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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.

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