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Review

Eubacterial rhodopsins — Unique photosensors and diverse ion pumps ☆

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ABSTRACT

Since the discovery of proteorhodopsins, the ubiquitous marine light-driven proton pumps of eubacteria, a large number of other eubacterial rhodopsins with diverse structures and functions have been characterized. Here, we review the body of knowledge accumulated on the four major groups of eubacterial rhodopsins, with the focus on their biophysical characterization. We discuss advances and controversies on the unique eubacterial sensory rhodopsins (as represented by *Anabaena* sensory rhodopsin), proton-pumping proteorhodopsins and xanthorhodopsins, as well as novel non-proton ion pumps. This article is part of a Special Issue entitled: Retinal Proteins — You can teach an old dog new tricks.

1. Introduction — functional and taxonomic diversity of eubacterial rhodopsins

For about thirty years, it was believed that prokaryotic microbial rhodopsins can be found exclusively in haloarchaea, where they can serve as light-driven proton or chloride pumps (bacteriorhodopsin, BR, and halorhodopsin, HR) or phototactic and photophobic receptors (sensory rhodopsins, SR-I and SR-II). But since the seminal discovery of eubacterial proton-pumping rhodopsins in marine picoplankton by metagenomic sequencing [1,2], our appreciation of the diversity and evolutionary plasticity of bacterial retinylidene proteins has been growing. Indeed, we learned that eubacterial rhodopsins can function not only as light-driven proton pumps, but also transport other ions and act as photosensors [3–10]. They can be found in almost every bacterial taxon, in many different ecological niches, and display unique structural features. To complicate the picture, frequent lateral gene transfers and duplications produced bacteria harboring several rhodopsin forms with different functions and origins, and made correlating taxonomic and functional groups an ambiguous task [11–15]. Additionally, close homologs of eubacterial rhodopsins have been recently found in *Archaea*, algae, and even viruses [11,14,16–19]. The goal of this review is to survey the latest developments in understanding structure and function of eubacterial rhodopsins.

While mere bioinformatic analysis of amino acid sequences of eubacterial rhodopsins does not always give us functional and structural insights, it may serve as an introduction to the field, showing the main trends. Fig. 1 delineates the four major clusters of eubacterial rhodopsins discussed in this review: photosensors (ASR-like cluster), proton pumps (PR-like and XR-like clusters), and novel non-proton ion pumps (NQ-type cluster). The tree shows only a small fraction of the available sequences (especially in the large XR-like and PR-like clusters) and does not include a number of eubacterial rhodopsins which do not fit into this rough classification. Namely, it does not include close homologs of haloarchaeal rhodopsins (e.g., from *Salinibacter* and *Rubrobacter*) [5,6,11,20], a unique proton pump from *Exiguobacterium* [21] (ER), as well as a number of other untypical rhodopsins. Each structural/functional cluster is taxonomically heterogeneous [12,14,15,22] and the names of several species can be found in two different clusters, reflecting the coexistence of multiple rhodopsin forms in the same host. Notable in this respect are rhodopsins from *Flavobacteria*, which form a separate (upper) branch in the PR-like cluster and have their counterparts in the NQ-type cluster (Fig. 1). To assist in understanding of the key differences between the major groups of eubacterial rhodopsins, Fig. 2 shows sequence alignments of transmembrane helices (B to G) of the representatives of each cluster and their halobacterial homolog, bacteriorhodopsin, highlighting the residues typical for each group.

The ASR-like cluster is the closest to BR in overal sequence similarity, but displays a number of unique residues (Figs. 1 and 2). It is formed by homologs of *Anabaena* sensory rhodopsin (ASR), a putative cyanobacterial photosensor [4]. The exact physiological role of ASR-like

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Abbreviations: ASR, *Anabaena* sensory rhodopsin; ASRT, *Anabaena* sensory rhodopsin transducer; AR, archaerhodopsin; BPR, blue-absorbing proteorhodopsin; BR, bacteriorhodopsin; ER, *Exiguobacterium* rhodopsin; FTIR, Fourier-transform infrared; GPR, green-absorbing proteorhodopsin; GR, *Gloeobacter* rhodopsin; HR, halorhodopsin; NMR, nuclear magnetic resonance; NQ, a new group of rhodopsins with Asn/Gln replacing carboxylic acids; PR, proteorhodopsin; RDs, rhodopsins; SR, sensory rhodopsin; XR, xanthorhodopsin

