most symporters that is often an asparagine among the uniporters, implying that this residue is key for proton coupling. Activity is fully abolished in a number of SP symporters upon mutation of this acidic residue [28,82–84]. Recent work with XylE and our own work with Mal11 have demonstrated that conversion of a symporter to a uniporter is not as simple as replacing this residue with a neutral amino acid, as symporters and uniporters have other structural features that affect the thermodynamics and function of the protein [85**]. For Mal11, two additional acidic residues in the central cavity have roles in proton coupling and must be neutral in order for coupling to be fully abolished [30*]. Furthermore, GLUT12 and the *Staphylococcus epidermidis* glucose transporter GlcP_{Se}, which contain an aspartate in the conserved position in TM1, can catalyze both uniport, symport, and partial coupling under different conditions [61*,82,86]. It has been suggested that GlcP_{Se} represents an evolutionary intermediate between uniporters and symporters, as it has a conserved proton-binding site but lacks the pK_a-modulating residue found in XylE [61*].

gradient (ΔpH) and membrane potential ($\Delta\Psi$); the proton motive force (Δp) is represented by the following equation:

$$\Delta p = \frac{\Delta\sim\mu_{H^+}}{F} = \Delta\Psi - \frac{2.3RT}{F} \Delta pH \quad (1)$$

where R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature in Kelvin, and F is the Faraday constant ($9.649 \times 10^4 \text{ C mol}^{-1}$). Naturally, Na^+ -coupled transporters are driven by the sodium motive force (Δs), and the values for the electrochemical sodium gradient ($\Delta\sim\mu_{\text{Na}^+}$) and transmembrane sodium gradient ($\Delta p\text{Na}$) may be substituted into Eq. (1). Also, note that the driving force of transport will change depending on the charge of the substrate and the stoichiometry between substrate and coupling ion [31]. For instance, anionic glutamate-proton symport (overall electroneutral transport) is driven only by the ΔpH , whereas lysine-proton symport is driven by two times $\Delta\Psi$ plus one time ΔpH . The two components ΔpH and $\Delta\Psi$ provide a thermodynamic basis for transport, but also act kinetically on transporters in different capacities. For instance, a recent comparison of lysine uptake into proteoliposomes containing proton-coupled lysine transporters from *Saccharomyces cerevisiae* (Lyp1) or *S. typhimurium* (LysP) showed that transport by Lyp1 was highly dependent on $\Delta\Psi$ regardless of whether a ΔpH was present, whereas transport by LysP could occur with only ΔpH in the absence of $\Delta\Psi$ [32].

Box 2 Comparison of coupling ions.

The predominant ions used for coupling in secondary transport are H^+ and Na^+ . There is no obvious trend relating the coupling ion with the transported solute; a transporter for a given solute may be proton-coupled in one organism and sodium-coupled in another, and both may even be present in the same organism [87]. Indeed, there does not appear to be any predominant energetic advantage to one over the other, except that membranes are more permeable to protons than to sodium ions and it thus is more costly to maintain a ΔpH than a $\Delta p\text{Na}$ especially at higher temperatures [88]. Ultimately, environmental factors such as temperature, salinity, and pH appear to be the most significant in influencing the evolution of cation specificity [87]. For instance, transport in marine organisms is typically coupled to Δs , whereas organisms growing at low pH values, which need to generate a large ΔpH to keep the internal pH around neutral, use the Δp . An additional consideration is that the concentration of protons (pH) can affect a proton-coupled transporter beyond coupling-site saturation, namely that any solvent-exposed titratable residue may become protonated or deprotonated. Such allosteric effects of pH can influence enzyme kinetics or protein stability, leading many secondary transporters to have bell-shaped pH-dependent activity profiles where, regardless of the magnitude of ΔpH , coupled transport rates diminish at high and low pH values [89].

Interestingly, the same family of transporters may include proton-coupled and sodium-coupled transporters, with some proteins able to use both ions interchangeably. This promiscuity has been observed in the mammalian sodium-glucose symporter SGLT1 [90] and perhaps the most striking example of this is the Glycoside-Pentoside-Hexuronide (GPH) family, which is part of the Major Facilitator Superfamily (MFS). Cation selectivity in this family can vary significantly between members and even for the same protein transporting different substrates. The *E. coli* transporter MelB catalyzes transport of melibiose using H^+ , Na^+ , or Li^+ but can only use the latter two for transport of lactose, and the *S. thermophilus* LacS can only catalyze proton-coupled symport of melibiose and lactose, among other substrates [91]. Biochemical data demonstrate that these cations compete for a single binding site in MelB [92] and the crystal structure of MelB from *S. typhimurium*, which shares cation selectivity and more than 85% sequence identity with the *E. coli* MelB [93], reveals cation-binding residues arranged in a trigonal bipyramidal geometry known to bind metals [25,94,95]. Additionally, single amino acid mutations can lead to shifts in cation selectivity or the introduction of leak pathways, as described in the main text [25,91]. The existence of families with both sodium and proton coupling led to the proposal that H_3O^+ could be the transported species rather than H^+ , or at least could play a role in protons binding to, or translocation through, the protein, due to its steric similarity to Na^+ [91,96,97].

