Crystallographic studies of the conformational changes that drive directional transmembrane ion movement in bacteriorhodopsin

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Abstract

Recent advances in the determination of the X-ray crystallographic structures of bacteriorhodopsin, and some of its photointermediates, reveal the nature of the linkage between the relaxation of electrostatic and steric conflicts at the retinal and events elsewhere in the protein. The transport cycle can be now understood in terms of specific and well-described displacements of hydrogen-bonded water, and main-chain and side-chain atoms, that lower the pKₐs of the proton release group in the extracellular region and Asp-96 in the cytoplasmic region. Thus, local electrostatic conflict of the photoisomerized retinal with Asp-85 and Asp-212 causes deprotonation of the Schiff base, and results in a cascade of events culminating in proton release to the extracellular surface. Local steric conflict of the 13-methyl group with Trp-182 causes, in turn, a cascade of movements in the cytoplasmic region, and results in reprotonation of the Schiff base. Although numerous questions concerning the mechanism of each of these proton (or perhaps hydroxyl ion) transfers remain, the structural results provide a detailed molecular explanation for how the directionality of the ion transfers is determined by the configurational relaxation of the retinal. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Bacteriorhodopsin, the light-driven pump in the cytoplasmic membranes of halobacteria [1–4], has posed a special challenge ever since its discovery three decades ago, because it promised to provide answers to the many unsolved questions of active ion transport. Indeed, this protein has become the best understood ion pump. In the last few years, particularly, the method developed to grow well-ordered crystals [5,6], led to unusually rapid progress in describing the atomic structure of this small membrane protein and its transport mechanism [7–12]. Understanding the light-driven transport at a truly molecular level is now within reach.

The central question in bacteriorhodopsin, as in other pumps, is how the driving reaction, in this case the multi-step relaxation in which the photoisomerized 13-cis,15-anti retinal returns to its initial all-trans configuration, causes the ion transfers in the protein that culminate in proton release at one surface and uptake at the other, against a transmembrane electrochemical gradient. The mechanism of pumps must include a ‘switch’, which determines the direction of the flow of the ion through the transport pathway across the protein. The nature of this switch is not well defined, however. It may reside in a single step, e.g., in a varying geometry of the retinal
Schiff base relative to the immediate proton acceptor and donor [13], or alternatively, or additionally, in other reactions of the transport cycle in which the varying proton affinities of the acceptors and donors play the decisive part [14,15]. Thus, movement of the protons may be controlled by either making and breaking the transfer pathways, or by increasing and decreasing the proton affinities of binding sites. A new structural approach, described here, together with the more conventional mutational and spectroscopic studies, has now begun to yield the kind of detailed mechanistic description that answers this and other conceptual questions of the transport mechanism.

2. Structure of the unilluminated (BR) state

It has been known from cryo-electron microscopy of the naturally occurring two-dimensional crystalline bacteriorhodopsin lattice, at increasing resolutions up to 3 Å [16–19], that the protein consists of seven transmembrane helices, A through G, that lie at various small angles to the membrane normal, and short interhelical loops and N- and C-termini. The all-trans retinal is in a transverse position near the middle of the interhelical cavity, bound to the ε-carbon of Lys-216 on helix G via a protonated Schiff base. The Schiff base, and the anionic Asp-85 and the protonated Asp-96, located in the extracellular...
and cytoplasmic regions, respectively, constitute the main components of the transport pathway. The extracellular region contains many polar side-chains, in contrast with the cytoplasmic region which appears to be predominantly hydrophobic.

