The role of protein-bound water molecules in microbial rhodopsins

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PROTEIN-BOUND INTERNAL WATER MOLECULES ARE ESSENTIAL FEATURES OF THE STRUCTURE AND FUNCTION OF MICRObial RHODOPSINS. Besides structural stabilization, they act as proton conductors and even proton storage sites. Currently, the most understood model system exhibiting such features is bacteriorhodopsin (BR). During the last 20 years, the importance of water molecules for proton transport has been revealed through this protein. It has been shown that water molecules are as essential as amino acids for proton transport and biological function. In this review, we present an overview of the historical development of this research on BR. We furthermore summarize the recently discovered protein-bound water features associated with proton transport. Specifically, we discuss a pentameric water/amino acid arrangement close to the protonated Schiff base as central proton-binding sites, a protonated water cluster as proton storage site at the proton-release site, and a transient linear water chain at the proton uptake site. We highlight how protein conformational changes reposition or reorient internal water molecules, thereby guiding proton transport. Last, we compare the water positions in BR with those in other microbial rhodopsins to elucidate how protein-bound water molecules guide the function of microbial rhodopsins. This article is part of a Special Issue entitled: Retinal Proteins – You can teach an old dog new tricks.

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1. Introduction: the family of microbial rhodopsins and bacteriorhodopsin

Microbial rhodopsins are a family of transmembrane proteins found in archaeabacteria, bacteria, eubacteria, and even simple eukaryotes like fungi [1]. Their functional repertoire ranges from proton [2] and cation pumping [3] to light sensing [4], osmotic pressure control via chloride pumping [5], and ion channel activity, allowing passive charge movement across the membrane [6,7]. Despite their large functional diversity, they all share a basic structural fold pattern of seven transmembrane helices (termed A to G) and a retinal molecule covalently attached to the protein via a protonated Schiff base formed with a lysine in helix G. In each protein, the retinal molecule undergoes a trans/cis isomerization, which drives BR through a set of conformational intermediates, named K, L, M, N, and O in order of their appearance [2]. A deeper understanding of this proton transport should allow a better understanding of ATPases [10–13], cytochrome C oxidase [14–16], and photosystem II [17–19], the central proteins in bioenergetics. Though the research on microbial rhodopsins has a long tradition, it recently experienced a renaissance with the discovery of the channelrhodopsins from Chlamydomonas reinhardii [6,7], which have become the corner stone of the fast-evolving field of optogenetics [20,21]. Moreover, the microbial rhodopsin subfamily of proteorhodopsins is found in microbial plankton in oceans worldwide, making it one of the major sources of biological light energy conversion on the planet [22–24].
2. Proton transport through water molecules in bacteriorhodopsin

2.1. Protonation changes of the aspartic acids 85 and 96 as catalytic residues on the release and uptake pathway

During the last 20 years, the importance of water molecules for proton transport in br was revealed. Indeed, water molecules are as essential as amino acids for proton transport and biological function [25]. However, before the involvement of water molecules in proton pumping through br was elucidated, proton transport via amino acids was discovered. Before the first three-dimensional structural models were resolved by electron microscopy [26], internal aspartic acids within the helical core of br were identified as temporary binding sites for protons during the photo cycle through the use of Fourier transform infrared (FTIR) spectroscopy and isotopic labeling [27]. However, isotopic labeling did not allow a specific assignment to individual residues and had a problem to distinguish between protonation changes and hydrogen bond changes. Using for the first time a combination of FTIR and site-directed mutagenesis, the absorbance bands of Asp85, Asp96 and Asp 115 were clear cut assigned, and also protonation changes were clearly distinguished from hydrogen bond changes [28–30]. In the next step, the respective protonation kinetics were determined using the first time-resolved FTIR experiments [31]. Earlier studies proposed [27,28] that the then so-called “Asp 1”, now identified as Asp96, deprotonates in the L intermediate and so either deduced a wrong placement of these amino acids [27] or the involvement of additional amino acids [28]. However, these studies were misleading and are now considered incorrect. Today, it is generally accepted that after the all-trans to cis isomerization, the protonated Schiff base protons its counterion Asp85 on the release pathway in the M intermediate, and that Asp96 is located on the uptake pathway and deprotonates in the N intermediate [29,31], which leads to the reprotonation of the Schiff base. The aspartic acids 85 and 96 and the retinal Schiff base are the catalytic key players of the proton transport mechanism, as for the first time mechanistically correct proposed by Gerwert et al. in [29,31].

