THE JOURNAL OF PHYSICAL CHEMISTRY B

 VOLUME 104, NUMBER 48, DECEMBER 7, 2000

FEATURE ARTICLE

Molecular Mechanism of Ion Transport in Bacteriorhodopsin: Insights from Crystallographic, Spectroscopic, Kinetic, and Mutational Studies

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Received: July 1, 2000; In Final Form: August 17, 2000

In the past few years, a multidisciplinary approach to the study of bacteriorhodopsin has provided answers to the most important questions about the photochemical transport cycle and yielded detailed molecular descriptions of several of its steps. The free energy gain after absorption of a photon is found to consist of electrostatic changes at the retinal Schiff base and steric conflicts with protein residues and bound water, introduced by photoisomerization of the retinal. Relaxation of the retinal chain, initially constrained by its binding site, is seen after deprotonation of the Schiff base. Structural rearrangements in the extracellular region, initiated by protonation of Asp-85 and mediated by Arg-82, cause release of a proton to the surface. Structural rearrangements in the cytoplasmic region, initiated by movement of the 13-methyl group of the retinal and mediated by Trp-182, then cause reprotonation of the Schiff base by Asp-96. The initial state is regained by reprotonation of Asp-96 from the cytoplasmic surface, reisomerization of the retinal, and deprotonation of Asp-85, that appear to utilize the same pathways of coupling.

Introduction

In bacteriorhodopsin, the light-driven pump in halobacterial membranes,[1−6] the thermal reconversion of the photoisomerized 13-cis,15-anti retinal to the initial all-trans is accompanied by a sequence of deprotonation and protonation reactions of side-chain groups (and probably also bound water) that culminate in the release of a proton to the extracellular surface and the uptake of another from the cytoplasmic side. The uptake and release of protons increase the transmembrane electrochemical H^+ gradient (protonmotive force) and result in the synthesis of ATP,[7−9] the extrusion of Na^+ from the cells,[10] and the inward transport of K^+ and nutrients[11−13] through chemiosmotic coupling. Thus, by translocating protons across the membrane, bacteriorhodopsin converts light energy into biologically usable currency. The molecular machinery that can accomplish such active transport is of great current interest and under intense investigation in bacteriorhodopsin, as well as in numerous other ion pumps driven by redox reactions[14−17] or the hydrolysis of ATP.[18] Another aspect of bacteriorhodopsin is its close structural relationship to the sensory rhodopsins,[19,20] that makes it of interest also to the wide field of receptors and transmembrane signaling.

Bacteriorhodopsin is a small integral membrane protein consisting of seven transmembrane helices, A through G, and short interhelical loops.[21] The transversely lying retinal, linked via a protonated Schiff base to Lys-216 of helix G, separates the extracellular region, with many polar side-chains and bound water molecules, from the cytoplasmic region that is more hydrophobic. The kinetic events that constitute the cyclic reaction after photoisomerization of the retinal, the "photocycle," are described by the intermediate states and the sequence and rates of their interconversions.[22,23] Measurements with time-resolved spectroscopy after pulse photoexcitation and stationary spectroscopy of photostationary states at cryogenic temperatures have defined the photocycle intermediates J, K, L, M, N, and O. The six intermediates exhibit distinct states of the chro-

10.1021/jp0023718 CCC: $19.00
Published on Web 10/25/2000

mophore with a red- or a blue-shift of its absorption maximum and are characterized at a molecular level by their FTIR,[24] resonance Raman,[25] and NMR[26] spectra. Because the number of the known physical processes (configurational changes of the retinal and the protein, changes in protonation states of residues, etc.) during the photocycle exceeds six, several intermediates must consist of sub-states. In some cases these have been suggested on logical grounds;[27] in others they have been identified also from spectroscopic and kinetic measurements.[22] Understanding of the mechanism of the transport means understanding, and in truly molecular detail, all of the intermediate states and their interconversions during the photocycle. It has been the hope of investigators that the many experimental and conceptual advantages of studying bacteriorhodopsin will produce not only a complete description of this protein, but solve also the general problem of how ion pumps work.

The development of a novel method[28,29] to grow bacteriorhodopsin crystals that diffract unusually well made it possible to determine the structure of the BR state[30−32] and some intermediates[33−35] at resolutions that allow increasingly detailed and far-reaching mechanistic conclusions. Together with the vast spectroscopic and mutational evidence already available and still accumulating, these studies are producing insights into the transport process at a level that was unimaginable only a few years ago. This short review recounts primarily these advances. Although it focuses mainly on our own results and interpretations, it is to be understood that all thinking about bacteriorhodopsin has been shaped by the contributions of many investigators, in the over 4000 extant publications over the last 30 years.

