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Minireview

Progress toward an explicit mechanistic model for the light-driven pump, bacteriorhodopsin

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Abstract Recent crystallographic information about the structure of bacteriorhodopsin and some of its photointermediates, together with a large amount of spectroscopic and mutational data, suggest a mechanistic model for how this protein couples light energy to the translocation of protons across the membrane. Now nearing completion, this detailed molecular model will describe the nature of the steric and electrostatic conflicts at the photoisomerized retinal, as well as the means by which it induces proton transfers in the two half-channels leading to the two membrane surfaces, thereby causing unidirectional, uphill transport.

Key words: Bacteriorhodopsin; Membrane protein; Proton transport; Bioenergetics

1. Introduction

Bacteriorhodopsin, the light-driven proton pump of halophilic archae [1,2], has long been viewed as the system which represents the principles of transmembrane ion pumps in their simplest possible form. In this small (26.5 kDa) integral membrane protein, photoisomerization of the all-*trans* retinal chromophore to 13-*cis*, 15-*anti* sets off a sequence of thermal reactions which regain the initial state in about 10 ms ('photocycle'). There being no chemical transformations during the photocycle, it is entirely the conformational changes of the retinal and the protein that modulate the p*K*s of various groups and facilitate translocation of the transported proton from the cytoplasmic to the extracellular side. Studies of this protein have offered the hope, therefore, to reveal the principles of energy coupling by ion pumps, free of complications from the often complex chemical reactions that drive them and from the necessity to consider the long-range interactions found in large proteins or protein assemblies.

An immense amount of work over the last 20 years, by many groups and with many different methods, has made good use of the conceptual and experimental advantages of this protein, and led to profound insights into how the protein functions. In the past few years, crystallographic structures at high resolution, for the unphotolyzed state as well as some of the photointermediates, added crucial new information that makes a comprehensive mechanistic model a realistic goal for the near future. The objective of this review is to summarize these recent findings, put them in the context of mutational and spectroscopic evidence, and offer the author's version of the mechanistic model that is emerging.

2. Structure of the protein

From cryo-electron microscopy of two-dimensional crystals, first with ≥ 3.5 Å [3] and then 3 Å [4] resolution, it was evident that there are seven transmembrane helices, A–G, at roughly normal to the membrane plane, with short interhelical loops and N- and C-termini. The all-*trans* retinal is linked via a protonated Schiff base to Lys-216 and lies in the interhelical area nearly parallel to the membrane plane. It is flanked by the proton acceptor and donor, Asp-85 and Asp-96, on its extracellular and cytoplasmic sides, respectively, the former being in its immediate vicinity and latter at an about 12 Å distance. The extracellular region contains the hydrophilic residues Asp-212, Arg-82, Glu-194 and Glu-204, that mutational studies (e.g. [5–9]) have shown to play roles in proton transport. In contrast, the cytoplasmic region lacks such an array of dissociable, or even polar residues. X-ray diffraction of three-dimensional (3D) crystals at 2.9 Å [10], 2.5 Å [11], 2.3 Å [12], 1.9 Å [13], and most recently at 1.55 Å [14], resolutions confirmed these features[1], and revealed the presence of numerous water molecules in the extracellular region that form, together with polar side-chains, a 3D hydrogen-bonded network. As expected, in the cytoplasmic region, much fewer bound water molecules were seen.

The active site[2] comprises water 402 that receives a hydrogen bond from the protonated Schiff base, and donates hydrogen bonds to the side-chain carboxyl oxygens[3] of the anionic Asp-85 and Asp-212. This arrangement (Fig. 1), together with additional hydrogen bonds of Asp-85 to water 401 and Thr-89, and of Asp-212 to Tyr-185, Tyr-57, and through water 406 to Arg-82, stabilizes the separated changes in this buried location. The network extends toward the extracellular surface through the hydrogen-bonded chain, Asp-85-water 401-water 406-Arg-82-water 403-water 404-Glu-194-Glu-204-

[1] There is good agreement on the positions of the protein atoms in [10,12–14], as well as on the positions of many of the bound water molecules in [13,14].

[2] Numbering for bound water as in [14].

