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Review

The role of protein-bound water molecules in microbial rhodopsins ☆,☆☆

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ABSTRACT

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 site, 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 archaebacteria, 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 after light activation. The question arises, how the different functions can be performed based on such a common structural motive? Do protein-bound water molecules play a decisive role to determine the respective function of the different proteins? Are protein-bound water molecules similarly distributed in the different proteins of the microbial rhodopsin family?

The prototype for biophysical research and the best characterized system among the microbial rhodopsins is the light-driven proton pump bacteriorhodopsin (bR) [2] from the archaebacterium *Halobacterium salinarum* (also called *Halobacterium halobium*), which was first discovered as part of the purple membrane [8,9]. It is a very well suited model system to investigate proton transport across membranes by proteins, as bR is highly stable under experimental conditions, and proton transport can be triggered by flash illumination. Upon light absorption in the light-adapted ground state (termed BR), the retinal chromophore undergoes an all-trans to 13-*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].

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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 pronation 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 13-*cis* isomerization, the protonated Schiff base protonates 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 pK_a 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 12 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: 1.43 Å, PDB ID: 1M0M [51]), and all later intermediate-state structural models are derived from mutant proteins (M_2: 2.00 Å, D96N mutant, PDB ID: 1C8S [52]; N: 1.62 Å, V49A mutant, PDB ID: 1P8U [53]; O: 2.00 Å, D85S/F219L double mutant, PDB ID: 1JV6 [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 [32,59–65] 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

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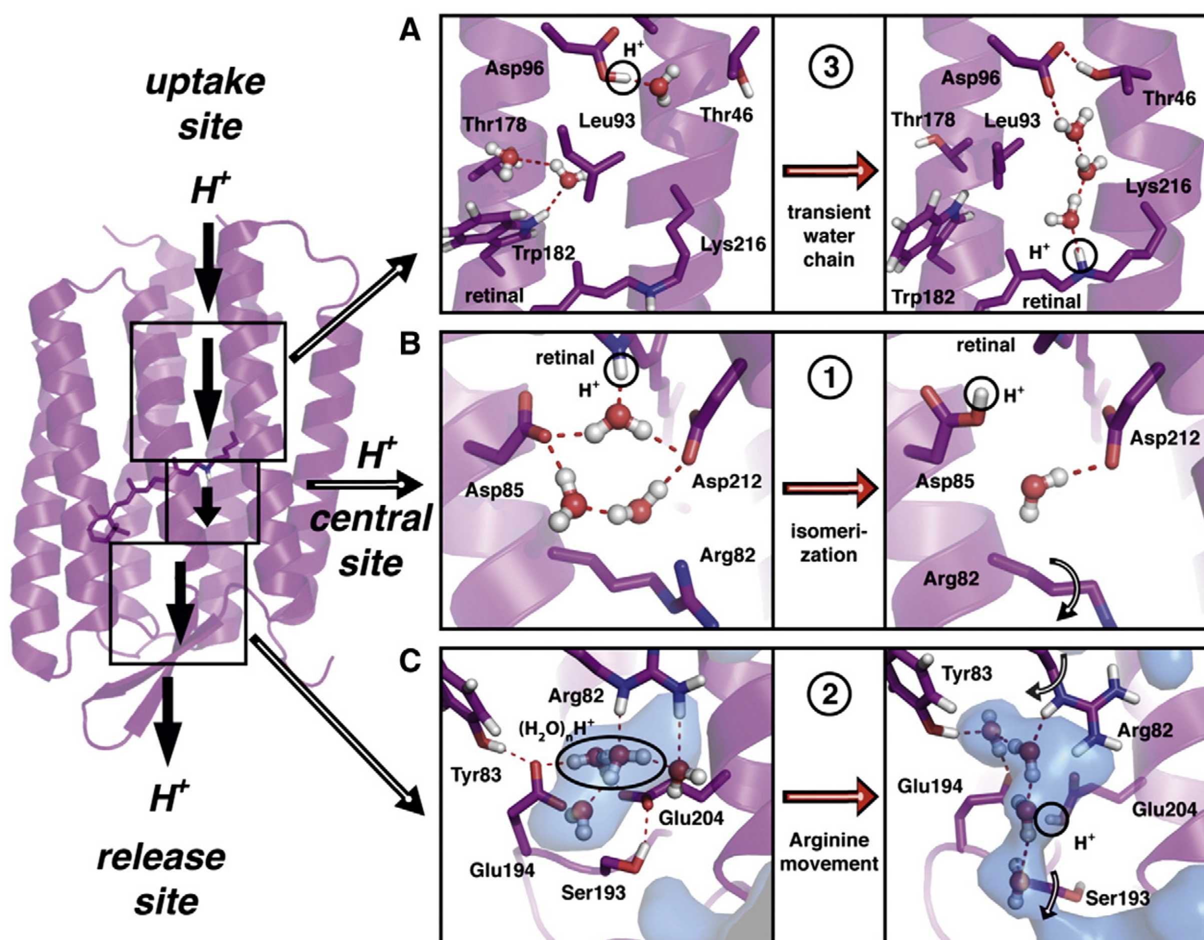

