

Proton reactions: From basic science to biomedical applications

Proton transfer is ubiquitous in biology. Specialized proteins—channels, pumps, and antiporters—transfer protons across cell membranes to establish proton electrochemical gradients needed to power energetically costly chemical reactions and perform myriad other tasks. Other proteins, enzymes, transfer protons locally during the chemical reaction they catalyze. How such proton transfers occur is a fundamental issue with implications for biomedical and biotechnological research. The *Biophysical Journal* special issue “Proton reactions: From basic science to biomedical applications” arises from the Biophysical Society conference that took place in Tahoe, California, in August 2023. This special issue contains 16 papers on provocative new developments in this exciting field. In the spirit of the conference, this special issue showcases diverse experimental and methodological approaches that together highlight the fundamental roles of protons in biology and how the importance of studying protons in biology furthers methodological developments. Topics addressed in the articles in the special issue include methodologies for experimental measurements of the proton motive force and using proton motive force estimations to understand how proton pumps work; advanced computational strategies to evaluate key properties of membrane transporters; water-mediated proton-transfer paths in complex protein environments and proton-coupled substrate transfer across biological membranes; the role of protein-water H-bond networks in membrane protein folding and function; proton channels, proton pumps, and lateral proton transfer across biomembranes; and protonation-coupled membrane receptors and protonation-coupled protein-membrane interactions.

The inner mitochondrial membrane hosts proton pumps that generate proton electrochemical gradients required for the function of ATP synthases and other proteins (1). As Kowaltowski and Abdulkader note in their review article, experimental measurements of the mitochondrial electrochemical gradient, or proton motive force, are of strong

and renewed interest because of the mitochondria being implicated in numerous human pathologies (2). The proton motive force, Δp , has two components, the chemical component, ΔpH , and the charge component, $\Delta\psi_m$, the transmembrane potential. With the ΔpH contributing between 3% and 32% to Δp , $\Delta\psi_m$ is the major determinant of the driving force for ATP synthesis (2), and the review article is primarily focused on how to measure, and how (and when) not to measure, $\Delta\psi_m$ by using fluorescent inner mitochondrial membrane probes. One potential caveat of $\Delta\psi_m$ measurements that use cationic lipophilic molecules as fluorescent probes is that they may accumulate within the mitochondria and interfere with chemical reactions, such that more accurate measurements are recommended (2).

NADH:ubiquinone oxidoreductase, also known as the respiratory complex I, is a large enzyme located in mitochondrial and bacterial membranes, where it couples the redox reaction of NADH oxidation and ubiquinone reduction (for which complex I uses two protons) with the pumping of four protons across the membrane; the resulting proton gradient will be used by ATP synthase. How this highly complex machinery works remains an open question. Crystal structures provide invaluable information about the architecture and key intramolecular interactions; however, as Wang and colleagues suggest in their article in this special issue (3), formation of proton-transfer paths may be only transient and require structural rearrangements. To decipher the mechanism by which complex I pumps protons, Wang and colleagues develop a computational strategy that makes it possible to identify potential proton-transfer channels (3). A Voronoi partitioning scheme applied to the protein structure reveals an extensive network of channels in which water molecules are then placed using the Dowsner++ software developed in their laboratory and molecular dynamics. This reveals an entire network of putative proton channels, some wide enough to host water; others could become open with minor structural rearrangements (3).

Phosphate-proton symporters are members of the major facilitator superfamily, which includes the well-studied lactose permease LacY from *Escherichia coli*, a protein that uses an alternate access mechanism for the cotransport

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of protons and galactopyranoside (4). Plants and fungi use phosphate-proton symporters for phosphate uptake. We know relatively little about the mechanism of action of these symporters. In this issue, Liu and colleagues develop a kinetic model to explain proton-coupled phosphate transport by PiPT, the phosphate transporter from *Piriformospora indica*, and eukaryotic fungus (5). The kinetic model by Liu and colleagues is based on extensive computations using distinct approaches, including multiscale reactive molecular dynamics combined with enhanced sampling to study proton release, quantum mechanical/molecular mechanical computations, classical mechanical simulations of the membrane-embedded transporter, and computations of the potential of mean force of proton transfer and phosphate release. The model reveals that the optimal pH for the functioning of PiPT depends on the concentration of phosphate at the extracellular side of the membrane and that the molecular origin of the optimal pH arises from “the optimal balance” between the protonation states of phosphate and a key aspartic residue (5).