Structure of the BR State

The outlines of the structure have been clear already from cryoelectron microscopy models at 3.0−3.5 Å resolution.[21,36,37] The pathway of the transported proton is defined by the interhelical space above and below the retinal Schiff base. The extracellular region contains the anionic Asp-85 and Asp-212 that together constitute the counterion to the positively charged protonated Schiff base. It is Asp-85, and not Asp-212, that will become protonated when the Schiff base deprotonates during the photocycle. The cytoplasmic region in turn contains the protonated Asp-96, which will be the proton donor to the Schiff base. The higher resolution models from X-ray diffraction,[30−32,38] from near 3 Å to most recently 1.55 Å resolution, established the positions of the hydrogen bonds in this structure and the locations of bound water. These revealed the nature of the active center and the putative pathways for coupling changes at the centrally located retinal and at the two membrane surfaces.

The stability of the active center, with its separated charges, is ensured by the intercalated water 402 that donates hydrogen-bonds to Asp-85 and Asp-212 and receives one from the Schiff base retylidene nitrogen.[31] As shown in Figure 1A, Asp-85 is further hydrogen-bonded to Thr-89 and to water 401. Asp-212 in turn is hydrogen-bonded additionally to Tyr-185 and Tyr-57. This arrangement explains why Asp-212 remains anionic after isomerization of the retinal: its pK_a is kept low (<2) by the firm hydrogen-bonds it receives form the bulky and immobile tyrosine groups. In the extracellular region the hydrogen-bonded chain, Asp-85−water 401−water 406−Arg-82−water 403−water 404−Glu-204−Glu-194−Ser-193 leads toward the surface (the last residue is not included in Figure 1). This chain will be the candidate for facilitating the release of a proton to the surface upon protonation of Asp-85 in the photocycle, and for transferring the proton from Asp-85 to the vacant proton release site at the end of the photocycle.[39,40]

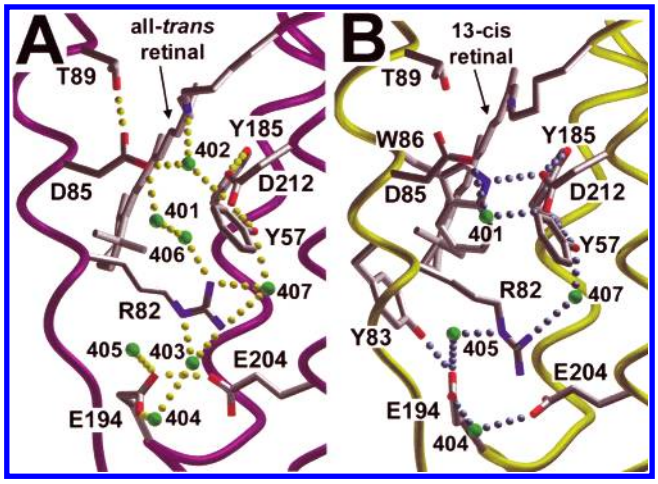

Figure 1. Crystallographic structure of the extracellular region of bacteriorhodopsin (A), and its changes in the M intermediate of the D96N mutant (B). Only the retinal and selected residues are included. Water molecules are labeled with their numbers and shown in green. Reprinted with permission from ref 34.

To the cytoplasmic side, a chain of covalent and hydrogen bonds connect the region of the retinal with Asp-96.[41] It consists of Trp-182−water 501−Ala-215 (C═O)−Lys-216 (C═O)−water 502−Thr-46 (C═O)−Thr-46 (OH)−Asp-96. This chain will be the candidate for lowering of the pK_a of Asp-96 during the photocycle and for linking the reisomerization of the retinal to the reprotonation of Asp-96. The main-chain of helix G assumes an unusual conformation at Lys-216, where the retinal is bound. The hydrogen-bonding pattern of the α-helix is interrupted by a single π-helical turn,[41] i.e., it contains a "π-bulge." This feature makes the main-chain of helix G a candidate for undergoing a local conformational shift of functional significance.

Conservation of Excess Free Energy: The K State

In the first stable state in the photocycle, the protein will have acquired the free energy gain that drives the recovery of the initial state, and provides for the transmembrane electrochemical gradient of protons produced in the process. Resonance Raman[42,43] and FTIR[44] spectra indicate that in the K state the retinal is in a twisted 13-cis configuration. It is tempting to speculate that at this stage the excess free energy is conserved in the distortion of the retinal evident from the large-amplitude C_{15}−H hydrogen out-of-plane (HOOP) vibrational bands. Steric and electrostatic conflict with residues that form the retinal binding site would make this a high-energy state. However, the structural model of the K state derived from X-ray crystallography[33] does not allow for such a conclusion, as the retinal configuration, not quite resolved at 2.1 Å resolution and 35% occupancy, was modeled as relaxed 13-cis,15-anti. On the other hand, large side-chain and main-chain atomic displacements that extend from Lys-216 to helix G were evident. Main-chain displacements of helix G at Lys-216, a feature of the M state,[35,45] seem to begin in the K state already. Near the Schiff base, no density for water 402 was reported, but Asp-85 was placed closer the retinylidene nitrogen.