Fig. 1. Proton binding and transfer involving protein-bound internal water molecules in bacteriorhodopsin. Temporal order of transfer events highlighted as numbers in circles. Positions of transferred protons before and after transfer highlighted as black circles. **A**: In the ground state (left), Asp96 and the Schiff base are separated by a hydrophobic barrier of 12 Å. Two water molecules W501 and W502 [48] are stored in this hydrophobic region close to Thr178 and Trp182. After deprotonation, the retinal relaxation around the single bonds triggers a relocation of these water molecules, which then form a transient linear chain between Asp96 and the Schiff base (right) in the M_2/N intermediate only. This induces proton transfer from Asp96 to the Schiff base in the M to N transition. **B**: Pentameric water arrangement close to the Schiff base in the BR ground state [48] (left) and in the M intermediate [52] (right). Hydrogen atom positions in the ground state were derived from MD simulations and spectroscopic investigations [66–68], as they cannot be observed in crystal structures; hydrogen positions in the M intermediate as illustration. Three water molecules (one of which contains a non-hydrogen bonded "dangling" O – H bond), the retinal Schiff base, Asp85, and Asp212 form a pentameric arrangement of strong hydrogen bonds in the BR ground state. This feature decays after retinal isomerization, and is not observed in crystal structures of later intermediates [52,53,70,71]. **C**: Proton release site in MD simulations [64] containing a protonated water cluster and the solvating amino acid residues [25,38,64]. A downward movement of Arg82, caused by the protonation of Asp85, moves the protonated water molecules towards Glu194 and Glu204 in the M intermediate. The glutamates become interchangeably protonated (here, only a protonation of Glu204 is indicated). The hydrogen bond between Glu204 and Ser193 is broken, and the Ser193 gate [64] opens at the release site. Internal and external water molecules merge and allow proton transfer to the bulk.

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 pK_a 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 pK_a of the proton release group [76,77], while it explains the pK_a reduction 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

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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, bR takes up several water molecules from the intracellular solvent [52,70]. This is consistent with the finding that, in an N′ 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? FTIR 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 N′ 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 Grotthuss-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 Asp85, 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 bR 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 funnel 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 bR 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 bR 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 back to the BR ground state contain the formation of such strongly hydrogen-bonded networks.

3. Protein-bound water molecules in other microbial rhodopsins

We have shown for bR 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 bR 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 bR-"similar" systems, namely archaerhodopsin 2, halorhodopsin, and sensory rhodopsin II and the bR-"unsimilar" systems xanthorhodopsin, *Anabaena* sensory rhodopsin, and channelrhodopsin. In the proton uptake path (Fig. 2A), both in the bR-"similar" and the bR-"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 bR-"similar" proteins sensory rhodopsin II, halorhodopsin, and archaerhodopsin 2 all possess a water distribution that is nearly identical to the arrangement in bR. 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

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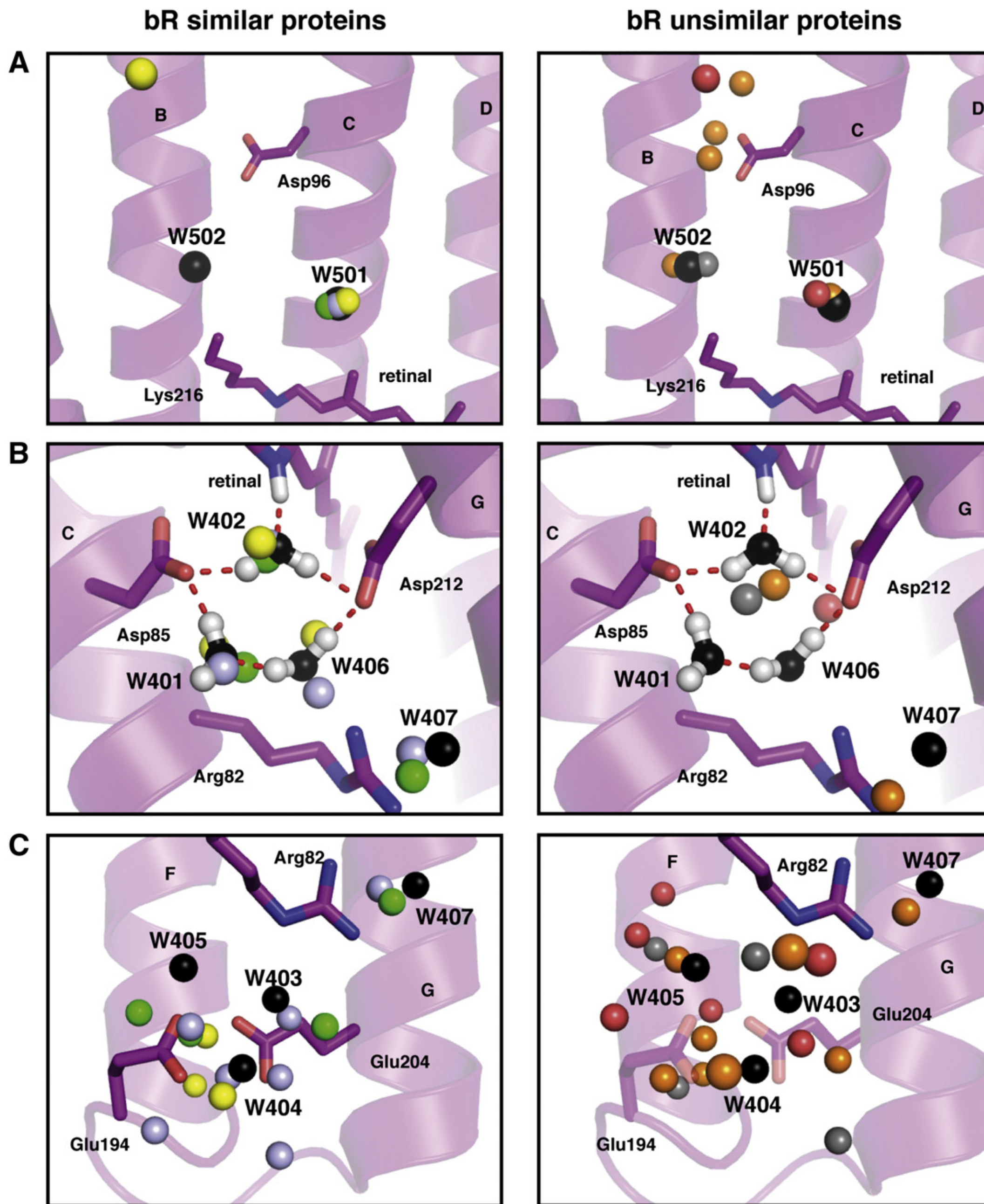