Surprisingly, Asp96 has an unusually high pKa in the ground state and is protonated [29,32]. It is deprotonated only in the N intermediate [31]. This result was highly controversial at the time of its publication in the early 1990s, because several groups ignored the N intermediate at that time, as it was not detectable by them. The proposal was supported by electrical charge displacement measurement, also showing the key role of Asp85 and Asp96 [33]. Mutation of these residues inhibits proton pumping. The first three-dimensional structural model of br [26] showed that Asp96 and the retinal Schiff base are separated by approximately 1.2 Å. This distance is too far to allow direct proton transfer, and the transfer would need to take place through a hydrophobic section of the protein core. Therefore, other molecular components in addition to amino acids are needed for proton transfer through the protein. Additionally, Asp85 could not be the proton release group, because at the same time that Asp85 becomes protonated, a proton is released to the external medium [34,35]. Furthermore, it was initially unclear what the proton storage position close to the extracellular protein surface (termed the proton release site) was constituted of; low-temperature FTIR studies suggested that it was Glu204 [36,37]. Because a protonated Glu204 in the ground state was not confirmed in room temperature measurements, an alternative was proposed: a protonated water cluster between Arg82, Glu194, and Glu204 as proton-release site [38], even though no water molecules were resolved by X-ray structure analysis at that time. Already in the 1980s, growing evidence from resonance Raman studies [39] and neutron scattering measurements [40–43] suggested that water molecules were somehow connected to proton pumping in br, possible through interactions between protein and internal water molecules during the photo-cycle. In the mid-1990s, especially Maeda, Kandori et al. performed FTIR measurements on br (reviewed in [44]), focusing on the spectral domain of O–H stretch vibrations between 3000 cm−1 and 4000 cm−1, and attributed changes in this domain to changes in protein-bound water molecules. Using point mutation analysis, it was possible to locate water molecule vibrations to positions within the protein that were close to the protonated Schiff base and Asp85/212 [45], the proton release site [38], and the proton uptake pathway [29,31,44]. Finally, in the first high-resolution X-ray crystallographic structures of br, several water molecules were observed at the proposed positions [46–48]. Nowadays, not only br ground state structures are available [46–48], but also structural models of photo cycle intermediate states [2]. These intermediate state structures significantly increase the knowledge on how water molecules are rearranged during the photo cycle. However, wild-type (WT) intermediate protein structures are only available for early photo cycle states (best resolutions: K: 1.43 Å, PDB ID: 1M0K [49]; L: 1.53 Å, PDB ID: 2NTW [50]; M: 1.43 Å, PDB ID: 1M0M [51]), and all later intermediate-state structural models are derived from mutant proteins (M2: 2.00 Å, D95N mutant, PDB ID: 1CBS [52]; N: 1.62 Å, V49A mutant, PDB ID: 1P8U [53]; O: 2.00 Å, D85S/F219L double mutant, PDB ID: 1V6 [54]). These mutations strongly affect both the photo cycle and the detailed protein structure, especially amino acid side chain orientation, and therefore cannot serve as correct models for the water distribution in the intermediates of wild type br. Furthermore, X-ray structures are derived from measurements performed in crystallized proteins at cryogenic temperatures, which implies that the water positions observed in such structural models may differ from those at room temperature under physiological conditions [55]. Furthermore, X-ray protein crystallography cannot resolve the positions of hydrogen atoms, as the resolution of the derived structural models usually is not sufficient to do so. FTIR measurements, however, can resolve hydrogen atoms, protons, and also the chemical environment in which they are found at physiological conditions [56]. Additionally, the atomic details of water distribution depend on the dynamics of the water molecules, which can be probed via Molecular Dynamics simulations [57,58]. Studies that combine these techniques [59–65] result in a strong synergy and have contributed towards a better understanding of the exact molecular details of the proton pathway in br in recent years. A major surprise coming from such studies was the confirmation that the proton stored at the release site is not fixed in a single binding site, i.e. an amino acid residue, but rather is delocalized over water molecules and solvated by the surrounding amino acids, Glu194/204, Tyr57, Tyr83, and Arg82, in form of a protonated water cluster solvated by amino acid residues [25,61].