The danger of uncontrolled transport

Thermodynamic equilibrium for the accumulation by a symport mechanism of a neutral substrate that is coupled with 1:1 stoichiometry to the electrochemical proton gradient is described by the following equation:

$$0 = \Delta p + Z \log \frac{[\text{solute}]_{in}}{[\text{solute}]_{out}} \quad (2)$$

where $Z = 60 \text{ mV}$. If Δp is -240 mV then 10^4 -fold accumulation of the solute inside the cell is possible if thermodynamic equilibrium is reached. For a protein with perfect coupling, where no substrate or co-substrate is

transported in the absence of the other, accumulation is concentration-independent. At low concentrations, this likely poses no threat to the cells and may be necessary for rapid metabolism. However, at high concentrations, if there are no intervening factors preventing uncontrolled uptake, a potentially lethal osmotic pressure can develop inside the cells. The osmotic pressure difference ($\Delta\pi$) can be calculated with the following equation:

$$\Delta\pi = RT([solute]_{in} - [solute]_{out}) \quad (3)$$

where $R = 0.08206 \text{ L atm/(mol K)}$ and T is absolute temperature (K). A difference in solute concentration between the inside and outside of the cell of 40 mM amounts to a pressure difference of about 1 atm, thus with 10^4 -fold accumulation and 0.05 mM of substrate on the outside the internal concentration would increase the internal concentration to 500 mM and generate an additional 12.2 atm of osmotic pressure.

The phenomenon of excessive accumulation, sometimes referred to as substrate-accelerated death, has been observed during uptake of glycerol [33] or maltose [30*,34] into *S. cerevisiae* and has also been reported in several bacteria for numerous metabolites [35–38]. To counter this, numerous regulation mechanisms are in place to control nutrient uptake at the levels of gene expression and transporter activity. Inactivation or removal of carriers from the membrane is common in many eukaryotic plasma membrane proteins; in *S. cerevisiae*, several sugar transporters undergo rapid inactivation and/or degradation in the presence of glucose [39–42] and several processes regulate the levels of amino acid permeases [43,44]. *E. coli* has similar mechanisms of catabolite-regulated inhibition and repression [45]. Finally, the substrate accumulated by the cell can act as an inhibitor of the transporter, a phenomenon known as *trans*-inhibition [46–48].

Leak pathways

Another strategy to avoid toxic solute levels is by introduction of a transmembrane leak pathway that permits efflux when the intracellular concentration becomes too high [49]. Indeed, a reduction in steady-state accumulation ratio ($[solute]_{in}/[solute]_{out}$) with increasing extracellular substrate concentration is well-documented, including for *E. coli* transport of thiogalactosides [50,51] and arabinose [52], hexose uptake in the eukaryotic *Chlorella vulgaris* [53], sugar transport in *Streptococcus thermophilus* [54], for maltose in yeast membrane vesicles [55], and for some amino acids in yeast [49] and cancerous mouse cells [56,57]. We distinguish between two types of leak pathways: those mediated by the transporter in question, termed ‘internal leaks’, and those that are not, or ‘external leaks’. External leaks may simply be passive diffusion of the substrate across the membrane or may involve other

transporters. Hereafter, we present in detail the internal leak pathways of transporters.

Internal leaks

In the absence of significant passive diffusion or additional transporters, one must conclude that translocation through the protein of interest is responsible for uncoupled efflux of substrate and the apparent reduction of the steady-state accumulation ratio from what would be predicted at thermodynamic equilibrium. Internal leak, also referred to as ‘slippage’, represents a deviation from perfect coupling. As discussed already (Figure 1), a canonical symporter should be able to alternate access between the two sides of the membrane only when neither substrate nor co-substrate are bound and when both substrate and co-substrate are bound, but never when only one is bound in the absence of the other (Figure 2a). Any transport of one without the other would create futile transport cycles and dissipate the electrochemical gradients generated by the cell, an energetic waste that would be disadvantageous in many circumstances. However, slippage could be crucial to proper cell function, or even survival, under conditions of transitory high intracellular accumulation or large transmembrane ion gradients by acting as a sort of safety valve [58]. A variable coupling mechanism may also serve the purpose of enabling additional transport capabilities, as appears to be the case for the POT family transporter PepT_{St} from *S. thermophilus*, which displays proton:peptide stoichiometry ranging from 3:1 to 5:1 depending on the substrate [59*].

The accumulation level achieved by cells or transporters analyzed in membrane vesicles is not necessarily a thermodynamic equilibrium, but rather represents a kinetic steady-state that results in less accumulation than what is predicted by the driving force (Figure 2e). Solute accumulation is therefore dependent on the driving forces acting upon the system, the magnitude of leak pathways, and the kinetic characteristics of the transporter. Mechanisms have been proposed to explain this kinetic steady-state, distinguishing between the two extremes of mobile binary complexes: enzyme-substrate (ES) and enzyme-co-substrate (EH) (Figure 2b and c) [60]. The ES-leak and EH-leak can exhibit overlapping phenotypes under certain conditions, namely that both leak types can transport substrate without co-substrate and *vice versa*. The two types of leaks are not necessarily mutually exclusive, which may further confound experimental interpretation (Figure 2d). Similar kinetic models have been proposed to explain the apparent interconversion between symport and antiport mechanisms in GlcP_{Sc} and EmrE (vide infra) [61*,62**].