The distortion of the retinal and the nature of its conflict with the binding site are more evident in the structure of the M states[35,45] that arise later in the cycle, as described below. From these results it could be concluded that the retinal does not assume a relaxed solution-like configuration until substantial changes occur in the protein. If these changes follow one another with progressively greater amplitudes until the N state is reached,

as they appear to be, the K state must contain an even more distorted retinal than that which was found in M.

Establishing the Conditions for Deprotonating the Schiff Base: The L State

In the L state the retinal is still twisted, as indicated by the high amplitude of the $C_{15}-H$ hydrogen-out-of-plane (hoop) vibrational band, and the low amplitude of the $C_{15}-H$ in-plane band.[46,47] Nevertheless, the configurational changes that store the excess free energy might be expected to have propagated more extensively to the protein than in the K state. Indeed, in the L state FTIR spectra demonstrate numerous protein changes, unexpectedly, in residues as far away as Asp-96. The peptide C=O stretching band assigned to Val-49 is shifted to a higher frequency,[48] and extensive changes of water O−H bands near Val-49, Thr-46 and Asp-96[48−50] suggest a series of rearrangements in the cytoplasmic region. According to one interpretation of the FTIR spectra, the higher frequency of its C=O stretch indicates that in L the hydrogen-bonding of the Asp-96 carboxyl group becomes weaker than in the BR state.[51] According to another, the Asp-96 carboxyl group becomes deprotonated.[52] Because these changes disappear in the M intermediate, they were proposed to represent a transient, globally strained state of the protein that is relieved upon protonation of Asp-85.[53]

At the active center, the perturbation of the Schiff base region will have upset the balance between destabilizing influences (electrostatic repulsion) and stabilizing factors (electrostatic attraction and hydrogen bonds). The possibility is now opened to decrease the number of buried charges by deprotonation of the Schiff base and protonation of one of the two neighboring anionic aspartates. Although the ensuing deprotonation of the Schiff base and the protonation of Asp-85 in the M intermediate are kinetically a single process (implying that a proton is simply transferred from the Schiff base to Asp-85), the mechanism for this crucial step in the transport is not yet clear. There are two simple alternatives.[35]

(1) Despite the rotation of the $C_{13}=C_{14}$ bond, at this time the retylidene N−H points still toward Asp-85, perhaps without water 402 interposed between them. This would require substantial distortion of the retinal. In fact, there is some evidence for this. The Schiff base frequency is higher in L than in the BR and K states, consistent with stronger hydrogen-bonding of the retylidene N−H with its counterion (i.e., Asp-85). In this case, a proton could be transferred directly from Schiff base to Asp-85, followed by relaxation of the Schiff base region of the retinal so as to turn the now uncharged retylidine nitrogen toward the cytoplasmic side.

(2) The retinal is relaxed sufficiently in the L state for the retylidene N−H to have turned toward the cytoplasmic side. Direct proton transfer between the Schiff base and Asp-85 in this configuration, that would have to occur an over > 4 Å distance and a tortuous path through space, seems unlikely. Participation of Thr-89 in such proton transfer is ruled out by the near-normal formation of the M state in the T89V mutant (Brown and Lanyi, unpublished results). Protonation of Asp-85 could be therefore by dissociation of water 402, which in this model would have to be still near its original location. The resulting hydroxyl anion would then move to the cytoplasmic side of the Schiff base and receive its proton. This alternative would be analogous to how chloride ion may be translocated at the same photocycle step in the chloride pump, halorhodopsin,[54,55] and in the D85T[56,57] and D85S[58] bacteriorhodopsin mutants.

With sufficient resolution, the crystallographic structure of the L state will decide between these, and perhaps other, alternatives. Recently, a 2.1 Å resolution map was reported for the "L intermediate" produced with green light illumination at 170 K,[59] but the spectrum of the crystal shows that it contains only a minority of the L state, rather than L with only a small contribution from M, as claimed.

Coupling the Relaxation of the Retinal to Directional Ion Movement: The M States

The deprotonation of the Schiff base that produces the M state(s) exhibits complex kinetics.[60−62] It was interpreted in terms of successive equilibration reactions, where the initial protonation equilibrium between the Schiff base and Asp-85 ($L \leftrightarrow M_1$) is followed by at least two shifts toward mixtures containing more of the deprotonated Schiff base. In one version of this model, first a change of the ΔpK_a between donor and acceptor causes the $M_1 \leftrightarrow M_2$ reaction, followed by proton release to the extracellular surface that causes the $M_2 \leftrightarrow M_2'$ reaction. The pK_a for proton release is 5−6.[61,63] At higher (i.e., physiological) pH the decay of M_2 becomes unidirectional and the Schiff base deprotonates virtually fully, while at lower pH the equilibrium proceeds only as far as the $M_1 \leftrightarrow M_2$ reaction allows.[61]