2.2. Proton transfer via water molecules on the release and uptake pathway

Fig. 1 shows the proton transfer mechanism in bacteriorhodopsin. In its ground state, br stores three protons (indicated by a circle), which take part in the proton transfer: one at Asp96 (Fig. 1A) at the uptake site, one at the protonated Schiff base (Fig. 1B) as a central binding site, and one delocalized proton with multiple binding sites at the proton release site (Fig. 1C). Of these three ground state-bound protons, two are stabilized by networks of water molecules and strong hydrogen bonds: the Schiff base bound proton, and the proton at the release site. The Schiff base-bound proton forms a strong hydrogen bond to a water molecule located directly between Asp85 and Asp212 [66–68]. This, together with two more water molecules and the two aspartates, forms a pentameric cyclic hydrogen bond network [25,69] (Fig. 1B). One of these water molecules exhibits an O–H bond, which is without a hydrogen bonding partner (a so-called “dangling” hydrogen bond). This strongly hydrogen bonded arrangement, together with the dangling bond, serves as energy storage for the stabilization of the protonated Schiff base [67]. Upon photo-isomerization, the Schiff base proton is transferred to Asp85 via the attached water molecule [29,31]. The pentameric arrangement then decays, thus stabilizing the protonation of Asp85: In four out of five M state structures, less water molecules are resolved, rendering the pentameric arrangement impossible [52,70,71]. Although even in one M intermediate structure (PDB ID: 1P8H [53]), the amount of water molecules below the Schiff base remains the same, their positions differ strongly from the pentameric arrangement. The structure and
energetics of this water arrangement have been well investigated both experimentally [25, 66, 68, 69] and theoretically [67, 72].

In the L to M transition, the neutralization at Asp85 weakens its electrostatic interaction with Arg82, which in turn moves closer towards Glu194 and Glu204 at the proton release site [32, 64] (Fig. 1C). The position of the arginine depends on the protonation state of Asp85 [32]. By the Arg82 movement, the pKa of the release group is reduced as elucidated in [32]. The proton stored at the release site is not bound to a single amino acid, as usual, but is delocalized by having multiple intermediate binding sites within a cavity that contains several water molecules and Tyr57, Arg82, Tyr83, Glu194, and Glu204 [25, 38, 61]. The movement of Arg82 changes the water molecule positions at the release site, and shifts the proton towards Glu194 and Glu204, which become interchangeably protonated in early M [64]. Thus, the delocalized proton becomes localized at the proton exit site by protonation of either of the glutamates. At pH 7 this is not observed, because the deprotonation of the glutamic acids is faster than the protonation. However by reducing the pH to 5, the proton release is delayed, and alternating protonation of Glu194 and Glu204 is observed [64] (in Fig. 1C, only a protonation of Glu204 is indicated). This protonation in turn leads to a weakening of the hydrogen bond between Ser193 and Glu204. In the ground state, Ser193 forms a barrier between the internal protein cavity and the extracellular bulk solvent [73]. The rupture of the hydrogen bond opens this Ser193/Glu204 “gate”, internal water molecules come into direct contact with external water molecules, and the stored proton is released to the extracellular medium in the L to M transition in a Grotthuss like mechanism [74, 75]. This is in agreement with the proposal, that Arg82 controls the pKa of the proton release group [76, 77], while it explains the pKa decrease by the water molecule relocation towards the glutamic acids induced by the Arg82 movement.

