Bacteriorhodopsin as a model for proton pumps

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According to a long-standing hypothesis, membrane pumps function by flip-flopping between two protein conformations that allow alternative access of the ion binding site to the two membrane surfaces. Site-specific mutagenesis, time-resolved spectroscopy and X-ray diffraction confirm this mechanism for bacteriorhodopsin, and implicate change of electrostatic interaction at the active site as the trigger for the global protein conformation change during the proton transport cycle.

THE interiors of cells and organelles are kept at compositions different from their exterior by active transport of ions across cellular and intracellular membranes. Such transport requires that a spontaneous reaction at the active site of a membrane enzyme be coupled to the unidirectional movement of an ion across the membranes so as to cause uptake at one surface and release at the other, even in the face of an opposing transmembrane electrochemical potential. In some proton pumps based on redox reactions, quinones act as mobile carriers for electrons and hydrogen within the membrane phase[1], but in others a proton wire[2] or alternating protein conformations[3-7] have been suggested. In the last mechanism, the chemical changes at the active site (the driving reaction) alter the conformation of the protein so as to regulate the conduction of the transported ion between its binding site and the two membrane surfaces. Such a relationship of the active site, the protein conformation, and the proton conduction pathways has been described so far only in bacteriorhodopsin.

Bacteriorhodopsin

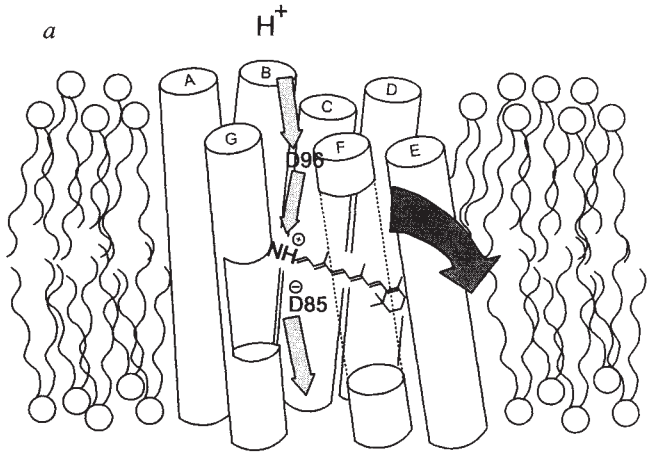
The light-driven proton pump of halobacteria[8,9] consists of seven transmembrane helices that enclose a cavity containing a retinal bound to the ε-amino group of Lys 216 through a protonated Schiff base (Fig. 1*a*). The structure is described by an atomic model based on a 3.5 Å resolution map from electron cryomicroscopy[10]. The trajectory of the transported proton is defined by the Schiff base and two aspartate residues, Asp 85 and Asp 96. After photoisomerization of the retinal from all-*trans* to 13-*cis* the Schiff base donates a proton to the anionic Asp 85, followed by its reprotonation from the initially protonated Asp 96. These internal proton transfers cause proton release at the extracellular surface and proton uptake at the cytoplasmic surface, and thereby result in the net translocation of a proton.

Many investigators have anticipated that if there are fundamental and common principles for ion pumps, the study of this small (M_r, 26K) protein would reveal them. Indeed, kinetic models and analyses of energy flow during the photocycle demonstrated[11-13] that, although initiated by photoexcitation, the ensuing thermal steps of the transport cycle are much like those in any complex enzyme reaction. Thus, although the proton transfers inside the protein take place near equilibrium, the proton release and uptake at the protein surfaces occur with transiently lowered and elevated pK_a values[14-16], respectively; that is, the proton electrochemical potential is created as the excess free energy acquired by the retinal chromophore is dissipated by protein residues. Also in analogy with enzymes, the protein conformation undergoes distinct changes during the reaction sequence[17-19]. The reaction pathway is illustrated in Fig. 2.