[3] For the sake of brevity, unless otherwise mentioned, the hydrogen-bonding of a residue will refer to its side-chain group.

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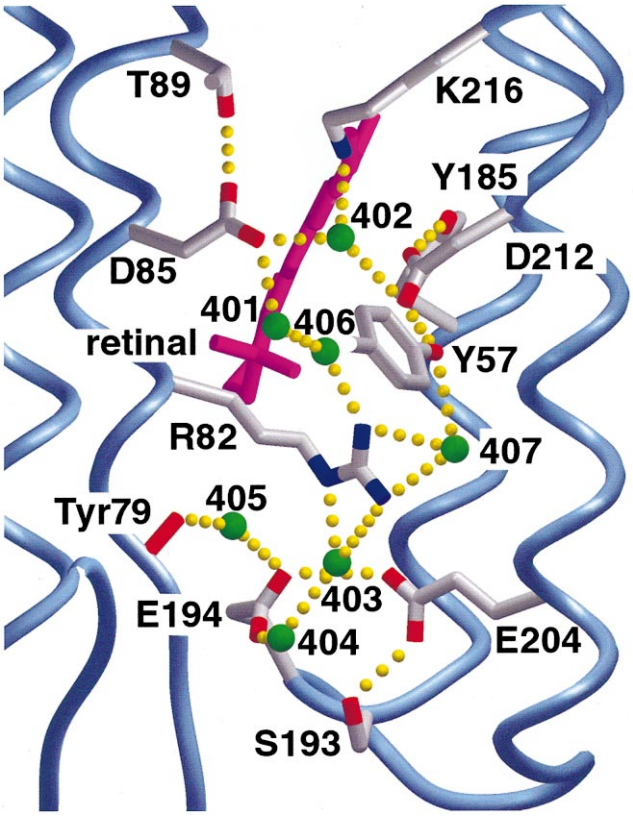

Fig. 1. Structure of the extracellular region of bacteriorhodopsin (coordinate file 1C3W, image reprinted with permission from [14]). The extracellular membrane surface is toward the bottom. View is from the direction of helix C. Hydrogen bonds visible from this angle are drawn as dashed yellow lines. The retinal is purple; water molecules are green.

Ser-193, and two other chains, Asp-212-water 406-Arg-82 and Asp-212-Tyr-57-water 407-Arg-82, that merge with it. This network must provide the means for the observed coupling of the protonation states of Asp-85 and the dissociable site near the extracellular surface that releases a proton to the surface during the photocycle [15–17]. It also provides a pathway for the proton liberated when Asp-85 dissociates in the last step of the cycle [18,19].

On the cytoplasmic side, only two water molecules are evident in functionally relevant positions [14]. Water 501 is hydrogen-bonded to the peptide C=O of Ala-215 on one hand, and the indole N of Trp-182 on the other. Water 502 is hydrogen-bonded to the peptide C=O of Lys-216 and to the peptide C=O of Thr-46. The participation of Ala-215 and Lys-216 in these bonds imparts a kink, in the form of a π-bulge, to helix G where the retinal is bound. This arrangement may provide a mechanism for the long-range coupling between Asp-96 and the retinal in the cytoplasmic region that occurs in the photocycle. Thus, because Thr-46 is hydrogen-bonded to Asp-96, the protonation state of Asp-96 could affect, though water 502, the conformation of helix G at the π-bulge. Through water 501 and Trp-182, the indole ring of which is in van der Waals contact with the 13-methyl group of the retinal, the disposition of the main-chain of helix G would then affect, in turn, the isomeric state of the chromophore.

The structure of the unphotolyzed, i.e. 'BR', state indicates that there is a fully formed pathway for proton conduction between the retinal Schiff base and the extracellular surface. The Schiff base is stabilized in its protonated state, in spite of this, by an extensive hydrogen-bonded network that lowers the proton affinity of the potential acceptors, Asp-85 and Asp-212. Loss of the Schiff base proton to the cytoplasmic direction is made infeasible by the lack of such a network in that region, as well as the very high proton affinity of Asp-96 which ensures that at physiological pH, it is already protonated and cannot be an acceptor. Obviously, as generally held, the hydrophobic cytoplasmic region is the primary barrier to dielectric breakdown via the interhelical cavity of the protein in the presence of a transmembrane electrical potential.