As this mechanism only allows the release of the proton to the outside of the protein, but no proton uptake, it functions like a rectifier, ensuring efficient vectorial proton pumping. Due to its similarity in function to a diode in electronics, the release site was named a “proton diode” [64]. This model of proton delocalization over water molecules and surrounding amino acids has been well established in experiments [25, 38, 61, 64, 73]. Its distinctive marker band observed in time-resolved FTIR spectra is a so-called “continuum band”, which is a characteristic feature for delocalized protons in strong hydrogen bonds, and is found in systems such as protonated water networks [78]. Such a continuum
band was experimentally found to disappear during the proton release into the extracellular medium and to be influenced by mutations of Tyr57, Arg82, Tyr83, Glu194, and Glu204 [25,38,61].

In contrast to experiments, biomolecular simulations can describe the protonated water cluster only in small model systems today. In QM/MM simulations, only part of the protein is described by Quantum Mechanics simulations within a so-called quantum box, whereas most of the protein is described by classical Molecular Dynamics. Recent simulations focus on the analysis of different QM boxes, which contain either only the water molecules at the release site [60,62,79], or the water molecules and Glu194/204 [80,81]. However, both these simulation systems resulted in a continuum absorbance, which matches the form of the experimentally observed one. In the first case, the proton is exclusively water-delocalized [60,62,79], and in the second case, the proton becomes delocalized exclusively between the two glutamates [80,81]. Therefore, the continuum absorbance alone cannot determine the distribution of the delocalized proton in detail. To do this discrimination, amino acids were experimentally identified, which affect the continuum absorbance upon mutation [61], and thus solvated the protonated water cluster. For correct system description, they therefore have to be taken into the QM box in simulations. We recently increased the size of the QM systems to include all these amino acids that solvate the protonated water cluster (S. Wolf, E. Freier, K. Gerwert, under review). It becomes obvious, that the continuum absorbance in the glutamate-only QM systems arises from artificial symmetry within the small QM boxes.

The proton becomes artificially shared in between the two glutamic acids only. As soon as the QM box size is increased by including additional amino acids, which regards the asymmetry of the protein environment, the proton becomes localized at Glu194. This is in contrast to the experimental findings. In summary, while experiments show a delocalization of the excess proton stored at the release site over water molecules and adjacent amino acids, the detailed description of the delocalization still poses a benchmark problem for biomolecular simulations.

The next step after proton release is the proton uptake, at which the Schiff base is reprotonated by Asp96. As mentioned previously, these two positions are separated by a hydrophobic barrier of about 12 Å length (Fig. 1A), which efficiently blocks any backflow of charges through the protein core in the protein ground state. Ground state structures [47,48] only show two water molecules in this part of the protein: one hydrogen bonded between Trp182 and the backbone carbonyl oxygen atom of Ala215, and the other one at a position close to Asp96. These two water molecules alone cannot form a chain to bridge the gap of 12 Å. It was argued that, to overcome this hydrophobic barrier, βr takes up several water molecules from the intracellular solvent [52,70]. This is consistent with the finding that, in an N2 crystal structure derived from the V49A mutant [53], five water molecules are present in this region and span Asp96 and the Schiff base, though the corresponding ground state structure of V49A exhibits only the two water molecules known from WT structures. Due to this difference in number, the V49A mutant actually seems to let water molecules invade into the protein core to form a water connection between Asp96 and the Schiff base. But does this invasion hold for the WT protein? FTR experiments showed already the presence of a third water molecule in the wild-type protein close to Trp182 and Ala215 [65] in the ground state. Molecular Dynamics simulations of a WT homology model derived from the N2 crystal structure [53], in which Ala49 is mutated back to Valine, proposed that these two water molecules, together with the one close to Asp96, could rearrange to form a linear chain of three water molecules, which can then connect Asp96 to the deprotonated Schiff base in the form of a “proton wire,” which can perform a Grothuss-like proton transfer [65]. Such a transient proton wire made out of water molecules was first identified in [65] in a membrane protein and might be present in other proton transporting proteins, thus representing a general important functional feature. This result is in agreement with findings from neutron-scattering experiments conducted on the D96N mutant, which found no significant water uptake during the photo cycle [42].

