
REVIEW

Photoreversible Reactions of Visual and Microbial Rhodopsins

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Received April 19, 2026

Revised May 26, 2026

Accepted May 30, 2026

Abstract—The review discusses experimental findings and theoretical studies of photoreversible transformations of retinal-containing proteins (rhodopsins). Understanding these processes is essential for elucidating the mechanisms of photochemical and conformational transformations of these proteins. Particular emphasis is placed on visual rhodopsins, as photoreversibility plays a crucial role in its physiological regeneration in the rhabdoms of invertebrates and the cone photoreceptors of the vertebrate retina. In recent years, interest in the mechanisms of photoreversible reactions in retinal-containing proteins has grown substantially, driven by their potential applications as active components in optoelectronic devices and biosensors.

DOI: 10.1134/S0006297926601267

Keywords: retinal-containing proteins, visual rhodopsin, chromophore, photoreversible reactions, photoisomerization, photoregeneration, dark adaptation

INTRODUCTION

The diversity of retinal-containing proteins (rhodopsins) is extremely large [1-12]. They are found in all domains of life. Type I (microbial) rhodopsins are characteristic of bacteria, archaea, lower eukaryotes, and giant viruses, while type II rhodopsins are characteristic of higher animals. Type I rhodopsins perform photoenergetic (ion pumps), photoreceptor (sensory rhodopsins and ion channels), and photoenzymatic functions, while type II rhodopsins perform primarily photoreceptor (visual and non-visual rhodopsins) and photoenzymatic functions. These types appear to have independent origins.

Each of the diverse functions of retinal-containing proteins is initiated by photoisomerization of the chromophore group of retinal – from all-*trans* to 13-*cis* in microbial rhodopsins and from 11-*cis* to all-*trans* in animal rhodopsins [1, 2]. The characteristics of this photochemical reaction were studied in detail after its discovery in the laboratory of J. Wald in the late 1950s in bovine visual rhodopsin [13].

It has now been established that this is a uniquely rapid and selective reaction, occurring with a high quantum yield in an electronically excited state [1-4, 8, 9]. Interactions between retinal and its protein environment (protein catalysis) play a major role in this reaction. The dynamics and sequence of formation of photoreaction products have been characterized in detail in both microbial and visual rhodopsins. The reverse photoreactions, initiated by the absorption of a second quantum of light by these products, have been described less thoroughly. However, they are of significant interest.

Reverse photoreactions of rhodopsins, initiated from the early products of the forward photoreaction, can serve as an important tool for studying the interactions of retinal with its immediate protein environment and conformational changes in the protein part of the molecule (opsin). Reverse photoreactions of some microbial rhodopsins, initiated at later stages, are used by the cell to more precisely discriminate between the wavelengths of incident light, which is important for its adaptation. In the case of visual rhodopsins, reverse photoisomerization of all-*trans* retinal to the 11-*cis* form is of fundamental physiological

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importance for photoreceptor cells – the rhabdoms of invertebrates and the cones of vertebrate retinas. This involves the return (regeneration) of rhodopsin to its original state, which is necessary for restoring normal physiological activity in the photoreceptor cell.

This review examines the forward and reverse photoreactions of visual rhodopsins, initiated at different stages of their photoconversion. A comparison is made of the reverse photoreactions of bovine visual rhodopsin and bacteriorhodopsin, initiated from the early products of their forward photoreactions. The review also discusses the light-dependent and light-independent pathways for regenerating visual rhodopsins.

PHOTOCHEMISTRY OF RETINAL-CONTAINING PROTEINS

Visual rhodopsins. Visual rhodopsins (type II rhodopsins) are transmembrane proteins that utilize light energy to initiate the phototransduction cascade. Light absorption is mediated by a chromophore group, 11-*cis* retinal, located in the chromophore-binding pocket of the apoprotein opsin. The chromophore is covalently bound to a lysine residue, forming a protonated Schiff base linkage (Fig. 1). Visual rhodopsins are members of the G protein-coupled receptor (GPCR) superfamily. The fundamental functional event in these proteins is retinal photoisomerization (Fig. 1), which occurs within 50-80 fs and proceeds with a quantum yield of 0.6-0.7. These exceptional parameters result from both intrinsic chemical properties of retinal and the influence of the surrounding protein environment. In contrast, when retinal is not embedded within a protein matrix, such as in the gas phase [14] or in a solution [15, 16], the photoisomerization reaction is approximately an order of magnitude slower and exhibits a substantially lower quantum yield of only 0.1-0.3 [17]. The primary role of the photoreaction is to store the energy of the absorbed photon in a form of a strained, highly twisted all-*trans* retinal configuration. Subsequent relaxation of such distorted chromophore releases the stored energy and induces structural rearrangements in its immediate protein environment, which triggers global structural changes in the rhodopsin molecule necessary for its functioning.

The most extensively studied visual pigments in vertebrates and invertebrates are bovine and octopus rhodopsins, respectively. Bovine rhodopsin is located in the outer segments of rod photoreceptor cells and is responsible for scotopic (twilight and low-light) vision. Octopus rhodopsin is found in the rhabdomeres of photoreceptor cells and serves as a primary visual

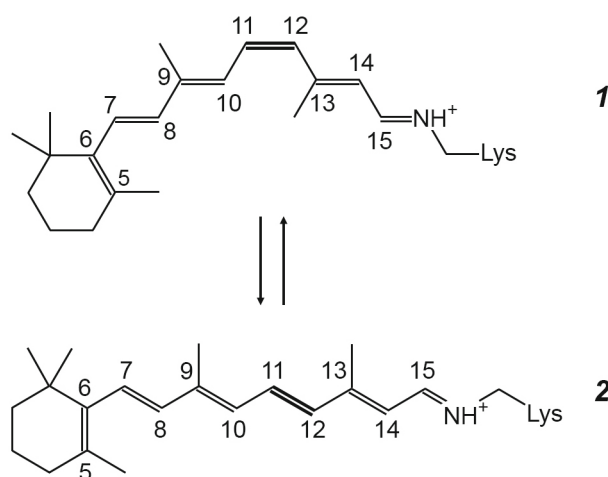


Fig. 1. Structures of the chromophore group in type II rhodopsins: protonated Schiff base of 11-*cis* and all-*trans* retinal in the rhodopsin dark state (1) and after photon absorption (2), respectively. Reactive C₁₁=C₁₂ double bond is shown in bold; photochemical transitions are indicated by arrows.

pigment mediating visual perception in this species. Visual rhodopsins of vertebrates are monostable proteins, meaning that under physiological temperatures in the dark, they exist in a single thermally stable state. Upon light absorption, these proteins undergo a sequence of mainly irreversible transformations, collectively referred to as photolysis (Fig. 2a). In contrast, invertebrate visual rhodopsins are bistable. When exposed to light, they form an alternative thermally stable state and can absorb a second photon, which reverts them to the original state (Fig. 2b). This mechanism enables physiological photoregeneration of visual rhodopsin in invertebrates.