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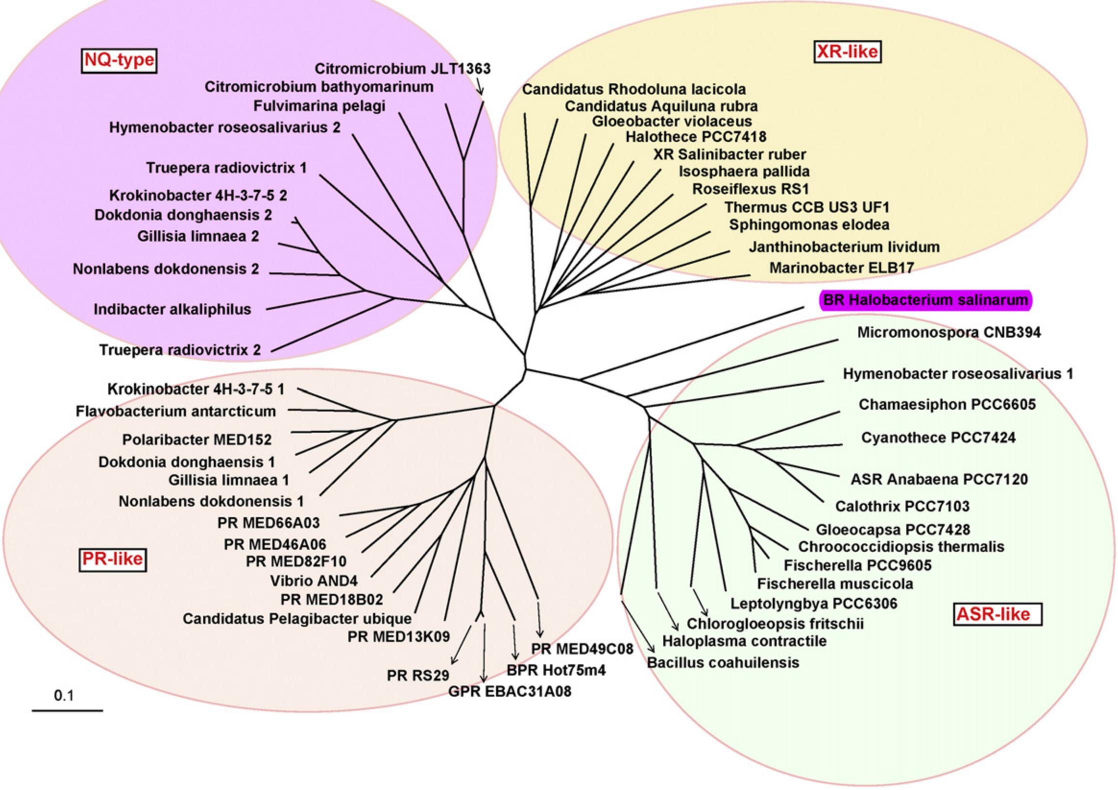
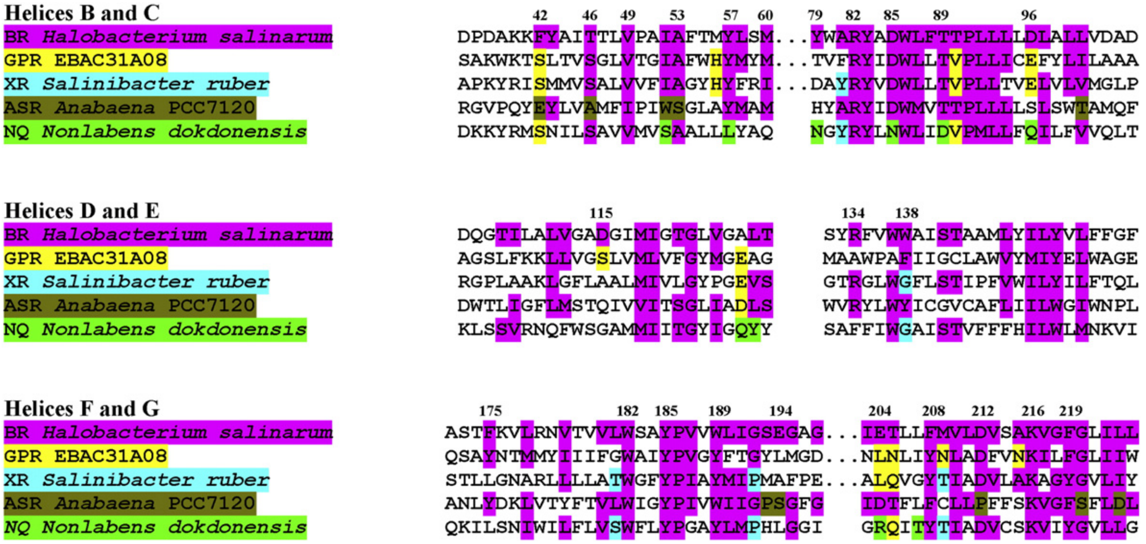


Fig. 1. The four major groups of eubacterial rhodopsins. Unrooted CLUSTALW [128] guide tree (rendered by TreeView [129]) of selected eubacterial rhodopsins from public genome databases (http://www.ncbi.nlm.nih.gov/protein and http://img.jgi.doe.gov), showing the four major structural/functional clusters and their distance from the halobacterial homolog BR (highlighted purple). The scale bar represents number of substitutions per site (0.1 indicates 10 nucleotide substitutions per 100 nucleotides).

proteins, recently named xenorhodopsins to reflect their taxonomic diversity [14], is not known, and they can be found outside of cyanobacterial clades as well. In Section 2, we will focus on ASR, the only member of this group characterized extensively. The taxonomically diverse XR-like and PR-like groups contain homologs of proton-pumping proteorhodopsin (PR) [1] and its dual-chromophore relative, xanthorhodopsin (XR) [23]. PR-like and XR-like proteins share a number of conserved residues, but do form distinct clusters [12,15,24], with XR-like proteins, which include numerous actinorhodopsins (coming from *Actinobacteria*), being closer to BR (Figs. 1 and 2). In Section 3, we will discuss the advances in characterization of PR, XR, and some of the related proteins. Finally, a new cluster of rhodopsins (named NQ-type [7,15]) will be briefly addressed in Section 4. NQ-type rhodopsins are the most similar to XR in overall sequence homology (Fig. 1), but have a very distinct amino acid pattern, in which