The last two missing proton transfer steps, which are the reprotonation of Asp96 from the intracellular solvent, and the reprotonation of the proton release site by Asp95, are still poorly understood, mostly due to insufficient information on the O intermediate structure and the protein motions during the respective intermediate transitions. However, it is intriguing that both steps seem to require water molecules. Structural analysis of the βr ground state showed a narrow tunnel leading from the intracellular surface to Asp96 [57], which is too narrow to allow water influx in the ground state. In late M, helix F moves away from the protein core [82–86], possibly broadens this tunnel and creates enough space to allow a contact between Asp96 and the intruding intracellular water molecules, thus facilitating Asp96 reprotonation [87]. For these protonation steps, two additional aspartates at the intracellular surface (Asp36 and Asp38) are believed to serve as temporary proton binding sites that guide protons into this water tunnel leading to Asp96 [88–90], and thus work like antennas for protons. The reestablishment of the proton release site by deprotonation of Asp85 is still the most mysterious of all protonation steps, as it requires a long-distance proton transfer and crossing the positive charge of Arg82. Early stages of this transfer have been investigated theoretically [91] and experimentally [92,93], and these investigations suggest an involvement of Asp212 in the process. How the barrier of the positive charge of Arg82 is overcome in the transfer step is currently unknown. Taken together, all five major proton transfer steps within βr actively employ water molecules, which form the medium for both proton transfer and storage. Water molecules therefore are as important for the proton transfer in βα as amino acids. It is interesting to note that the first steps through the M intermediate consist of the destruction of strongly hydrogen-bonded networks, whereas the remaining steps from M to the βr ground state contain the formation of such strongly hydrogen-bonded networks.

3. Protein-bound water molecules in other microbial rhodopsins

We have shown for βr that the proton transfer mechanism depends on highly specific positions of water molecules within the protein. In the following, we investigate how well conserved water positions are among microbial rhodopsins, and whether this spatial conservation corresponds to functional conservation. Fig. 2 compares the water distribution within the crystal structures of βr to those in the analogous proton pumps archaerhodopsin 2 [94] and xanthorhodopsin [95], of the photoreceptors sensory rhodopsin II [96] and Anabaena sensory rhodopsin [97], the chloride pump halorhodopsin [5], and the ion channel channelrhodopsin [98]. We distinguish in Fig. 2 between the βr-“similar” systems, namely archaerhodopsin 2, halorhodopsin, and sensory rhodopsin II and the βr-“unsimilar” systems xanthorhodopsin, Anabaena sensory rhodopsin, and channelrhodopsin. In the proton uptake path (Fig. 2A), both in the βr-“similar” and the βr-“unsimilar” systems water molecules between Asp96 and the Schiff base (termed W501 and W502 [48]), are highly conserved. The “unsimilar” proteins show some additional water molecules at positions close to Asp96. However, one has to take into account that the amino acids surrounding these water positions differ between the respective proteins, which in turn affects the protein–water interactions and thus water positions.

All proteins contain water molecules at the positions of the pentameric arrangement below the Schiff base [Fig. 2B: W401, W402, and W406 [48]]. The βr-“similar” proteins sensory rhodopsin II, halorhodopsin, and archaerhodopsin 2 all possess a water distribution that is nearly identical to the arrangement in βr. However, the “unsimilar” systems do not exhibit such an arrangement at all. For proton pumps, it appears that this pentameric arrangement of water molecules is a crucial feature, which determines their function [66,68,99]. Sensory rhodopsin II also becomes a proton pump if it is uncoupled from its transducer [1]. Xanthorhodopsin may differ in the exact
The largest differences in water positions are found at the proton release site (Fig. 2C; W403, W404, W405, and W407 [48]). In the “similar” structure set, water molecules show a general agreement with regard to the space they occupy in bR, which is found surrounding Glu194 and Glu204. Of these “similar” proteins, only archaerhodopsin 2 [100] exhibits an early proton release like bacteriorhodopsin [101], which is a distinct evidence for an intact proton release group [25,61,64]. The “unsimilar” proteins exhibit water positions that are completely different than those found in bR. Here, water molecules are distributed across the complete extracellular half of the protein. This is due to the fact that these proteins do not contain the glutamate pair of the bR release group. It indicates that the delocalized protonate pair of the bR release site might be a specific

pumping mechanism from the other pumps because of its dual chromophore and a slightly different transmembrane fold [95].