Vertebrate visual rhodopsin. Let's consider the stages of photolysis of vertebrate (bovine) visual rhodopsin in more detail. Following the photoinduced 11-*cis* → all-*trans* isomerization of the retinal chromophore, the protein progresses through a series of intermediate conformational states. In the final stage of photolysis, the Schiff base linkage between retinal and opsin is hydrolyzed, leading to the release of all-*trans* retinal from the chromophore-binding pocket and its subsequent removal from the photoreceptor cell. Rhodopsin intermediate states or products of photoconversion have been characterized using a variety of spectroscopic techniques, including low-temperature absorption spectrophotometry, time-resolved absorption and fluorescence spectroscopy (Fig. 2a).

The *cis* → *trans* photoisomerization of retinal in bovine rhodopsin proceeds with a quantum yield of 0.65-0.67 [27, 28] and is completed within 50-80 fs [29-33]. This process leads to the formation of the first photoproduct, photorhodopsin, through a transition from the first excited electronic state (S₁)

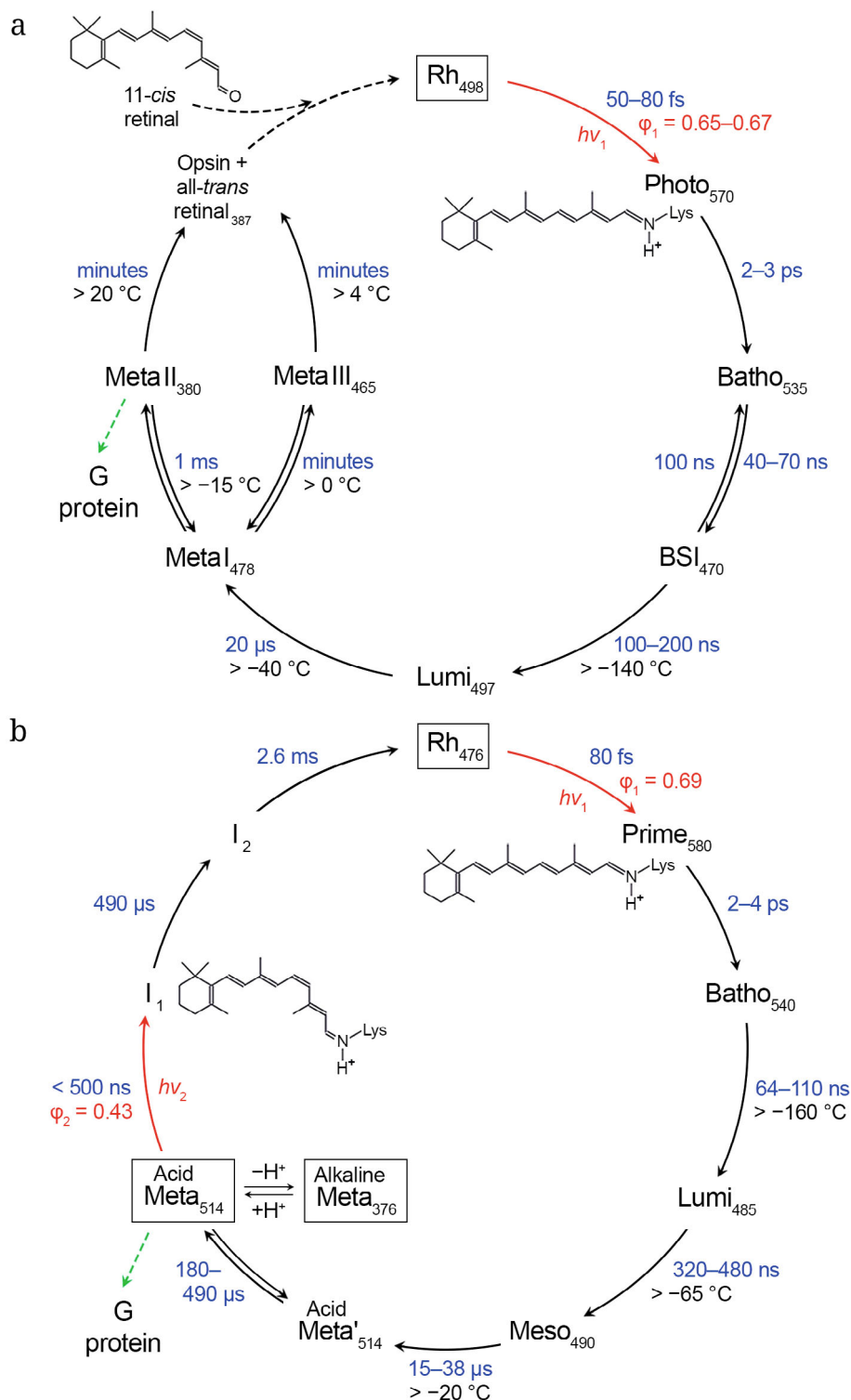


Fig. 2. Photoactivated processes in visual rhodopsins: a) photolysis of monostable bovine rhodopsin [2, 7, 18-20]; b) photocycle of bistable octopus rhodopsin [21-26]. The schemes show the sequence of intermediate states formed during rhodopsin photoactivation, including the room temperature maxima of their α -absorption bands, characteristic formation times, and minimum temperatures at which these intermediates were detected by low-temperature spectrophotometry. In panel (b), the minimum temperature values are given for squid visual rhodopsin [18]. Photochemical transitions are indicated by $h\nu$ symbols together with respective quantum yields (ϕ). Rhodopsin, dark state of the visual pigment; Photo, photorhodopsin; Batho, bathorhodopsin; BSI, blue shifted intermediate (intermediate with the absorption spectrum shifted to the blue region); Lumi, lumirhodopsin; Meta, metarhodopsin; Prime, primerhodopsin; Meso, mesorhodopsin; Meta', short-lived metarhodopsin; I_1 and I_2 , intermediate products of phototransition from acid metarhodopsin to octopus rhodopsin. States stable at physiological temperatures are framed. See the text for details.