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Helices B and C
                                 42  46 49  53  57 60    79 82  85  89      96
BR Halobacterium salinarum      DPDAKKFYAITTLVPAIAFTMYLSM...YWARYADWLFTTPLLLLDLALLVDAD
GPR EBAC31A08                   SAKWKTSLTVSGLVTGIAFWHYMYM...TVFRYIDWLLTVPLLICEFYLILAAA
XR Salinibacter ruber           APKYRISMMVSALVVFIAGYHYFRI...DAYRYVDWLLTVPLLTVELVLVMGLP
ASR Anabaena PCC7120            RGVPQYEYLVAMFIPIWSGLAYMAM   HYARYIDWMVTTPLLLLSLSWTAMQF
NQ Nonlabens dokdonensis        DKKYRMSNILSAVVMVSAALLLYAQ   NGYRYLNWLIDVPMLLFQILFVVQLT

Helices D and E
                                            115                   134 138
BR Halobacterium salinarum      DQGTILALVGADGIMIGTGLVGALT   SYRFVWWAISTAAMLYILYVLFFGF
GPR EBAC31A08                   AGSLFKKLLVGSLVMLVFGYMGEAG   MAAWPAFIIGCLAWVYMIYELWAGE
XR Salinibacter ruber           RGPLAAKLGFLAALMIVLGYPGEVS   GTRGLWGFLSTIPFVWILYILFTQL
ASR Anabaena PCC7120            DWTLIGFLMSTQIVVITSGLIADLS   WVRYLWYICGVCAFLIILWGIWNPL
NQ Nonlabens dokdonensis        KLSSVRNQFWSGAMMIITGYIGQYY   SAFFIWGAISTVFFFHILWLMNKVI

Helices F and G
                                175      182 185 189  194     204 208 212 216 219
BR Halobacterium salinarum      ASTFKVLRNVTVVLWSAYPVVWLIGSEGAG...IETLLFMVLDVSAKVGFGLILL
GPR EBAC31A08                   QSAYNTMMYIIIFGWAIYPVGYFTGYLMGD...NLNLIYNLADFVNKILFGLIIW
XR Salinibacter ruber           STLLGNARLLLLATWGFYPIAYMIPMAFPE...ALQVGYTIADVLAKAGYGVLIY
ASR Anabaena PCC7120            ANLYDKLVTYFTVLWIGYPIVWIIGPSGFG   IDTFLFCLLPFFSKVGFSFLDL
NQ Nonlabens dokdonensis        QKILSNIWILFLVSWFLYPGAYLMPHLGGI   GRQITYTIADVCSKVIYGVLLG
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Fig. 2. Characteristic amino acid sequence signatures of the main clusters of eubacterial rhodopsins. Partial CLUSTALW [128] alignment of amino acid sequences of helices B–G of bacteriorhodopsin and representatives of the four major clusters. Residues conserved in BRs are highlighted purple, in PR-like cluster — yellow, in XR-like cluster — cyan, in ASR-like cluster — tan, and in the NQ-type cluster — green. The functionally/structurally important residues are numbered according to the *H. salinarum* BR sequence.

primary proton donors and acceptors of XR (Glu and Asp) are replaced with Gln and Asn (Fig. 2), reflecting the changes in ion specificity of these transporters [7,9].

2. Eubacterial photosensors

While some eubacteria (e.g., *Salinibacter ruber*) were found to have halobacterial-like sensory rhodopsins interacting with membrane-embedded Htr-type transducers [6,25], such examples are relatively rare. Some of proteorhodopsins, possessing extremely slow photocycles and/or weak proton transport, were suggested to perform photosensory functions as well [26,27]. Finally, based on bioinformatic analyses showing the lack of key residues necessary for proton transport, a number of other eubacterial rhodopsins were suspected to perform photosensory functions [3,8,28,29], but none has been demonstrated so far. In view of this, ASR-like proteins remain the strongest candidates for eubacterial sensory rhodopsins with distinct non-archaeal phototransduction mechanism, but the details of this mechanism are far from being established [14,30,31].

When ASR was discovered in the genome of cyanobacterium *Anabaena* (*Nostoc*) PCC7120 [4,32], it was suggested to coexpress with a transducer, 126 amino-acid protein named ASRT. ASRT, whose gene follows that of ASR, turned out to be a soluble tetrameric protein of predominantly β-stranded structure [33,34], which does not belong to any known class of transducers. It was shown to interact with ASR in vitro by several techniques, including pull-down assays, surface plasmon resonance, isothermal titration calorimetry, visible spectroscopy (affecting the photocycle, dark-adaptation, and absolute spectra of ASR), and transient grating experiments [4,33,35,36]. The transient grating experiments showed that ASRT is released upon the activation of ASR (more precisely, its all-*trans*-retinal form) [36], in line with an earlier proposal that such a release would be necessary to modulate interaction of ASRT with DNA and regulate expression of photosynthesis-related genes [37]. Indeed, it was shown by gel-shift assays, chromatin immunoprecipitation, isothermal titration calorimetry, and solution NMR that ASRT can bind DNA and is possibly a novel transcription factor [34]. Interestingly, the DNA binding occurs on the flexible partially disordered face of ASRT, which was also suggested to participate in the interaction with ASR [33,34], and induces some loop ordering, suggesting an induced-fit mechanism.