The released proton originates not from the now protonated Asp-85,[64−66] but from a site near the extracellular surface. An inherent link between the protonation of Asp-85 and dissociation of another group (and *vice versa*) is established by the anomalous titration behavior of Asp-85.[67,68] Asp-85 exhibits two apparent pK_a values in the BR state, but only one in mutants of Arg-82, Glu-194, and Glu-204, i.e., of the residues necessary for proton release. Accordingly, as Asp-85 becomes protonated, the site dissociates and releases its proton to the surface. Importantly, the greater the difference between the pK_a for release and the pH, the more the pK_a of Asp-85 will rise and the protonation equilibrium mixture will contain more of the deprotonated Schiff base. This would be the reason for the M rise component associated with the $M_2 \leftrightarrow M_2'$ reaction. The identity of the proton release site is not known. Glu-204,[69] Glu-194 *plus* Glu-204,[38] a water molecule,[31,70] and a hydrogen-bonded continuum in this region[71,72] have been suggested.

This mechanism, gleaned earlier from spectroscopic, kinetic, and mutational evidence, requires a molecular rationale, as does the reprotonation of the Schiff base by Asp-96, in the $M \rightarrow N$ step later in the photocycle. The answer to both of these questions can be given from the crystallographic structures reported for two different M states. Both were produced in photostationary states by illuminating bacteriorhodopsin crystals of mutants in which one or another kind of M state is stabilized. It is assumed in this approach, that these states are relevant to the M configurations in the wild-type cycle. The structure of an M state from the wild-type protein has been reported also,[73] although to lower resolution (2.25 vs. 1.8 Å) and from partial occupancy (35% vs. nearly 100%). Most of the changes resemble those detected in the extracellular region of D96N[45] and the cytoplasmic region of E204Q.[35] The M state that accumulates in the E204Q mutant,[35] in which proton release is blocked, should correspond, roughly, to M_2. The M that accumulates in the D96N mutant,[45] in which the proton donor to the Schiff base is removed, should correspond, in turn, to M_2' or a late (or last?) M state. Indeed, the two structures show progressive relaxation of the distorted 13-cis,15-anti retinal to a configuration similar to what it would assume in free solution. *The ensuing changes in the protein reveal the way the photoisomerized retinal causes proton release on the extracellular side and initiates the reprotonation of the Schiff base by Asp-96 in the next step of the photocycle.*

In both M states the C_{13}═C_{14} bond, but not the C_{15}═N bond, of the retinal has rotated so as to turn the Schiff base nitrogen toward the cytoplasmic side, i.e., away from Asp-85. As shown in Figure 1B, water 402 is not evident in the structure, and water 401 has moved to bridge the protonated Asp-85 and the unprotonated Asp-212. The now anionic state of Asp-212 is further stabilized by a new hydrogen-bond with the indole nitrogen of Trp-86. There is no density for water 406. Thus, the result of the changes at the Schiff base is that the connection of Asp-85 with Arg-82 is lost. The guanidinium group of Arg-82 is displaced by about 1.6 Å toward the extracellular surface, and its positive charge approaches the Glu-194/Glu-204 region. This is undoubtedly the way Arg-82 mediates the coupling between the protonation states of Asp-85 and the proton release site. This idea is strongly supported by the fact[35] that the displacement of Arg-82 occurs in the M states of both D96N (where proton release is normal) and E204Q (where proton release is blocked). It is therefore the events at the Schiff base that are the cause of the movement of Arg-82, and not the negative charge that develops at Glu-194 and Glu-204 upon proton release.

The involvement of the numerous water molecules in coupling Asp-85 to Arg-82, and Arg-82 to Glu-194 and Glu-204, is demonstrated by the large (> 6) deuterium kinetic isotope effect on the last phase of the rise of the M state.[63,74−76] This step is unique in the photocycle in exhibiting a strongly curved proton inventory plot that implies cooperative breaking of numerous hydrogen bonds. The isotope effect of this step is about 2× greater in the E204Q mutant,[76] consistent with the observed more complex geometry of bound water in the extracellular region of this mutant and the rearrangement of more water molecules in the M state.[35]

There are progressive changes of the retinal chain in the two M states that appear to represent relaxation of a strained configuration. In the earlier M (from E204Q), the 13-methyl group of the retinal is pushed in the cytoplasmic direction by 0.6 Å. The structural changes near the retinal and in the cytoplasmic region are shown in Figure 2. This movement continues and produces a 1.3 Å displacement in the latter M (from D96N). As a result, the chain buckles progressively upward and assumes the shape of the relaxed, bent 13-cis,15-anti configuration. This movement is made possible by the fact that the indole ring of Trp-182, which contacts the 13-methyl group, is pushed upward and its hydrogen bond with water 501 is broken. The peptide C═O of Ala-215 and Lys-216, in the π-bulge of helix G, are displaced, with a corresponding movement of water 502 and the connected peptide C═O of Thr-46. The result of this cascade of movements is that the side-chain of Thr-46 moves away from Asp-96. Another series of rearrangements initiated by the upward buckling of the retinal is the repacking of the (mostly) hydrophobic side chains between helices F and G, with the result that Asp-96 moves away from Thr-46.

