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VISUAL AND ARCHAEAL RHODOPSINS: SIMILARITIES, DIFFERENCES AND CONTROVERSY

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Abstract: Rhodopsins are currently known to belong to two distinct protein families. The visual rhodopsins, found in eyes throughout the animal kingdom, are photosensory pigments. Archaeal rhodopsins, found in extreme halophiles, function as light-driven proton pumps (bacteriorhodopsins), chloride ion pumps (halorhodopsins), or photosensory receptors (sensory rhodopsins). Light absorption by rhodopsins triggers their characteristic photoconversion extending into the (milli)second time range. There are three main paradigms of rhodopsins photoconversion. (1) Initiation of the *trans-cis* isomerization is the very primary consequence of light absorption. (2) Rhodopsins store light energy via the charge-separation mechanism (the charge of Schiff base is separated from its counterion). (3) Full *trans-cis* isomerization of the chromophore is a prerequisite for the full biological activity of rhodopsins. These paradigms will be questioned.

Key Words: Bacteriorhodopsin, Rhodopsin, Primary Processes

INTRODUCTION

Retinal is a chromophore that binds integral membrane proteins (opsins) to form light-absorbing pigments called rhodopsins. Rhodopsins are currently known to belong to two distinct protein families [1, 2]. The visual rhodopsins, found in eyes throughout the animal kingdom, are photosensory pigments. Archaeal rhodopsins, found in extreme halophiles, function as light-driven proton pumps (bacteriorhodopsins), chloride ion pumps (halorhodopsins), or photosensory receptors (sensory rhodopsins). The two protein families show no significant sequence similarity, and may have different origins. They do, however, share identical topologies characterized by seven transmembrane α-helices that form a pocket in which retinal is covalently linked, as a protonated Schiff base, to a

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Abbreviations used: bR - bacteriorhodopsin; Rh - rhodopsin; 14-F-bR - 14-fluorobacteriorhodopsin; bR_{redu} - bacteriorhodopsin with reduced retinal-protein linkage.

lysine in the seventh transmembrane helix. Light absorption (femtosecond time scale) by rhodopsins triggers their characteristic photoconversion extending into the (milli)second time range. How do rhodopsins translate femtosecond events into (milli)second responses? This question is still open [3, 4].

It is generally accepted that the photoconversion of rhodopsins reflects the conversion of light energy into structural transformations in the chromophore (11-*cis*-all-*trans* in visual pigments or all-*trans*-13-*cis* in archaeal rhodopsins) and in its surrounding protein matrix, which are responsible for the biological activity of the pigments [5-7]. The consecutive transformations are called intermediates: I, J, K, L, M, N, O in the bacteriorhodopsin (bR) photocycle and photo-, batho-, lumi-, metharhodopsin in rhodopsin (Rh) photoconversion. There are two paradigms of rhodopsins photophysics, which define the first steps of photoconversion. (1) The initiation of the *trans-cis* isomerization (torsional motion about a "critical" double bond) is the very primary consequence of light absorption in rhodopsin systems [8-11]. (2) Rhodopsins store the light energy via primary charge-separation mechanism (the charge of the Schiff base is separated from its counterion) as a result of the *trans-cis* chromophore isomerization [11-14]. Finally, the third paradigm combines chromophore isomerization with rhodopsins biological activity: (3) full *trans-cis* isomerization of the chromophore is a prerequisite for the initiation of the full photoconversion, i.e., for full rhodopsins biological activity [1, 3, 4, 7].

The arguments which bring these paradigms into question are presented below.

THE PARADIGMS OF VISUAL AND ARCHAEAL RHODOPSINS

Paradigm (1): the initiation of the *trans-cis* isomerization is the primary consequence of light absorption in rhodopsin systems

The predominant working hypothesis in retinal proteins has been that all protein changes are exclusively due to a *cis-trans* or *trans-cis* C=C, or C=N, isomerization of the retinal moiety [8, 10, 15]. In other words, it is commonly postulated that transient, light-induced, conformational changes in the protein can only be induced as a result of the isomerization of the embedded chromophore around a "critical" double bond. The time for reaching the first intermediate in a configuration different to that of the ground state has been estimated to be about 200 fs for vertebrate Rh [8] and about 500 fs for bR [15, 16], although it was recently demonstrated that the time constant of the first intermediate in the bR photoreaction could be about 200 fs [17].