The type(s) of leak can be elucidated with detailed kinetic analysis of the protein using different modes of transport (uptake, efflux, and exchange) at a range of substrate concentrations, pH values, and magnitudes of

Figure 2

Dissecting leak pathways in symporters. **(a)** A well-coupled symporter with random order of substrate (S) and co-substrate (H) binding can only make the transition between outward-facing (subscript 'o') and inward-facing (subscript 'i') when neither or both substrates are bound. Leak pathways exist when a transporter can make this transition in a binary complex with either **(b)** the substrate (ES-leak) or **(c)** the coupling substrate (EH-leak). **(d)** A transporter in which both binary complexes can re-orient between inward- and outward-facing conformations. **(e)** Symporter-

driving force [60,63]. A symporter with partial coupling (ES or EH leak) retains the concentrative transport ability of the well-coupled protein but may have less accumulation (Figure 2e). One way to distinguish between the two is that accumulation displays opposite dependence on pH (Figure 2f). An ES leak leads to a sigmoidal decrease in steady-state accumulation level with increasing pH due to the decreased flux through Cycle B (Figure 2b), whereas an EH leak causes a sigmoidal increase in accumulation as function of pH. This is a consequence of the reduced flux through the uncoupled leak pathway due to the lower concentration of protons. Ideally, accumulation by a well-coupled symporter is not influenced by the pH, but allosteric effects of H^+ binding to non-catalytic sites may influence transport (see in Box 2). Additionally, under steady-state conditions of substrate transport (i.e. zero net flux), ES and EH leak transporters have proton leaks that are influenced by the concentration of substrate (Figure 2g). Transporters with an ES leak cannot transport the proton in the absence of substrate, and thus the ion leak increases with increasing substrate concentration as flux through Cycle B (Figure 2b). On the other hand, a symporter with an EH leak has a constitutive proton leak in the absence of substrate due to flux through Cycle C, which is reduced with increasing substrate concentration as flux increases through Cycle D (Figure 2c).

Alteration of leak pathways in symporters

Mutagenesis is an effective tool in the study of coupled transport (Box 1). Mutants with different uncoupled phenotypes increase our understanding of leak pathways and the nature of coupling. LacY is undoubtedly the most extensively mutated secondary transporter and its leak mutants have been categorized based on the observed phenotypes: firstly transport of sugar without protons; secondly transport of protons in the absence of sugar; thirdly proton slippage in the presence of sugar [64]. Mutants of the first phenotype are characterized by a lack of lactose accumulation (energy-coupled transport) while downhill transport is retained, but it is not always clear whether they are ES-leak or EH-leak based on current experimental evidence. The second category, containing mutants including LacY-A177V, can be considered EH-leak mutants and are identified by a reduced Δp in cells expressing these mutant transporters [65]. The third category, best represented by LacY-A177V/K319N, likely fits the ES-leak type model in which sugar is transported into the cell together with protons but subsequently effluxes without protons, leading to reduction

in the sugar concentration gradient but also of the Δp [66,67].

Although many wild-type symporters are well coupled and show no signs of significant leak pathways, substrate slippage is not uncommon. The bacterial proton-galactoside symporter LacS has been shown to contain an ES-leak that is augmented by mutation of Glu-379 [54,63]. LacS-E379D and E379A/Q all have normal downhill uptake but have reduced (E379D) or abolished (E379A/Q) substrate accumulation, thus displaying characteristic uncoupled transport. The hexose transporter from *C. vulgaris*, Hup1, has also been suggested to contain a native substrate leak [68]. Interestingly, coupling is fully abolished in the presence of the antibiotic nystatin, which interacts with sterols in the membrane but does not form pores [69–71]. This may imply the native leak of Hup1 is associated with the presence or absence of sterols. Mutation of a conserved aspartate to glutamate does not amplify the effects of the ES leak as in LacS; rather, Hup1-D44E causes a large shift in the pH dependence of activity from an optimum of pH 4.5 to about pH 7.0 [72]. Cation slippage has been observed in a number of wild-type eukaryotic secondary active transporters [58]. The proton-coupled metal ion transporter DCT1 displays variability in its coupling stoichiometry, ranging from unity to 18 protons per Fe^{2+} [58,73]. Impressively, the single mutation F227I reduced slippage of H^+ without affecting metal transport [74]. By contrast, the low-level proton slip mediated by the human folate symporter PCFT (SLC46A1) significantly increased with the mutation H247A [75]. The opposite was found for several single mutations in LacY, which cause a proton slip in an otherwise well-coupled transporter [76,77].