3. Proton transfer steps in the photocycle

Absorption of a photon will cause isomerization of the retinal and change this structure. The photochemical cycle that ensues is described by the sequence of the intermediates J, K, L, M, N and O. Although from kinetic measurements it is clear that the photocycle is more complex, to a first approximation it is described by the scheme, BR-hν → J → K ↔ L ↔ M_1 → M_2 ↔ N ↔ O → BR (with the possibility of more substates, as reviewed in [20]). Vibrational spectra have indicated that a torsion of the polyene chain of the 13-*cis* retinal in the K state relaxes in L. In the L state, the displacement of the Schiff base will have disrupted hydrogen bonds and destabilized the separated charges at the active site. There is FTIR evidence in fact [21], for a global rearrangement of the hydrogen-bonded network that has effects as far away as Asp-96. As a consequence, the p*K* of the Schiff base becomes lower and the p*K* of Asp-85 increases, and a protonation equilibrium develops between the Schiff base and Asp-85. According to kinetic data [22–24], this generates a mixture of L and M_1, in which the protonated Schiff base (the L state) still dominates. Protonation of Asp-85 is linked to the release of a proton to the extracellular surface [15,16], and at pH well above the p*K* for the release (p*K* = 5–6), the protonation equilibrium will be shifted far toward full deprotonation of the Schiff base. Thus, the proton release shifts the next step, the M_1 to M_2 transition, toward M_2 [24–26]. At pH < 5, proton release does not occur at this time, and the L state persists along with M_1 and M_2 during the further progress of the photocycle [24,27,28].

Because the p*K* of Asp-85 at this time is high [29], if the Schiff base is to be reprotonated, it cannot occur from this residue as donor. There is evidence [30] that a large-scale conformational change in the cytoplasmic region [31–35] causes both the lowering of the p*K* of Asp-96 and the establishment of a proton conductive pathway, so as to reprotonate the Schiff base, in the M_2 to N reaction, from the cytoplasmic side. The protonation of the Schiff base removes a large barrier to the rotation of the C_{13}-C_{14} double bond [36], but the thermal reisomerization from 13-*cis* to a twisted all-*trans* that follows, i.e. the N to O reaction, is linked to a rise of the p*K* of Asp-96 and its reprotonation from the cytoplasmic surface. Re-establishement of the initial state at the Schiff base occurs in the O to BR reaction, when Asp-85 dissociates and reprotonates the site that had released the proton, and the retinal chain then relaxes to a non-strained all-*trans* configuration [18,37].

4. Structural changes in the intermediate states

X-ray diffraction of the K intermediate at 2.1 Å resolution, produced in a photostationary state at a cryogenic temperature, revealed movement of Asp-85 toward the Schiff base and displacement of water 402, although only disorder along the polyene chain of the retinal [38]. As expected, the changes identified at this early stage of the photocycle occur in the immediate vicinity of the retinal.

The description of the structural changes in the L state that follows K will be crucial for understanding where the Schiff base proton moves and how Asp-85 becomes protonated in the L to M_1 reaction. It is not yet available at the time of this writing, but cannot be far in the future. On the other hand, the structure of the M intermediate was determined already [39], so far to 2 Å resolution, after trapping the photostationary state of the D96N mutant, by rapidly cooling to 100 K. Once in the M state (a late substate, which is M_2 or M_N), the density map shows the retinal to be clearly 13-*cis*, 15-*anti*, which causes the electron pair of the unprotonated retinylidene nitrogen to point away from Asp-85 and toward the hydrophobic region of Val-49 and Leu-93. Further, the isomerization around the C_{13}-C_{14} double bond introduces a curvature in the polyene chain, with the region of the 13-methyl group experiencing the greatest displacement, and toward the cytoplasmic direction. Correspondingly, the side-chains of Trp-86 to the extracellular side, and Trp-182 to the cytoplasmic side, both move away, and the side-chain of Leu-93 rotates, to accommodate this distortion.

