**Minireview**

**Mechanism of Ion Transport across Membranes**

**BACTERIORHODOPSIN AS A PROTOTYPE FOR PROTON PUMPS**

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The high resolution structures now available for mitochondrial ATPase (1) and mammalian and bacterial cytochrome c oxidases (2, 3) have raised hopes that the long sought description of the proton translocation mechanism in transmembrane pumps might be near. According to the simplest version of the alternating access hypothesis (reviewed in Refs. 4–6), such transport is based on the energy-dependent cycling through two protein conformations in which the access of a single ion binding site changes from one membrane side to the other. It has been difficult to put this idea to a test because most proton pumps are large or multisubunit proteins in which the site of ion translocation is at some distance from the chemical reactions that drive it, and the chemical reactions are themselves complex. If there is a general mechanism in proton pumps, clues to it are more likely to come from a simpler system. Recent progress with bacteriorhodopsin, a small retinal protein in which the thermal reisomerization of photoisomerized retinal drives the proton transport, has yielded a step-by-step mechanism for the translocation cycle. The principles and perhaps some of the details in this mechanism, described briefly below, may prove to apply to other ion pumps. They seem relevant also to signal receptors (7).

**Structure and Photochemical Reaction Cycle**

Bacteriorhodopsin is a seven-helix transmembrane protein, with an all-trans-retinal lying at a small angle to the membrane surface and linked via a protonated Schiff base to Lys216 near the middle of helix G (Fig. 1). Most of the structure has been known at 3.5-Å resolution from two-dimensional crystals (8), and more recently the entire structure was described at 3.0 Å (9). Higher resolution from three-dimensional crystals, at 2.5 Å is now available (10). The interhelical cavity is divided by the Schiff base into extracellular and cytoplasmic “half-channels” that together describe the trajectory of the transported proton. The extracellular half-channel contains numerous charged or hydrogen-bonding residues, whereas the cytoplasmic region is simpler and mostly hydrophobic. In the first transport event after absorption of a photon, the Schiff base proton is mobilized by photoisomerization of the retinal to 13-cis,15-anti, and transferred to Asp85 in the extracellular region, causing the release of a proton to the surface. The Schiff base is then reprotonated from Asp96 from the cytoplasmic side. Asp96 in turn is reprotonated from the surface. These proton transfers together add up to translocation across the membrane. They and other reactions of the retinal and the protein during the cycle have been measured by various spectroscopic methods and consist of the interconversions of the intermediate states designated as J, K, L, M, N, and O, and substates of several of these. Much effort has been expended to describe these reactions and the protein residues involved (4, 11–15). But to understand bacteriorhodopsin as a proton pump we must know also what determines the rates of the proton transfers and how the pKₐ’s and the geometry of the donors and acceptors change so as to give them a cytoplasmic-to-extracellular direction.

**Partial Reactions of the Photocycle Observed without Illumination**

Although the proton transport is the consequence of a “photocycle,” mechanistic clues have been gained recently from reactions of the unphotolyzed protein that correspond to various single photocycle steps. First, the observation of a biphasic titration curve for Asp85 suggested that its pKₐ is linked to the protonation of another residue (16, 17) that turned out to be Glu204 (18–20) perhaps together with liganded water but certainly other residues near the extracellular surface, such as Glu194. The nature of the linkage is that protonation of Asp85 will cause deprotonation of the Glu204 site and vice versa. This is the kind of coupling that would cause proton release at the extracellular surface after proton transfer from the Schiff base.
to Asp$^{85}$ (21–25) even though Asp$^{85}$ itself remains protonated until the end of the photocycle (26–28). Conversely, once the proton is released at a pH higher than the pK$_A$ of the Glu$^{204}$ site, the pK$_A$ for Asp$^{85}$ will rise. Under physiological conditions, where the difference between the pH and the pK$_A$ for the release is large, the proton release will shift the protonation equilibrium between Asp$^{85}$ and the Schiff base toward virtually complete and unidirectional proton transfer.