When antiporters become symporters or vice versa

A well-coupled antiporter should be immobile in its conformational transitions except in the presence of one of the two substrates. Just like for symporters, the canonical is not always the reality, and loose coupling may give rise to promiscuous functions. In the bacterial Cl^-/H^+ antiporter CLC-ec1, variable uncoupling is observed in mutants of the ion-binding residue Tyr-445, ranging from almost total abolishment of proton transport (Y445G) to almost no effect on stoichiometry (Y445F), all of which have comparable Cl^- transport rates as the wildtype [78]. Additionally, the D367N mutant of the Na^+ /multidrug antiporter NorM of *Vibrio parahaemolyticus*

catalyzed uptake of substrate against its electrochemical gradient leads to accumulation of the substrate inside a membrane-bound compartment. The dashed orange line and dashed grey line indicate the thermodynamic equilibrium that may be reached by a well-coupled symporter and a uniporter, respectively. A symporter with an internal leak may reach a lower accumulation level. The presence of a leak pathway (ES or EH) does not lead to thermodynamic equilibrium but instead to a kinetic steady state, as indicated by the dashed yellow line. **(f)** Accumulation by transporters with ES or EH leak are oppositely affected by pH. A well-coupled symporter should not be influenced by pH, as shown by the orange line. **(g)** Under steady-state conditions of substrate transport (i.e. zero net flux), constitutive proton leaks through transporters with ES or EH leaks are influenced differently by the concentration of substrate. A well-coupled symporter should have no substrate or proton leakage, and thus is not shown. The content of this figure has been adapted from [60].

causes partial uncoupling of Na⁺ and ethidium antiport, leading to a sodium leak in the presence of ethidium bromide and a diminished uphill ethidium efflux activity [79]. It has also been reported that deletion of the coupling Glu in EmrE E14D renders the transporter a uniporter [80]. Even more strikingly it has been proposed that wildtype EmrE performs H⁺-substrate symport *in vitro* [62^{••}]. Furthermore, the W63G mutation of EmrE performs both H⁺/erythromycin antiport and bis-Tris propane import, such that the transport mechanism depends on the substrate [81]. Apparent antiport has also been reported for sugar symporter GlcP_{sc} *in vitro* when conditions are favorable, that is, an inwardly-directed sugar gradient and an outwardly-directed proton gradient [61[•]]. In conclusion, even some wildtype transporters can depending on conditions perform symport, antiport or uniport, which is most probably a consequence of their only loosely-coupled transport mechanism [61[•], 62^{••}]. It is unclear whether this behavior serves a biological purpose or is just a mechanistic feature that is not relevant under physiological conditions.

Conclusion

Secondary active transporters are thermodynamic machines that in many cases convert an electrochemical ion gradient into a substrate or product gradient. While they are driven thermodynamically, kinetic information is necessary to fully understand the mechanism of energy coupling. When combined with the growing number of transporter structures, and in particular the same transporter in multiple conformations, detailed mechanistic models become much more attainable. Some systems are well coupled, but many transporters have leak pathways that may serve important biological functions, such as acting as a release for dangerously high intracellular solute levels, or may exist as evolutionary remnants of an energetically-coupled ancestral protein. It is imaginable that strict coupling of symport and antiport is not required in the cell and therefore is not subject of evolutionary optimization. Loose coupling on the other hand may be a kinetic advantage leading to faster transport at the expense of lower accumulation potency [62^{••}]. The leak pathways can be manipulated by mutagenesis to increase or decrease their impact under various conditions. Additional research in the future should focus on acquiring a better understanding of the differences between homologous uniporters, symporters, and antiporters to gain insight into coupling mechanisms and allow for the engineering of interconversion between transport mechanisms.

Transmembrane movement of molecules is obligatory for many bio(nano)technological purposes. Nutrient uptake is obviously a prerequisite for any bioprocess, and bio-conversion of an added substrate to product must begin with uptake into the cell. Understanding the kinetics and energetics of nutrient and end-product transporters will

therefore improve metabolic modeling and engineering of cellular systems. As the understanding of transport mechanisms advances, engineering transporters to eliminate 'futile cycles' that deplete energy reserves will become more feasible, which is important in optimizing industrial strains for higher yield without compromising cell physiology. This may also permit the engineering of cells for (more efficient) product excretion.

Conflict of interest statement

Nothing declared.

Acknowledgments

This work was carried out within the BE-Basic R&D Program, which was granted a FES subsidy from the Dutch Ministry of Economic affairs, agriculture and innovation (EL&I), Netherlands. The research was also funded by a NWO TOP-PUNT, Netherlands (project number 13.006) grant. The authors thank Dirk-Jan Slotboom for critical reading of the manuscript.