The synthesis of all these data allows building a very simplistic hypothetical scheme of the phototransduction cascade of ASR (Fig. 3), even though this scheme can be challenged on several grounds. First, it was noted that ASR/ASRT interaction cannot be universal for all of ASR-like proteins, as no other xenorhodopsin was found to coexist with an ASRT-like transducer and, conversely, there are many examples of ASRT-like proteins in rhodopsin-less bacteria [14,30]. It was suggested that physiological role of ASRT-like proteins may be binding of sugars [30], while that of standalone ASR-like proteins is unclear, but is most likely photosensory [14]. However, our most recent analysis (L.S. Brown, unpublished) of publicly available eubacterial genomes (http://img.jgi.doe.gov) shows that the coexistence of ASR-like and ASRT-like proteins in *Anabaena* sp. PCC7120 is not unique. Out of 14 eubacterial species shown in the ASR-like cluster in Fig. 1, three cyanobacterial species (*Anabaena* sp. PCC7120, *Chroococcidiopsis thermalis*, and *Chamaesiphon* sp. PCC6605, all from different families) have ASR-like and ASRT-like genes following each other, and two other cyanobacterial species (both from the *Fischerella* genus) have both genes, but in different genomic regions. This fact does not disprove the hypothesis about alternative functions of ASR-like and ASRT-like proteins in some bacteria, but lends credibility to the idea of their common photosensory role in cyanobacteria. Second, it was recently shown that ASR alone can regulate (repress) gene expression in *Escherichia coli* via its C-terminal region [31], while ASRT may merely modulate this interaction, suggesting an alternative model of the ASR signaling cascade. In our opinion, this result does not prove the direct interaction of ASR with DNA, unless its interaction with any of *E. coli* transcription factors (or other proteins affecting gene expression) can be excluded.

Despite the lack of clarity on the physiological role of ASR, there is a wealth of structural and spectroscopic information showing its

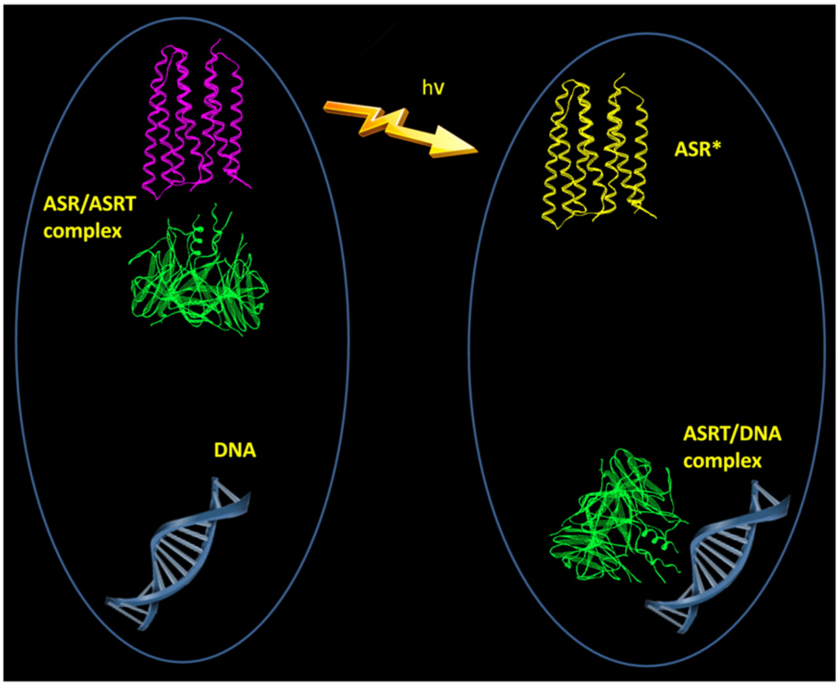

Fig. 3. A hypothetical phototransduction cascade of ASR [34,36,37]. In the dark, ASRT (green) is bound to ASR (purple), while light-activated ASR (yellow) releases ASRT to bind DNA, interacting with promoter regions of several genes and regulating their expression. The protein images were constructed from the X-ray structures of ASR and ASRT (PDB ID: 1XIO and 2II7) [33,38] using SWISS-PDB viewer [130].

uniqueness among microbial rhodopsins [35,38–41]. The X-ray structure of ASR (PDB ID: 1XIO) [38] as well as solid-state NMR studies [39,42] present a protein of essentially BR-like architecture consistent with their significant primary structure similarity (Fig. 1). Both proteins are bundles of seven transmembrane helices with a β-hairpin in the B–C loop, and both are prone to trimerization. On the other hand, the X-ray structure revealed an unusually polar cytoplasmic half of the protein, very different from that in BR and other proton-pumping rhodopsins, where it is mainly hydrophobic and has very few bound water molecules (Fig. 4) [38,41,43]. In ASR, several polar sidechains (including conserved Asp-217 and Ser-214, Figs. 2 and 4) along with a few water molecules, form a hydrogen-bonded network connecting the retinal Schiff base with the cytoplasmic surface, which could be presumably used for ASRT activation [38,41,44,45]. On the other side of the Schiff base, its counterion complex, consisting of two aspartic acids (Asp-85 and Asp-212) in BR, is rearranged, which is mainly caused by Asp-212 being replaced by a proline (Figs. 2 and 4). Low-temperature FTIR showed that ASR does not possess strongly hydrogen-bonded water believed to be required for proton pumping [46–48], while solid-state NMR showed very different chemical shifts for the carboxylic counterions of BR and ASR [39]. In agreement with these structural observations, several FTIR studies confirmed that the first light-driven proton transfer from the retinal Schiff base does not proceed towards its carboxylic counterion (Asp-75, homologous to Asp-85 of BR) [44,45,49]. This is consistent with the hydration switch model [50], which states that for the efficient proton transfer from the Schiff base to Asp-85 in BR, hydration of Asp-85 should be switched to Asp-212, which is missing in ASR. Interestingly, when Pro-206 of ASR (homologous to Asp-212 of BR) was mutated back into Asp, the outward-directed proton-pumping ability of ASR was not observed [51].