to the ground state (S_0). This extremely fast transition is enabled by a conical intersection between the S_1 and S_0 potential energy surfaces, which provides an efficient pathway for the ultrafast conversion of photon energy into chemical energy [3, 34, 35]. In reactions of this type, the product is formed as a result of coordinated (coherent) movement of nuclei in the chromophore. The dynamics of coherent reactions is highly sensitive to the chromophore structure, its local environment, and initial conditions of photoexcitation. The protein environment of retinal actively modulates this process by tuning the absorption maximum as well as the selectivity, rate, and quantum yield of photoisomerization. In this way, the protein environment effectively catalyzes such ultrafast and highly efficient photochemical reaction. Steric constraints within the chromophore-binding site and electrostatic interactions between retinal and surrounding amino acid residues, particularly the negatively charged counterion Glu113, play an important role in this regulation [2, 7].

Photorhodopsin is finally formed within 200 fs [36]. It contains vibrationally excited all-*trans* retinal that remains highly distorted within the confined volume of the binding pocket [37, 38]. Subsequent intermediates, bathorhodopsin [30, 33, 36, 39] and the blue-shifted intermediate (BSI) [19, 40, 41], arise during vibrational relaxation of the chromophore and its “straightening” in the β -ionone ring region. Later stages of rhodopsin photolysis involve conformational rearrangements of opsin and include the formation of lumirhodopsin and metarhodopsins I, II, and III (Fig. 2a) [2, 7, 20]. Metarhodopsin II is the key signaling state, as it is capable of activating the G protein transducin. Its formation is accompanied by deprotonation of the retinal Schiff base and proton transfer to the acceptor residue Glu113, which triggers major conformational changes in rhodopsin. On a minute timescale, metarhodopsin I can reversibly convert into metarhodopsin III through the thermal *anti* $\rightarrow$ *syn* isomerization of the $C_{15}=N$ Schiff base linkage (Fig. 1) [42]. Metarhodopsins II and III subsequently decay slowly through hydrolysis of the Schiff base linkage, yielding the apoprotein opsin and all-*trans* retinal.

Invertebrate visual rhodopsins. The photoreactions of invertebrate visual rhodopsins have been investigated primarily in cephalopod mollusks [21-26, 43-46]. The photocycle of octopus rhodopsin is illustrated in Fig. 2b. Its early photochemical events closely resemble those observed in vertebrate rhodopsins. Following photon absorption, chromophore photoisomerization proceeds coherently within ~80 fs [26] with a quantum yield of 0.69 [22]. This process generates the first photoproduct, primerhodopsin, which is analogous to the vertebrate photorhodopsin and converts to bathorhodopsin within 2-4 ps [46].

Subsequent intermediates arise from progressive retinal planarization and accompanying conformational changes in opsin. These transitions lead to the formation of lumirhodopsin, mesorhodopsin, short-lived and ultimately thermally stable acid metarhodopsins [21, 23-25]. Acid metarhodopsin represents the signaling state of the photocycle; however, unlike vertebrate metarhodopsin II, it retains a protonated Schiff base chromophore, which likely contributes to its enhanced stability and resistance to photolysis [47]. At elevated pH, acid metarhodopsin can convert to its alkaline form through the Schiff base deprotonation. Photon absorption by acid metarhodopsin initiates the reverse photoreaction, regenerating the original rhodopsin state. Importantly, this reverse transition does not trigger photoreceptor cell excitation (see the section “Photoreversibility at late stages of rhodopsin conversion”).

Microbial rhodopsins. The primary photoreaction of microbial rhodopsins (all-*trans* $\rightarrow$ 13-*cis* isomerization of retinal) occurs on a slightly longer timescale than in visual rhodopsins, typically within 300-800 fs, and proceeds with a quantum yields of 0.3-0.7 [2, 3, 8]. Subsequent processes are closed in a cycle, with one of the last stages involving thermal retinal re-isomerization. This photocycle has been characterized in greatest detail for bacteriorhodopsin from the haloarchaeon *Halobacterium salinarum* [48]. The early intermediates *J* and *K* of this bacteriorhodopsin are considered analogs of photorhodopsin and bathorhodopsin, respectively [9, 49-52]. A central intermediate of the bacteriorhodopsin photocycle is the long-lived *M* state, which is essential for the protein's photoenergetic function as a light-driven proton pump. The created proton gradient supplies the motive force for ATP synthesis [53]. Formation of the *M* intermediate is accompanied by deprotonation of the retinal Schiff base and substantial conformational rearrangements within the seven-transmembrane α -helical protein scaffold, reminiscent of structural changes associated with the formation of metarhodopsin II in vertebrate visual rhodopsins.

In some microbial rhodopsins, the reverse photoconversion from 13-*cis* to all-*trans* retinal is also physiologically important. It enables these proteins to function as photochromic sensors for regulating phototactic responses and gene expression [54-58].

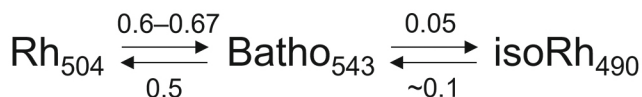
PHOTOREVERSIBILITY OF RHODOPSIN PHOTOCONVERSION PRODUCTS

Retinal-containing proteins exhibit photoreversibility, i.e., the ability to return to their original state upon light absorption by the products of their photoconversion. This reverse photoreaction is known

as photoregeneration. Photoreversibility is observed both *in vitro* [9, 33, 59-61] and *in vivo* [62-66].

Photoreversibility at early photochemical stages of rhodopsin conversion. Photoreversibility at early photochemical stages has been investigated experimentally mainly in bovine visual rhodopsin [9, 33, 66-77] and bacteriorhodopsin [33, 60, 78-84], as well as studied using computational modeling methods [82, 85-87].