Fig. 2. Protein-bound water distribution in crystal structures of other microbial rhodopsins in comparison with water positions in bR (PDB ID: 1C3W [48]; protein in purple, water molecules in black). Key residues of bR shown as sticks to provide a visual reference to compare water positions in respect to the protein. Please take into account that such key amino acids may differ in the other proteins. Water molecule positions of bR are taken from PDB ID: 1C3W [48]. Left side: Proteins with internal water distributions similar to bR (halorhodopsin, sensory rhodopsin II, archaerhodopsin 2). Right side: Proteins with water positions different from those in bR (*Anabaena* sensory rhodopsin, xanthorhodopsin, channelrhodopsin). Water molecule oxygen positions in the different proteins are highlighted as colored spheres. Light blue: sensory rhodopsin II (PDB ID: 1H68 [96]); green: archaerhodopsin 2 (PDB ID: 1VGO [94]); yellow: halorhodopsin (PDB ID: 1E12 [5]); orange: *Anabaena* sensory rhodopsin (PDB ID: 1XIO [97]); red: channelrhodopsin (PDB ID: 3UG9 [98]); gray: xanthorhodopsin (PDB ID: 3DDL [95]). White spheres: proton positions in the bR pentameric arrangement (see Fig. 1B). **A**: proton uptake pathway. The "similar" protein/water systems do not exhibit water molecules close to Asp96, but water molecules between Asp96 and the Schiff base (termed W501) are highly conserved. The "unsimilar" protein/water systems *Anabaena* sensory rhodopsin, channelrhodopsin, and xanthorhodopsin also show conserved water molecules at positions W501 and W502. In addition, they exhibit more water molecules close to Asp96. **B**: Pentameric arrangement including the protonated Schiff base. The "similar" systems all possess a water distribution that is nearly identical to the water positions in bR (W401, W402, and W406), while the "unsimilar" systems do not exhibit such an arrangement. **C**: Proton release site. The "similar" structure set shows a general agreement regarding the space occupied by water molecules in bR, which are found surrounding the positions of Glu194 and Glu204 in bR, though not at the identical water positions of bR (W403, W404, W405, and W407). The "unsimilar" proteins exhibit water positions in completely different regions than those found in bR.

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

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 proton release site might be a specific

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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 I/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 D85T 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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References