The combined outcome of these changes is that the direct hydrogen bond between the side chains of Asp-96 and Thr-46 is broken, and water 504 is interposed between them.[35] Because in the BR state Thr-46 is hydrogen bonded also to the peptide C═O of Phe-42, the OH of Asp-96 must be the hydrogen-bond donor to Thr-46. This will elevate the pK_a of the carboxyl and ensure that Asp-96 is protonated. In the BR state its pK_a is at least as high as 11.[77] The appearance of water 504 makes it possible to delocalize the proton of Asp-96, and thereby lower the pK_a in the M → N reaction that follows. Earlier results had indicated that the barrier to deprotonation of Asp-96 consists mainly of the electrostatic cost of separating the proton from the aspartate anion.[78] Water 504 will have lowered this activation enthalpy.

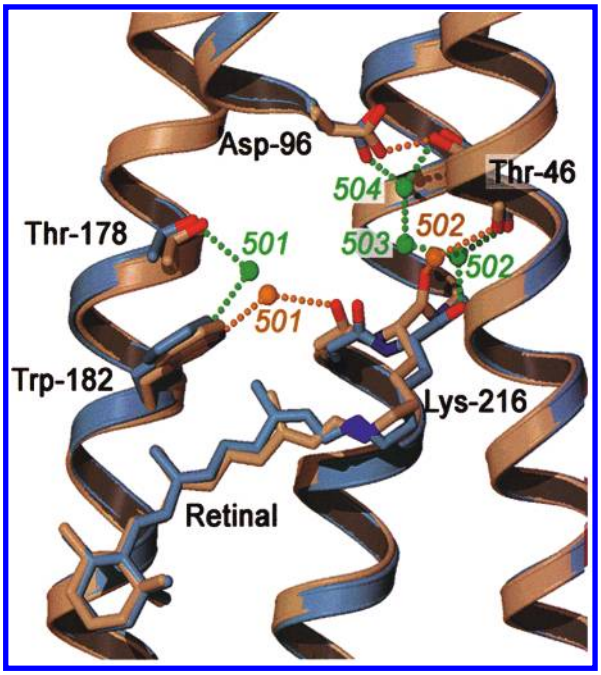

Figure 2. Crystallographic structure of the cytoplasmic region of bacteriorhodopsin, and its changes in the M intermediate of the E204Q mutant. Only the retinal and selected residues are included. The BR state is shown in beige, and its water molecules and hydrogen bonds are gold color. The M state is shown in blue, and its water molecules and hydrogen bonds are green. Reprinted with permission from ref 35.

Breaching the Hydrophobic Barrier: The N State

The kinetics suggest[79,80] that decay of the M state is through a protonation equilibrium that develops between Asp-96 and the Schiff base, with a $\Delta pK_a \leq 1$ in favor of the protonated Schiff base. There are independent estimates for the pK_as of both the Schiff base and Asp-96. In the D96N mutant, in which direct proton equilibration of the Schiff base with the bulk can be facilitated with azide, the pK_a of the Schiff base was found to be 8.3.[81] From the dependence of the ratio of the accumulated amounts of the N and O states on pH, the pK_a of Asp-96 was estimated to be 7−7.5,[82−86] in agreement with the value predicted from the M/N equilibrium.

It is generally assumed that it is a hydrogen-bonded chain of water molecules that mediates the transfer of a proton from Asp-96 to the Schiff base. The structure of the M states does not contain such a chain, and neutron diffraction maps of D96N in projection ruled out a net increase of hydration.[87] If the two water molecules not observable in the extracellular region of the M state are in fact absent, this would allow the gain of additional waters in the cytoplasmic region. In the M from E204Q (in which the cytoplasmic region is the same as in the wild-type) and in the M state of the wild type,[73] there are several new water molecules in this region (in addition to one present in the BR state already) that could be regarded as the beginnings of a chain. This putative proton-transfer pathway extends from Asp-96 to water 504, water 503, and water 502, reaching as far as the peptide C═O of Lys-216 which is about 7 Å from the Schiff base. A somewhat different proton conduction pathway of water molecules (that utilizes a 3.7 Å long hydrogen bond and passes through the C═O of Ala-215) was suggested for the M of the wild-type, and brings this network within 5.9 Å

of the Schiff base.[73] Presumably, the chain will be completed only in the next step of the photocycle. In this case, insertion of water is that will limit the rate of the formation of N.