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and optimized feature of bR and bR-like proteins, and that the other proton pumps might use a localized proton release site, a protonated amino acid. In summary, the conservation of water molecule positions in microbial rhodopsins is indeed coupled to conservation of protein function.

The involvement of water molecules in the proton transfer within channelrhodopsin II [102] is especially interesting due to its importance in the field of optogenetics [20,21]. The recently solved crystal structure of a channelrhodopsin II chimera contains numerous water molecules in the extracellular half of the protein [98], and their positions are in general agreement to those in bR. Because the protein was only crystallized in its inactive, closed state, little is known about the position of the open pore on the intracellular side. Similar to bR in its ground state, channelrhodopsin exhibits a hydrophobic barrier in the intracellular half of the protein. It is generally believed that changes in protein conformation induce a rearrangement of internal water molecules to overcome this hydrophobic barrier and thereby opening a connection between intracellular solvent and water molecules in the extracellular half of the protein, which ultimately results in a pore for proton and cation transport. The pore in channelrhodopsin II is proposed to be located between helices A, B, C and G, which is in contrast to the proton pathway in bR, located between helices C, D, F and G. This proposed location of the pore, based on homology modeling [103], is nicely supported by the high-resolution X-ray structural model [98]. This structural model also reveals numerous hydrophilic residues to line the conducting pore within channelrhodopsin II. Interestingly, one of them, Glu90, which is located close to the Schiff base, is deprotonated during the ChR2 photo cycle [103]. Consequently, point mutation experiments show that this deprotonation is crucial for the ion selectivity [103] as well as photocycle kinetics of the channel [104,105]. Furthermore, Molecular Dynamics simulations show that the deprotonation of Glu90 leads to a rearrangement of internal water molecules, which might contribute to the pore formation and the ion selectivity in channelrhodopsin [103]. In general, proton transfer reactions in channelrhodopsins are comparable to bR [106] in the absence of a membrane potential [107,108], though the detailed timing and order of protonation events might differ in channelrhodopsin II and bR [105]. The detailed molecular reaction mechanisms remain to be elucidated. With the rise of optogenetics [20,21], research on microbial rhodopsins has entered a new phase, the customization of optogenetic tools through tailored mechanistic changes. It is already known that bR can be converted into a chloride pump [109,110] by a single DBST mutation, which creates a chloride binding site at the position of amino acid 85, as it is found in halorhodopsin [5]. Furthermore, bR can be converted into a sensory rhodopsin [111,112] through only three mutations (A215T, P200T/V210Y). Last, Anabaena sensory rhodopsin has been converted into an inverse proton pump [113]. This functional convertibility coincides with the conservation of water positions in these three proteins, as we highlighted above. In this respect, one approach to perform this customization is to specifically analyze and possibly alter the strength of the protein–internal water–hydrogen bonds, and correlate protein function with it [68,99].

4. Conclusions

In this review, we highlight the role and importance of water molecules in the structure and function of microbial rhodopsins. They store and transfer protons, and this seems to be a general functional feature, which is found not only in bR, but also in other microbial rhodopsins. The water distribution seems decisive at least for the function as a proton pump. Of special interest is the pore formation by water rearrangement within channelrhodopsins. Additionally, the lessons learned from bR might play an important role in our understanding of the function of ATPases [10–13], cytochrome c oxidase [14–16,114], or photosystem II [17–19], all of which either exhibit or may exhibit water wires [115] and proton storage by water molecules [114].
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