[1] J.L. Spudich, H.K. Jung, Microbial rhodopsins: phylogenetic and functional diversity, in: W.R. Briggs, J.L. Spudich (Eds.), Handbook of Photosensory Receptors, Wiley-VCH, Weinheim, 2004, pp. 1–24.
[2] J.K. Lanyi, Bacteriorhodopsin, Annu. Rev. Physiol. 66 (2004) 665–688.
[3] K. Inoue, H. Ono, R. Abe-Yoshizumi, S. Yoshizawa, H. Ito, K. Kogure, H. Kandori, A light-driven sodium ion pump in marine bacteria, Nat. Commun. 4 (2013) 1678.
[4] R. Moukhametzianov, J.P. Klare, R. Efremov, C. Baeken, A. Göppner, J. Labahn, M. Engelhard, G. Büldt, V.I. Gordeliy, Development of the signal in sensory rhodopsin and its transfer to the cognate transducer, Nature 440 (2006) 115–119.
[5] M. Kolbe, H. Besir, L.-O. Essen, D. Oesterhelt, Structure of the light-driven chloride pump halorhodopsin at 1.8 Å resolution, Science 288 (2000) 1390–1396.
[6] G. Nagel, D. Ollig, M. Fuhrmann, S. Kateriya, A.M. Musti, E. Bamberg, P. Hegemann, Channelrhodopsin-1: a light-gated proton channel in green algae, Science 296 (2002) 2395–2398.
[7] O.A. Sineshchekov, K.-H. Jung, J.L. Spudich, Two rhodopsins mediate phototaxis to low-and high-intensity light in *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 8689–8694.
[8] D. Oesterhelt, W. Stoeckenius, Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*, Nat. New Biol. 233 (1971) 149–152.
[9] A.E. Blaurock, W. Stoeckenius, Structure of the purple membrane, Nat. New Biol. 233 (1971) 152–155.
[10] R.A. Capaldi, R. Aggeler, P. Turina, S. Wilkens, Coupling between catalytic sites and the proton channel in F1F0-type ATPases, Trends Biochem. Sci. 19 (1994) 284–289.
[11] W. Junge, Nature's rotary electromotors, Science 308 (2005) 642–644.
[12] T. Murata, I. Yamato, Y. Kakinuma, A.G. Leslie, J.E. Walker, Structure of the rotor of the V-type Na^+-ATPase from *Enterococcus hirae*, Science 308 (2005) 654–659.
[13] T. Meier, P. Polzer, K. Diederichs, W. Welte, P. Dimroth, Structure of the rotor ring of F-type Na^+-ATPase from *Ilyobacter tartaricus*, Science 308 (2005) 659–662.
[14] M.K. Wikstrom, Proton pump coupled to cytochrome C oxidase in mitochondria, Nature 266 (1977) 271–273.
[15] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 Å resolution of cytochrome C oxidase from *Paracoccus denitrificans*, Nature 376 (1995) 660–668.
[16] S. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, Proton-pumping mechanism of cytochrome C oxidase, Annu. Rev. Biophys. 40 (2011) 205–223.
[17] J.P. McEvoy, G.W. Brudvig, Water-splitting chemistry of photosystem II, Chem. Rev. 106 (2006) 4455–4483.
[18] T.J. Meyer, M.H.V. Huynh, H.H. Thorp, The possible role of proton-coupled electron transfer (PCET) in water oxidation by photosystem II, Angew. Chem. Int. Ed. 46 (2007) 5284–5304.
[19] Y. Umena, K. Kawakami, J.-R. Shen, N. Kamiya, Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å, Nature 473 (2011) 55–60.
[20] K. Deisseroth, Optogenetics, Nat. Methods 8 (2010) 26–29.
[21] O. Yizhar, L.E. Fenno, T.J. Davidson, M. Mogri, K. Deisseroth, Optogenetics in neural systems, Neuron 71 (2011) 9–34.
[22] L. Gómez-Consarnau, J.M. González, M. Coll-Lladó, P. Gourdon, T. Pascher, R. Neutze, C. Pedrós-Alió, J. Pinhassi, Light stimulates growth of proteorhodopsin-containing marine Flavobacteria, Nature 445 (2007) 210–213.
[23] D.B. Rusch, A.L. Halpern, G. Sutton, K.B. Heidelberg, S. Williamson, S. Yooseph, D. Wu, J.A. Eisen, J.M. Hoffman, K. Remington, K. Beeson, B. Tran, H. Smith, H. Baden-Tillson, C. Stewart, J. Thorpe, J. Freeman, C. Andrews-Pfannkoch, J.E. Venter, K. Li, et al., The Sorcerer II Global Ocean Sampling expedition: northwest Atlantic through eastern tropical Pacific, PLoS Biol. 5 (2007) e77.
[24] L. Gómez-Consarnau, N. Akram, K. Lindell, A. Pedersen, R. Neutze, D.L. Milton, J.M. González, J. Pinhassi, Proteorhodopsin phototrophy promotes survival of marine bacteria during starvation, PLoS Biol. 8 (2010) e1000358.
[25] F. Garczarek, K. Gerwert, Functional waters in intraprotein proton transfer monitored by FTIR difference spectroscopy, Nature 439 (2005) 109–112.
[26] R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann, K.H. Downing, Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy, J. Mol. Biol. 213 (1990) 899–929.
[27] M. Engelhard, K. Gerwert, B. Hess, F. Siebert, Light-driven protonation changes of internal aspartic acids of bacteriorhodopsin: an investigation of static and time-resolved infrared difference spectroscopy using [$^{4-13}$C]aspartic acid labeled purple membrane, Biochemistry 24 (1985) 400–407.