The entry of additional water molecules into the cytoplasmic region could be related to the large-scale conformational changes evident in low-resolution difference Fourier maps, which consist of the outward tilt of the cytoplasmic part of helix F and less defined changes at helices B, C, and G.[88−91] A similar structure, but to 3.2 Å resolution and in three dimensions, was described for the unilluminated D96G/F171C/F219L triple mutant, where, apparently, an M-like conformation is assumed even when the retinal is all-trans.[92] The rigid-body tilt of helix F is described also by decreased spin−spin interaction between pairs of labels placed at the ends of helices A and F, and helices D and F.[93] These occur in the N, but not the M, state. A tilt of helix F, which begins a few residues above Trp-182, was noted, however, although with low amplitude, already in the structure of the M from E204Q.[35] Helix F in the M state of D96N is locally disordered between Val-177 and the cytoplasmic surface,[45] presumably the consequence of a continuing tilt that disrupts the crystal packing in this region. The location of the "hinge" for the tilt somewhat above Trp-182 suggests the repacking of side-chains between helices F and G on the cytoplasmic side of the retinal as its cause.

At this time in the photocycle, in the N intermediate, the retinal is in a relaxed 13-cis,15-anti configuration,[94] and the Schiff base and Asp-85 are both protonated, whereas Asp-96 is deprotonated. The conflicts of the retinal binding site and the photoisomerized retinal are now fully resolved. This is indicated by the surprising observation that in the D85N/F42C mutant, in which at pH > 8 the same charge state exists as in the N intermediate, the retinal rapidly and spontaneously isomerizes to 13-cis,15-anti in the dark.[95] In fact, except for the absence of the C═O stretch band of Asp-85, the FTIR spectrum of the D85N/F42C mutant is identical to that of a genuine N state. The characteristic amide band in the mutant indicates that the similarity extends to the well-known protein conformational change of the N state as well. It appears therefore, that unlike the earlier photocycle steps that are driven by the relaxation of the retinal, the steps after N that lead back to the BR state must be driven by the relaxation of the protein. The first of these steps is the reprotonation of Asp-96 from the cytoplasmic surface.

The pH independence of the protonation equilibrium that develops between Asp-96 and the Schiff base indicates that at the time of the M ↔ N reaction Asp-96 cannot exchange protons with the bulk phase. This is difficult to reconcile with the proposed "open" conformation in the cytoplasmic region in the M state, which would allow water entry and proton traffic to the Schiff base and thereby provide the switch function for the pump.[92] Rather, access will have to be newly created during the decay of N to reprotonate Asp-96. How this occurs is not yet clear, and little information is available for the structural difference between M and N. At 3.5 Å resolution and in projection, differences were described by one group[96] but not another.[89] Spin-label studies with membrane suspensions do suggest some kind of conformational differences between M and N.[93,97−99] It has been suggested that the four aspartic acid residues at the cytoplasmic surface, Asp-36, Asp-38, Asp-102, and Asp-104, act as a collecting funnel that channels the proton to Asp-96,[100−103] but replacing these residues with asparagine had only minor effect on (and except for D36N accelerated rather than inhibited) the rate of the protonation reaction.[104] It now appears that in a narrow pH region, well below the physiological range, the surface aspartates or glutamates do become transiently protonated (our unpublished experiments), even though this is not the rate-limiting step in the proton transfer to Asp-96.

It appears that the reisomerization of the retinal to all-trans requires that Asp-96 become reprotonated, or at least be a neutral species (as in the D96N mutant). This was suspected for some time from the concurrence of the two events in the photocycle and from the fact that an all-trans retinal containing intermediate with unprotonated Asp-96 has never been found. The link was explicitly demonstrated by the finding of the reverse transition that required no illumination: when the pH was raised from 6 to 8 so as to dissociate Asp-96 in the D85N/F42C mutant, the retinal isomerized within a few tens msec from all-trans (and 13-cis,15-syn) to 13-cis,15-anti.[105] The reason for this coupling must be that the cascade of displacements that links the upward buckling of the retinal chain to the deprotonation of Asp-96 needs to be reversed during the decay of the N state. Indeed, replacement of the residues between the 13-methyl group of the retinal and Asp-96, i.e., Trp-182, Phe-219, Val-49, Thr-90, or Leu-93, with smaller or less hydrophobic residues leads to greatly slowed (by as much as 1000×) reisomerization of the retinal.[106−109]