References

- Jardetzky O: **Simple allosteric model for membrane pumps.** *Nature* 1966, **211**:969-970.
- Shi Y: **Common folds and transport mechanisms of secondary active transporters.** *Annu Rev Biophys* 2013, **42**:51-72.
- Forrest LR, Krämer R, Ziegler C: **The structural basis of secondary active transport mechanisms.** *Biochim Biophys Acta* 2011, **1807**:167-188.
- Drew D, Boudker O: **Shared molecular mechanisms of membrane transporters.** *Annu Rev Biochem* 2016, **85**:543-572.
- Gadsby DC: **Ion channels versus ion pumps: the principal difference, in principle.** *Nat Rev Mol Cell Biol* 2009, **10**:344-352.
- Yan N: **Structural biology of the major facilitator superfamily transporters.** *Annu Rev Biophys* 2015, **44**:257-283.
- Yan N: **A glimpse of membrane transport through structures—advances in the structural biology of the GLUT glucose transporters.** *J Mol Biol* 2017, **429**:2710-2725.
- A comprehensive review of the GLUT transporters with a detailed comparison of known structures in different conformations and highlighting the differences between the mechanics of uniport and symport.
- Weyand S, Shimamura T, Yajima S, Suzuki S, Mirza O, Krusong K, Carpenter EP, Rutherford NG, Hadden JM, O'Reilly J *et al.*: **Structure and molecular mechanism of a nucleobase-cation-symport-1 family transporter.** *Science* 2008, **322**:709-713.
- Ressl S, Terwisscha van Scheltinga AC, Vonrhein C, Ott V, Ziegler C: **Molecular basis of transport and regulation in the Na⁺/betaine symporter BetP.** *Nature* 2009, **458**:47-52.
- Shimamura T, Weyand S, Beckstein O, Rutherford NG, Hadden JM, Sharples D, Sansom MSP, Iwata S, Henderson PJF, Cameron AD: **Molecular basis of alternating access membrane transport by the sodium-hydantoin transporter Mhp1.** *Science* 2010, **328**:470-473.
- Perez C, Koshy C, Yildiz Ö, Ziegler C: **Alternating-access mechanism in conformationally asymmetric trimers of the betaine transporter BetP.** *Nature* 2012, **490**:126-130.
- Penmatsa A, Gouaux E: **How LeuT shapes our understanding of the mechanisms of sodium-coupled neurotransmitter transporters.** *J Physiol (Lond.)* 2014, **592**:863-869.
- Yernool D, Boudker O, Jin Y, Gouaux E: **Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*.** *Nature* 2004, **431**:811-818.

14. Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E: **Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter.** *Nature* 2007, **445**:387-393.
 15. Reyes N, Ginter C, Boudker O: **Transport mechanism of a bacterial homologue of glutamate transporters.** *Nature* 2009, **462**:880-885.
 16. Jensen S, Guskov A, Rempel S, Hänel I, Slotboom DJ: **Crystal structure of a substrate-free aspartate transporter.** *Nat Struct Mol Biol* 2013, **20**:1224-1226.
 17. Guskov A, Jensen S, Faustino I, Marrink SJ, Slotboom DJ: **Coupled binding mechanism of three sodium ions and aspartate in the glutamate transporter homologue GltTk.** *Nat Commun* 2016, **7**:13420.
 18. Canul-Tec JC, Assal R, Cirri E, Legrand P, Brier S, Chamot-Rooke J, Reyes N: **Structure and allosteric inhibition of excitatory amino acid transporter 1.** *Nature* 2017, **544**:446-451.
 19. Garaeva AA, Oostergetel GT, Gati C, Guskov A, Paulino C, Slotboom DJ: **Cryo-EM structure of the human neutral amino acid transporter ASCT2.** *Nat Struct Mol Biol* 2018, **25**:515-521.
 20. Călinescu O, Fendler K: **A universal mechanism for transport and regulation of CPA sodium proton exchangers.** *Biol Chem* 2015, **396**:1091-1096.
 21. Kunji ERS, Aleksandrova A, King MS, Majd H, Ashton VL, Cerson E, Springett R, Kibalchenko M, Tavoulari S, Crichton PG et al.: **The transport mechanism of the mitochondrial ADP/ATP carrier.** *Biochim Biophys Acta* 2016, **1863**:2379-2393.
 22. Driessen AJ, Poolman B, Kiewiet R, Konings W: **Arginine transport in *Streptococcus lactis* is catalyzed by a cationic exchanger.** *Proc Natl Acad Sci U S A* 1987, **84**:6093-6097.
 23. Driessen AJ, Molenaar D, Konings WN: **Kinetic mechanism and specificity of the arginine-ornithine antiporter of *Lactococcus lactis*.** *J Biol Chem* 1989, **264**:10361-10370.
 24. Heinz E, Geck P, Wilbrandt W: **Coupling in secondary active transport. Activation of transport by co-transport and/or counter-transport with the fluxes of other solutes.** *Biochim Biophys Acta* 1972, **255**:442-461.
 25. Ethayathulla AS, Yousef MS, Amin A, Leblanc G, Kaback HR, Guan L: **Structure-based mechanism for Na⁺/melibiose symport by MelB.** *Nat Commun* 2014, **5**.
 26. Sun L, Zeng X, Yan C, Sun X, Gong X, Rao Y, Yan N: **Crystal structure of a bacterial homologue of glucose transporters GLUT1-4.** *Nature* 2012, **490**:361-366.
 27. Quistgaard EM, Löw C, Moberg P, Trésaugues L, Nordlund P: **Structural basis for substrate transport in the GLUT-homology family of monosaccharide transporters.** *Nat Struct Mol Biol* 2013, **20**:766-768.
 28. Wisedchaisri G, Park M-S, Iadanza MG, Zheng H, Gonen T: **Proton-coupled sugar transport in the prototypical major facilitator superfamily protein XylE.** *Nat Commun* 2014, **5**:4521.
 29. Parker JL, Li C, Brintha A, Wang Z, Vogeley L, Solcan N, Ledderboge-Vucinic G, Swanson JMJ, Caffrey M, Voth GA et al.: **Proton movement and coupling in the POT family of peptide transporters.** *Proc Natl Acad Sci U S A* 2017, **114**:13182-13187.
- The authors combine biochemical and biophysical data with molecular dynamics to demonstrate differences in proton-coupling between members of the POT family.
30. Henderson R, Poolman B: **Proton-solute coupling mechanism of the maltose transporter from *Saccharomyces cerevisiae*.** *Sci Rep* 2017, **7**:14375.
- A biochemical study revealing that the proton-coupled maltose transport by Mal11 is retained when one or two acidic residues central cavity are mutated, but that mutation of all three yields a fully uncoupled maltose uniporter. Also, demonstrates cell lysis upon uncontrolled uptake of maltose.
31. Poolman B, Konings WN: **Secondary solute transport in bacteria.** *Biochim Biophys Acta* 1993, **1183**:5-39.
 32. Bianchi F, Klooster JSVT, Ruiz SJ, Luck K, Pols T, Urbatsch IL, Poolman B: **Asymmetry in inward- and outward-affinity constant of transport explain unidirectional lysine flux in *Saccharomyces cerevisiae*.** *Sci Rep* 2016, **6**:31443.
 33. Indge K, Seaston A, Eddy AA: **The concentration of glycine by *Saccharomyces uvarum*: role of the main vacuole and conditions leading to the explosive absorption of the amino acid.** *J Gen Microbiol* 1977, **99**:243-255.
 34. Postma E, Verduyn C, Kuiper A, Scheffers WA, Van Dijken JP: **Substrate-accelerated death of *Saccharomyces cerevisiae* CBS 8066 under maltose stress.** *Yeast* 1990, **6**:149-158.
 35. Postgate JR, Hunter JR: **Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates.** *J Gen Microbiol* 1964, **34**:459-473.
 36. Strange RE, Dark FA: **"Substrate-accelerated death" of *Aerobacter aerogenes*.** *J Gen Microbiol* 1965, **39**:215-228.
 37. Strange RE, Hunter JR: **"Substrate-accelerated death" of nitrogen-limited bacteria.** *J Gen Microbiol* 1966, **44**:255-262.
 38. Calcott PH, Postgate JR: **On substrate-accelerated death in *Klebsiella aerogenes*.** *J Gen Microbiol* 1972, **70**:115-122.
 39. Brondijk TH, van der Rest ME, Pluim D, de Vries Y, Stingl K, Poolman B, Konings WN: **Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae*.** *J Biol Chem* 1998, **273**:15352-15357.
 40. Medintz I, Wang X, Hradek T, Michels CA: **A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity.** *Biochemistry* 2000, **39**:4518-4526.
 41. Horak J, Wolf DH: **Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination, endocytosis, and degradation in the vacuole.** *J Bacteriol* 1997, **179**:1541-1549.
 42. Horak J: **Regulations of sugar transporters: insights from yeast.** *Curr Genet* 2013, **59**:1-31.
 43. Lin CH, MacGurn JA, Chu T, Stefan CJ, Emr SD: **Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface.** *Cell* 2008, **135**:714-725.
 44. Hinnebusch AG, Natarajan K: **Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress.** *Eukaryot Cell* 2002, **1**:22-32.
 45. Görke B, Stülke J: **Carbon catabolite repression in bacteria: many ways to make the most out of nutrients.** *Nat Rev Microbiol* 2008, **6**:613-624.
 46. Adelman JL, Ghezzi C, Bisignano P, Loo DDF, Choe S, Abramson J, Rosenberg JM, Wright EM, Grabe M: **Stochastic steps in secondary active sugar transport.** *Proc Natl Acad Sci U S A* 2016, **113**:E3960-6.
 47. Johnson E, Nguyen PT, Yeates TO, Rees DC: **Inward facing conformations of the MetNI methionine ABC transporter: implications for the mechanism of transinhibition.** *Protein Sci* 2012, **21**:84-96.
 48. Gerber S, Comellas-Bigler M, Goetz BA, Locher KP: **Structural basis of trans-inhibition in a molybdate/tungstate ABC transporter.** *Science* 2008, **321**:246-250.
 49. Eddy AA: **Slip and leak models of gradient-coupled solute transport.** *Biochem Soc Trans* 1980, **8**:271-273.
 50. Buttin G, Cohen GN, Monod J, Rickenberg HV: **Galactoside-permease of *Escherichia coli*.** *Ann Inst Pasteur (Paris)* 1956, **91**:829-857.
 51. Kepes A: **Kinetic studies on galactoside permease of *Escherichia coli*.** *Biochim. Biophys Acta* 1960, **40**:70-84.
 52. Novotny CP, Englesberg E: **The L-arabinose permease system in *Escherichia coli* B/r.** *Biochim Biophys Acta* 1966, **117**:217-230.
 53. Komor E, Haass D, Komor B, Tanner W: **The active hexose-uptake system of *Chlorella vulgaris*. Km-values for 6-deoxyglucose influx and efflux and their contribution to sugar accumulation.** *Eur J Biochem* 1973, **39**:193-200.