Author's personal copy

[28] M.S. Braiman, T. Mogi, T. Marti, L.J. Stern, H.G. Khorana, K.J. Rothschild, Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartic acid residues 85, 96, and 212, Biochemistry 27 (1988) 8516–8520.
[29] K. Gerwert, B. Hess, J. Soppa, D. Oesterhelt, Role of aspartate-96 in proton translocation by bacteriorhodopsin, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 4943–4947.
[30] T. Marinetti, S. Subramaniam, T. Mogi, T. Marti, H.G. Khorana, Replacement of aspartic residues 85, 96, 115, or 212 affects the quantum yield and kinetics of proton release and uptake by bacteriorhodopsin, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 529–533.
[31] K. Gerwert, G. Souvignier, B. Hess, Simultaneous monitoring of light-induced changes in protein side-group protonation, chromophore isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 9774–9778.
[32] D. Bashford, K. Gerwert, Electrostatic calculations of the pK_a values of ionizable groups in bacteriorhodopsin, J. Mol. Biol. 224 (1992) 473–486.
[33] H.J. Butt, K. Fendler, E. Bamberg, J. Tittor, D. Oesterhelt, Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump, EMBO J. 8 (1989) 1657–1663.
[34] S. Grzesiek, N.A. Dencher, Time-course and stoichiometry of light-induced proton release and uptake during the photocycle of bacteriorhodopsin, FEBS Lett. 208 (1986) 337–342.
[35] J. Heberle, D. Oesterhelt, N.A. Dencher, Decoupling of photo- and proton cycle in the Asp85->Glu mutant of bacteriorhodopsin, EMBO J. 12 (1993) 3721–3727.
[36] R.H. Lozier, W. Niederberger, R.A. Bogomolni, S. Hwang, W. Stoeckenius, Kinetics and stoichiometry of light-induced proton release and uptake from purple membrane fragments, *Halobacterium halobium* cell envelopes, and phospholipid vesicles containing oriented purple membrane, BBA-Bioenergetics 440 (1976) 545–556.
[37] L.S. Brown, J. Sasaki, H. Kandori, A. Maeda, R. Needleman, J.K. Lanyi, Glutamic acid 204 is the terminal proton release group at the extracellular surface of bacteriorhodopsin, J. Biol. Chem. 270 (1995) 27122–27126.
[38] R. Rammelsberg, G. Huhn, M. Lübben, K. Gerwert, Bacteriorhodopsin's intramolecular proton-release pathway consists of a hydrogen-bonded network, Biochemistry 37 (1998) 5001–5009.
[39] P. Hildebrandt, M. Stockburger, Role of water in bacteriorhodopsin's chromophore: resonance Raman study, Biochemistry 23 (1984) 5539–5548.
[40] G. Papadopoulos, N.A. Dencher, G. Zaccai, G. Büldt, Water molecules and exchangeable hydrogen ions at the active centre of bacteriorhodopsin localized by neutron diffraction, J. Mol. Biol. 214 (1990) 15–19.
[41] U. Lehnert, V. Réat, M. Weik, G. Zaccai, C. Pfister, Thermal motions in bacteriorhodopsin at different hydration levels studied by neutron scattering: correlation with kinetics and light-induced conformational changes, Biophys. J. 75 (1998) 1945–1952.
[42] M. Weik, G. Zaccai, N.A. Dencher, D. Oesterhelt, T. Hauß, Structure and hydration of the M-state of the bacteriorhodopsin mutant D96N studied by neutron diffraction, J. Mol. Biol. 275 (1998) 625–634.
[43] G. Zaccai, Moist and soft, dry and stiff: a review of neutron experiments on hydration-dynamics–activity relations in the purple membrane of *Halobacterium salinarum*, Biophys. Chem. 86 (2000) 249–257.
[44] H. Kandori, Role of internal water molecules in bacteriorhodopsin, BBA-Bioenergetics 1460 (2000) 177–191.
[45] H. Kandori, Y. Yamazaki, J. Sasaki, R. Needleman, J.K. Lanyi, A. Maeda, Water-mediated proton transfer in proteins: an FTIR study of bacteriorhodopsin, J. Am. Chem. Soc. 117 (1995) 2118–2119.
[46] E. Pebay-Peyroula, G. Rummel, J.P. Rosenbusch, E.M. Landau, X-ray structure of bacteriorhodopsin at 2.5 Å from microcrystals grown in lipidic cubic phases, Science 277 (1997) 1676–1681.
[47] H. Belrhali, P. Nollert, A. Royant, C. Menzel, J.P. Rosenbusch, E.M. Landau, E. Pebay-Peyroula, Protein, lipid and water organization in bacteriorhodopsin crystals: a molecular view of the purple membrane at 1.9 Å resolution, Structure 7 (1999) 909–917.
[48] H. Luecke, B. Schobert, H.-T. Richter, J.-P. Cartailler, J.K. Lanyi, Structure of bacteriorhodopsin at 155 Å resolution, J. Mol. Biol. 291 (1999) 899–911.