Distinct changes are seen also at the Schiff base [39]. Either as the cause, or the consequence, of the deprotonation of the Schiff base, water 402 is no longer evident in this region, and water 401 is displaced from its position in the BR state so as to form a hydrogen bond with both Asp-85 and Asp-212. Water 406, that bridges water 401 and Arg-82 in the BR state, is absent. Asp-85, now protonated, is moved away from the Schiff base, and no longer forms a hydrogen bond with Thr-89. On the other hand, Asp-212, which remains anionic, retains its hydrogen bonds to Tyr-185 and Tyr-57 and forms a new one with the indole nitrogen of Trp-86. Although it is not yet clear which of these changes occurred at what step of the photocycle between the K and the M states, the side-chain displacements near the retinal chain, and at least some of the rearrangements of side-chains and water at the Schiff base, do reveal the steric and electrostatic conflicts which make M and the earlier photointermediates high-energy states.

Structural changes associated with release of a proton to the surface upon protonation of Asp-85 are also evident in the M state [39]. The side-chain of Arg-82 is displaced toward the extracellular direction, probably the result of the break of two of the three hydrogen bonds the guanidinium group forms with waters 406 and 407 in the BR state (Fig. 1). If this is so, the shuttling of the positively charged side-chain of Arg-82 [40] acts as the means of coupling the proton release site to Asp-85. With such coupling, the proton should be released from the vicinity[4] of Glu-194 and Glu-204, where the negative charge created would be stabilized by the positive charge of Arg-82. The region of the two glutamic acid residues is rearranged in M [39]. Water molecule 403 is not evident, and waters 404 and 405 are displaced. The side-chains of Glu-204 and Glu-194 rotate out of hydrogen-bonding distance, and they are now bridged by water 404. Glu-194 forms a new hydrogen bond with Tyr-83.

In contrast, there is no hydrogen-bonded chain evident in the M state (at least in the M of the D96N mutant) that would constitute a proton conducting pathway between the cytoplasmic surface and the Schiff base. A hint of the possibility of building such a path is the observation [39] that the side-chains of Leu-93 and Phe-219, that occlude the path in the BR state, appear to rotate away. As no other alternative suggests itself, presumably the passage of a proton is limited by a chain of water molecules that is formed only during the M to N transition, or by mobile water that is transiently intercalated into a tortuous path between donor and acceptor. If this is so, these water molecules might be observed in the structure of the N state. The cytoplasmic ends of helices F and G, although not B and C that are also suggested by projection maps, must play a role in this, because they appear disordered in the present structural data for M. There is an indication, from the displacement of the main-chain of helix F at the last two well-defined residues, Val-177 and Asn-176, that there may be an outward tilt of this helix, as inferred earlier from structural [31–35] as well spectroscopic [41,42] data. However, if there is such a tilt, it begins well to the cytoplasmic side of Tyr-185 and Pro-186.

5. The emerging model for the energetics and the mechanism of the transport

From the foregoing, it should be clear that for bacteriorhodopsin the two fundamental problems of ion pumps are now nearing solution. They are: (a) how excess free energy is conserved and transmitted within the protein and (b) how the alternating access of an occluded ion binding site to the two membrane surfaces is assured during the transport cycle.

The sequence of events during the photocycle, given in detail above, not only describes the path of proton translocation across the membrane, but identifies the cause of each reaction in the preceding step. It is intuitively obvious that the conservation of the energy gained from light is initially in the form of a steric and electrostatic conflict of the photoisomerized retinal chromophore with its binding site. The nature of this conflict is now indicated by the displacements of side-chains, water molecules and charges in the immediate vicinity of the retinal, observed in the M state [39]. Later in the photocycle, however, the excess free energy will come to reside in more remote regions of the protein. This is illustrated by a recent experiment [43]. When Asp-85 was changed to an asparagine, and an additional mutation was introduced to lower the p*K* of Asp-96, raising the pH to 9 created a state analogous to the N intermediate of the photocycle, where residues 85 and 96 are neutral and anionic, respectively. In this state, produced now without illumination, the retinal was found to assume the 13-*cis*, 15-*anti* configuration, normally reached only through photoisomerization, and the protein conformation, as defined by FTIR amide bands, was like that in the N intermediate. Thus, one would conclude that once Asp-85 is protonated and Asp-96 deprotonated in the photocycle, the

[4] The origin of the released proton is still not established, but lack of a negative C=O stretch band from a protonated COOH after proton release indicates that it cannot be simply Glu-194 or Glu-204 [17].