The consequences of replacing various residues have been previously explored by kinetic spectroscopy in the visible and the infrared. Much of this work was directed towards identifying the path of the transported proton and describing the kinetics of the reaction cycle (the 'photocycle'). The switch in the connectivity of the Schiff base from Asp 85 to Asp 96 was recognized as the equivalent of the alternating access in other pumps[11], and identified as a unidirectional step between the deprotonation and the subsequent reprotonation of the Schiff base[13]. More recent studies have been on the origins of the protein conformation change in the photocycle, and the way it is linked to this reprotonation switch. The properties of a group of mutated proteins with the proton acceptor Asp 85 replaced suggested that coulombic interaction between the positively charged proton donor and the negatively charged proton acceptor at the active site regulates the global protein conformation[20]. The unphotolysed state of the protein is stabilized by the binding energy of a complex formed by the Schiff base, Asp 85, a few other charged residues, and liganded water. In this state (conformation E) the Schiff base proton is connected to Asp 85, and thus the extracellular side. Photoisomerization of the retinal[21] displaces the Schiff base relative to Asp 85, and the new geometry, as well as bond torsions along the retinal chain immobilized by residues in the binding pocket, favours transfer of the Schiff base proton to the aspartate. Once the Schiff base/Asp 85 ion pair is thereby converted to a neutral pair, the protein relaxes into its default conformation (conformation C). Difference projection maps from X-ray and electron diffraction indicate that the conformation change at this time in the cycle is mostly a tilt of helix F at the cytoplasmic surface away from the centre of the seven-helical structure, and reorganization of the cytoplasmic side of helix G[17-19]. In conformation C the Schiff base is connected to the cytoplasmic side[20], and its access to Asp 85, and therefore to the extracellular side, is broken. The electrostatic nature of the trigger for the transition from conformation E to C is demonstrated by the finding that it occurs not only in the photocycle of the wild-type protein but also in the unilluminated D85N mutant when the pH is raised to deprotonate the Schiff base[20].

Proton transport in bacteriorhodopsin is therefore accomplished according to the alternating access model of ion pumps. Unexpectedly, the initial state of the polypeptide chain is an inherently high-energy conformation, and it is the release of this conformational energy upon the initial proton transfer from Schiff base to Asp 85 that provides for the reprotonation switch. This means that at the end of the photocycle, part of the excess free energy acquired will be retained in the protein. Such a thermodynamic cycle is not unlike what was recently suggested for myosin–actin interaction on ATP binding and hydrolysis[22].

It appears that in bacteriorhodopsin the protein conformation alternates between two states according to the presence or absence of a local interaction at the active site. Is this a general principle? Certainly, in this respect, bacteriorhodopsin is similar to visual and sensory rhodopsins. The former initiates the cascade of visual transduction when its Schiff base is deprotonated in the meta II photointermediate, and thereupon binds transducin. Replacement of the anionic Glu 113 (the equivalent of



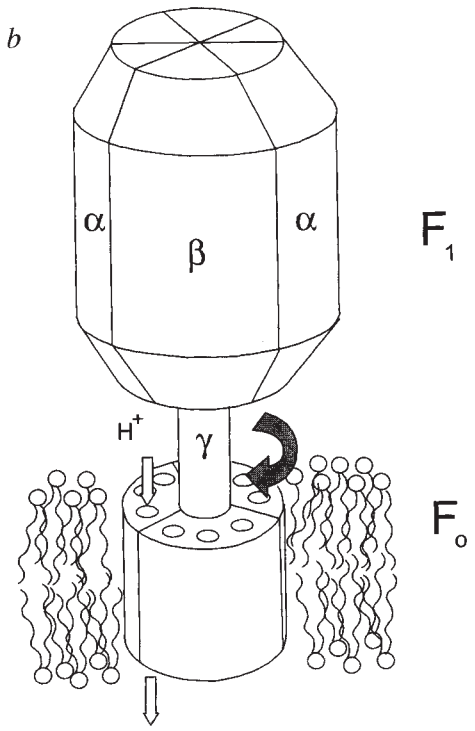


FIG. 1 Schematic drawings of bacteriorhodopsin (*a*) and the F_0F_1-ATPase (*b*). The pathways of protons are indicated by straight arrows, the proposed tilt of helix F and rotation of the γ-subunit by curved arrows.

Asp 85 in bacteriorhodopsin) with glutamine, or the positively charged Lys 296 (the equivalent to Lys 216 in bacteriorhodopsin, that is, the Schiff base) with glycine, resulted in constitutive binding of transducin[23]. The contribution of electrostatics at the active site to the stability of the overall structure is emphasized by the finding that when the coulombic interaction was made weaker by moving the anion one helical turn away from the Schiff base, photoisomerization of the retinal (from 11-*cis* to all-*trans* in vertebrate rhodopsins) caused transducin binding even without deprotonation of the Schiff base[24]. Remarkably, the sensory rhodopsin I of the halobacteria that normally functions as a light-receptor with no transport function, uses an aspartate near the retinal to transport protons, like bacteriorhodopsin, in the absence of its transducer protein[25]. Because the rhodopsins seem to be prototypes for biogenic amine-binding receptors, or perhaps even for the entire G-protein linked receptor superfamily[26,27], this kind of behaviour might represent a general mechanism for conformational control.