Low-temperature studies of rhodopsin reverse photoreactions. The first studies of the early stages of rhodopsin photolysis employed low-temperature spectrophotometry, infrared (IR) spectroscopy, and Raman spectroscopy. These approaches are based on the ability to stabilize individually each product of rhodopsin photolysis (except for photorhodopsin and BSI) at cryogenic temperatures, when the thermal energy is insufficient to overcome the activation barriers required for progression to subsequent states (Fig. 2, a and b). Under such conditions, rhodopsin is typically studied in detergent extracts. Irradiation generates a photostationary mixture containing the original state and the products of both forward and reverse photoreactions, with their relative proportions determined by the temperature and illumination conditions. For example, bovine rhodopsin irradiated at liquid nitrogen temperature (-196°C) with light in the 450-500 nm range yields a mixture of rhodopsin ($\lambda_{\text{max}} = 504$ nm), bathorhodopsin ($\lambda_{\text{max}} = 543$ nm), and isorhodopsin ($\lambda_{\text{max}} = 490$ nm), which contain 11-*cis*, all-*trans*, and 9-*cis* retinal, respectively [67-69, 71-73, 75, 88] (Scheme 1). Illumination with longer-wavelength light (550-600 nm) shifts the equilibrium toward rhodopsin and isorhodopsin, whose relative abundance (about 90 : 10) reflects the quantum yields of their formation from bathorhodopsin (0.5 and 0.05, respectively) [71]. These low-temperature experiments on forward and reverse photoreactions provided some of the earliest evidence that the primary event in vision involves retinal isomerization and that bathorhodopsin already contains all-*trans* retinal [67]. These observations were later corroborated by Raman spectroscopy [89], as well as by spectrophotometric and picosecond spectroscopic studies of rhodopsin reconstituted with retinal analogs [90, 91].



Scheme 1. Phototransitions occurring in the bovine visual rhodopsin at -196°C under steady-state illumination. Rh₅₀₄, dark state of the visual rhodopsin; Batho₅₄₃, bathorhodopsin; isoRh₄₉₀, isorhodopsin. Subscripts indicate the low-temperature absorption maxima; the quantum yields of the corresponding phototransitions are indicated next to the arrows [68, 71].

When lumirhodopsin was excited at low temperatures, formation of rhodopsin was observed together with a minor fraction of isorhodopsin [67, 70, 72], as confirmed by IR spectroscopy [75] and HPLC analysis [69]. However, the photoregeneration of rhodopsin from lumirhodopsin is less efficient than from bathorhodopsin [69, 72], despite a reported quantum yield of 0.5 [70]. Reverse photoreactions at later stages of rhodopsin photolysis will be discussed in the section "Photoreversibility at late stages of rhodopsin conversion."

Investigation of reverse photoreactions of rhodopsin and bacteriorhodopsin using time-resolved spectroscopy. The photoreversibility of rhodopsin from bathorhodopsin was first demonstrated in real time using the nanosecond flash photolysis method [92]. Excitation with laser pulses (532 and 605 nm) separated by 93 ns led to a significant decrease in bathorhodopsin absorption, indicating reverse phototransition to rhodopsin. Subsequently, femtosecond studies with a time resolution of 300 fs [74] demonstrated the possibility of reverse phototransition not only from bathorhodopsin, but also from photorhodopsin. More detailed investigations using femtosecond absorption spectroscopy with a 30-fs time resolution [33, 76, 77] employed two excitation pulses at 500 and 620 nm with delays of 0.2-3.8 ps. It was shown that the phototransition from bathorhodopsin or photorhodopsin to rhodopsin occurred without formation of isorhodopsin or other side products (Scheme 2). The quantum yields of reverse transitions from photorhodopsin and bathorhodopsin were similar (0.14 and 0.16, respectively) and significantly lower than those demonstrated by low-temperature methods (Scheme 1). The time of the photoreaction initiated from photorhodopsin was comparable to the time of forward photoreaction, consistent with theoretical calculations [85, 86]. Finally, the possibility of *in vitro* ultra-fast reverse phototransitions in the visual rhodopsin molecule [76, 77] was confirmed by elegant experiments conducted at a femtosecond time resolution *in vivo* on dark-adapted mouse eyes in [66].



Scheme 2. Reverse photoreactions of bovine visual rhodopsin initiated from primary products at room temperature [29, 76, 77, 86]. Rh₄₉₈, dark state of visual rhodopsin; Photo₅₇₀, photorhodopsin; Batho₅₃₅, bathorhodopsin. Subscripts indicate the absorption maxima; solid arrows show phototransitions; dashed arrow indicates thermal transition. The corresponding transition times and quantum yields are shown next to the arrows.

Table 1. Quantum yields (ϕ) and characteristic times for *cis* $\rightarrow$ *trans* and *trans* $\rightarrow$ *cis* photoisomerization of retinal with protonated Schiff base in a free form and as a component of bovine and octopus visual rhodopsins and bacteriorhodopsin at room temperature

Retinal in various environments	Forward photoreaction		Reverse photoreaction	
	ϕ	<i>t</i> , ps	ϕ	<i>t</i> , ps
	11- <i>cis</i> $\rightarrow$ all- <i>trans</i>		all- <i>trans</i> $\rightarrow$ 11- <i>cis</i>	
Free form	0.18-0.24* [17, 96]	0.4** [14]	0.14-0.25* [17]	3** [14]
Bovine rhodopsin	0.65-0.67 [27, 28]	0.05-0.08 [29, 31-33]	0.16 [77]	< 0.1 [77]
Octopus rhodopsin	0.69 [22]	0.08 [26]	0.36*** [71]	–
	all- <i>trans</i> $\rightarrow$ 13- <i>cis</i>		13- <i>cis</i> $\rightarrow$ all- <i>trans</i>	
Bacteriorhodopsin	0.64 [79]	0.45-0.5 [33, 49-51]	0.81-1 [33, 83, 84]	0.19-0.3, 1.1-1.7, 11-16 [83, 84]

Note. Parameters of reverse photoreactions of rhodopsin and bacteriorhodopsin are given for reactions were initiated from bathorhodopsin and *K* products, respectively.