However, for the last ten years, arguments have been collected bringing into question simple *trans-cis* isomerization of the chromophore as a primary event in rhodopsins photophysics. For example, Song and El-Sayed have stated that the primary step in bacteriorhodopsin photoconversion is a bond stretch rather than an angle twist of its retinal excited-state structure [18]. Patzelt *et al.* have stated that the change of the N-H dipole is the primary cause of energy storage and its release in all subsequent steps in the catalytic cycle [19]. Also, our early

work [20] with bR and 14-F-bR questioned the postulate about *trans-cis* isomerization as a primary event in bR photophysics. However, the time resolution of the femtosecond apparatus did not allow us to register signals faster than 300 fs.

In recent experiments (K. Bryl and K. Yoshihara, unpublished results), we obtained much faster components of fs signals (about 20-30 fs) than those ascribed to primary events in the bR photoreaction [16, 17]. No reasonable explanation for the obtained results is available so far. Fortunately, we can refer to early theoretical considerations which advocated the induction of protein conformational changes by charge redistribution in the excited retinal chromophore [21-23]. Moreover, one can find suggestions in the literature that the bR protein is activated via a mechanism that does not require chromophore double bond (C_{13}=C_{14}) isomerization. The most plausible suggestion is that protein activation is a result of charge delocalization in the excited state of the chromophore [24]. We hope that our currently performed experiments will help to clarify the problem of charge redistribution as a primary event in rhodopsins [K. Bryl and K. Yoshihara, unpublished results]. The background of these

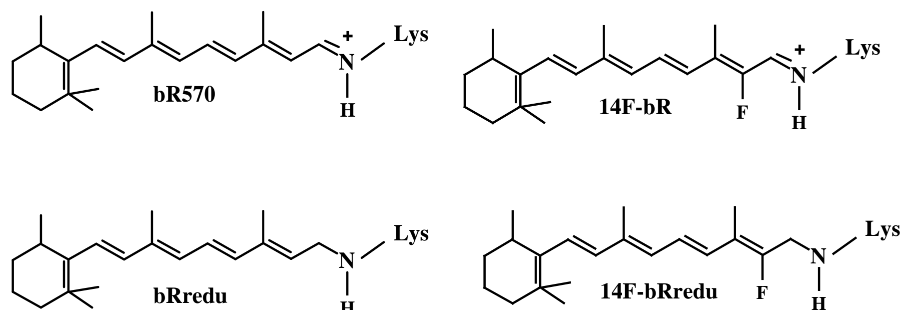

Fig. 1. Native (bR570) and artificial chromophores employed for probing the primary events in bR.

experiments is as follows. In the ground state, the positive charge of the chromophore is localized around the Schiff base nitrogen. Upon excitation, the positive charge moves toward the ionone ring. Accordingly, any perturbations which stabilize this charge delocalization lead to a smaller energy gap between the ground and excited states, resulting in a red shift of the absorption maximum [25].

On the basis of this background, one should expect that: (a) such a perturbation would make the transition connected with charge separation slower); (b) light-induced conformational changes should be absent in a reduced pigment in which the polyene chain is symmetrical, and therefore, following light absorption, significant electronic charge redistribution should be highly unlikely; and (c) upon the induction of a charge asymmetry in the reduced chain by appropriate

substitution of the polyene, the light-induced conformational changes should be restored. Fig. 1 shows native bR (bR570) and artificial chromophores employed for probing the primary events in bR.

The differences between the femtosecond responses of wild bR and artificial bR, in which retinal was substituted with 14-F-retinal are shown in Fig. 2. The calculated times are as follows: 30 and 500 fs for bR, 600 and 1200 fs for 14-F-bR.