Instead of the BR-like light-driven proton transfer from retinal to Asp-75, it was suggested that the Schiff base proton is transferred in the cytoplasmic direction, protonating Asp-217 and/or Glu-36 (Fig. 4) [44,45], and consistent with the photoelectric measurements [27]. On the other hand, later photoelectric measurements conducted on the full-length ASR (as opposed to the earlier measurements performed on the C-terminally truncated species) suggested that the direction of proton transfers may depend on the presence of the C-terminus [52]. It appears that the Schiff base proton transfer vectoriality strongly depends on a subtle charge balance and geometry of carboxylic residues in ASR. For example, the replacement of the counterion Asp-75 with Glu favors the extracellular direction, as does the mutation of the putative cytoplasmic proton acceptor (Asp-217 to Asn) [52]. The introduction of Asp-86 instead of Ser on the cytoplasmic side (at a site homologous to that of Asp-96 of BR, Fig. 4) biases protons to move in the cytoplasmic direction, while the replacement of Asp-217 by Glu results in a full-scale inward-directed transmembrane proton transport [43,52]. Interestingly, the difference in the photocycle between the full-length and C-terminally truncated ASR was observed only in *E. coli* cells but not in the purified protein [45,52], which may suggest that the C-terminus interacts with some partners in the cytoplasm, in line with the recent gene expression studies [31]. To sum up, a general consensus may be that the cytoplasmic surface of ASR (and presumably its transducer) can communicate with the Schiff base region through the extended unique cytoplasmic hydrogen-bonded network (Fig. 4), but the pattern of proton transfers is highly labile [43,44,52]. Photoactivation of ASR leads to significant conformational changes, as can be judged from FTIR signatures of the protein itself and its associated lipids [44,45,49], as well as from the strongly enhanced H/D exchange of the backbone detected by solid-state NMR [53]. Apparently, the interplay of the light-driven proton transfers and the conformational changes causes activation of pre-bound ASRT, most probably in the M photointermediate of the photocycle of the all-*trans*-ASR [36,44], in a process which is very likely to involve both the cytoplasmic hydrogen-bonded network and the C-terminus of ASR.

An alternative proposal for the signaling mechanism of ASR is based on the balance of its two stable dark states, those with all-*trans* and 13-*cis* retinal [38,54]. This proposal stems from another very interesting feature of ASR, its unusual photochemistry, namely, its apparent photochromism [35,38,55], reminiscent of animal rhodopsins. Unlike most microbial rhodopsins, which either do not display any light/dark adaptation or transition from a mixture of all-*trans* and 13-*cis* states to mostly all-*trans*, ASR has predominantly all-*trans*-retinal in its dark-adapted state and transitions to a mixture dominated by 13-*cis* species upon light-adaptation [35,55]. The 13-*cis* species can be photoconverted back to all-*trans* so that the isomeric composition of light-adapted ASR depends on the wavelength of illumination [35,38], making it a bistable system. Surprisingly, it appears that neither of the dark states has a true photocycle, as photoactivation of each of the species results in full conversion to the alternative isomeric state (all-*trans* to 13-*cis* and 13-*cis* to all-*trans*), a hallmark of genuine photochromism [55–57]. Similar to BR, the primary photochemistry and the early intermediates of all-*trans* and 13-*cis* ASR are structurally and kinetically different [54,58]. It also

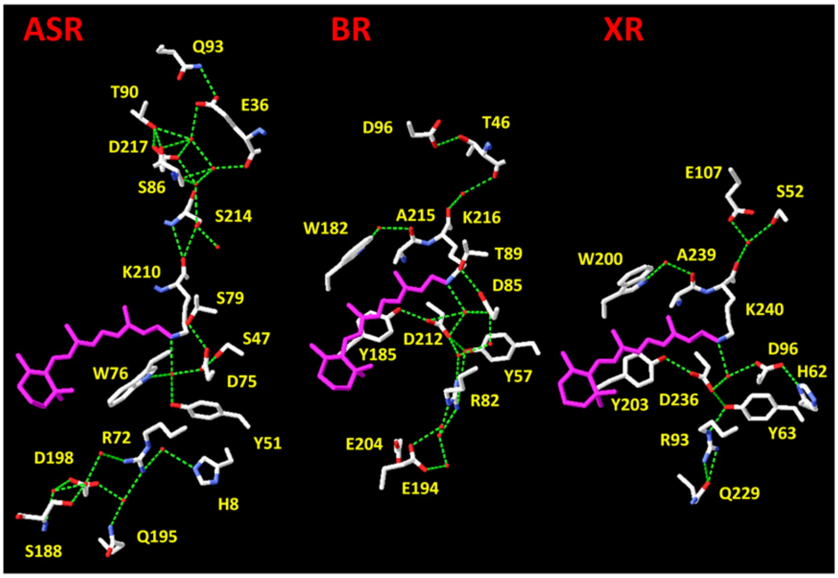

Fig. 4. Comparison of structural organization of the hydrogen-bonded networks in BR (PDB ID: 1C3W [90]), ASR (PDB ID: 1XIO [38]), and XR (PDB ID: 3DDL [62]). Structures are rendered by SWISS-PDB viewer [130], water molecules are shown as red spheres, hydrogen bonds as green dashed lines, retinal is purple; the extracellular sides are at the bottom.