*, in solvents; **, in vacuum; ***, for squid rhodopsin at 77 K.

Experimental studies of reverse photoreaction were also carried out on bacteriorhodopsin, starting from the first products of forward photoreaction *J* and *K* [33, 83, 84]. Reverse phototransition from *K* was demonstrated with a substantially higher quantum yield than that observed in visual rhodopsin, reaching 0.81 in [33] and ~1 in [79, 80, 83, 84, 93]. In [84], the dynamics of reverse photoreaction from *K* was investigated following excitation conducted 5 ps after the first laser pulse. The excited state *K** was found to decay through three channels with characteristic lifetimes of 0.19, 1.1, and 16 ps, ultimately regenerating the original state of bacteriorhodopsin. Comparable kinetic behavior was observed upon excitation of *K* at a time delay of 60 ps [83]. The heterogeneity of the excited state *K** might be determined by the heterogeneity of its chromophore-binding site, arising from the presence of multiple configurations with different nature of retinal–protein interactions, as it was shown for the microbial rhodopsin KR2 from the bacterium *Krokinobacter eikastus* [94, 95]. Since the forward photoreaction is dominated by a single ultrafast component (~0.5 ps) [33, 49-51] and has a lower quantum yield of 0.64 [79], it was concluded that the reverse photoreaction initiated from the product *K* is more efficient, albeit not necessarily faster in kinetics.

The quantum yields and the formation times for the primary products of forward and reverse photoreactions in visual rhodopsin and bacteriorhodopsin are presented in Table 1.

The dynamics of forward and reverse photoreactions of visual rhodopsin and bacteriorhodopsin from their primary products have been investigated in detail using quantum mechanical/molecular mechanical (QM/MM) calculations [82, 86]. In [86], it was demonstrated that the reverse photoreaction initiated from bathorhodopsin involves a reversal motion of the retinal chromophore, including torsional deformations along the C₁₃=C₁₄, C₁₁=C₁₂, and C₉=C₁₀ bonds in directions opposite to those observed during the forward photoreaction. The quantum yields of both forward and reverse photoreactions were found to depend on the phase and amplitude of some vibrations of the retinal at the moment of transition from the S₁ excited electronic state to the ground state. These are hydrogen-out-of-plane (HOOP) vibrations at the carbon atoms C₁₁ and C₁₂.

The authors of [82] compared the photoexcited states of visual rhodopsin, bacteriorhodopsin, and their primary photoproducts – bathorhodopsin and *K* intermediate. Using available X-ray structures, they calculated the absorption spectra of these species and identified the retinal vibrational modes active in the S₀ $\rightarrow$ S₁ transition, and therefore, governing the earliest stages of the excited state dynamics. Analysis of the obtained data revealed that photoexcitation of rhodopsin, bacteriorhodopsin, and the *K* intermediate selectively enhances the activity of the vibrational modes directly involved in photoisomerization. These modes are associated with the C₁₁=C₁₂ bond

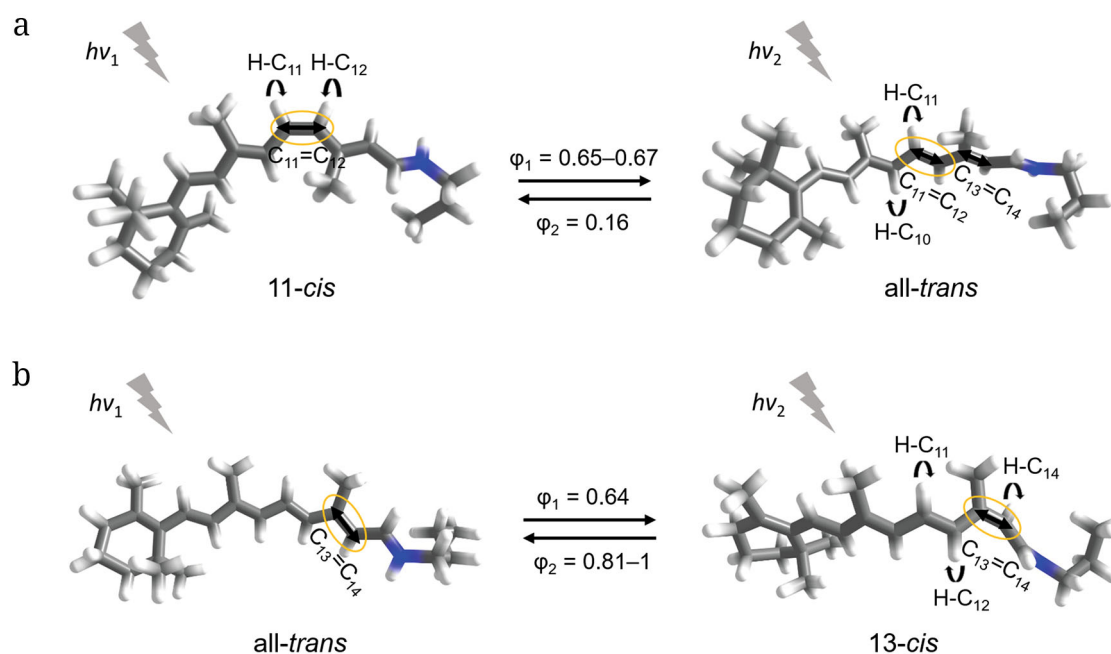


Fig. 3. Comparison of the vibrational activity of the protonated Schiff base of retinal in the initial state of rhodopsin and in its primary photoproduct immediately after excitation to the S_1 electronic state [82]: a) bovine visual rhodopsin (left) and its product bathorhodopsin (right); b) bacteriorhodopsin (left) and its product K (right). The arrows in the structures indicate the most active high-frequency valence C=C vibrations of the retinal polyene chain and HOOP vibrations; the photoisomerizing $C_{11}=C_{12}$ (a) and $C_{13}=C_{14}$ (b) bonds are shown with an oval; the quantum yields of forward and reverse photoreactions are given next to the arrows indicating the phototransitions.

