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Xanthorhodopsin: Proton pump with a carotenoid antenna

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Abstract. Retinal proteins function as photoreceptors and ion pumps. Xanthorhodopsin of *Salinibacter ruber* is a recent addition to this diverse family. Its novel and distinctive feature is a second chromophore, a carotenoid, which serves as light-harvesting antenna. Here we discuss the properties of this carotenoid/retinal complex most relevant to its function (such as the specific binding site controlled by the retinal) and its relationship to other retinal proteins (bacteriorhodopsin, archaerhodopsin, proteorhodopsin and retinal photoreceptors of archaea and eukaryotes). Antenna addition to a retinal protein has not been observed among the archaea and emerged in bacteria apparently in response to environmental conditions where light-harvesting becomes a limiting factor in retinal protein functioning.

Keywords. Light energy transfer, salinixanthin, proton transport, carotenoid binding.

Introduction

Xanthorhodopsin (XR) is a novel retinal protein of eubacterium *Salinibacter ruber*, which uses a carotenoid molecule to collect more light for proton pumping [1]. It appears to be the simplest bioenergetic machine, which utilizes excited-state energy transfer. This article summarizes the basic features of this recently described novel system [1–4], and its relevance to other retinal proteins.

The vast majority of energy resources on Earth originate from light captured by living organisms and transformed in the processes of photosynthesis. The efficiency of light absorption in these systems depends on an optical cross-section high enough to capture photons at the light flux available from the Sun. The most common pigments, chlorophyll, retinal, carotenoids and flavins, are highly colored chromophores. However, because there is an upper limit to the extinction coefficients of organic molecules, and their spectra are not always optimized for sunlight, evolution has augmented energy-transducing photosystems with additional pigments that function as antennae. Tens and hundreds of pigment molecules (mostly chlorophylls and carotenoids) in the light-harvesting complexes of chloroplasts and chromatophores of photosynthetic bacteria function together as antennae, absorbing light and funneling excitation energy to the reaction centers to initiate electron transfer linked to transmembrane proton transport and thereby to the biosynthesis of high-energy substances.

In 1971, an alternative and much simpler way of light energy conversion was discovered in the haloarchaea [5]. All the processes from light absorption to transmembrane proton transport are performed by a single protein, bacteriorhodopsin (BR), containing all-trans retinal as a chromophore. Several varieties of BR, and other related retinal proteins capable of light-induced chloride transport [6] (halorhodopsin) and control of cell motility (sensory rhodopsin I [7] and II [8]) have

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been since discovered in the archaea (reviewed in [9, 10]).

For almost three decades, it was generally thought that retinal-based energy conversion is restricted to archaea and extreme conditions of high light intensity and high salt. This view was changed recently by a series of discoveries. A gene homologous to BR was found in uncultured marine proteobacteria from the Pacific Ocean [11]. Expression of this gene in *E. coli* produced a retinal protein named proteorhodopsin (PR), capable of transmembrane proton transport. Numerous PR genes were found in different aquatic conditions [12–14]. Moreover, retinal proteins with homology to BR and bearing all-trans retinal as a chromophore (type 1 retinal proteins [15]) have subsequently also been discovered in different groups of eukaryotes, fungi and algae. Some of them are apparently proton pumps, as *Leptosphaeria* rhodopsin [16], while others are sensors as rhodopsin of *Chlamydomonas* [17, 18]. Only a few of those proteins were isolated from original sources. Some were expressed in *E. coli* but many are still not available.

Earlier observations on energy transfer to retinal and possible involvement of carotenoids

Unlike photosynthetic units, most known retinal proteins do not involve excitation energy transfer to retinal except from aromatic amino acid residues in the retinal binding site. No energy transfer occurs between the common carotenoid of archaea, bacterioruberin, and BR [19, 20] or even archaerhodopsin which interacts with this carotenoid [2, 3, 21]. The much-disputed explanation for the bilobe circular dichroism (CD) spectrum of the purple membrane by an exciton interaction within the BR trimer [22], which would result in energy transfer between the three retinal molecules, disagreed with a number of experimental results [23]. However, there are several cases when energy transfer from accessory pigments to retinal is involved in photoreception. Absorption of light by retinol or 3-hydroxyretinol, bound to insect rhodopsin, was reported to extend the range of vision to the ultraviolet [24]. Another interesting case, a derivative of chlorophyll that increases the sensitivity to red light apparently through chlorophyll to retinal energy transfer, was reported in the photoreceptor of the deep-sea dragon fish [25]. In salamander retinas a similar phenomenon is observed upon addition of chlorin [26].