13-*cis*, 15-*anti* retinal is better accommodated in the protein than the all-*trans*. The structural changes of the side-chains that contact the retinal observed in the M state [39] hint at the reason for why this happens. It is not obvious, however, exactly how the proton transfers that change the protonation states of Asp-85 and Asp-96 occur during the rise and the decay of the M state. It seems likely that water 402 plays a critical role in deprotonating the Schiff base and protonating Asp-85, but how the proton moves in this reaction, and whether water 402 dissociates in this process, is not yet clear.

On the other hand, the way excess free energy is dissipated in the cycle seems to be clear. In the photocycle at physiological pH (between pH 7 and 8), nearly all free energy gain is dissipated at steps where protons move downhill: at proton release to the extracellular surface ($pH > pK_{release}$, [24]), at proton uptake from the cytoplasmic surface ($pH < pK_{uptake}$, [44]), and at the last proton transfer step of the cycle, from Asp-85 to the proton release site ($pK_{Asp-85} < pK_{acceptor}$, [18,19]). The first two ensure a high efficiency for energy coupling, as protons are released and taken up by sites with p*K*s well below and above the pH of the medium, respectively. The last ensures that proton back-pressure at maximal transmembrane proton gradient does not cause the accumulation of intermediate states, and the initial state can fully recover.

The access change in the pump is the second question of importance. Kinetically, the protonation switch, from the extracellular to the cytoplasmic side, is represented by the $M_1 \rightarrow M_2$ step, which is a unidirectional reaction under some conditions but an equilibration under others [24–26,28]. In principle, the switch may be a combination of changes of the p*K*s of the proton acceptor and donor, and the making and breaking of proton conductive pathways to the extracellular and cytoplasmic directions. In the 'local-access' model, a general scheme suggested by the phenotype of the D85N/D96N mutant [45], once the retinal is in the 13-*cis*, 15-*anti* configuration, local proton transfer at the Schiff base is possible in both extracellular and cytoplasmic directions. The actual directions of the proton movements are determined, therefore, by the changing proton conductivities of the two half-channels that lead to the membrane surfaces. In the extracellular region, the conductivity is regulated through the p*K* of Asp-85 which depends on the position of the side-chain of Arg-82 and proton release. The observation that when the pH is below the p*K* for proton release, the $L \leftrightarrow M_1$ equilibrium, and therefore access to the extracellular side, is retained throughout the photocycle [25,27,28], strongly supports this. In the cytoplasmic region, the conductivity is regulated though the p*K* of Asp-96 and the formation of a postulated, but so far not actually observed, hydrogen-bonded chain of water molecules. The changed structure of the M state in the extracellular region identified the way this half-channel participates in the switch. The structural changes in the cytoplasmic region offer fewer clues [39], but there can be no doubt that this region is modulated for the passage of a proton when N is formed.

On the other hand, the kinetic data allow for factors in the access change, additional to the one related to proton release, that raises the p*K* of Asp-85 and thereby shifts the $M_1 \leftrightarrow M_2$ equilibrium toward M_2 [24–26]. At physiological pH, that is well above the p*K* for proton release, the kinetics determined from spectra in the visible yield an M_2/M_1 equilibrium ratio of ≥ 300 [24], consistent with the observed -160 mV electrical potential that can balance the electrogenic event of the $M_1 \rightarrow M_2$ reaction [26], although much greater than the ratio estimated from Raman spectra [27]. At pH below the p*K* for release, the calculated M_2/M_1 ratio is about 30 [24]. The reason for this forward bias in the M_2/M_1 equilibrium, not dependent on proton release, is not yet clear, and might reside in a geometry change at the Schiff base [33].

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