in rhodopsin and the $C_{13}=C_{14}$ bond in bacteriorhodopsin and the K intermediate [87]. Simultaneous in-phase excitation of these reactive vibrational modes may facilitate phase synchronization as the molecule passes through the S_1/S_0 conical intersection, thereby contributing to the high quantum yields of the forward photoreactions of rhodopsin and bacteriorhodopsin, as well as of the reverse photoreaction of bacteriorhodopsin originating from the K intermediate (Table 1) [3, 35]. This effect arises from specific interactions between retinal and its immediate protein environment in the chromophore-binding pocket of opsin. The vibrational modes activated upon excitation of rhodopsin are shown in Fig. 3a (left) and include high-frequency valence vibrations of the $C_{11}=C_{12}$ bond together with HOOP vibrations of hydrogen atoms attached to carbons C_{11} and C_{12} . The corresponding modes for bacteriorhodopsin and its K intermediate are presented in Fig. 3b. Notably, in the K intermediate, excitation induces not only reactive vibrations associated with the $C_{13}=C_{14}$ bond but also HOOP vibrations at the $C_{11}=C_{12}$ bond (Fig. 3b, right). This additional vibrational activity may underlie a complex mechanism of reverse photoreaction in bacteriorhodopsin, as mentioned above [83, 84].

In bathorhodopsin, photoexcitation non-selectively activates vibrations of the $C_{11}=C_{12}$ and $C_{13}=C_{14}$ bonds and HOOP vibrations of hydrogen atoms at the

carbon atoms C_{10} and C_{11} (Fig. 3a, right panel; see also [82]). Bathorhodopsin does not exhibit a dominant high-frequency vibrational mode; instead, its excited state is characterized by the HOOP vibrations at two different double bonds, which may reduce the coherence of retinal intramolecular motions during the reverse photoreaction, thereby contributing to a low quantum yield of bathorhodopsin phototransition to rhodopsin (Table 1).

Thus, both experimental studies and QM/MM simulations of forward and reverse photoreactions indicate a more finely tuned interaction between retinal and its protein environment in visual rhodopsin than in bacteriorhodopsin [9, 82]. One manifestation of this optimization is a substantially lower probability of reverse photoreactions (which are functionally unfavorable for phototransduction) in visual rhodopsin compared to bacteriorhodopsin. These differences in the photophysical behavior of retinal isomers in visual (type II) rhodopsin and bacteriorhodopsin (type I rhodopsin) are likely related to their distinct biological functions. In bacteriorhodopsin, an efficient reverse photoreaction from the K intermediate is not essential for the proton-pumping activity during the photocycle. In contrast, suppression of reverse photoisomerization in the primary photoproducts of visual rhodopsin – photorhodopsin and bathorhodopsin – enhances the reliability of the forward

photoreaction that initiates the phototransduction cascade.

Photoreversibility at late stages of rhodopsin conversion. *Reverse photoreactions of vertebrate rhodopsins.* The products of photoconversion of vertebrate visual rhodopsin formed within the time ranges from microseconds to seconds also retain photoactivity [42, 64, 65, 72, 75, 88, 97-106]. However, photoregeneration of rhodopsin from these later intermediates is considerably less efficient than from earlier photoproducts because it requires selective all-*trans* → 11-*cis* isomerization in the altered chromophore-binding site, followed by substantial conformational rearrangements of opsin. For example, bathorhodopsin generated by irradiation of bovine rhodopsin at -196°C can be converted back to rhodopsin almost quantitatively upon re-irradiation with long-wavelength light (see "Photoreversibility at early photochemical stages of rhodopsin conversion"). In contrast, photoregeneration from metarhodopsin II in bovine and frog rhodopsins reaches no more than 50%, as demonstrated both *in vitro* at -20°C [97] and in single photoreceptor cells at physiological temperatures [105]. The efficiency of rhodopsin regeneration from metarhodopsin III is even lower (less than 37% depending on temperature) [42]. Studies examining the effects of duration and intensity of irradiation light on rhodopsin bleaching, conducted both *in vitro* [107] and *in vivo* [62, 63], have shown that a brief light flash (≤1 ms) never bleaches more than 50-75% of rhodopsin, irrespective of light intensity. Based on these observations, it was concluded that the photolysis products formed before metarhodopsin II are completely photoreversible, whereas later intermediates exhibit only partial photoreversibility. Importantly, regenerated rhodopsin remains physiologically functional. Furthermore, experiments in bovine rod outer segments [72] demonstrated that recovery of a functional rhodopsin, not amenable to phosphorylation, occurs much less efficiently from metarhodopsins I and II than from the earlier intermediates bathorhodopsin and lumirhodopsin.

Using low-temperature IR spectroscopy of rod outer segments [75] and flash photolysis of detergent extracts of bovine rhodopsin [100], metarhodopsin I was shown to be isochromic, i.e., existing in two spectrally indistinguishable forms. One of these forms is capable of regenerating into rhodopsin with the formation of isorhodopsin and several minor photoproducts, whereas the other lacks photoreactivity. Subsequent studies of photoreactions of frog metarhodopsin I by absorption spectrophotometry at -25°C further demonstrated the formation of two isochromic rhodopsin species, one of which remained stable upon warming to room temperature, while the other underwent decay [101, 102].

Photon absorption by metarhodopsin II results predominantly in the formation of metarhodopsin III, together with smaller amounts of rhodopsin and isorhodopsin, as has been observed in both detergent extracts of rhodopsin and suspensions of photoreceptor membranes [64, 98, 104, 106, 108]. Similar data have been reported for albino rat eyes [64] and intact frog rod cells [105]. Irradiation of metarhodopsin III in bovine photoreceptor membranes primarily drives its conversion to metarhodopsin II via metarhodopsin I, while producing lesser amounts of rhodopsin and possibly isorhodopsin [42, 88, 104]. These findings indicate that, during reverse photoreactions initiated at the later stages of rhodopsin photolysis (beginning with metarhodopsin II), the *anti* ↔ *syn* photoisomerization of the C₁₅=N Schiff base becomes the dominant photochemical process, whereas the *trans* → *cis* photoisomerization at the C₁₁=C₁₂ and/or C₉=C₁₀ bonds plays a comparatively minor role (Fig. 1).

It should be noted that the data on photoreversible reactions of metarhodopsins remain contradictory. For example, the restoration of the absorption band in the 500 nm region is not necessarily associated with the return of rhodopsin to a physiologically active state capable of initiating phototransduction [20, 72], contrary to the assumptions made in the earliest studies of rhodopsin reverse photoreactions. Resolving this issue requires the application of various methods, including HPLC analysis, IR spectroscopy, and time-resolved laser spectroscopy (for details, see [7]).