In fact, the idea of active-site stabilization of protein conformation is implicit in models proposed for a wide variety of ionic pumps. This is illustrated in two pumps of greater complexity than bacteriorhodopsin.

Cytochrome *c* oxidase and F_0F_1-ATPase

Cytochrome *c* oxidase[28–32] is a representative of a large group of terminal oxidases of the respiratory chain. In these enzymes four electrons are used sequentially to reduce O_2. Four protons react with the dioxygen to form water, and another four are translocated across the membrane[3]. The structure is known to about 15 Å resolution[33]. It is large and complex in eukaryotes (M_r about 200K, 13 subunits), but simpler in prokaryotes which contain only subunits I–III (ref. 34). Subunits I and II are sufficient for both oxygen reduction and proton transport. Subunit II contains Cu_A, the entry point for electrons from cytochrome *c*. Subunit I contains 12 putative transmembrane helices, labelled I to XII, and two metal centres: haem *a* and haem a_3, the Cu_B binuclear centre. The metal centres are located near the cytoplasmic side, and oriented so that the path of the electrons is roughly parallel to the plane of the membrane. The transport of protons occurs in two steps after the binuclear centre is partially reduced and dioxygen is bound to haem a_3. Many mutations along the hydrophilic surface of helix VIII abolish oxygen reduction and transport but do not reveal which groups carry the protons to and from the active site, and bind protons at the binuclear centre[31]. Replacement of Asp 134 located in the loop between the matrix ends of helices II and III abolishes transport but not the redox reaction[35]. Thus, it is not yet clear how the electron transfer steps and the proton transfer steps are coupled, and whether this coupling is direct or indirect. Most proposed models of proton translocation are based on shuttling of ligands to and from haem a_3 or Cu_B. In a proposed direct coupling model[36], for example, His 284 coordinated to Cu_B plays the role of such a ligand, carrying 2×2 protons per cycle across the internal barrier.

Schemes that account for the proton transport in terms of alternating input and output states in the reaction cycle make use of many observations that the protein can assume two conformations[37]. Although direct evidence for protein conformation changes during the reaction cycle is lacking, the kinetics indicate[38] that as the enzyme undergoes two cycles of electron transfer there must be an input conformation that allows the reduction of haem *a* and the uptake of two protons from the matrix side, and an output conformation that allows the release of two protons to the cytoplasmic side at the binuclear centre.

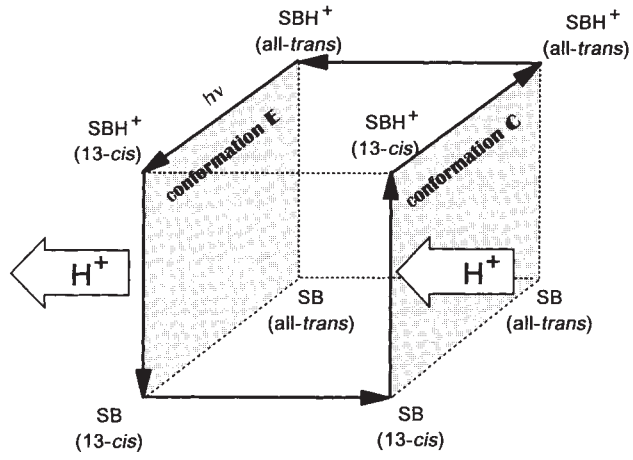


FIG. 2 The bacteriorhodopsin photoreaction cycle, drawn similarly to the 'cubic scheme' for cytochrome c oxidase[3,30]. The retinal is either all-*trans* or 13-*cis*, the Schiff base protonated (SBH^+) or unprotonated (SB), and the protein conformation is either E (with extracellular access of the Schiff base proton) or C (with cytoplasmic access).