X-Ray diffraction of three-dimensional crystals, at better than 2 Å resolution, located the ordered water molecules inside the protein and identified the functionally important hydrogen-bonds [9,11]. The structure, shown in Fig. 1a (from [9]), contains important clues to the changes that determine the direction of the ion transfer events in the transport cycle. The positively charged protonated Schiff base is stabilized in the immediate vicinity of the anionic Asp-85 and Asp-212 side-chains by water 402 which is coordinated by these three groups. This region is stabilized also by hydrogen-bonds of Asp-85 and Asp-212 to Thr-89, Tyr-185, Tyr-57, and water molecules 401 and 406. An extensive 3-dimensional hydrogen-bonded network, in which Arg-82, Glu-194, Glu-204, Ser-193 and water molecules 403, 404, 405 and 407 also participate, connects Asp-85 with the extracellular surface. This network constitutes a coupling pathway which facilitates long-distance interaction between the buried retinal region and the extracellular proton release site, as discussed below. The cytoplasmic region provides a hydrophobic barrier to ion traffic when the chromophore is not cycling. This region also contains a coupling pathway, that extends from Trp-182 via water 501, the C=O of Ala-215, two peptide bonds in a π-bulge configuration, the C=O of Lys-216, water 502, the C=O of Thr-46, and OG1 of Thr-46, to Asp-96. Along with a repacking of side-chains between helices F and G, the movement of this chain of covalent and hydrogen-bonds facilitates the long-distance interaction between the retinal and the cytoplasmic proton donor, Asp-96, as discussed below.

3. Kinetic description of the transport cycle

The photocycle, initiated by the photoisomerization of the retinal, is described as a reaction sequence of the J, K, L, M, N and O intermediates [20–22]. These states differ from one another in the configuration of the retinal and in the protonation states of key residues, in ways that are now fairly well understood from various spectroscopic approaches such as Fourier transform infrared (FTIR) [23,24], resonance Raman [25], and nuclear magnetic resonance [26]. It is intuitive that the cycle should consist of events coupled to the release of electrostatic and steric conflicts that arise upon photoisomerization of the retinal in the locally restrictive environment of its binding site, and later spread to distant regions of the protein. At the kinetic level, these events are described as interconversions of the intermediate states, while keeping in mind that they consist of changes of the pKₐ's of dissociable residues that constitute the driving force for the ion transfers, and changes in their geometry and hydration that provide the required pathways for the ion transfers.

The changed steric relationship of the numerous charged groups at the retinal Schiff base in the J, K, and then the L, states results in the deprotonation of the Schiff base and the protonation of Asp-85. In the L state, FTIR spectroscopy has detected distinct changes of the hydrogen-bonding of protein groups and water [27]. The protonation equilibrium that first develops generates what is kinetically defined as a mixture of the L and M₁ states [28]. In a model proposed to explain this [29], M₁ is converted to M₂ and then to M₂', the second step being coupled to release of a proton to the extracellular surface.² This scheme had its origin in the pH dependence of the L and M kinetics, but titration experiments in the dark had shown directly [30] that protonation of Asp-85 is indeed coupled to deprotonation of another group. The biphasic titration of Asp-85 yields pKₐ's for this group when Asp-85 is unprotonated or protonated, that correspond remarkably well to the pKₐ's for the putative proton release group in the BR state and during the photocycle (about 9 and 5, respectively). This group, which will release a proton to the extracellular surface during the photocycle, is still not identified, although Glu-194, Glu-

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¹ The numbering of water molecules is according to that in the 1.55 Å resolution structure [9].

² Alternatively, the model might have M₁ ↔ M₂ linked to proton release, followed by the M₂' state. The kinetics do not favor one model over the other [29].
204 and/or hydrogen-bonded water have been implicated, [31–35]. The two reactions between L and M2', particularly the second one that is linked to the better understood proton release [29], raise the pKa of Asp-85 and shift proton equilibrium toward nearly full deprotonation of the Schiff base. This will block reprotonation of the Schiff base from the extracellular side.

Instead, the Schiff base is reprotonated by Asp-96, a residue located to the cytoplasmic side, in the reaction that produces the N state. In the N intermediate in the wild type, under physiological conditions, Asp-85 and the Schiff base are protonated (uncharged and positively charged, respectively) and Asp-96 is deprotonated (negatively charged). At this stage, the retinal is evidently no longer in conflict with its binding site because when this constellation of three charge states is engineered into the protein, the retinal is thermally converted from the all-trans to a stable photoisomerized 13-cis,15-anti configuration [36]. Now this state, otherwise the result of photoisomerization, is assumed without illumination upon raising the pH so as to deprotonate Asp-96. The results demonstrate directly that, similarly to the extracellular region where there is coupling of the protonation states of groups [30,33], a coupling mechanism exists also in the cytoplasmic region that links the isomeric state of the retinal with the protonation state of Asp-96.