[49] B. Schobert, J. Cupp-Vickery, V. Hornak, S. Smith, J. Lanyi, Crystallographic structure of the K intermediate of bacteriorhodopsin: conservation of free energy after photoisomerization of the retinal, J. Mol. Biol. 321 (2002) 715–726.
[50] J.K. Lanyi, B. Schobert, Structural changes in the L photointermediate of bacteriorhodopsin, J. Mol. Biol. 365 (2007) 1379–1392.
[51] J.K. Lanyi, B. Schobert, Crystallographic structure of the retinal and the protein after deprotonation of the Schiff base: the switch in the bacteriorhodopsin photocycle, J. Mol. Biol. 321 (2002) 727–737.
[52] H. Luecke, B. Schobert, H.-T. Richter, J.-P. Cartailler, J.K. Lanyi, Structural changes in bacteriorhodopsin during ion transport at 2 Å resolution, Science 286 (1999) 255–260.
[53] B. Schobert, L.S. Brown, J.K. Lanyi, Crystallographic structures of the M and N intermediates of bacteriorhodopsin: assembly of a hydrogen-bonded chain of water molecules between Asp-96 and the retinal Schiff base, J. Mol. Biol. 330 (2003) 553–570.
[54] S. Rouhani, J.-P. Cartailler, M.T. Facciotti, P. Walian, R. Needleman, J.K. Lanyi, R.M. Glaeser, H. Luecke, Crystal structure of the D85S mutant of bacteriorhodopsin: model of an O-like photocycle intermediate, J. Mol. Biol. 313 (2001) 615–628.
[55] V.A. Lórenz-Fonfría, Y. Furutani, H. Kandori, Active internal waters in the bacteriorhodopsin photocycle a comparative study of the L and M intermediates at room and cryogenic temperatures by infrared spectroscopy, Biochemistry 47 (2008) 4071–4081.
[56] K. Gerwert, C. Kötting, Fourier transform infrared (FTIR) spectroscopy, Encyclopedia of Life Sciences (ELS), John Wiley & Sons, Ltd., Chichester, UK, 2010, (http://www.els.net [doi: http://dx.doi.org/10.1002/9780470015902.a0003112.pub2]).
[57] C. Kandt, J. Schlitter, K. Gerwert, Dynamics of water molecules in the bacteriorhodopsin trimer in explicit lipid/water environment, Biophys. J. 86 (2004) 705–717.
[58] S. Grudinin, G. Büldt, V. Gordeliy, A. Baumgaertner, Water molecules and hydrogen-bonded networks in bacteriorhodopsin—molecular dynamics simulations of the ground state and the M-intermediate, Biophys. J. 88 (2005) 3252–3261.
[59] M. Engels, K. Gerwert, D. Bashford, Computational studies of the early intermediates of the bacteriorhodopsin photocycle, Biophys. Chem. 56 (1995) 95–104.
[60] R. Rousseau, V. Kleinschmidt, U.W. Schmitt, D. Marx, Assigning protonation patterns in water networks in bacteriorhodopsin based on computed IR spectra, Angew. Chem. Int. Ed. 43 (2004) 4804–4807.
[61] F. Garczarek, L.S. Brown, J.K. Lanyi, K. Gerwert, Proton binding within a membrane protein by a protonated water cluster, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 3633–3638.
[62] G. Mathias, D. Marx, Structures and spectral signatures of protonated water networks in bacteriorhodopsin, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 6980–6985.
[63] M. Baer, D. Marx, G. Mathias, Theoretical messenger spectroscopy of microsolvated Hydronium and Zundel cations, Angew. Chem. Int. Ed. 49 (2010) 7346–7349.
[64] S. Wolf, E. Freier, M. Potschies, E. Hofmann, K. Gerwert, Directional proton transfer in membrane proteins achieved through protonated protein-bound water molecules: a proton diode, Angew. Chem. Int. Ed. 49 (2010) 6889–6893.
[65] E. Freier, S. Wolf, K. Gerwert, Proton transfer via a transient linear water-molecule chain in a membrane protein, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 11435–11439.
[66] M. Shibata, T. Tanimoto, H. Kandori, Water molecules in the Schiff base region of bacteriorhodopsin, J. Am. Chem. Soc. 125 (2003) 13312–13313.
[67] S. Hayashi, E. Tajkhorshid, H. Kandori, K. Schulten, Role of hydrogen-bond network in energy storage of bacteriorhodopsin's light-driven proton pump revealed by ab initio normal-mode analysis, J. Am. Chem. Soc. 126 (2004) 10516–10517.
[68] Y. Furutani, M. Shibata, H. Kandori, Strongly hydrogen-bonded water molecules in the Schiff base region of rhodopsins, Photochem. Photobiol. Sci. 4 (2005) 661.
[69] H. Kandori, Y. Shichida, Direct observation of the bridged water stretching vibrations inside a protein, J. Am. Chem. Soc. 122 (2000) 11745–11746.