The second observation, made from x-ray diffraction, was that deprotonation of the Schiff base of the D85N mutant by raising the pH in the dark caused the protein to assume an equilibrium mixture of conformations that exhibit structural changes seen otherwise only in the M photointermediate (29). In the D85N/D96N double mutant the equilibrium contained a large amount of the M-like conformation even with the Schiff base protonated. The pK$_A$s for the protonated Schiff base and the changes in crystallographic parameters for D85N were the same, and the isomeric composition of the retinal was indifferent to the shift of protein structure (30). As expected from this, the pK$_A$ of the Schiff base in D85N was nearly unchanged when the retinal was replaced with an analogue locked in the all-trans configuration. These observations provided a hint to the cause of the proton transfer switch in the photocycle that allows reprotonation of the Schiff base from the cytoplasmic side; if it is a result of the shift of the global protein conformation, it depends on deprotonation of the Schiff base, i.e. loss of interaction between the protonated Schiff base and its complex counterion rather than directly on the isomeric state of the retinal.

In the third study (31), mutations in the extracellular proton channel were shown to cause parallel decreases in the rate of the final photocycle step and the deprotonation of Asp$^{85}$ in pH jump experiments in the dark. The correlation of the rates over 3 orders of magnitude suggested that the loss of proton from Asp$^{85}$ to the Glu$^{204}$ site or directly to the medium depending on jump experiments in the dark. The correlation of the rates over

![Minireview: Transport Mechanism in Bacteriorhodopsin](image)

**Mechanism of Transport**

These recent results contributed some missing pieces in the puzzle and helped to refine the proton transport mechanism that follows. The active site consists of the protonated Schiff base and the anionic Asp$^{85}$ and bound water (35–39) that stabilizes the buried charges. Photosomeroserization of the retinal from all-trans to 13-cis,15-anti changes this geometry (40, 41) and redistributes the π-electron system along the retinal chain (42, 43), causing the pK$_A$ difference between the Schiff base and Asp$^{85}$ to narrow from about 5 to <1 (44). Fig. 1 shows schematically the proton transfer steps in the early and late phases of the transport cycle that ensues. As Asp$^{85}$ becomes protonated, linkage of the pK$_A$s of Asp$^{85}$ and the Glu$^{204}$ site (Fig. 1, blue arrow in left panel) causes the pK$_A$ of the Glu$^{204}$ site to drop from 9 to about 5. As a result, Glu$_{204}$ protonates Glu$_{194}$, and the latter releases the proton to the surface. At neutral (physiological) pH release of the proton to the extracellular surface is strongly favored. This in turn causes the pK$_A$ of Asp$^{85}$ to further rise (17, 19, 46), and its protonation equilibrium with the Schiff base shifts toward nearly full proton transfer (24). This tran-

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11 Much indirect evidence indicates that there is specifically bound water near the Schiff base and Asp$^{85}$, and its hydrogen bonding undergoes changes throughout the photocycle. Water near the Schiff base and Asp$^{85}$ was suggested to mediate the initial proton transfer (41, 70). Hydrogen-bonded water molecules that connect Asp$^{85}$ to Glu$^{204}$ in structures calculated from molecular dynamics (71, 72) may play the major role (19) in the

**Role for Bound Water in Proton Transfers**

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linked $pK_a$ of Asp$^{85}$ and the Glu$^{204}$ site. The changed access of the Schiff base to Asp$^{96}$ has been suggested to be through reorganized water molecules at the active site (73). Water molecules between the Schiff base and Asp$^{96}$ were postulated to mediate proton transfer across the 12-Å distance in the interior of the protein (8, 9). The effects of osmotic agents on this reaction (63) suggested that first an increased, then a decreased, amount of bound water near Asp$^{96}$ associated with the formation and the closing of a cleft at the cytoplasmic surface is what regulates the proton affinity of this residue as a proton donor to the Schiff base and then an acceptor from the cytoplasmic surface. Finally, in the last step of the sequence proton is lost from Asp$^{85}$ to Glu$^{204}$ most likely through the mediation of the hydrogen-bonded water chain that connects them. Indeed, the large $D_2O$ isotope effect (5–10-fold) associated with both proton release and the deprotonation of Asp$^{85}$ that takes place later (31) strongly suggest that bound water plays an important role in these processes.