4. Conversion of free energy gain by the retinal into vectorial ion movement

The ion transfer steps of the photocycle together add up to the full translocation of a proton across the width of the protein that spans the membrane. All steps are understood in terms of simple acid/base chemistry inside the protein and at its two surfaces, but how and why they occur after photoisomerization of the retinal has not been as clear. The recently determined crystallographic structures of two of the M states [10,43] now reveal how the only locally vectorial C13–N–C14 bond-rotation in the retinal is translated into larger changes that cause the directional movement of the transported ion across the full width of the membrane.

Comparison of the crystallographic structures of the two M states, produced in photostationary states at ambient temperature from the E204Q [43] and the D96N [10] mutants, reveal differences suggestive of an ‘early’ (or pre-proton release) and a ‘late’ (or post-proton release) state, with deprotonated Schiff base. They should resemble, although not necessarily identical with, the kinetically defined M2 and M2' (or M1 and M2) states, respectively. In these states the retinal undergoes two kinds of progressive changes: (a) the Schiff base turns away from the region of water 402 (now absent), Asp-85 and Asp-212, and the apex of CE-NZ-C15 angle points toward the cytoplasmic direction; and (b) the constrained retinal chain, initially linear and extended in spite of the rotation of the C13–N–C14 bond, assumes the upward buckling at C13 expected in a relaxed 13-cis,15-anti configuration. These two changes, which occur in response to the photoisomerization of the retinal, have functionally important conformational consequences in the extracellular and cytoplasmic regions of the protein.
In order to separate the local effects of the mutations that stabilize the two M states from the desired properties of the M state of the wild-type protein, Fig. 1b shows a composite of the structures of the M states from D96N (for the extracellular region) and E204Q (for the cytoplasmic region). To the extracellular side of the retinal, the displacement of the Schiff base nitrogen in the earlier M (and maybe in the states before it) causes disruption of the balance of hydrogen-bonds of water 402, and this water is no longer evident in the structure [10,43]. Water 401 moves to bridge Asp-85 (now protonated) and Asp-212, and importantly, water 406 is absent. The side-chain of Arg-82 thereby loses hydrogen-bonding, as well as electrostatic interaction, with the region of Asp-85, and flips toward the extracellular surface, NZ becoming displaced along the \( z \)-axis by as much as 1.6 Å. It interacts now more intimately with the two carboxyl groups implicated in proton release, Glu-194 and Glu-204, as suggested earlier on logical grounds and from modeling [44,45]. The shuttling of the positively charged guanidinium group of Arg-82 between the ‘up’ and ‘down’ positions appears to be the means of coupling protonation of Asp-85 to deprotonation of the proton release site. Because the movement of Arg-82 occurs also when the proton release is blocked by the E204Q mutation [43], its cause cannot be the negative charge that would develop at the surface from the proton release. The cause of the displacement of Arg-82 must be the protonation of Asp-85. This finding therefore establishes the events at the Schiff base as the cause of proton release to the extracellular surface.

In contrast with the changes at the Schiff base, the displacements of the retinal chain occur later, during the lifetime of the M state. In the earlier and late M states the 13-methyl group moves upward by 0.6 and 1.3 Å, respectively (Fig. 1b shows the retinal in the first of these states). The end-to-end distance of the chain becomes accordingly progressively shorter, and the angle at C13 decreases from the distorted 126° to its expected lower value, indicating that the retinal assumes, increasingly, the bent shape of the 13-cis,15-anti configuration when unhindered, as in solution. The relaxation of the extended, strained chain is made possible by the fact that the indole ring of Trp-182 sets into motion two parallel series of events.

(1) Water 501 remains hydrogen-bonded to the indole N of Trp-182, but its hydrogen-bond to the C=O of Ala-215 is broken. Because of this, and because of distortion of the side-chain of Lys-216, the \( \pi \)-bulge region of the main-chain of helix G (Ala-215 and Lys-216) moves, and water 502, hydrogen-bonded to the C=O of Lys-216, is displaced along with the C=O of the connected Thr-46. OG1 of Thr-46 is thereby moved away from Asp-96.