[70] H. Luecke, B. Schobert, J.-P. Cartailler, H.-T. Richter, A. Rosengarth, R. Needleman, J.K. Lanyi, Coupling photoisomerization of retinal to directional transport in bacteriorhodopsin, J. Mol. Biol. 300 (2000) 1237–1255.
[71] K. Takeda, Y. Matsui, N. Kamiya, S.-I. Adachi, H. Okumura, T. Kouyama, Crystal structure of the M intermediate of bacteriorhodopsin: allosteric structural changes mediated by sliding movement of a transmembrane helix, J. Mol. Biol. 341 (2004) 1023–1037.
[72] M. Baer, G. Mathias, I.-F.W. Kuo, D.J. Tobias, C.J. Mundy, D. Marx, Spectral signatures of the pentagonal water cluster in bacteriorhodopsin, Chemphyschem 9 (2008) 2703–2707.
[73] S. Wolf, E. Freier, K. Gerwert, How does a membrane protein achieve a vectorial proton transfer via water molecules? Chemphyschem 9 (2008) 2772–2778.
[74] C. de Grotthuss, Sur la Décomposition de l'eau et des corps qu' elle tient en dissolution à l'aide de l'électricité galvanique, Ann. Chim. LVIII (1806) 54.
[75] N. Agmon, The Grotthuss mechanism, Chem. Phys. Lett. 244 (1995) 456–462.
[76] S.P. Balashov, R. Govindjee, M. Kono, E. Imasheva, E. Lukashev, T.G. Ebrey, R.K. Crouch, D.R. Menick, Y. Feng, Effect of the arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle, Biochemistry 32 (1993) 10331–10343.
[77] R.V. Sampogna, B. Honig, Electrostatic coupling between retinal isomerization and the ionization state of Glu-204: a general mechanism for proton release in bacteriorhodopsin, Biophys. J. 71 (1996) 1165–1171.
[78] J.M. Headrick, E.G. Diken, R.S. Walters, N.I. Hammer, R.A. Christie, J. Cui, E.M. Myshakin, M.A. Duncan, M.A. Johnson, K.D. Jordan, Spectral signatures of hydrated proton vibrations in water clusters, Science 308 (2005) 1765–1769.
[79] R. Rousseau, V. Kleinschmidt, U.W. Schmitt, D. Marx, Modeling protonated water networks in bacteriorhodopsin, Phys. Chem. Chem. Phys. 6 (2004) 1848–1859.
[80] P. Phatak, N. Ghosh, H. Yu, Q. Cui, M. Elstner, Amino acids with an intermolecular proton bond as proton storage site in bacteriorhodopsin, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 19672–19677.
[81] P. Goyal, N. Ghosh, P. Phatak, M. Clemens, M. Gaus, M. Elstner, Q. Cui, Proton storage site in bacteriorhodopsin: new insights from quantum mechanics/molecular mechanics simulations of microscopic pK(a) and infrared spectra, J. Am. Chem. Soc. 133 (2011) 14981–14997.
[82] S. Subramaniam, M. Gerstein, D. Oesterhelt, R. Henderson, Electron diffraction analysis of structural changes in the photocycle of bacteriorhodopsin, EMBO J. 12 (1993) 1–8.
[83] S. Subramaniam, A.R. Faruqi, D. Oesterhelt, R. Henderson, Electron diffraction studies of light-induced conformational changes in the Leu-93 ->Ala bacteriorhodopsin mutant, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1767–1772.
[84] T. Rink, M. Pfeiffer, D. Oesterhelt, K. Gerwert, H.-J. Steinhoff, Unraveling photoexcited conformational changes of bacteriorhodopsin by time resolved electron paramagnetic resonance spectroscopy, Biophys. J. 78 (2000) 1519–1530.
[85] J. Heberle, Proton transfer reactions across bacteriorhodopsin and along the membrane, BBA-Bioenergetics 1458 (2000) 135–147.
[86] N. Radzwill, K. Gerwert, H.-J. Steinhoff, Time-resolved detection of transient movement of helices F and G in doubly spin-labeled bacteriorhodopsin, Biophys. J. 80 (2001) 2856–2866.
[87] B. Schätzler, N.A. Dencher, J. Tittor, D. Oesterhelt, S. Yaniv-Checover, E. Nachliel, M. Gutman, Subsecond proton-hole propagation in bacteriorhodopsin, Biophys. J. 84 (2003) 671–686.
[88] L.S. Brown, R. Needleman, J.K. Lanyi, Functional roles of aspartic acid residues at the cytoplasmic surface of bacteriorhodopsin, Biochemistry 38 (1999) 6855–6861.
[89] E. Nachliel, M. Gutman, J. Tittor, D. Oesterhelt, Proton transfer dynamics on the surface of the late M state of bacteriorhodopsin, Biophys. J. 83 (2002) 416–426.
[90] H. Saitô, S. Yamaguchi, K. Ogawa, S. Tuzi, M. Márquez, C. Sanz, E. Padrós, Glutamic acid residues of bacteriorhodopsin at the extracellular surface as determinants for conformation and dynamics as revealed by site-directed solid-state ^{13}C NMR, Biophys. J. 86 (2004) 1673–1681.

