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Commentary

Transport bicycles

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Like a Hollywood action film, the *Escherichia coli lac* permease has a few stars and many extras. The paper by Kaback (1) in this issue of the *Proceedings* points out that only 4 of 417 residues are irreplaceable actors in the mechanism that transduces a proton electrochemical gradient into a lactose concentration gradient. These four are two pairs of interacting residues: Glu-269 in membrane-spanning segment 8 (M8) and His-322 (M10), and Arg-302 (M9) and Glu-325 (M10). Mutation of any one of these residues eliminates proton-driven uptake of lactose. Mutation of only one, Glu-325, eliminates all partial transport functions involving protons. A specific substrate binding site with alternating high and low affinity is an indispensable part of every active transport mechanism. In the *lac* permease, Glu-325 is such a site for H^+, with changing pK_a. Glu-325 may also participate in a second indispensable part of the mechanism, alternating access to the two sides of the membrane.

Uphill transport of one substrate (the driven substrate) can be coupled to the dissipation of an electrochemical potential gradient of another substrate (the driving substrate), to an exergonic chemical reaction, or to a photoreaction. In all cases, the driven substrate is taken up from a low concentration (or relatively low electrochemical potential) on one side of the membrane and bound to a high-affinity site; the driving energy is used both to raise the free energy of the binding complex—it is converted to a low-affinity state—and to switch the access of the site to the other side of the membrane, to which the substrate is released into a high concentration (or relatively high electrochemical potential). When the free energy source is the downhill transport of a driving substrate, it must be taken up by its own specific site in a low-affinity state, from the side of the membrane on which its electrochemical potential is high, and released from its site in a high-affinity state, to the side on which its electrochemical potential is low. In general, the "driven cycle" of affinities and accessibilities is tightly coupled to the "driving cycle" of chemical or photochemical reactions or of downhill transport. Characteristically, under the appropriate transmembrane gradients, coupled transporters can be reversed; the substrate normally driven becomes the driver.

The structural basis for coupled transport is best understood in the light-driven proton pump, bacteriorhodopsin (2–4). The Schiff base formed between retinal and Lys-216, in the middle of the membrane-spanning domain, is the primary H^+-binding site. The light-driven isomerization of all-*trans* to 13-*cis* retinylidene powers the pump. On photoisomerization of the retinylidene, the pK_a of the protonated Schiff base drops by about 7 units. The proton is transferred to the extracellular side of the membrane, not in one big jump, but in three steps, and each of these steps involves changes in proton affinities. With the drop in the pK_a of the Schiff base, the pK_a of Asp-85 rises markedly, possibly because of a drop in the local dielectric constant (5), and a proton is transferred from the Schiff base to Asp-85. By interactions in which Arg-82 has a major role, protonation of Asp-85 leads to a decrease in the pK_a of a residue closer to the extracellular side of the membrane, identified as Glu-204 (6–8). Glu-204, which was protonated in the previous photocycle, now loses its proton to the extracellular side of the membrane. The pK_a of the Schiff base rises, and it is reprotonated from the intracellular side by transfer from Asp-96, the pK_a of which has fallen. The pK_a of Asp-96 rises again, it is reprotonated from the cytoplasm, and retinylidene isomerizes back to all-*trans*. With the requisite mirror changes in pK_a, Asp-85 transfers its proton to Glu-204, and thus the transport cycle completes the transfer of one proton from the cytoplasm to the extracellular medium.

What determines the direction of this transport? Are gates involved? Directionality must arise primarily from the switching of the accessibility of the Schiff base coordinated with the alternating of its proton affinity. How the accessibility is determined, however, is not known. All four helices that line the proton pathway in bacteriorhodopsin participate in structural changes, and these are likely to be involved in the coordinated changes in the accessibility from the two sides and in the affinities of the primary and secondary proton binding sites (2, 9). One possibility for gates would be the binding sites themselves, for if the transport were single-file, strictly from cytoplasm to Asp-96 to Schiff base to Asp-85 to Glu-204 to extracellular medium, then an occupied site with a high pK_a could act as a closed gate. After photoisomerization of the retinylidene, the Schiff base could only transfer its proton to Asp-85 in the extracellular direction because Asp-96 would be protonated already. Also, Glu-204 could transfer its proton only to the extracellular medium and not to the protonated Asp-85. The protonation of Glu-204 by transfer from Asp-85, and not from the relatively acidic extracellular medium, however, would require another means to make the first occur much faster than the second. Similar considerations apply to the proton transfer from Asp-96 to the Schiff base and not to the relatively alkaline cytoplasm. We fall back on the movements of the helices lining the pathway to create and remove obstacles to diffusion.

A clear example of a gate was found in the photoactive yellow protein (PYP), a cytosolic blue-light photoreceptor of *Ectothiorhodospira halophila* (10). PYP contains a 4-hydroxycinnamyl chromophore that photoisomerizes during the photocycle. As determined by time-resolved, multiwavelength Laue x-ray diffraction of PYP, in the trans state of the chromophore, the chromophoric phenolic oxygen is buried and deprotonated! In the excited *cis* state, the anionic phenolic oxygen moves toward the surface of the protein, and a covering Arg residue (the "arginine gateway") also moves, allowing solvent exposure and protonation of the oxygen. "X-ray movies" (when available) tend to limit speculation.