appears that the M intermediate with deprotonated retinal Schiff base is formed only as a result of the photoreaction of all-*trans*-ASR [44,49,55,56]. This finding bridges the two hypotheses on the nature of the signaling state of ASR, as the ratio of 13-*cis* and all-*trans* stable species will affect the accumulation of the M state responsible for ASRT release.

3. Eubacterial proton pumps

Notwithstanding the existence of a few very close homologs of BR in some eubacterial species [11,20], most eubacterial proton pumps, such as those in PR-like and XR-like clusters (Fig. 1), possess very distinct structural features (Figs. 2 and 4). Even though the count of known PR-like and XR-like sequences goes by thousands [12,59–61], only a few proteins have been characterized in-depth by structural or spectroscopic techniques. So far, we are aware only of one high-resolution X-ray structure of a eubacterial proton pump, XR (PDB ID: 3DDL [62], Fig. 4), however, two more unreleased structures are listed in the Protein Data Bank (BPR, blue-absorbing PR, and ER). Additionally, a solution NMR structure of GPR (green-absorbing proteorhodopsin) in detergent micelles has been solved [63], complemented by a number of solid-state NMR studies [64,65] supported by AFM, electron microscopy, mass spectrometry, and electron paramagnetic resonance [66–69]. GPR is the most studied eubacterial rhodopsin in terms of its photochemistry and transport activity, even though spectroscopic studies of XR, BPR and several other PRs, XR-like GR (*Gloeobacter* rhodopsin), and unique ER are available as well [21,26,70–73]. The proton-pumping activity was demonstrated by direct measurements of light-induced pH changes or photocurrents in the native host cells, *E. coli* cells, liposomes, or oocytes for GPR, BPR, a number of other PR variants, as well as for XR, GR, and ER [1,21,23,27,43,74,75]. It should be mentioned that proton-pumping by PR-like proteins remains somewhat controversial in several respects. First, there is a dispute on whether GPR reverses direction of its proton transport at acidic pH [27,75,76] and on possible physiological significance of such reversal. Second, initially, it was not clear if proton transport by PR-like proteins is physiologically relevant, but lately it was shown that PR-based photosynthesis becomes important for cell survival mostly under the conditions of energy starvation, similar to the situation with BR in *Halobacteria* [77,78]. Finally, even though proton pumping was demonstrated for BPR, its extremely slow photocycle combined with unique spectral features raises a possibility that it is a photosensory rather than ion-transporting rhodopsin [27,71,72,79].

As mentioned above, despite having many common motifs in the primary structure, XR-like and PR-like proteins can be separated into distinct clusters (Figs. 1 and 2) [12,15,24], with a few rhodopsin species being intermediate between the two [12,13]. It was suggested that some of the unique structural features in the XR-like proteins originate from the need to accommodate a carotenoid antenna, the second chromophore first observed for XR [23]. Unlike some haloarchaeal rhodopsins in which bound carotenoids play a structural role (e.g., bacterioruberin between monomers of archaerhodopsin-2 [80]), XR uses its carotenoid (salinixanthin) as a light-collecting antenna, one end of which is located very close to the retinal chromophore (Fig. 5) [62,81]. While *Gloeobacter* rhodopsin (GR), a cyanobacterial homolog of XR, was shown to bind carotenoids as well, we do not know if all XR-like proteins do the same, even though it is suggested by the primary structure analysis [82,83]. In particular, conserved Trp-138 of BR is usually replaced by a small Gly in XR-like proteins (Fig. 2), making space for the carotenoid ring [82,84].

Similar to BR, eubacterial proton pumps are prone to oligomerization and formation of 2D crystals, but both the oligomeric state and lattice parameters are strongly dependent on the conditions. For example, the lattice in lipid- or polymer-based 2D crystals of GPR can be either rectangular or hexagonal [67,85,86]. Solubilized GPR was shown to form oligomers ranging from dimers to hexamers depending on the detergent [68,69], but is mostly hexameric (with occasional pentamers) in lipid-containing 2D crystals [66], while mutant BPR turned out to be pentameric in 3D crystals [87]. Solubilized GR showed similar multiplicity of oligomeric states, which was strongly pH-dependent, favoring oligomers at alkaline pH [88]. The radially symmetric organization of hexamers and pentamers of PR, different from trimers of archaeal rhodopsins and ASR, was suggested to be functionally important for the optimal absorption of light [66]. The different organization of oligomers of BR-like proteins and ASR on the one hand and XR-like proteins on the other hand is confirmed by the opposite signs of their CD spectra in the visible range [42,88,89].