Protons released by a proton pump may dwell on the membrane surface before equilibration in the aqueous solution (6,7); protons may also migrate along the membrane for long distances, on the order of tens of micrometers (8). This process of lateral proton transfer is essential for bioenergetics because the membrane may function as “a proton-conducting link between membrane-spanning proton transporters” (9). Now, Variyam and colleagues combine simulations with steady-state and time-resolved spectroscopy experiments to track excited-state proton transfer; they find that the structure of the membrane, which depends on the lipid membrane composition, significantly influences proton diffusion around the probe (10). In membranes composed of phosphatidylcholine (PC) and phosphatidic acid (PA), the efficiency of proton transfer had an anomalous dependence on the PC:PA ratio, possibly because of the effect of PA lipids on the membrane structure and water wires at the membrane interface (10).

The voltage-gated proton channels are unique in that they use the same four-transmembrane helical scaffold for voltage sensing, gating, and proton transfer (11,12), and their voltage-dependent gating depends on the pH gradient such that channel opening results in acid extrusion (13). Proton selectivity requires an internal aspartic acid located at approximately the middle of the membrane plane (14). Voltage sensing appears to be mediated by several Arg side chains that move in response to a depolarizing membrane potential (15). The special issue contains three original research articles on H_v1 and one article on the recently discovered HCNL1, a tetrameric voltage-gated ion channel that, similar to H_v1, uses the same helical scaffold to sense voltage and conduct protons but has an additional nonconducting pore domain (16).

Working with human H_v1, Ayuyan and colleagues (17) report experimental evidence that stomatin, a protein found

in cholesterol-rich raft domains, interacts with H_v1, resulting in the localization of H_v1 within cholesterol-rich domains. This hypothesis explains the paradoxical observation that cholesterol influences H_v1 function in excised membrane patches but not in whole cells. Using their data from site-directed mutagenesis, electrophysiology, membrane patches with different cholesterol contents, channel coimmunoprecipitation, and Western blotting, Ayuyan and colleagues propose that H_v1 and stomatin form cholesterol-independent complexes that accumulate in cholesterol-dependent lipid domains, which provides a novel mechanism for its subcellular localization (17). On the basis of biochemistry and electrophysiology experiments on H_v1 from the sea urchin *Strongylocentrotus purpuratus*, SpH_v1, Okochi and colleagues (18) demonstrate that dimerization of SpH_v1 occurs in the endoplasmic reticulum. They further show that H_v1 must dimerize before it becomes glycosylated and that the ion channel spending more time in the endoplasmic reticulum was associated with its becoming glycosylated; this observation indicates that dimerization of H_v1 may have an important role in protein biosynthesis (18). Shen and colleagues (19) study H_v1 from yet another perspective, that of advanced computational approaches. The authors used a membrane-embedded hybrid-solvent continuous constant pH molecular dynamics approach developed in their laboratory to probe the dynamics and protonation states of human H_v1. They started from closed and open models of the channel derived previously by another laboratory using simulations of the channel under constant uniform electric fields (20). A quintessential feature of H_v1 channels is the sensitivity of gating to pH. In a study of snail H_v1, anomalously weak sensitivity to intracellular pH (pH_i) was localized to a specific His residue (21). Mutating the corresponding His in human H_v1 recapitulated the pH_i-sensing defect, identifying this residue as a key pH_i sensor (22). The continuous constant pH molecular dynamics simulations by Shen and colleagues identify the same His residue as a pH_i sensor of H_v1 by showing that its protonation differs in closed or open states (19). For HCN1, which is less studied than H_v1 because of its recent discovery, Kuwabara and colleagues use electrophysiology, two-electrode voltage clamp, and voltage-clamp fluorometry to show that Zn²⁺ ions inhibit the channel whether applied to intracellular or extracellular solutions (23). They identify an important difference between HCN1 and H_v1: the latter is inhibited primarily by Zn²⁺ binding at the extracellular side, whereas HCN1 is inhibited more potently from the inside (23).

The mechanism by which electrochemical potentials affect the properties of ion channels is a key question of general interest to ion channel research. Carlsen and colleagues (24) use theoretical voltage-responsive kinetic models to study chemically and electrically driven ion channels, with a focus not on reproducing experimental current-voltage curves but rather on the molecular origin of ion

channel rectification (i.e., the preference of an ion channel for conductance in one particular direction). The authors find that rectification arises from asymmetries in the potential of mean force; particularly important are the relative stabilities of the ion binding sites and bulk solutions and the voltage sensitivities of the rate-limiting step, ion release or ion uptake (24).

Stochastic titration constant pH simulations are used by Oliveira and colleagues (25) to study the protonation-coupled conformational switching of the diphtheria toxin translocation domain. Upon internalization and encountering an acidic environment in the endosome, this toxin undergoes a pH-dependent conformational change that allows the translocation domain to insert into the membrane and the catalytic domain to enter the cytosol (26). The extensive computations reported here on wild-type and four mutant proteins identify the pK_a values and functional roles of His and Glu side chains from the protonation sequence of the toxin translocation domain and suggest that the protonation dynamics of the toxin's translocation domain is such that the protein avoids accidental protonation-coupled refolding at neutral pH (25).