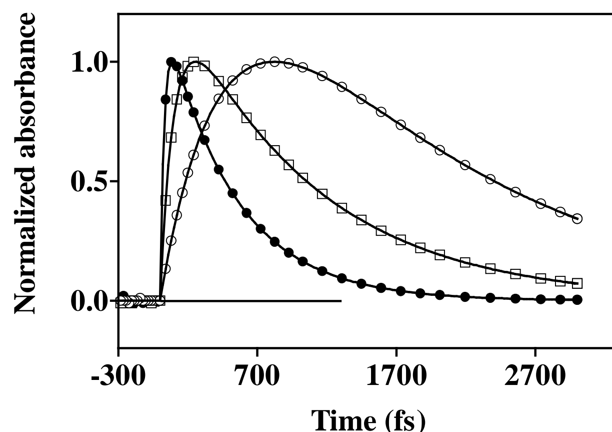

Fig. 2. The transient absorbance change of bacteriorhodopsins at 460 nm. Solid circle, bacteriorhodopsin; open circle, bacteriorhodopsin with 14-Fluoro-retinal; open square, bacteriorhodopsin with reduced Schiff base double bond and with 14-Fluoro-retinal. Solid line: fit to the experimental points.

A highly electronegative fluorine atom prevents the positive charge of the Schiff base from moving towards ionone ring in the excited state. Due to this screening, the primary charge separation along the retinal becomes more difficult, which is reflected in the slowing down of the fast component of femtosecond signal (from 30 fs to about 600 fs) with a smaller effect on slower component (from 500 fs to about 1200 fs).

Reduction of the retinal-protein Schiff base linkage with sodium borohydride leads to a symmetric polyene, covalently bound to the protein via a -CH_2-NH-bond (Fig. 1, bRredu). This bR did not show a femtosecond signal at 460 nm. However, incorporation of a fluorine atom close to the Schiff base linkage (Fig. 1, 14F-bRredu), reinstating charge delocalization, restores femtosecond signals (Fig. 2) with a time of 100 and 1000 fs. These results indicate that femtosecond absorption signals can be ascribed to two clearly different processes: a very fast process, which may suggest charge redistribution along the chromophore, and a slower process, which has already been interpreted as chromophore *trans-cis* isomerization [16, 26].

It might be very instructive to perform similar experiments for Rh. However, so far we have not succeeded in recording clear transient absorbance changes of 11-*cis*-F-Rh similar to those obtained for F-bR. Therefore, we can conclude that the paradigm about *trans-cis* isomerization as a primary event in the light induced conversion of retinal proteins is not valid as a general one, at least for bacteriorhodopsin.

Paradigm (2): Light-induced primary charge separation of the protonated Schiff base and its counterion, as a result of *cis-trans* photoisomerization of the chromophore, is the mechanism of energy storage in rhodopsins

In retinal proteins, absorption of a photon by the retinal induces a *cis-trans* (or *trans-cis*) isomerization. This step is associated with the initial storage of a substantial fraction of the photon energy and results in several distinct intermediates characterized by an altered protein conformation [27].

The overall potential of the protein-chromophore system is given in [13] as:

$$V^M = V_{intra}{}^M + V_{cons} + V_{elect}$$

where $V_{intra}{}^M$ is the intramolecular potential of the isolated chromophore in the Mth electronic state, V_{cons} is the potential of the steric constraint, and V_{elect} is the electrostatic interaction between the chromophore and the protein. The energy accumulated in the form of electrostatic interaction constitutes the largest amount of total energy. Therefore, it is considered the main reservoir of rhodopsins energy needed for their biological activity [11-14].