**Nature of the Active Site**

The Schiff base and its acceptor appear to constitute the single active site in this proton pump. The function of the rest of the protein may be simply to alternately deliver to and remove a proton from this site. If this is true, we should distinguish between an active site specific for the initial and critical ion transfer and a nonspecific matrix that responds to it passively by redirecting its access to either membrane surface.

There is support for this view from an observed change in the ion specificity of bacteriorhodopsin. Upon replacing Asp$^{85}$ with a threonine, the equivalent residue in the light-driven chloride pump halorhodopsin (74), bacteriorhodopsin acquired the spectroscopic properties and the chloride transport activity (that proceeds in the opposite direction from the proton transport) of halorhodopsin (75). Although the chloride transport mechanism in this recombinant bacteriorhodopsin and in halorhodopsin (76–79) is not yet well understood, it must involve binding of chloride at the active site and its translocation to the cytoplasmic domain. Evidently, the alternating pathways of access in this protein allow the movement of not only protons but also chloride.

Haloforms, in turn, can be made to transport protons. This will occur in a two-photon reaction in which the deprotonated 13-cis and protonated all-trans-retinal Schiff bases are forcibly interconverted, with an accompanying protonation and deprotonation that results in net translocation of charge (80). Analogously to the mutational change of specificity of bacteriorhodopsin, haloforms also becomes a proton pump when an artificial proton acceptor, the azide anion, occupies a site near the Schiff base where otherwise the transported chloride is bound (81). Thus, when provisions are made for a proton acceptor to the Schiff base, the access change and the conduction pathways for chloride will also accommodate protons.

The hypothesis that the functioning of bacteriorhodopsin depends less on proton conduction pathways to and from the surface than on the initial proton transfer at the active site is supported by the remarkable fact that of the many hundreds of site-specific mutations studied (12, 14, 15) none inactive transport entirely. Those that have the greatest effect prevent proton transfer from the Schiff base to Asp$^{85}$, e.g., by replacing the aspartate with a neutral residue or by keeping it permanently protonated. However, even in the former case where transport of the kind in the wild type is inaccessible, proton transport is observed under some conditions (57, 58). Two other mutations that are less disruptive are also worthy of note. In the first, E204Q, proton release to the extracellular surface upon protonation of Asp$^{85}$ is abolished. Although proton release is delayed until the end of the photocycle when the low $pK_a$ of Asp$^{85}$ is reestablished, the direction of the proton transfers is preserved (18, 19). In the second, D96N, reprotonation of the Schiff base and the subsequent proton uptake from the cytoplasmic surface are hindered. Protonation of the Schiff base is directly from the surface but again with the correct directionality (62, 83, 84). Thus, the proton conduction pathways to both membrane surfaces can be seriously perturbed without loss of transport activity. On the other hand, interference with these pathways does affect the turnover rate of the pump, and the rate of proton transport at high light intensities will be decreased. At physiological pH the slowing of the photocycle in D96N amounts to 2–3 orders of magnitude, as the rate-limiting step becomes the inefficient capture of a proton at the cytoplasmic surface (63). Replacement of residues at the cytoplasmic surface, such as Asp$^{85}$, that might also have roles in proton conduction causes a severalfold slowing of the photocycle (45). Similarly, at the extracellular surface, the E204Q mutation causes decrease of the turnover by 1–2 orders of magnitude, as the loss of the proton of Asp$^{85}$ becomes rate-limiting (18, 31).

Avoiding such problems must be the rationale for the evolution of the proton transfer pathways described above. Indeed, the arrangement of acidic residues in the structure suggests that multiple pathways may exist for both release and uptake of protons at the two surfaces (9).

**Conclusions**

Thus, the transport in bacteriorhodopsin is explained by an alternating access mechanism. The change in the access of the occluded proton binding site occurs in sequential steps. In the first and last steps the direction of the transfer of proton between the Schiff base and the two membrane surfaces is determined by the magnitude and sign of a changing $ApK_a$ relative to proton acceptor and donor groups. Although this would ensure, by itself, the direction of the transport, there may be in addition a change of the local geometry to switch the connectivity of the Schiff base. The lesson for other pumps may be that the local ion transfer reactions, which deliver the transported ion to the active site and remove it, are more crucial and more stringently regulated than the conduction pathways between the binding site and the two membrane surfaces.

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