54. Foucaud C, Poolman B: **Lactose transport system of *Streptococcus thermophilus*. Functional reconstitution of the protein and characterization of the kinetic mechanism of transport.** *J Biol Chem* 1992, **267**:22087-22094.
 55. Van Leeuwen CC, Weusthuis RA, Postma E, Van den Broek PJ, Van Dijken JP: **Maltose/proton co-transport in *Saccharomyces cerevisiae*. Comparative study with cells and plasma membrane vesicles.** *Biochem J* 1992, **284**(Pt. 2):441-445.
 56. Heinz E, Mariani HA: **Concentration work and energy dissipation in active transport of glycine into carcinoma cells.** *J Biol Chem* 1957, **228**:97-111.
 57. Hacking C, Eddy AA: **The accumulation of amino acids by mouse ascites-tumour cells. Dependence on but lack of equilibrium with the sodium-ion electrochemical gradient.** *Biochem J* 1981, **194**:415-426.
 58. Nelson N, Sacher A, Nelson H: **The significance of molecular slips in transport systems.** *Nat Rev Mol Cell Biol* 2002, **3**:876-881.
 59. Parker JL, Mindell JA, Newstead S: **Thermodynamic evidence for a dual transport mechanism in a POT peptide transporter.** *eLife* 2014, **3**.
- Biophysical study showing variable stoichiometry of proton-peptide symport depending on the substrate transported.
60. Lolkema JS, Poolman B: **Uncoupling in secondary transport proteins. A mechanistic explanation for mutants of lac permease with an uncoupled phenotype.** *J Biol Chem* 1995, **270**:12670-12676.
 61. Bazzone A, Zabadne AJ, Salisowski A, Madej MG, Fendler K: **A loose relationship: incomplete H⁺/sugar coupling in the MFS sugar transporter GlcP.** *Biophys J* 2017, **113**:2736-2749.
- The authors show that GlcP_{se} is a loosely-coupled glucose-proton symporter and can carry out apparent symport, antiport, or uniport depending on the conditions.
62. Robinson AE, Thomas NE, Morrison EA, Balthazor BM, Henzler-Wildman KA: **New free-exchange model of EmrE transport.** *Proc Natl Acad Sci U S A* 2017, **114**:E10083-E10091.
- The authors use NMR to probe the conformational changes in the presence and absence of EmrE substrates and demonstrate that "forbidden" changes for a canonical antiporter are in fact possible for this protein, indicating this transporter should be able to carry out symport, antiport, and uniport under the right conditions.
63. Poolman B, Knol J, Lolkema JS: **Kinetic analysis of lactose and proton coupling in Glu379 mutants of the lactose transport protein of *Streptococcus thermophilus*.** *J Biol Chem* 1995, **270**:12995-13003.
 64. Varela MF, Wilson TH: **Molecular biology of the lactose carrier of *Escherichia coli*.** *Biochim Biophys Acta* 1996, **1276**:21-34.
 65. King SC, Wilson TH: **Characterization of *Escherichia coli* lactose carrier mutants that transport protons without a cosubstrate. Probes for the energy barrier to uncoupled transport.** *J Biol Chem* 1990, **265**:9645-9651.
 66. Brooker RJ: **An analysis of lactose permease "sugar specificity" mutations which also affect the coupling between proton and lactose transport. I. Val177 and Val177/Asn319 permeases facilitate proton uniport and sugar uniport.** *J Biol Chem* 1991, **266**:4131-4138.
 67. Eelkema JA, O'Donnell MA, Brooker RJ: **An analysis of lactose permease "sugar specificity" mutations which also affect the coupling between proton and lactose transport. II. Second site revertants of the thiodigalactoside-dependent proton leak by the Val177/Asn319 permease.** *J Biol Chem* 1991, **266**:4139-4144.
 68. Tanner W: **The *Chlorella* hexose/H⁺-symporters.** *Int Rev Cytol* 2000, **200**:101-141.
 69. Komor B, Komor E, Tanner W: **Transformation of a strictly coupled active transport system into a facilitated diffusion system by nystatin.** *J Membr Biol* 1974, **17**:231-238.
 70. Opekarová M, Tanner W: **Nystatin changes the properties of transporters for arginine and sugars. An in vitro study.** *FEBS Lett* 1994, **350**:46-50.
 71. Opekarová M, Urbanová P, Konopásek I, Kvasnicka P, Strzalka K, Sigler K, Amler E: **Possible nystatin-protein interaction in yeast plasma membrane vesicles in the presence of ergosterol. A Förster energy transfer study.** *FEBS Lett* 1996, **386**:181-184.
 72. Will A, Grassl R, Erdmenger J, Caspari T, Tanner W: **Alteration of substrate affinities and specificities of the *Chlorella* hexose/H⁺ + symporters by mutations and construction of chimeras.** *J Biol Chem* 1998, **273**:11456-11462.
 73. Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA: **Cloning and characterization of a mammalian proton-coupled metal-ion transporter.** *Nature* 1997, **388**:482-488.
 74. Nevo Y, Nelson N: **The mutation F227I increases the coupling of metal ion transport in DCT1.** *J Biol Chem* 2004, **279**:53056-53061.
 75. Unal ES, Zhao R, Chang M-H, Fiser A, Romero MF, Goldman ID: **The functional roles of the His247 and His281 residues in folate and proton translocation mediated by the human proton-coupled folate transporter SLC46A1.** *J Biol Chem* 2009, **284**:17846-17857.
 76. Franco PJ, Brooker RJ: **Functional roles of Glu-269 and Glu-325 within the lactose permease of *Escherichia coli*.** *J Biol Chem* 1994, **269**:7379-7386.
 77. Matzke EA, Stephenson LJ, Brooker RJ: **Functional role of arginine 302 within the lactose permease of *Escherichia coli*.** *J Biol Chem* 1992, **267**:19095-19100.
 78. Walden M, Accardi A, Wu F, Xu C, Williams C, Miller C: **Uncoupling and turnover in a Cl⁻/H⁺ exchange transporter.** *J Gen Physiol* 2007, **129**:317-329.
 79. Otsuka M, Yasuda M, Morita Y, Otsuka C, Tsuchiya T, Omote H, Moriyama Y: **Identification of essential amino acid residues of the NorM Na⁺/multidrug antiporter in *Vibrio parahaemolyticus*.** *J Bacteriol* 2005, **187**:1552-1558.
 80. Yerushalmi H, Schuldiner S: **An essential glutamyl residue in EmrE, a multidrug antiporter from *Escherichia coli*.** *J Biol Chem* 2000, **275**:5264-5269.
 81. Brill S, Falk OS, Schuldiner S: **Transforming a drug/H⁺ antiporter into a polyamine importer by a single mutation.** *Proc Natl Acad Sci U S A* 2012, **109**:16894-16899.
 82. Iancu CV, Zamoon J, Woo SB, Aleshin A, Choe J-Y: **Crystal structure of a glucose/H⁺ symporter and its mechanism of action.** *Proc Natl Acad Sci U S A* 2013, **110**:17862-17867.
 83. Caspari T, Stadler R, Sauer N, Tanner W: **Structure/function relationship of the *Chlorella* glucose/H⁺ symporter.** *J Biol Chem* 1994, **269**:3498-3502.
 84. Henderson PJ, McDonald TP, Steel A, Litherland GJ, Cairns MT, Martin GE: **The variability of kinetic parameters for sugar transport in different mutants of the galactose-H⁺ symport protein, GalP, of *Escherichia coli*.** *Biochem Soc Trans* 1994, **22**:643-646.
 85. Ke M, Yuan Y, Jiang X, Yan N, Gong H: **Molecular determinants for the thermodynamic and functional divergence of uniporter GLUT1 and proton symporter XylE.** *PLoS Comput Biol* 2017, **13**:e1005603.
- A computational study in which the authors model the thermodynamics of a symporter and a uniporter and identify several amino acid changes that lead to interconversion between the two transport mechanisms.
86. Wilson-O'Brien AL, Patron N, Rogers S: **Evolutionary ancestry and novel functions of the mammalian glucose transporter (GLUT) family.** *BMC Evol Biol* 2010, **10**:152.
 87. Lolkema JS, Speelmans G, Konings WN: **Na⁺-coupled versus H⁺-coupled energy transduction in bacteria.** *Biochim Biophys Acta* 1994, **1187**:211-215.
 88. Speelmans G, Poolman B, Abee T, Konings WN: **Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions.** *Proc Natl Acad Sci U S A* 1993, **90**:7975-7979.