Overall architecture of XR and GPR monomers is close to that of the archaeal proton-pumping rhodopsins, but the length and the tilt of the helices, as well as the length and the structure of the loops varies quite dramatically (Fig. 5) [62,63]. Most prominently, the length and the position of the β-hairpin in the BC loop differs in XR, opening up a hydrophilic cavity on the extracellular side, which propagates towards the Schiff base [62]. In addition, hydrogen-bonded networks on the cytoplasmic and especially on the extracellular side have very different organization in archaebacterial and eubacterial proton pumps, as can be judged from the X-ray structures of BR and XR [62,90] (Fig. 4) and deduced from the biophysical data on GPR and GR [71,91–95].

The extracellular hydrogen-bonded network, which in the case of BR includes a pentagonal water-containing cluster near the Schiff base and extends towards the extracellular surface towards the Glu-194/Glu-204 dyad (the proton-releasing complex) via several additional water molecules, looks quite different in the case of XR [62,90] (Fig. 4). First, it seems to have preserved only one water molecule, coordinated by the Schiff base and the two counterion aspartates and believed to be important for proton-pumping functionality [83,96]. In support of the existence of strongly hydrogen-bonded water near the Schiff base, two related eubacterial proton pumps, GPR and GR, were found to show its infrared signature upon photoisomerization of retinal [92,97]. Second, among the most prominent structural signatures on the extracellular side (Fig. 2) of both XR-like and PR-like proteins (and also found in ER) is a unique counterion/primary proton acceptor complex, where the homolog of Asp-85 of BR interacts with a histidine of helix B (His-75/Asp-97 in GPR, or His-62/Asp-96 in XR, Fig. 4) [62,94,98,99]. This interaction of the carboxylic counterion with histidine is believed to modulate the pKa of the former, but is not solely responsible for much higher apparent pKa of the proton acceptor of eubacterial proton-pumping rhodopsins compared to that of BR [70,73,74,99,100]. Different structure of the counterion complex in the PR-like and XR-like proteins is also reflected in much smaller spectral shifts of the visible absorption upon its protonation compared to BR (~40 nm in BR vs ~20 nm in GPR and just a few nm in XR and GR [70,73,100]). Third, possibly as a result of the introduction of a histidine into the counterion complex, the homolog of Arg-82 of BR, which interacts with the Schiff base counterion and couples it to the proton-releasing complex [101–103], is located farther away from the Schiff base in XR (Fig. 4). Accordingly, it was found that this arginine has much weaker coupling to the counterion of GPR and ER [95,99]. Finally, instead of the terminal Glu-194/Glu-204 pair which was suggested to play the key role in proton release in BR [104,105], XR and GPR have non-protonatable residues (Figs. 2 and 4). The extracellular hydrogen-bonded network in XR is terminated by Gln-229, which is conserved in PR-like proteins as Asn. PR-like proteins (but not XR-like ones) have two more conserved Asn residues in helix G (Asn-226 and Asn-230 in GPR), which, along with Tyr-223 and Glu-142, were proposed to be a part of the extracellar proton-conducting pathways [71,93,106]. Glu-142 is conserved in PR-like and XR-like proteins, and was proposed to serve as an alternative proton acceptor in BPR [71]. On the other hand, it was found to be protonated in GPR, where it can possibly serve as an alternative proton-releasing group [71,106,107].

In general, so-called “early” submillisecond proton release typical for BR under physiological conditions is not characteristic for eubacterial proton pumps. Usually, their order of proton release and uptake

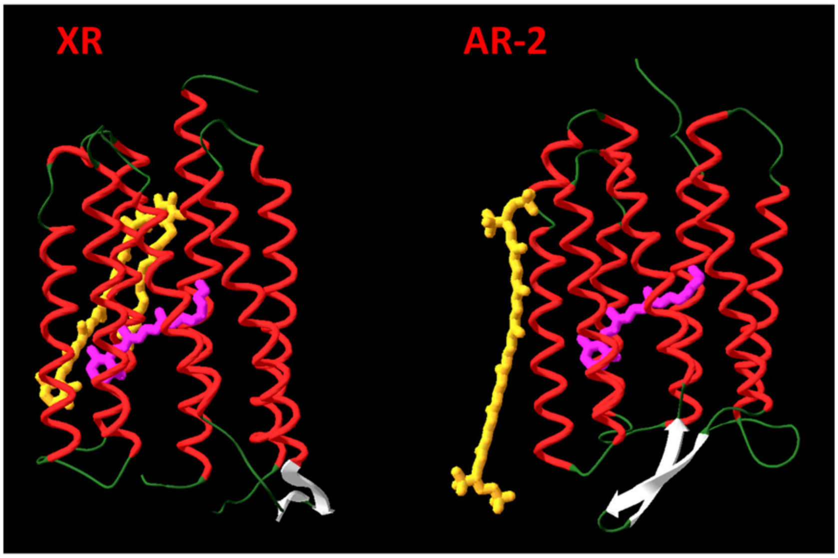

Fig. 5. Backbone structure and location of bound carotenoids of archaeal (AR-2) and eubacterial (XR) rhodopsins (PDB ID: 2EI4 [80] and 3DDL [62]) rendered by Swiss-PDB viewer [130]. The cytoplasmic sides are on top, α-helices are shown in red, β-strands in white, unstructured segments in green, all-*trans*-retinal chromophore with the connected Lys side-chain in purple, and bound carotenoids in orange (bacterioruberin of AR-2 and salinixanthin of XR).

is reversed, as was shown for GPR, XR, GR, and ER, with the release occurring on a millisecond time scale, reminiscent of mutants or low pH conditions of BR, where the proton release complex is disabled or cannot deprotonate [62,73,99,100,108]. It should be noted, that the fast proton release preceding the proton uptake has been observed for GPR at high pH (above 9.5) [108] and in DHPC micelles at pH 9.5 [109], but it is not clear if this fast release can occur under the physiological conditions. There is no clarity on the source of the released proton, with the candidates being the primary proton acceptor (homolog of D85 of BR) with or without its histidine partner, the histidine itself (e.g., His-75 of GPR), homologs of Arg-82 of BR, and homologs of Glu-142 of GPR [62,94,106,110].