Fig. 3 shows a simple model of the mechanism of energy accumulation in Rh in the form of charge separation energy. The charge separation is a result of *cis-trans* photoisomerization of the chromophore. Photoisomerization increases the distance between the protonated nitrogen of the Schiff base and a negatively charge protein group (counterion), thus storing electrostatic energy [14, 28]. The amount of electrostatic energy stored by this mechanism can be calculated for assumed models by microscopic dielectric approaches [13]. It was demonstrated that a change in the distance between the charges (e.g., the NH^+ and COO^- ion pair, with a concomitant shift of the protonated Schiff base from a polar environment to a nonpolar environment) can store more than 30 kcal/mol [13, 14].

These theoretical calculations were performed without knowing the structure of the proteins, without evidence that bR and Rh have a counterion for the protonated Schiff base. Recent crystallographic studies have confirmed correctness of the above assumption [29-31]. Fig. 4 shows the schemata of a cross-section of bovine Rh and bR demonstrating the close vicinity of the protonated Schiff base and its counterion (Glu113 in Rh and Asp85 in bR).

In vertebrate visual pigments, a glutamic acid serves as a negative counterion to the positively charged chromophore, a protonated Schiff base of retinal. When photoisomerization leads to the Schiff base deprotonating, the anionic glutamic acid becomes protonated, forming a neutral species that activates the visual

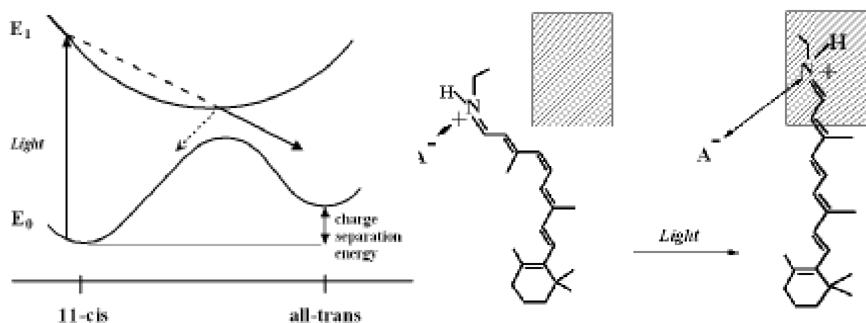

Fig. 3. Light-induced charge separation as a result of *cis-trans* photoisomerization of the chromophore in bovine rhodopsin. Photoisomerization increases the distance between the protonated nitrogen of the Schiff base and a negatively charged protein group (counterion A^-), thus storing electrostatic energy. The cross-hatched region indicates a nonpolar environment.

cascade. In bR, deprotonation/protonation of Schiff base-counterin pairs is a key step in proton pumping.
But, it has recently been demonstrated that in invertebrate (octopus) rhodopsins, there is no anionic group serving as a counterion [32]. However, we have observed the absorption signals in the femtosecond and picosecond time scale, indicating an existence of primary processes in octopus Rh [33]. The kinetics and the number of signal components were different from those for bovine Rh. We obtained two components with times of 400 fs and 2 ps.

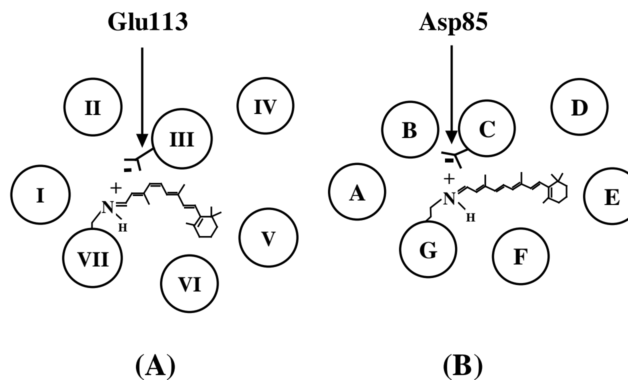

Fig. 4. The projection model of bovine rhodopsin (A) and bacteriorhodopsin (B). The seven transmembrane helices (denoted I-VII and A-G for rhodopsin and bacteriorhodopsin, respectively) are supposed to have a clockwise sequential order. The chrompohores are bound via protonated Schiff base linkage: 11-*cis* retinal is bound to Lys296 (A), while all-*trans* retinal is bound to Lys216. The model of rhodopisn adapted from [29], and the model of bacteriorhodopsin from [31].