G-protein-coupled receptors (GPCRs) are seven-helical membrane proteins that mediate cellular signal transduction. The human genome has ~800 GPCR sequences (27), and about one third of drugs currently approved by the US Food and Drug Administration target a GPCR (28). The best-studied GPCRs are class A, which include the two receptors discussed in this special issue: visual rhodopsin and the μ opioid receptor. The review article by Stein (29) discusses the influence of pH on the functioning of the μ opioid receptor and what this could mean for drug design. Injury and disease can be associated with acidic pH, which in turn can influence the protonation states of both ligands and receptors (29). In the case of the μ opioid receptor, this is particularly important because a negatively charged Asp side chain at the receptor ligand-binding site recognizes the positive charge at the tertiary amine group of an opioid drug. The review article by Stein (29) highlights the importance of protonation state considerations for fentanyl and a fluorinated fentanyl derivative, ($\pm$)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide, which is a potentially safer alternative to fentanyl. The review article by Bachler and Brown (30) focuses on the key role of water in the functioning of visual rhodopsin. The authors interpret hydrostatic and osmotic pressure data to suggest that the osmotic pressure acts primarily on the bulk-like waters and that the hydrostatic pressure acts on the waters that interact with the receptor; receptor activation associates with an increase in the number of weakly bound waters and enhanced protein conformational flexibility (30).

Aquaporins (AQPs) are membrane proteins known for their function as water transporters; in the mammalian kidney, for example, AQP1 is essential for concentrating urine. AQP1 is a homotetramer, with each of the monomers

providing a water channel (31). AQP1 is an extremely interesting example of a protein studied intensively to understand how it actually avoids proton transport (see, e.g., (32–37)). In this special issue, Drewniak and colleagues combined experiment with computation to study the role of a conserved H-bond network in the stability and tetramerization of AQP1 (38). H-bonding residues of interest were individually mutated to Ala, and a Val residue to Pro, and the stability of AQP1 was studied with Fourier transform infrared spectroscopy (FTIR spectroscopy), hydrogen/deuterium exchange, differential scanning calorimetry, dynamic light scattering, and tryptophan fluorescence spectroscopy. The authors report that Ala replacements of the selected residues lead to partial instability or unfolding, can even disrupt the oligomerization, and can associate with drastic rearrangements of the protein's protein-water H-bond network, which could have more general implication for a role of conserved H-bond networks as key determinants of the native fold and oligomerization of AQPs (38).

Microbial rhodopsins are perhaps among the best-studied proton-binding proteins and serve as model systems to study general physical-chemical principles of ion transport and signaling across cellular membranes, and some of them are also used as tools to control the transmembrane potential in optogenetics applications. Microbial rhodopsins are seven-helical membrane proteins that bind a retinal chromophore via a protonated Schiff base (as is also the case for the visual rhodopsin discussed by Bachler and Brown in this special issue (30)). These proteins are highly versatile because they can function as ion pumps (protons, chloride, and sodium), ion channels, sensors, or, as multidomain proteins, as enzymes (39). Two papers in this special issue report studies of microbial rhodopsins. In work highlighted in the New and Notable by Brown (40), Sugimoto and colleagues (41) study with FTIR spectroscopy a microbial rhodopsin known as “TAT rhodopsin,” named after the Thr-Ala-Thr motif on the third transmembrane segment of the protein. An intriguing aspect of the wild-type TAT rhodopsin is that after photoisomerization of the protonated form of the retinal and formation of an early photocycle intermediate called “K,” the protein reverts to its resting state, apparently without any productive photocycle. The T82D mutation, which introduces an Asp near the retinal Schiff base, allows the protein to visit the M intermediate with a deprotonated retinal Schiff base, and the FTIR spectroscopy data from Sugimoto and colleagues identify the Asp (D82) that functions as proton acceptor and the Glu that donates a proton to re-protonate the retinal Schiff base (41). The paper by Okuyama and colleagues (42) presents systematic electrophysiology experiments on nine distinct microbial proton-pumping rhodopsins to decipher how $\Delta\psi$ and ΔpH influence their pumping activities. The authors use the measured current-voltage curves to estimate the driving force of each of the studied pumps; an extremely interesting

observation is that pumps from different organisms have different proton-pumping properties, which the authors suggest could indicate adaptations to the environments in which the organisms live (42).

The collection of original research and review articles included in this special issue showcase exciting developments in experimental and computational studies of proton-transfer reactions in biology.

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