Recovery of the Initial Conformation: The O State

Isomerization of the retinal to all-trans might be expected to restore the geometry of the Schiff base region to nearly its initial state, but strong hoop bands[65,110] indicate twist of the chain. These disappear only upon recovery of the BR state, initiated by deprotonation of Asp-85. That this deprotonation limits the rate of the decay of the O state is indicated by the fact that in a variety of mutants of extracellular residues the rate of O decay is linearly related, over 3 orders of magnitude, to the rate of the deprotonation of Asp-85 in the dark after a pH shift from 2 to 6.[39] Thus, at physiological pH, where proton release will have occurred earlier in the cycle, Asp-85 will reprotonate the vacant proton release site. At pH <6, a proton is released during the decay of O, from Asp-85 either via the proton release group or directly. In either case, the proton follows the same path as the earlier released proton, because mutation of Glu-194 or Glu-204, that blocks proton release earlier in the cycle, greatly slows the decay of the O state.[69,111,112] However, little is known about this pathway, because the structure of O is not yet known. The appearance of a C═O stretch band of COOH, and the disappearance of a symmetrical C═O band from COO^- concurrently with deprotonation of Asp-85[113] indicated that an acidic residue might be the initial proton acceptor to Asp-85. [^{13}C] labeling had shown that this residue is an aspartate, and as the bands were unaffected by mutation of Asp-96 and Asp-115, it seems likely that the residue is Asp-212. In the M state, Asp-85 and Asp-212 are bridged by the hydrogen-bonded water 401.[45] If Asp-212 were transiently protonated during the decay of the O state, the path of the proton, on its way toward the extracellular surface, would include this residue.

Redundancies and Variabilities in the Transport Mechanism

Although the photocycle steps are now basically understood, and several of them in considerable molecular detail, it is clear that the transport process cannot be fully described without considering the many additional alternative modes of transport that come into play under nonphysiological conditions, and in some mutants. Light-driven transport of some kind occurs at pH as low as 1 and as high as 11, and virtually no single-residue

replacement, including mutation of Asp-85 and Asp-96, fully abolishes all transport activity. In most cases what appears to be inhibition of the transport is, in fact, merely the slowing of the overall turnover of the photocycle, and the rate of the pump is unaffected under light-limiting conditions. In principle, therefore, the translocation itself is not inhibited. In other cases, some of which are discussed below, however, there are interesting changes in the transport mechanism.

Because the proton release group has a pK_a of about 9 in the BR state and decreases to about 5 during the photocycle, one might expect that outside this pH range the photocycle would be perturbed as release of a proton during the rise of the M state is absent. It is indeed true that at pH > 9 the release site has no proton to release,[114,115] and at pH < 5 it will remain protonated during the cycle.[61] However, the rise of M is not slowed when the proton release is blocked in this way, or by mutation of Arg-82, Tyr-57, Glu-194 and Glu-204. At low pH (pH $\ll$ pK_a for proton release), at least, it has been demonstrated that while the $M_2 \leftrightarrow M_2' + H^+$ equilibrium (which favors M_2') is not established, the $M_1 \leftrightarrow M_2$ reaction favors M_2 sufficiently to allow nearly full deprotonation of the Schiff base. The persistence of the L state under these conditions[61] does not seem to be a hindrance to transport. Transport is accomplished by releasing a proton, from Asp-85 to the extracellular surface, at the end of the photocycle, i.e., after uptake of a proton at the cytoplasmic side. Thus, instead of a net loss of a proton from the protein in the M and N states, there is a net gain of a proton during the O state.

Given the important role played by Asp-85 and Asp-96 in the transport, it might be expected that the D85N/D96N mutant, and its variants, would be completely inactive. However, when the retinal Schiff base is initially unprotonated, this mutant was found to exhibit light-driven proton transport that amounted to a few percent of the wild-type.[116,117] A thorough study of this process, which utilized both visible and vibrational (resonance Raman and FTIR) spectroscopy and explored proton release and uptake also upon pH jump in the dark in membrane sheets and in membrane vesicles, yielded the unexpected result that a net translocation of protons is possible by a much simpler mechanism than in wild-type bacteriorhodopsin.[118] In this mechanism the extracellular and cytoplasmic regions contribute nothing more than static half-channels between the active center and the membrane surfaces. Schematically, the transport is explained by the facts that (i) the proton conductivity is less in the extracellular than in the cytoplasmic half-channel and (ii) when the retinal is all-trans the Schiff base proton has local access exclusively to the extracellular side, but after photoisomerization it acquires concurrent access to both sides. Thus, in the photocycle, the Schiff base first gains a proton, preferentially from the cytoplasmic side, and its reprotonation allows its reisomerization to all-trans, whereby it loses its proton, obligatorily to the extracellular side. Although not very effective, this is a very simple mechanism with few structural requirements. It could be a model for the evolutionary ancestor of ion pumps.