The F_0F_1-ATPase of the mitochondrial membrane represents a group of proteins with very complex architecture that can function either as ATP syntheses driven by proton-motive force or proton pumps driven by ATP hydrolysis[39–42]. The total M_r is ~500K. The dissociable, water-soluble, bulb-shaped F_1 complex contains subunits α, β, γ, δ and ε (with a stoichiometry 3:3:1:1:1), whereas the membrane-immersed F_0 complex contains numerous kinds of small hydrophobic peptides, in particular an estimated 9–12 copies of the *c* subunit (Fig. 1*b*). The structure of most of F_1 is now known to 2.8 Å resolution[42]. In the pump mode the single reaction cycle consists of the hydrolysis of 3 ATP molecules by F_1, and the translocation of 9–12 protons by F_0. A great deal of kinetic, and now also structural, evidence indicates the existence of three interacting hydrolytic sites, one on each β-subunit, and the sequential changes of these sites as they progress through the non-binding, the ATP-binding and the ADP + P_i-binding states, each 120° out of phase with the others[41–44]. The F_0 complex has been visualized as a rotor assembly of subunits, with 9–12 stops that allow an interrupted flow of protons[45]. The transient binding of these protons may be to Asp 61 of each *c* subunit[40]. The separation of the functions of the F_1 and F_0 complexes is suggested by the existence of an analogous but Na^+-translocating ATPase[46], and the fact that a hybrid protein containing F_1 from the proton pump and F_0 from the sodium pump transports Na^+ (ref. 47). Transmission of free energy between F_1 and F_0 is through a slender stalk that connects them, by what appears to be rotation of the γ-subunit to connect, one by one, with the β-subunits according to whether their hydrolytic sites are empty or contain ATP, or ADP + P_i[48,49]. This long-range conformational change would allow the directional transfer of three or four protons across the membrane for each third of the cycle.

Discussion

The common element in these three pumps is that the measured or postulated alternative protein conformations are regulated by interaction of the active site with the rest of the protein. The results with bacteriorhodopsin suggest that it is feasible for local changes at the active site upon energization to affect the overall protein structure. Thus, the group transfer energy of the chemical reaction (ATP hydrolysis, electron and/or proton transfer and so on) may be sufficient to shift one structural alternative to the other. In the simpler pumps the excess free energy is either generated at the binding site (in bacteriorhodopsin), or transferred directly to the ion binding site (in cytochrome *c* oxidase). In more complex pumps the transmission of free energy from the site(s) of the driving reaction to the site of ion translocation must be through structures that offer mechanical coupling of conformation changes. When the number of ions transported per cycle is large the transducer appears to be a rotor, with multiple binding sites, that turns to expose one site at a time to the membrane surfaces (in the F_0F_1-ATPase). Thus, at one extreme, ion pumps appear to be based on principles akin to muscle contraction, but at the other they resemble molecular motors otherwise used for locomation, as in bacterial flagella.

In evaluating the generality of this hypothesis, we will need to know more about the nature and energetics of the protein conformational changes. Efforts are underway to describe the conformational change in bacteriorhodopsin at a higher resolution and in three dimensions, and to decide whether it consists of flip-flopping between two alternatives or of a series of conformational shifts that originate at the active site and spread progressively over the protein, as suggested in a different context for myoglobin[50]. In the F_0F_1-ATPase the structural differences among the three α/β subunits, and their connection to the γ-subunit, are well documented[40], and further work will reveal how these are linked to the states of the nucleotide binding sites and the position of the proposed rotor of the F_0 complex. In cytochrome oxidase there is only indirect evidence for an important conformational change.

The second question concerns the energetics of the alternating conformations. The results with bacteriorhodopsin suggest that the connection between the active site and the protein conformation might be primarily electrostatic. Both the redox states of the iron and copper centres and the protonation states of the proton transfer groups in cytochrome *c* oxidase, for example, in the histidine cycle[36], and the binding, hydrolysis, and release of ATP and ADP in the F_0F_1-ATPase[41] will be affected by the net charge and the geometry of the charges at the active site.

Despite the many uncertainties discussed above, it appears that a common mechanism for energy coupling in proton pumps (and signal transduction in receptors) is beginning to emerge. Results with bacteriorhodopsin, the simplest of the pumps, confirm the long-held alternating access hypothesis of active ion translocation, and attribute the conformational transition of the protein to changes in electrostatic interactions at the active site. The continued elucidation of these principles, as well as the mechanistic details unique to each protein, should provide a much improved understanding of ion pumps within the next few years. □

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ACKNOWLEDGEMENTS. The author's research is supported by the NIH, DOE and NSF. He thanks R. G. Gennis, R. Henderson, B. Malmström, T. Poulis, B. Rosen, J. E. Walker, S. H. White and M. Wikström for valuable suggestions.