The component in the femtosecond time scale may indicate *cis-trans* isomerization. This advocates *cis-trans* isomerization as a common event for all rhodopsin-like systems. Surprisingly, we did not observe signals corresponding to charge redistribution along the chromophore. Does this mean a different primary process in invertebrate rhodopsins? An important consequence of the lack of a counterion is that it disproves the simple version of the charge-separation hypothesis for the storage of the photon's energy in the primary photoproduct of rhodopsins. If there is no counterion, there can be no energy storage by charge separation. The "old" paradigm of rhodopsins photophysics should be changed.

Hence, what is the mechanism of light energy storage in invertebrate rhodopsins? Should we find another paradigm?

Paradigm (3): Full *trans-cis* chromophore isomerization is a prerequisite for the full biological activity of rhodopsins

The experiments with artificial bacteriorhodospins, in which the C_{13}=C_{14} flexibility of the chromophore is limited or isomerization is blocked, provide information about the functional role of *trans-cis* configuration in their activity.

The comparison of the early dynamics of the native all-*trans* pigment bR570 with those of bR5.12, in which isomerization around the critical C_{13}=C_{14} bond is blocked into an all-*trans* configuration by a five-membered ring is shown in Fig. 5 (A). Nearly identical spectral evolution was observed in both native and artificial systems over the first 100-200 fs. Native bR goes through a regular photocycle. Instead, in artificial systems, all the changes were arrested beyond the 200 fs, reverting uniformly to the initial ground state [34]. It was suggested that these modifications should lead to the blockage of bR proton pumping activity, stressing the crucial importance of isomerization in the photoreactivity of bacteriorhodopsin [35].

Our currently performed experiments demonstrate that, irrespective of previous expectations, this bR analogue displays limited pumping activity. However, a very simple model system for ATP production (liposomes containing modified bRs and ATP-ase in liposomes [36, 37]) reveals no ATP production (Fig. 5 (B)).

These results call for a major revision of the models previously put forward for the primary events in bR and requirement of *trans-cis* retinal isomerization as a prerequisite for bR biological activity. We suggest that the term "bR activity" should refer to two activities: bR acticity itself, which means proton pumping, and the activity leading to ATP production, which can be called biological activity.

It is surprising that blocking the *trans-cis* chromophore isomerization does not block proton pumping. This proton pumping activity is limited but still exists. However, ATP production in a model system (liposomes containing ATP-ase and modified bR) is blocked. This may mean that the primary charge separation (dipole generation along the polyene chain but not *trans-cis* isomerization) induces a small but important change in the geometry of the groups around the

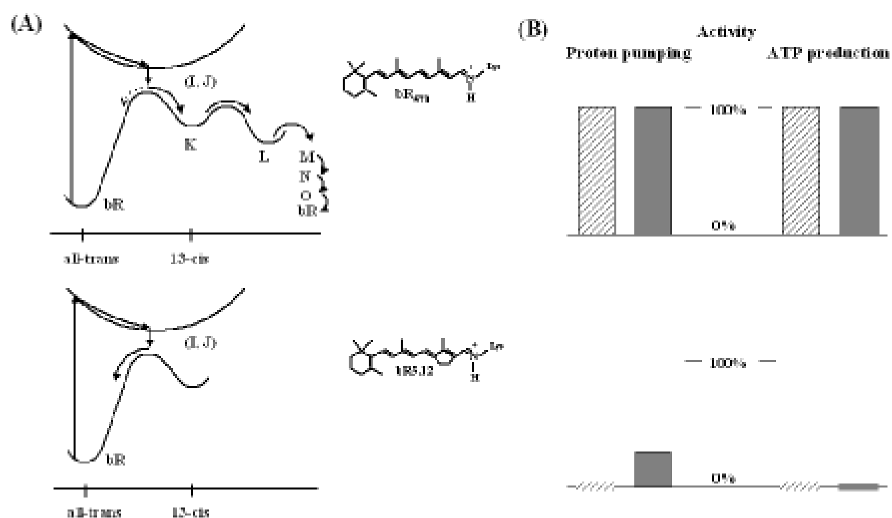

Fig. 5. Schematics of the grand- and excited-state potential surface along the *trans-cis* torsional coordinates of bacteriorhodopsins (A) and a comparison of their biological activities (B). Cross hatched rectangle – expected value, grey rectangle – observed value.