(2) The upward movement of Trp-182 is accompanied by repacking of the mostly hydrophobic side-chains (of Leu-93, Leu-181, Thr-178, and Phe-219) between helices F and G. The changed geometry of side-chain packing moves the carboxyl of Asp-96 away from Thr-46.

As a result, the direct hydrogen-bond between the side-chains of Thr-46 and Asp-96 is broken, and a new water, water 504, is intercalated between them. This will allow lowering of the \( pK_a \) of Asp-96, and it becomes the proton donor to the Schiff base. The results establish, therefore, that the relaxation of the retinal chain is the cause of proton movement across the cytoplasmic region, from Asp-96 to the Schiff base. They establish also the structural basis for the reverse, the coupling of the reprotonation of Asp-96 to the reisomerization of the retinal.

A large amount of information on the phenotypes of various mutants support the existence of coupling links in both extracellular and cytoplasmic regions. The pathway of coupling in the extracellular region is confirmed by the consequences of residue replacements at Arg-82 (R82A or Q), Tyr-57 (Y57F), Glu-194 (E194C or Q), or Glu-204 (E204Q), which either abolish proton release or greatly shift its \( pK_a \) [31,32,34,46-48]. The pathway of coupling in the cytoplasmic region is confirmed by the extremely slow decay of the N state (by three orders of magnitude) when any of six residues between the 13-methyl group of the retinal and Asp-96, or near Asp-96, i.e., Trp-182, Thr-46, Phe-171, Phe-42, Thr-90, and Leu-93, is replaced with a smaller or non-hydrogen-bonding residue [49-53]. This is as expected if N decay, where reisomerization of the retinal depends on reprotonation of Asp-96, were slowed by the greatly lowered proton occupancy of Asp-96 in these mutants.
5. Mechanism of local proton/hydroxyl transfers, as still unsolved questions

Although the driving force for the ion transfers in the extracellular and cytoplasmic regions are now understood in terms of the gradual response of the retinal binding site to the changing retinal configuration, how these ion transfer events occur is not clear [54].

Deprotonation of the Schiff base and protonation of Asp-85 appear kinetically as a single step, but direct proton transfer between them is only one of the alternatives. It would require that the retinal be highly strained at the Schiff base, so as to connect it with Asp-85 in spite of the $C_{13} = C_{14}$ bond isomerization. Another possibility is that water 402 dissociates in the L to M reaction, and in a concerted reaction protonates Asp-85 while the OH moves to the cytoplasmic side and receives the proton of the Schiff base. This mechanism would be analogous to Cl transport in halorhodopsin [55,56] or the D85T mutant of bacteriorhodopsin [57], where movement of an anion from the extracellular side of the protonated Schiff base to its cytoplasmic side is an unavoidable step of the transport cycle.

In the M state the beginnings of a hydrogen-bonded chain of water molecules between Asp-96 and the Schiff base is already evident, consisting of a network of water 502, 503, and 504, hydrogen-bonded to each other and to main-chain and side-chain atoms [43]. However, because this chain does not reach the Schiff base, it seems that the rate-limiting step in the reprotonation must be the conformational change that brings at least two water molecules into this region to complete it.

The means for reprotonating Asp-96 are as yet unclear. It must be the result of a conformational shift that allows accessibility, not present until this time, between Asp-96 and the cytoplasmic surface. Also unclear is the pathway the proton takes in the extracellular region during O decay. Understanding these steps requires that structures for the N and O states be determined.

6. Perspectives

Solving the transport mechanism in bacteriorhodopsin means answering two questions: (1) how the photoisomerized retinal causes timed changes of the $pK_a$ of the appropriate protonatable groups, and (2) how protons (or hydroxyl ions) are transferred from site to site in response to these changes of proton affinities. Unexpectedly, we have been able to answer the first question without having complete answers to the second. The crystallographic results on different M states have provided conclusive supporting evidence for the earlier proposed flip/flop of Arg-82 as mediator of proton release to the extracellular surface, and contributed new evidence for how movement of the retinal is coupled to decrease of the proton affinity of Asp-96, and therefore to reprotonation of the retinal Schiff base. Further progress, which will complete the molecular description of this pump, will require structural understanding of the other intermediates involved in transport steps, i.e., L, N and O.

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References