89. Poolman B, Driessen AJ, Konings WN: **Regulation of solute transport in streptococci by external and internal pH values.** *Microbiol Rev* 1987, **51**:498-508.
90. Hirayama BA, Loo DD, Wright EM: **Protons drive sugar transport through the Na⁺/glucose cotransporter (SGLT1).** *J Biol Chem* 1994, **269**:21407-21410.
91. Poolman B, Knol J, van der Does C, Henderson PJ, Liang WJ, Leblanc G, Pourcher T, Mus-Veteau I: **Cation and sugar selectivity determinants in a novel family of transport proteins.** *Mol Microbiol* 1996, **19**:911-922.
92. Damiano-Forano E, Bassilana M, Leblanc G: **Sugar binding properties of the melibiose permease in Escherichia coli membrane vesicles. Effects of Na⁺ and H⁺ concentrations.** *J Biol Chem* 1986, **261**:6893-6899.
93. Guan L, Nurva S, Ankeshwarapu SP: **Mechanism of melibiose/cation symport of the melibiose permease of Salmonella typhimurium.** *J Biol Chem* 2011, **286**:6367-6374.
94. Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E: **Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters.** *Nature* 2005, **437**:215-223.
95. Kovalevsky AY, Hanson BL, Mason SA, Yoshida T, Fisher SZ, Mustyakimov M, Forsyth VT, Blakeley MP, Keen DA, Langan P: **Identification of the elusive hydronium ion exchanging roles with a proton in an enzyme at lower pH values.** *Angew Chem Int Ed Engl* 2011, **50**:7520-7523.
96. Boyer PD: **Bioenergetic coupling to protonmotive force: should we be considering hydronium ion coordination and not group protonation?** *Trends Biochem Sci* 1988, **13**:5-7.
97. Smirnova I, Kasho V, Sugihara J, Choe J-Y, Kaback HR: **Residues in the H⁺ translocation site define the pKa for sugar binding to LacY.** *Biochemistry* 2009, **48**:8852-8860.