The implication of this mode of transport to the highly evolved wild-type mechanism is that the direction of the translocation is determined by the asymmetry of the protein to either side of the active center, rather than merely by the local geometry at the Schiff base. The significance of local geometry is, rather, to allow access to both extracellular and cytoplasmic directions. Indeed, two-photon experiments with residue 85 now as aspartate confirmed that the predicted concurrent access of the Schiff base to both directions persists in the photocycle when the pH is low enough to prevent proton release to the extracellular surface.[119] The mechanism that was proposed from these observations was termed the "local-access" model.[118] According to this model, the proton transfer at the active center is inherently bi-directional, and its directionality is determined by the changing "proton conductivities" of the half-channels to the two surfaces during the transport cycle. From crystallographic comparisons of the M states,[35] the molecular mechanism by which the proton translocation acquires direction in this way is now becoming clear. On the other hand, there are other, also structurally based, proposals for the mechanism that establishes directionality of the transport. From small-molecule structures of retinal analogues it was suggested that the retinal assumes a less curved configuration when deprotonated, moving the Schiff base nitrogen about 0.7 Å in the cytoplasmic direction.[92] This would reduce its accessibility to Asp-85, as required. From a crystallographic study of an early intermediate it was suggested that the main-chain of helix C (with Asp-85) first approaches the Schiff base and then retracts,[59] thereby inhibiting reprotonation of the Schiff base by its proton acceptor. Last, it was suggested that the directionality of the transport originates from the reorientation of the Schiff base away from the hydrogen-bonded extracellular network, which breaks its connection to Asp-85.[73]

The sequence similarity between bacteriorhodopsin, the outward directed proton pump, and halorhodopsin, the inward directed chloride ion pump, had suggested that these proteins, found in the same halophilic archae, might be related in a functional sense as well. Unexpectedly, it requires very little manipulation to interconvert the ion specificities of the two pumps. The residue equivalent to Asp-85 is a threonine in halorhodopsin, and the D85T bacteriorhodopsin mutant is an inward directed chloride pump,[56,57] as is D85S.[58] The protonation of Asp-85 in wild-type bacteriorhodopsin is equivalent to transfer of a chloride ion from the extracellular to the cytoplasmic side of the Schiff base in D85T, because both result in the release of a proton to the surface, which is blocked by the E204Q mutation.[58] In fact, all that might be needed for chloride transport is that Asp-85 be protonated. At pH < 2 the finding of photoelectric current with chloride/bromide but not proton/deuteron specificity had suggested that anion transport might occur.[120]

On the other hand, mutation of the threonine to aspartate does not convert halorhodopsin into a proton pump. Specificity four protons can be conferred on halorhodopsin by the weak acid, azide, instead, which will bind to the chloride site in the extracellular region near the Schiff base and the threonine.[121] During the photocycle azide becomes protonated in the same way as Asp-85 does in bacteriorhodopsin, while another azide molecule appears to shuttle protons in the cytoplasmic side so as to reprotonate the Schiff base, thereby completing the proton transport across the protein. Thus, an exogeneous proton acceptor/donor converts halorhodopsin into a proton pump. The close functional relationship between the two pumps indicates, perhaps, a fundamental similarity of the machinery needed for translocating protons and chloride ions (or hydroxyl and chloride ions, as discussed above).

Another variant of this proton transporter family is sensory rhodopsin. The primary structures of these retinal proteins are homologous to bacteriorhodopsin, even though they function not as pumps but as phototactic receptors in the archaea[122] and it seems also in eukaryotes.[123] Unexpectedly, when one of them, SRI, is free of its otherwise tightly bound transducing protein, it functions as a light-driven proton transporter.[124,125] As for Asp-85 in bacteriorhodopsin, the transport requires that the

counterion, Asp-76, be initially uprotonated (here it has a much higher pK_a than in the other protein) so it can function as a proton acceptor. As in the D96N mutant of bacteriorhodopsin,[78,126,127] reprotonation of the Schiff base is highly pH dependent, suggesting lack of an internal proton donor. These observations have raised the interesting question of whether the transport function of SRII is related to its signaling or is a relic of its evolutionary relationship to bacteriorhodopsin.

Prospects

In the past few years, our understanding of bacteriorhodopsin has reached a mature stage that invites reflection as to what more we need to know. It seems that the information now available is far more extensive than for other membrane ion pumps, and it extends to both reaction mechanism and structure. This review suggests that, although there are still many outstanding and important unsolved problems, they concern details rather than principles, and can be asked in very specific and focused ways. For the most part, what is still missing for a comprehensive description of the light-driven proton transport are the high-resolution crystallographic structures of the K, L, N, and O intermediates. These will provide answers to the following final questions: (i) In what way is the photoisomerized retinal distorted in the K state before its conflict with the binding site is resolved as it transfers its excess free energy to the protein? (ii) What is the pathway of the proton of the retinal Schiff base as the latter becomes deprotonated during the L to M reaction? (iii) What is the proton release site? (iv) Is the reprotonation of the Schiff base facilitated by a fully formed hydrogen-bonded chain that reaches from Asp-96 to the Schiff base in the N intermediate, and how is this chain formed through the large-scale conformational change at helix F? (v) What is the pathway of the proton as it passes from Asp-85 to the proton release site at the extracellular surface in the O to BR reaction, the last step of the photocycle?

Acknowledgment. The author is supported by grants from the National Institutes of Health (GM 29498) and the Department of Energy (DEFG03−86ER13525).

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