Author's personal copy

[91] P. Phatak, J.S. Frähmcke, M. Wanko, M. Hoffmann, P. Strodel, J.C. Smith, S. Suhai, A.-N. Bondar, M. Elstner, Long-distance proton transfer with a break in the bacteriorhodopsin active site, J. Am. Chem. Soc. 131 (2009) 7064–7078.
[92] C. Zscherp, J. Heberle, Infrared difference spectra of the intermediates L, M, N, and O of the bacteriorhodopsin photoreaction obtained by time-resolved attenuated total reflection spectroscopy, J. Phys. Chem. B 101 (1997) 10542–10547.
[93] A.K. Dioumaev, L.S. Brown, R. Needleman, J.K. Lanyi, Fourier transform infrared spectra of a late intermediate of the bacteriorhodopsin photocycle suggest transient protonation of Asp-212, Biochemistry 38 (1999) 10070–10078.
[94] N. Enami, K. Yoshimura, M. Murakami, H. Okumura, K. Ihara, T. Kouyama, Crystal structures of archaerhodopsin-1 and -2: common structural motif in archaeal light-driven proton pumps, J. Mol. Biol. 358 (2006) 675–685.
[95] H. Luecke, B. Schobert, J. Stagno, E.S. Imasheva, J.M. Wang, S.P. Balashov, J.K. Lanyi, Crystallographic structure of xanthorhodopsin, the light-driven proton pump with a dual chromophore, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 16561–16565.
[96] A. Royant, P. Nollert, K. Edman, R. Neutze, E.M. Landau, E. Pebay-Peyroula, J. Navarro, X-ray structure of sensory rhodopsin II at 2.1-Å resolution, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 10131–10136.
[97] L. Vogeley, O.A. Sineshchekov, V.D. Trivedi, J. Sasaki, J.L. Spudich, H. Luecke, Anabaena sensory rhodopsin: a photochromic color sensor at 2.0 Å, Science 306 (2004) 1390–1393.
[98] H.E. Kato, F. Zhang, O. Yizhar, C. Ramakrishnan, T. Nishizawa, K. Hirata, J. Ito, Y. Aita, T. Tsukazaki, S. Hayashi, P. Hegemann, A.D. Maturana, R. Ishitani, K. Deisseroth, O. Nureki, Crystal structure of the channelrhodopsin light-gated cation channel, Nature 482 (2012) 369–374.
[99] K. Saito, H. Kandori, H. Ishikita, Factors that differentiate the H-bond strengths of water near the Schiff bases in bacteriorhodopsin and anabaena sensory rhodopsin, J. Biol. Chem. 287 (2012) 34009–34018.
[100] E.P. Lukashev, R. Govindjee, M. Kono, T.G. Ebrey, Y. Sugiyama, Y. Mukohata, pH dependence of the absorption spectra and photochemical transformations of the archaerhodopsins, Photochem. Photobiol. 60 (1994) 69–75.
[101] L. Zimanyi, G. Varo, M. Chang, B. Ni, R. Needleman, J.K. Lanyi, Pathways of proton release in the bacteriorhodopsin photocycle, Biochemistry 31 (1992) 8535–8543.
[102] G. Nagel, T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann, E. Bamberg, Channelrhodopsin-2, a directly light-gated cation-selective membrane channel, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13940–13945.
[103] K. Eisenhauer, J. Kuhne, E. Ritter, A. Berndt, S. Wolf, E. Freier, F. Bartl, P. Hegemann, K. Gerwert, In channelrhodopsin-2 Glu-90 is crucial for ion selectivity and is deprotonated during the photocycle, J. Biol. Chem. 287 (2012) 6904–6911.
[104] E. Ritter, K. Stehfest, A. Berndt, P. Hegemann, F.J. Bartl, Monitoring light-induced structural changes of channelrhodopsin-2 by UV–visible and Fourier transform infrared spectroscopy, J. Biol. Chem. 283 (2008) 35033–35041.
[105] V.A. Lórenz-Fonfría, T. Resler, N. Krause, M. Nack, M. Gossing, G. Fischer von Mollard, C. Bamann, E. Bamberg, R. Schlesinger, J. Heberle, Transient protonation changes in channelrhodopsin-2 and their relevance to channel gating, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E1273–E1281.
[106] O.A. Sineshchekov, E.G. Govorunova, J. Wang, H. Li, J.L. Spudich, Intramolecular proton transfer in channelrhodopsins, Biophys. J. 104 (2013) 807–817.
[107] K. Feldbauer, D. Zimmermann, V. Pintschovius, J. Spitz, C. Bamann, E. Bamberg, Channelrhodopsin-2 is a leaky proton pump, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 12317–12322.
[108] M. Nack, I. Radu, B.-J. Schultz, T. Resler, R. Schlesinger, A.-N. Bondar, C. Del Val, S. Abbruzzetti, C. Viappiani, C. Bamann, E. Bamberg, J. Heberle, Kinetics of proton release and uptake by channelrhodopsin-2, FEBS Lett. 586 (2012) 1344–1348.
[109] J. Sasaki, L. Brown, Y. Chon, H. Kandori, A. Maeda, R. Needleman, J. Lanyi, Conversion of bacteriorhodopsin into a chloride ion pump, Science 269 (1995) 73–75.
[110] J. Tittor, U. Haupts, C. Haupts, D. Oesterhelt, A. Becker, E. Bamberg, Chloride and proton transport in bacteriorhodopsin mutant D85T: different modes of ion translocation in a retinal protein, J. Mol. Biol. 271 (1997) 405–416.
[111] Y. Sudo, J.L. Spudich, Three strategically placed hydrogen-bonding residues convert a proton pump into a sensory receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16129–16134.
[112] E.N. Spudich, G. Ozorowski, E.V. Schow, D.J. Tobias, J.L. Spudich, H. Luecke, A transporter converted into a sensor, a phototaxis signaling mutant of bacteriorhodopsin at 3.0 Å, J. Mol. Biol. 415 (2012) 455–463.
[113] A. Kawanabe, Y. Furutani, K.-H. Jung, H. Kandori, An inward proton transport using anabaena sensory rhodopsin, J. Microbiol. 49 (2011) 1–6.
[114] J.M.J. Swanson, C.M. Maupin, H. Chen, M.K. Petersen, J. Xu, Y. Wu, G.A. Voth, Proton solvation and transport in aqueous and biomolecular systems: insights from computer simulations, J. Phys. Chem. B 111 (2007) 4300–4314.
[115] J. Koepke, E. Olkhova, H. Angerer, H. Müller, G. Peng, H. Michel, High resolution crystal structure of *Paracoccus denitrificans* cytochrome C oxidase: new insights into the active site and the proton transfer pathways, BBA-Bioenergetics 1787 (2009) 635–645.