Earlier work suggested involvement of carotenoids in photoreception in archaea [27], based on the presence of several maxima in the blue-green wavelength range of the action spectrum. This, however, needed further investigation in view of the presence of sensory rhodopsin II (phoborhodopsin) with a complex vibronic spectrum from its retinal chromophore [28–30].

The possible role of carotenoids and/or flavins as antenna pigments in photoreception was proposed [31] to explain the observed highly structured action spectrum for phototaxis and photoelectric responses in the unicellular alga *Haematococcus pluvialis* [32]. This organism exhibits sensory signal transduction mechanism similar to that in *Chlamydomonas reinhardii* [33, 34], in which two retinal proteins act as photoreceptors [18]. Future studies might clarify whether the photoreceptor of *H. pluvialis* has a carotenoid antenna. The discovery of XR [1] provides evidence that such an antenna can function efficiently.

Features of xanthorhodopsin

XR was isolated from the extremely halophilic eubacterium *S. ruber* which was found in salt ponds in Spain, where it shares its environment with archaea [35]. Partial genome sequencing indicated the presence of a halorhodopsin-like gene in *Salinibacter* [36]. Examination of light-induced changes of proton concentration in cell vesicle suspensions led us to the conclusion that the cells express mostly a proton pump similar to BR. The complete genome of *Salinibacter* became available by that time at the Institute for Genomic Research gene bank, and it indeed contained genes homologous to a proton pump (BR and PR), in addition to halorhodopsin and two sensory rhodopsin genes, described later in detail [37].

The primary structure of XR, confirmed with amino acid sequencing [1], exhibits slightly more homology to BR than PR (58 *versus* 48 identical residues); however, the internal proton donor is Glu as in PR, and one of two glutamic acids that constitute the proton release group in BR [38] is missing, as in PR. This indicates that the sequence of events leading to proton release during the transport cycle in this pigment should be similar to that observed in PR [39]. The novel member of microbial retinal protein family showed several features different from those both in BR and PR. Thus, protonation of the counter-ion to the Schiff base at low pH is accompanied by a large, approximately 30-nm red shift in BR, PR and sensory rhodopsins. In XR, however, only a small, 3–5-nm red shift is observed, indicating a different arrangement of the Schiff base environment [4]. Interestingly, recently a similarly small shift was observed also for *Anabena* sensory rhodopsin [40]. The pK_a of the counterion in XR (~6.0) is nearer to that in PR (7.5) than BR (2.6) [4].

Isolation of a retinal protein from a cultured eubacteria is still a rare event [41], but that was not the only

interesting feature of the new protein. The main surprise was that XR contains a second chromophore, the carotenoid salinixanthin, the main carotenoid in this organism [42].

The structure of this C40 carotenoid (Fig. 1) has several distinctive features: a keto group in the ring, which is known to be involved in carotenoid binding by forming a hydrogen bond in other carotenoproteins ([43–45]); a glycoside, which is likely to be located at the surface of the membrane, and an acyl tail, which is probably immersed in the hydrophobic core of the lipid bilayer of the membrane (or in the membrane protein).

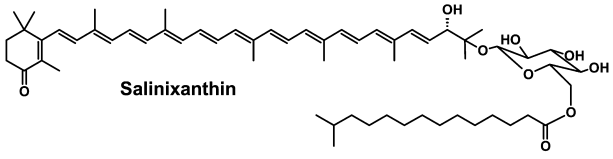

Figure 1. Chemical structure of carotenoid salinixanthin from *Salinibacter ruber* (from [42]).

Several different tests indicated tight and specific association of the carotenoid with the protein and intimate interaction with the retinal. Hydrolysis of the Schiff base with hydroxylamine produced not only changes in the broad retinal absorption band but also large changes of carotenoid bands (broadening and decrease in extinction) [1, 3]. These changes are reversible upon reconstitution of the retinal protein with all-trans retinal. Similar changes occur transiently during the photocycle of XR [1, 3] in response to charge movements and conformational changes of the retinal and the protein during proton transport. These observations showed that binding of retinal to the apoprotein strongly affects binding of the carotenoid. It restricts carotenoid conformational motions, both of its conjugated chain and its ring and the interaction between the two chromophores changes during the photocycle.