The mechanism of proton pumping by bacteriorhodopsin is not likely to be based on the unique properties of protons in binding or diffusion. Halorhodopsin is a related protein also found in *Halobacterium salinarum.* On photoisomerization of all-*trans* retinylidene to 13-*cis* retinylidene, halorhodopsin pumps Cl^- from the extracellular side to the intracellular side of the membrane. In halorhodopsin, a Thr replaces bacteriorhodopsin Asp-85, and the Schiff base does not deprotonate on photoisomerization of the retinylidene; rather, Cl^- is likely

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bound by the positively charged Schiff base and H-bonded to the Thr hydroxyl. The Asp-85 → Thr mutant of bacteriorhodopsin, like halorhodopsin, transports Cl^- from the extracellular side to the intracellular side of the membrane (11). Under certain conditions, the same mutant will also pump protons, but from the intracellular to the extracellular side (4). Conversely, when azide is bound to the Cl^--binding site of halorhodopsin from *Natronobacterium pharaonis*, this protein undergoes a bacteriorhodopsin-like photocycle in which H^+ is pumped from the intracellular side to the extracellular side (12). Thus, the same pathway can be used by a cation and an anion, but transport is in opposite directions. A kinetic model, in which the rates of ion uptake and loss by the Schiff base and the rates of accessibility changes are independent and the rates of these two processes for H^+ and for Cl^- are different, can account for the opposite directions of transport (4). It comes down to whether the transfer occurs before or after the switch in accessibility. This model could be extended to include secondary binding sites on either side of the Schiff base that also switch affinities and accessibilities.

The versatility of proton transporters is evident also in the F_1F_o–ATP synthases (13). These convert a proton electrochemical potential gradient generated by electron transport into the synthesis of ATP. The extramembranous hexameric head of F_1 synthesizes ATP, the membrane-embedded F_o complex transports protons, and the two are connected by a stalk. The downhill flux of protons through F_o is coupled to the rotation of the head relative to the stalk. This rotation cycles the binding specificities of the three pseudosymmetrical binding sites in the F_1 head from empty to ADP plus P_i to ATP, thereby driving the synthesis and release of ATP (14–16). The head and stalk form the tiniest known rotary motor: the γ subunit of the stalk has been observed to rotate at 4 Hz relative to the F_1 head, powered by the hydrolysis of ATP (17, 18). The F_o **c** subunit, which is present in 9 to 12 copies, is small and hydrophobic. In chloroform–methanol–water, it forms a hairpin of two helices (13). Asp-61, in one of the helices, is a crucial residue for H^+ transport and is predicted to cycle between high and low pK_a values. Arg-210 and other residues in F_o subunit **a** may interact with subunit **c** Asp-61 during proton translocation. Once again, with small adjustments, this transporter will run on the electrochemical potential gradient of another ion, in this case Na^+ (19).

Are coupled transporters channels or carriers? This is not a question of whether transporters are like gramicidin A, the prototypic channel, or like valinomycin, the prototypic diffusing carrier. Neither *lac* permease nor bacteriorhodopsin moves across the membrane. Furthermore, segments of transmembrane pathways are evident in the high-resolution structure of bacteriorhodopsin (2). In the high-resolution structure of cytochrome *c* oxidase, specifically in subunit I, which forms the core of the complex and is responsible for the redox-coupled pumping of protons, three channels are seen (20, 21). The question is what controls the alternating access of the substrate binding sites in the transport pathway. Does the binding site move across a permeability barrier no matter how thin (carrier mechanism), or does substrate hop from site to site with accessibility controlled by gates (channel mechanism)? Evidence for a channel mechanism, access to which is controlled by gates, was obtained in the Na^+- and Cl^--coupled transporter for the neurotransmitter γ-aminobutyric acid. Patch-clamp recording revealed unitary current events that are the signature of the brief openings of a single channel (22). The open probability for these events was about 10^{-6}, and they occurred on average once per 500 transport cycles. They may have been the manifestation of the rare state of the transporter when all (at least two) gates were open simultaneously.

The water-accessible channels through transporters and the binding sites in these channels are being mapped by the mutation of residues in membrane-spanning segments to Cys and to His and by testing the accessibility of these substituted residues to charged, hydrophilic reagents or metal ions. In UhpT, the antiporter in *E. coli* that exchanges glucose-6-phosphate for phosphate, M7, 1 of 12 membrane-spanning segments, was mutated and probed with p-chloromercuribenzenesulfonate (23). Of 29 Cys-substitution mutants tested, 2 residues were accessible only from the inside, 4 were accessible only from the outside, and 6 in the middle were accessible from both sides. The middle 6 mutants were protected from reaction by glucose-6-phosphate. These results allow the interpretation that M7 is exposed in the pathway for glucose-6-phosphate and that the protectable residues are associated with the binding site(s) (with the caveat that protection could be indirect), which are alternately accessible from the two sides of the membrane. In *lac* permease, Cys-148 (M5) and Cys-substitution mutants of Met-145 (M5) and of Val-264, Gly-268, and Asn-272 (M8) are protected by β-galactosides against reaction with *N*-ethylmaleimide (1). Cys-148 is accessible from both sides of the membrane. These residues are likely to be exposed in the pathway for lactose and to contribute to its binding site(s). Furthermore, the binding of Mn^{2+} by pairs of substituted His residues established not only the proximity of residues but also the accessibility of the residues to a hydrophilic probe.

If the lactose pathway in *lac* permease is lined by M5 and M8 and the H^+ pathway is lined by M9 and M10 (1), then there may be two separate pathways, one for the driving substrate and one for the driven substrate, each with gates, binding sites, and a cycle of alternating affinities and accessibilities. The wondrous subtlety of such pathways and linked cycles deserves and requires our close attention and our greatest ingenuity, so evident in the work discussed here.

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