Photoreversible reactions occurring at different stages of rhodopsin photolysis provide a valuable tool for investigating light-induced conformational changes in the protein. For example, in our studies of the rhodopsin activation mechanism using saturation-transfer electron paramagnetic resonance (EPR) spectroscopy with spin labels covalently attached to the hydrophilic sulfhydryl groups of Cys140 and Cys316 located in the second and third cytoplasmic loops, respectively, we were the first to observe photoreversible changes in the conformational mobility of opsin [109-111]. The mobility of the labels increased during rhodopsin transition to metarhodopsin II and decreased during the reverse metarhodopsin II phototransition to a mixture of products, including photoregenerated rhodopsin and metarhodopsin III. These observations indicated that light activation exposes opsin regions involved in G protein binding. Subsequent EPR studies, particularly those employing pulsed EPR techniques, together with numerous complementary approaches, have revealed not only rearrangements of the cytoplasmic loops but also movements of the transmembrane α-helices, which are critical elements of signal transmission from activated rhodopsin to the G protein (see review [7]).

Reverse photoreactions of invertebrate rhodopsins. Bistable rhodopsins of invertebrates are capable of efficient photoregeneration, which is initiated when a second photon is absorbed by the thermally stable acid metarhodopsin (Fig. 2b). In invertebrates, photoreversibility has been extensively characterized in the rhodopsins of the octopus, squid, and jumping spider (JSR1) [22, 25, 112-116]. In octopus rhodopsin, sequential blue- and orange-light irradiation at room temperature initiated multiple rounds of rhodopsin photoconversion to acid metarhodopsin and back on both minute [112] and second [113] timescales. The quantum yield of the reverse photoreaction from acid metarhodopsin to rhodopsin (0.43 for octopus rhodopsin) was lower than that of the forward photoreaction [22].

Higher time-resolution experiments have revealed that photoregeneration of invertebrate rhodopsins involves one photochemical step followed by two thermal (dark) reactions [25, 115, 116]. The initial photochemical event rapidly reisomerizes retinal to the 11-*cis* configuration, generating an intermediate with the absorption spectrum closely resembling that of the dark-state rhodopsin [25]. Two subsequent thermal transitions (formation of the second product and of the original state of rhodopsin) are thought to reflect conformational rearrangements in protein regions distant from the chromophore. In octopus rhodopsin, these reactions proceed within 490 μ s and 2.6 ms [25], whereas in JSR1 they are markedly faster, occurring within 2 μ s and 60 μ s, respectively [116]. In squid rhodopsin, these thermal transitions are accompanied by deprotonation and subsequent reprotonation of the retinal Schiff base [115].

It was shown that if octopus rhodopsin undergoes a significant increase in volume during the forward photoreaction, reflecting formation of the functional metarhodopsin state that initiates phototransduction, the reverse photoreaction is accompanied by a decrease in the protein volume back to its original value [25]. Importantly, the reverse photoreaction does not trigger the phototransduction cascade [117, 118].

The photoreactions of squid alkaline metarhodopsin have also been investigated [114]. Upon illumination, this protein forms a photoproduct that slowly reverts to rhodopsin in the dark. However, prolonged repeated irradiation can drive the protein back to the metarhodopsin state.

Reverse photoreactions of microbial rhodopsins. Photoreversibility (13-*cis* $\rightarrow$ all-*trans* transition) on the microsecond-to-millisecond timescale has also been studied in type I rhodopsins, including bacteriorhodopsin [60, 61], as well as in photochromic sensory rhodopsins (SRIs) from halophilic archaea and bacteria [56, 57], cyanobacterial sensory rhodopsin from *Anabaena* sp. (ASR) [54, 55], and channelrhodopsins

from the green alga *Chlamydomonas reinhardtii* and related species (ChR1 and ChR2) [56, 58]. In bacteriorhodopsin, reverse photoreactions initiated from the *M*, *N*, and *O* intermediates were found to be highly complex, proceeding through the formation of multiple transient states [60, 61].

PHOTOREVERSIBILITY OF RHODOPSIN IN VIVO AND REGENERATION OF VISUAL RHODOPSINS

Phototransduction in both invertebrates and vertebrates is initiated by the photoisomerization of 11-*cis* retinal to its all-*trans* configuration. This photochemical event induces conformational changes in opsin, triggering a signaling cascade that transmits and amplifies the light signal and ultimately leads to the generation of the photoreceptor potential. Because phototransduction relies on this forward photoreaction, all-*trans* retinal must subsequently be converted back to the 11-*cis* form, and rhodopsin must be restored to its original functional state through a process known as regeneration.

Two distinct strategies have evolved to regenerate the thermodynamically less favorable 11-*cis* retinal of visual pigments. The first, and evolutionarily more ancient, strategy utilizes light energy to drive retinal re-isomerization, as observed in many invertebrate rhodopsins. A critical requirement of this mechanism is preservation of the covalent linkage between all-*trans* retinal and opsin following photoactivation. Absorption of the second photon converts the chromophore back to the 11-*cis* configuration, thereby promoting restoration of the native opsin conformation (see "Photoreversibility at late stages of rhodopsin conversion").

The second strategy for restoring 11-*cis* retinal has evolved in response to the loss of retinal from rhodopsin as a result of photolysis, which is characteristic of vertebrate visual pigments. After hydrolysis of the covalent bond between opsin and all-*trans* retinal, the chromophore dissociates from opsin and is ultimately removed from the photoreceptor cell. The key innovation of this evolutionarily more recent strategy is the development of a light-independent biochemical pathway – the visual (retinoid) cycle, which involves retinal pigment epithelium (RPE) cells and, potentially, Müller glial cells. This cycle mediates the multistep regeneration of the visual pigment dark-adapted state by converting retinal back to its 11-*cis* configuration required for the chromophore function. Because rhodopsin-containing photoreceptor discs in rod outer segments have a lifespan of approximately 10-12 days, individual rhodopsin molecules can repeatedly absorb photons, undergo

photolysis, and be regenerated. The evolution of this complex and highly reliable regeneration mechanism has enabled vertebrates to minimize the accumulation of “errors” in structures of both retinal and opsin that could arise during photocycles in invertebrates. Indeed, in many invertebrates, retinal undergoes repeated photoisomerization while remaining associated with the same rhodopsin molecule. Such cycling may promote the formation of side products containing alternative retinal isomers, potentially inducing nonphysiological conformational changes in opsin. In vertebrates, dissociation of all-*trans* retinal from opsin permits chromophore regeneration in the visual (retinoid) cycle, ensuring that retinal is re-isomerized to the 11-*cis* form required for proper assembly and function of the chromophore-binding site in opsin.