The functional role of the tight carotenoid-retinal interaction became clear from the action spectrum for proton transport. It exhibits sharp carotenoid bands at approximately 460, 489, and 522 nm, along with a broad retinal band at ~560 nm [1, 2], which indicated that light absorbed by a carotenoid as well as by retinal is utilized for proton transport (Fig. 2). Given the close similarity of XR to the other bacterial rhodopsins, there is no reason to suppose that in this protein salinixanthin is directly involved in the proton transport mechanism. Rather, it must be an antenna, which supplies energy to retinal through excitation energy transfer. The carotenoid/retinal stoichiometry is near one. From comparison of the absorption spectrum and the action spectrum, the efficiency of energy transfer in the excited state was calculated to be about 40 % [1]. In light-harvesting complexes of photosynthetic organisms efficiency of energy transfer from carotenoids to chlorophyll varies from 25 % to 100 % [46].

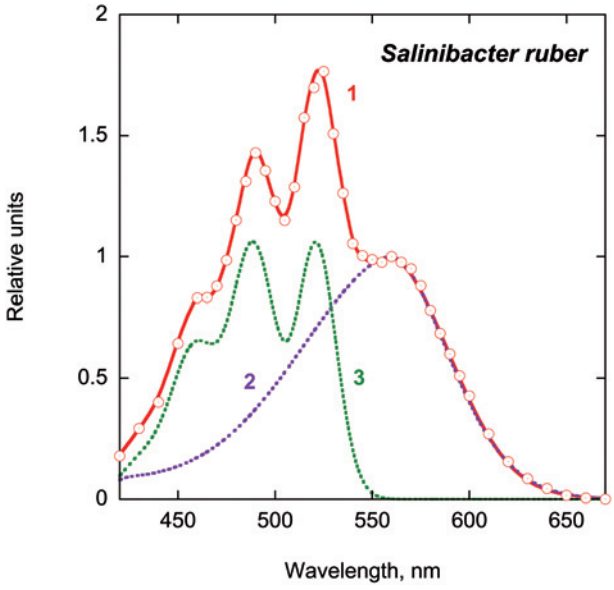

Figure 2. Action spectrum for light-induced proton transport in *Salinibacter ruber* (1) and its retinal (2) and carotenoid components (3), obtained by measurements of photoinhibition of cell respiration (from [1, 2]).

Energy transfer from carotenoids to chlorophyll is a well-studied process in several light-harvesting complexes (reviewed in [47]). XR is the first system where carotenoid to retinal energy transfer can be examined in detail. The discovery of XR posed questions on the origin of this protein, the spatial arrangement of the carotenoid in it, and the involvement of the second chromophore in the photocycle and proton transfer reactions.

Relative geometry of the salinixanthin and retinal binding sites holds the key to the function

The principal question at this time is where the carotenoid is located in this small heptahelical membrane protein. The crystal structure of BR shows that the retinal lies transversely at about the center of the protein, in the interhelical region, linked to a lysine on helix G *via* a Schiff base [48]. A similar position for the retinal is expected for XR. Because the energy level of the first excited state S_1 of the carotenoid lies below that of the retinal, energy transfer will be restricted to be from higher levels, probably from the second excited state S_2 populated during light absorption

(direct transition S_0 to S_1 is forbidden for symmetry reason). S_2 has a very short lifetime (typically 100–200 fs) [47]. Thus, the relatively high efficiency for the energy transfer implies that the carotenoid and the retinal are close to one another and with a favorable orientation of the conjugated side chains that allows dipolar interaction. The latter is expected to be maximal for the parallel and co-linear orientation of the transition dipole moments (oriented along the conjugated chain). However, this geometry might be difficult to realize with the two relatively large chromophores buried in a relatively small protein like XR. This consideration implies that orientation and position of salinixanthin might be influenced not only by factors that affect the efficiency of energy transfer, but also by steric and energetic considerations that dictate positions consistent with the structural requirements of the retinal protein and that do not interfere with proton transport. Does the carotenoid simply bind to the hydrophobic surface of the opsin at the lipid-protein interface, or is there a specific binding site? Optical activity acquired by the carotenoid when bound to the opsin, and its dependence on retinal, strongly suggest the latter. XR can be compared with archaerhodopsin in which a carotenoid (bacterioruberin) has also been found, but in that protein it does not serve an antenna function [2, 21]. In solution, neither salinixanthin nor bacterioruberin exhibit CD bands in the visible wavelenghts, but the CD spectra of XR and archaerhodopsin contain large contributions from these carotenoids, indicating that they become chiral when bound [3]. This would arise either by an asymmetry of the polyene configuration in a constrained binding site, or interaction with asymmetric groups of the protein.