Schiff base, which is a cause of proton transfer in the photoexcited protein [7]. But such a proton pumping activity is not sufficient for ATP-ase stimulation. This suggests that full *trans-cis* isomerization guarantees proper bR proton pumping activity, effective enough for ATP production.

The experiments performed with rhodopsins formed from retinal analogs in which the 11-ene is fixed in the *cis* configuration by ring structures of various size have demonstrated how limited chromophore flexibility affects rhodopsin activity [38, and literature therein]. Irradiation of the native rhodopsin elevates it to the excited state, which is first converted to photorhodopsin and then to bathorhodopsin. When the 11-ene is rigidly fixed by the five-membered ring, the excitation merely leads to an excited state that reverts to the ground state.

Fixation of the *cis* 11-ene with a seven-membered ring, which allows some flexibility around the double bond, results in formation of a photorhodopsin-like intermediate through the excited state. When the flexibility of the *cis* 11-ene is further increased by replacing a seven-membered ring with an eight-membered ring, the formation of the two intermediates corresponding to photorhodopsin and bathorhodopsin is observed. Therefore they concluded that full *cis-trans* chromophore isomerization is required for the initiation of the photocycle, i.e., for full rhodopsins biological activity (although the biological activity has not been examined). Instead, Jang *et al.* [39] demonstrated that rhodopsin regenerated with a ring-constrained 11-*cis*-retinal analog (six-membered) undergoes photoisomerization; however, it remains marginally biologically active because isomerization occurs without the chromophore-induced

conformational change of the opsin moiety. Recent studies by Kuksa *et al.* [40] strengthened these observations. They demonstrated that the 11-*cis*-7-ring-Rh does not activate G-protein *in vivo* and *in vitro*, and that it does not isomerize along other double bonds. By contrast, the 11-*cis*-6-ring-Rh modestly activates phototransduction *in vivo* and at low pH *in vitro*. It was stated that full activation is not achieved because isomerization does not induce a complete set of conformational rearrangements of Rh [40]. Furthermore, illumination of the active forms of rhodopsin generates a product that is in an inactive conformation, but has an all-*trans*-retinal chromophore in the binding pocket [41]. This suggests that, to regenerate rhodopsin, the chromophore must be subjected to the enzymatic process known as the retinoid cycle, which produces 11-*cis*-retinal, which can combine with opsin to form rhodopsin rather than to simple photoreversal of the configuration of the chromophore in the binding site of rhodopsin.

It may be concluded that incomplete *cis-trans* retinal isomerization itself is required for full rhodopsin biological activity. Already mentioned papers [39-41] clearly demonstrate that “full” *cis-trans* retinal isomerization should only be considered in the context of chromophore-protein interaction: chromophore isomerization that is responsible for the chromophore-induced complete set of conformational rearrangements of the opsin is a prerequisite for full Rh biological activity.

Finally, it can be stated that the paradigm “full *cis-trans* retinal isomerization is a prerequisite for the full activity of rhodopsins” must be also verified.

SUMMARY

Rhodopsins (and rhodopsin-like systems) have been the subject of very intensive investigations for last thirty-five years. The unquestionable successes of the first decades of investigations led to the egoistic opinion that we were able to truly describe the structure and mechanisms of the action of different retinal-based systems (the formulation of the so-called rhodopsin paradigms). However, recent years have brought us the lesson of humiliation: arguments have accumulated against the principal rhodopsins paradigms. The arguments based on our research supplement this opinion. We are returning to the basic questions which were asked at the beginning of “rhodopsin research”.

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