It should be noted that both strategies can co-exist within the same organism. Reverse *trans* → *cis* photoisomerization is also present in vertebrates, where it contributes to the regeneration of cone visual pigments (photoreceptors of daylight vision) [119]. However, this is indirect photoregeneration, as it involves a specialized non-visual protein, retinal G protein-coupled receptor (RGR), which acts as a photoisomerase in Müller glial cells of the retina and RPE cells [120]. The involvement of non-visual retinal proteins in rhodopsin photoregeneration has long been recognized. A classic example is retinochrome in cephalopod mollusks, which functions as a photoisomerase [121, 122]. Notably, retinochrome is phylogenetically related to the vertebrate RGR protein [119].

Thus, the evolutionary landscape of light-dependent and dark regeneration of visual pigments can be summarized as follows:

- a) direct photoregeneration of invertebrate rhodopsin, in which a second photon is absorbed by the stable photoproduct (acid metarhodopsin), restoring the original state;
- b) indirect photoregeneration of invertebrate rhodopsin mediated by retinochrome, a non-visual retinal protein that catalyzes the light-driven conversion of all-*trans* retinal back to the 11-*cis* configuration;
- c) dark regeneration of vertebrate visual pigments via the enzymatic visual (retinoid) cycle (see review [123]);
- d) indirect photoregeneration of vertebrate cone visual pigments catalyzed by RGR (see review [123]).

The physiological significance of indirect photoregeneration of cone visual pigments lies in the requirement for rapid restoration of their native state, enabling sustained phototransduction during the daytime and under continuously bright lighting conditions. In this process, 11-*cis* retinal is supplied

to cones from two principal sources: Müller glia of the retina and RPE cells. However, in both rods and cones, the primary pathway responsible for slow but complete dark adaptation is the classical visual (retinoid) cycle, in which the RPE plays a central role.

Notably, *in vivo* and *in situ* experiments conducted in rabbits and albino rats have demonstrated direct photoregeneration of rhodopsin from early photointermediates, including metarhodopsin I (see “Photoreversibility at late stages of rhodopsin conversion”) [62-65]. These findings show that following a sufficiently brief light flash, irrespective of its intensity, approximately 25-50% of rhodopsin in the retinas of the studied animals remained in the dark state. This indicates the existence of an additional, rapid mechanism for restoring the physiologically active dark state of the visual pigment, which can operate under conditions of high light intensity in living organisms. However, the extent to which this photoregenerative mechanism contributes to rhodopsin recovery under natural lighting conditions in vertebrates remains unclear.

CONCLUSION

This review examines reverse photoreactions in visual rhodopsins (type II), as well as in selected type I rhodopsins, as “tools” for investigating both forward and reverse photochemical pathways and associated conformational rearrangements of these proteins.

Although reverse photoreactions initiated from the earliest photointermediates of forward rhodopsin conversion occurring on the femtosecond to picosecond timescales are not considered physiologically relevant, their experimental and theoretical analysis provides important insights into the mechanism of retinal photoisomerization and chromophore-protein interactions that enable the protein to “catalyze” this exceptionally rapid and efficient process. These studies are therefore crucial for understanding the fundamental photobiological principles governing light energy conversion in retinal-binding proteins that mediate both photoinformational and photoenergetic functions.

Reverse photoreactions originating from later stages of the rhodopsin photocycle can serve as markers of changes in chromophore-protein interactions and of protein (opsin) conformational rearrangements that ultimately lead to the formation of the signaling state.

The physiological significance of photoreversible reactions in restoring the native state of visual pigments in both vertebrates and invertebrates is evident. While the mechanism of rhodopsin photo-

regeneration in invertebrates is relatively well characterized, several key questions remain unresolved regarding the photoregeneration of vertebrate cone photoreceptors (see review [123]). In particular, the relationship between the classical dark visual (retinoid) cycle of rhodopsin regeneration occurring in the RPE and the distinct light-dependent and dark regeneration pathways operating within the neural part of the retina remains poorly understood.

From an applied perspective, retinal-containing proteins are regarded as promising biomaterials for molecular electronics and optoelectronics, owing to their chemical stability, functional photocycles, and photoreversible reactions. In particular, they can function as molecular photoswitches, based on the reversible light-induced isomerization between the *cis* and all-*trans* forms of retinal. For instance, bacteriorhodopsin has been used as a holographic medium for processing optical information in high-speed biochips. The development of logic elements and memory devices modeled on rhodopsin-based systems is currently actively discussed. In this context, the ultrafast photoreversibility of both visual and microbial rhodopsins, occurring on the femtosecond to picosecond timescale, is of particular interest.

In conclusion, studying photoreversible reactions in rhodopsins remains important not only for fundamental understanding in photochemistry, biophysics, and visual physiology, but also for applied research in molecular electronics and optoelectronic technologies.

Abbreviations

BSI	blue shifted intermediate of visual rhodopsin
HOOP	hydrogen-out-of-plane
MM/QM	hybrid method of molecular mechanics and quantum mechanics
RPE	retinal pigment epithelium

Acknowledgments

The authors express their gratitude to A. V. Bochenkova and P. A. Kusochev for their assistance in creating the Figure 3 and discussing review sections on the theoretical calculations.

Contributions

M.A.O. developed the study concept; M.A.O., O.A.S., and T.B.F. wrote and edited the manuscript.

Funding

The work was supported by the Ministry of Science and Higher Education of the Russian Federation (project no. 122041400102-9) and the State Assignment to the Lomonosov Moscow State University.

Ethics approval and consent to participate

This work does not contain any studies involving human or animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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