Consistent with energy migration from carotenoid to retinal, strong retinal-carotenoid interaction is observed in XR but not in archaerhodopsin. In XR but not in archaerhodopsin, removal of the retinal by splitting the Schiff base bond with hydroxylamine dramatically affects the carotenoid spectrum: its vibronic bands become broader and it now resembles the spectrum of free carotenoid. Likewise, hydroxylamine bleaching abolishes the induced chirality in XR but only partially reduces it in archaerhodopsin, consistent with the close retinal-carotenoid interaction required for antenna function [3].

High resolution X-ray structure would certainly verify the predictions of close proximity and deviation from parallel orientation of the two chromophores, and provide other details on binding. Additional information will come from examination of the excited states of the salinixanthin and XR in steady-state fluorescence and time resolved experiments, which might be a source of valuable data on the mutual orientation of the two chromophores, the distance between them from the overlap integrals of donor emission and acceptor absorption, and shed light on the mechanism of energy transfer from carotenoid to retinal. Work on both of these approaches by the authors is in progress.

In XR, the single antenna per molecule seems a more primitive arrangement than in photosynthetic complexes, where collection of light is ensured by large arrays of antenna molecules that funnel energy to the reaction center. The gains also appear to be more modest. Given the extinction coefficients of carotenoid and retinal in XR and the efficiency of the transfer, the antenna increases the optical cross-section by only a factor of about 2. Further, in archaerhodopsin the carotenoid has no antenna function. How and why did these structures evolve? Currently only a hypothetical answer can be given to this question. It is likely that initially the binding of a carotenoid to the retinal proteins of archaea (*e.g.*, archaerhodopsin) served a photoprotective and maybe a structural role; the former was shown for *H. salinarum* [49]. Unbound retinal absorbing at 360 nm and probably the M intermediate with deprotonated Schiff base might photosensitize formation of singlet oxygen [50]. The excited states of singlet oxygen [51], free retinal and the short wavelength photocycle intermediate M can be quenched and deactivated by carotenoids through energy transfer to their singlet and triplet excited states [46, 52, 53]. The light harvesting function found in *Salinibacter* became important under conditions of low light intensity in either deep water or optically dense culture. An interesting question is whether and how the futile energy transfer from the S_1 of the retinal chromophore to lower lying S_1 of salinixanthin is prevented. At this time, again, only a hypothetical answer can be given, as no direct measurements have been published. The efficiency of resonance energy transfer depends on the overlap of the emission spectrum of the donor and absorption band of the acceptor. The S_0 to S_1 transition is symmetry forbidden and the absorption bands corresponding to this transition in long chain carotenoids exhibit extremely small extinction coefficient (reviewed in [47]). This is probably the reason why the efficiency of energy transfer from S_1 of the retinal chromophore to S_1 of salinixanthin is not expected to be large enough to lower the quantum yield for proton transport (provided that energy transfer in XR occurs according to the Förster mechanism [54, 55] or other mechanism involving dipole-dipole interaction).

The origin and relationship of XR with other microbial retinal proteins is an intriguing question. Retinal proteins have an ancient origin [56]. Gene transfer between archaea and eubacteria is likely to be involved in exchange of retinal proteins [37, 57, 58],

but XR does not have an immediate counterpart among archaeal retinal proteins, which lack an antenna. Interestingly, the XR gene exhibits greatest homology with rhodopsin of cyanobacteria *Gloeobacter violaceus* [37, 58]. This suggests that it is a product of evolution in the bacterial domain, where we expect that other proteins similar to XR will be found.

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