The cytoplasmic side of eubacterial retinal proton pumps is mainly hydrophobic, similar to BR, and in the case of XR contains only two water molecules [62] (Fig. 4), keeping the pKa of the carboxylic proton donor high [70]. The proton-donating Asp-96 of BR in complex with Thr-46 is usually replaced by Glu/Ser pair in XR-like and PR-like rhodopsins (Figs. 2 and 4), which has somewhat different hydrogen-bonding geometry. It was suggested that proton-conducting pathways coupling the proton donor and the Schiff base in the second half of the photocycle, in part pre-exist in the dark state of XR [62,83]. In agreement with this, it was shown that homologous GR has an unusually strong coupling of the retinal and the proton donor [73,92]. Interestingly, the proton-pumping rhodopsin ER, which does not belong to either XR or PR cluster, has a lysine in place of the carboxylic proton donor, displaying the photocycle kinetics comparable to those of other eubacterial proton pumps [21,99] and reprotonating the Schiff base by yet unknown mechanism. To couple the carboxylic proton donor with the Schiff base, BR undergoes a substantial light-induced conformational rearrangement, involving changes in some helical tilts and entrance of water [111–113]. If the hypothesis on the pre-coupling of the proton donor with retinal is true, one may expect that the extent of the light-induced conformational changes may be smaller in eubacterial proton pumping rhodopsins than in BR. This idea is not supported by the results of wide-angle X-ray scattering (WAXS), suggesting similar conformational changes for detergent-solubilized BR and GPR [114]. In agreement with this, combined EPR and ODNP (Overhauser dynamic nuclear polarization) study showed substantial conformational changes in the EF loop of GPR [115], similar to those in BR [116–119]. On the other hand, recent solution NMR experiments detected only very small conformational changes induced by light in solubilized GPR, even though this could be possibly a result of the kinetically disadvantaged accumulation of the relevant photointermediates with changed conformation [120].

4. Other ion pumps of eubacteria

Until very recently, the only known non-proton retinylidene ion pumps of eubacteria were close homologs of haloarchaeal chloride pump, halorhodopsin (HR), probably obtained via lateral gene transfer, e.g., in *Salinibacter ruber* [6,11]. But, this year, several papers noting the existence of a peculiar XR-related group of eubacterial rhodopsins, in which the primary proton acceptor and donor (Asp and Glu) were replaced by Asn and Gln (the NQ-type group in Fig. 1), came out [7,9,15]. It was found that some rhodopsins of this type, as was shown for proteins from *Nonlabens* (K.-H. Jung, personal communication) and *Krokinobacter* [9], can pump sodium (and lithium) ions in the extracellular direction, but do not transport potassium ions. The latter rhodopsin also shows residual proton-pumping activity in the absence of sodium [9]. Besides having the primary carboxylic proton donor and acceptor of helix C replaced by Asn and Gln, most of the NQ-type proteins have a new Asp residue inserted between them, replacing conserved Thr-89 of BR (Fig. 2) and forming so-called NDQ motif. It appears that light-induced proton transfer from the Schiff base to this new proton acceptor, manifested by a formation of the M-like intermediate, is required for sodium transport, both in *Nonlabens* [121] and *Krokinobacter* [9]. Interestingly, the conserved glutamate near the extracellular surface of PR-like and XR-like proteins (Glu-142 of GPR), suggested to participate in proton transfers [71,106,107], is replaced by Gln as well, hinting that it may be involved in sodium translocation, along with a number of other unique polar residues (Fig. 2).

The existence of eubacterial rhodopsins performing light-driven sodium extrusion shows that, analogous to F/V-ATPases, proton-pumping microbial rhodopsins have their sodium-pumping homologs, often present in the same host, which has a fundamental importance for understanding the relationship between proton- and sodium-based bioenergetics [122,123]. The coexistence of sodium- and proton-pumping rhodopsins is logical in view of the recently shown light-enhanced expression of Na^+ pumping NADH-quinone oxidoreductase together with PR in *Dokdonia*, suggesting the importance of sodium extrusion during the light-stimulated growth in addition to protons [124]. We believe that discovery of sodium-pumping eubacterial rhodopsins will be followed by many more surprises, as can be expected from the existence of other untypical sequences of rhodopsins with yet unknown functions.

Intriguingly, a subgroup of the NQ-type rhodopsins forming the upper branch in Fig. 1 does not possess the NDQ motif, having replaced the putative proton-accepting aspartate with threonine, and forming the NTQ motif. In what could be regarded as an astonishing demonstration of parallelism between archaeal and bacterial rhodopsins, at least one of the members of this group (*Fulvimarina*) was shown to transport chloride ions [125]. Such change in the ion specificity upon the replacement of carboxylic acid with threonine is reminiscent of chloride-pumping mutants of BR mimicking halorhodopsin [126,127].

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