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# The role of retinal light induced dipole in halorhodopsin structural alteration

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ABSTRACT

**The present work studies the mechanism of light induced protein conformational changes in the over-expressed mutant of halorhodopsin (phR) from *Natronomonas pharaonis*. The catalytic effect of light is reflected in accelerating hydroxyl amine reaction rate of light adapted phR. Light catalysis was detected in native phR but also in artificial pigments derived from tailored retinal analogs locked at the crucial $C_{13}$=$C_{14}$ double bond. It is proposed that the photoexcited retinal chromophore induces protein concerted motion that decreases the energy gap between reactants ground and transition states. This energy gap is overcome by coupling to specific protein vibrations. Surprisingly, the rate constants show unusual decreasing trend following temperature increase both for native and artificial pigments.**



## 1. Introduction

Halorhodopsin (HR), a member of archaeal rhodopsins contains retinal chromophore bound via a protonated Schiff base to the ε-amino group of a lysine residue. The photoisomerization reaction around the $C_{13}$=$C_{14}$ double bond triggers a series of conformational changes by which a chloride ion is transported [1–3]. The HR homologs from *Halobacterium salinarum* (shR) and *Natronomonas pharaonis* (phR) were extensively studied [4–10]. These HR homologs investigated so far differs in many aspects. They have low sequence identity (55%) which is subsequently reflected in profound differences in their ion specificity, spectroscopic property and kinetic behavior. The photocycle of phR consists of four distinct intermediates (K, L1, L2 and O) whereas the O intermediate is undetectable in the case of the shR for kinetic reasons [11–18].

It is widely accepted that all light-induced protein conformational alterations in retinal proteins are initiated by photoisomerization of the retinal chromophore. Alternatively, it has been suggested that protein conformational alterations can be induced by the large light induced charge redistribution developed in the retinal chromophore following light absorption [19–22]. This suggestion was supported by atomic force sensing and ESR experiment conducted in bacteriorhodopsin (bR) indicating that protein conformational alterations are induced even in artificial locked bR pigments derived from synthetic retinal analogs in which isomerization around the crucial $C_{13}$=$C_{14}$ double bond is prevented [23,24]. Moreover, the light catalyzed cleavage of retinal protonated Schiff base bond by hydroxylamine was used to support the proposal that bR protein can experience light induced conformational alterations without double bond isomerization [25].

Here we present the possibility that the over-expressed mutant of halorhodopsin from *N. pharaonis* can catalyze chemical reaction which is not associated with retinal double bond isomerization in presence of light. To that end we have followed the rate of hydroxyl amine reactions in dark and under illumination with the native protein as well as with different non-isomerizable artificial pigments, derived from synthetic retinals with "locked" $C_{13}$=$C_{14}$ double bond preventing its isomerization (Fig. 1). The results indicate that indeed the chemical reaction is light catalyzed even though double bond isomerization is prevented. It is proposed that following retinal light excitation the protein experiences a concerted motion that decreases the energy separation between the reactants and products. Once the separation matches the energy of a long lived protein vibration, it gradually couples the reactants into the products.

*Author contributions:* SD obtained the data. AH synthesized the retinal analogs. MS and SD designed the experiments and wrote the manuscript.

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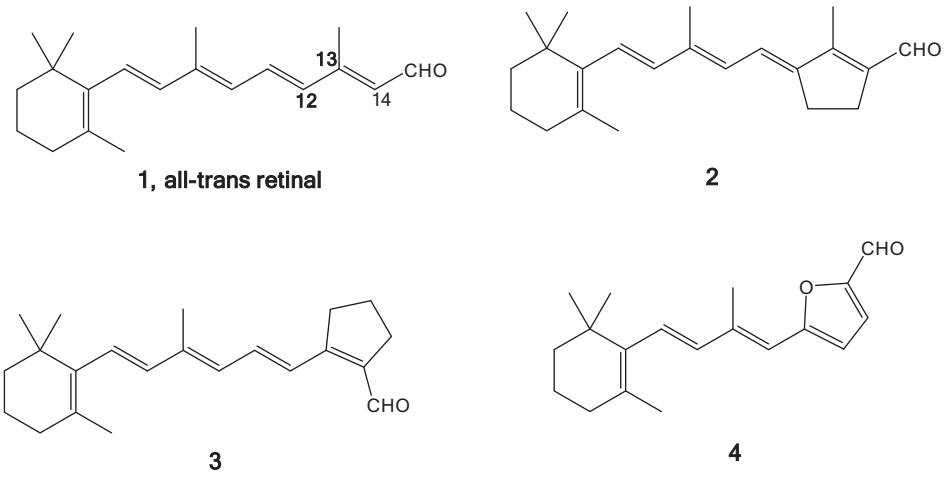


**Fig. 1.** Chemical structures of all-*trans* retinal and the synthetic locked retinals.

## 2. Materials and methods

### 2.1. phR membrane preparation

The KM-1 strain of *N. pharaonis* was grown in a culture medium (pH 9) as described by Ihara et al. [11]. The phR membranes were isolated using a reported procedure [11]. Finally the membranes were suspended in 100 mM NaCl and were centrifuged several times and were kept in 100 mM NaCl.

### 2.2. Preparation of the apoprotein

Apoprotein was prepared by incubating phR membranes with freshly prepared hydroxyl amine (pH 7.2), and irradiation for 2 h with a Schott 250 W cold light source (Carl Zeiss Microscopy, Jena, Germany) equipped with a heat-absorbing filter, and an optic fiber (level 4B). The light was filtered through a long pass cut off filter with a $\lambda$ of >550 nm (Schott, Mainz, Germany). The samples were dialyzed against double distilled water.

### 2.3. Reconstitution of the non-isomerizable artificial pigments

Apoprotein was incubated overnight with different tailored retinal analogs bearing a rigid cyclic structures at the $C_{13}$=$C_{14}$ position at 25 °C in a medium containing 100 mM NaCl. The artificial pigments are characterized by absorption spectra.

### 2.4. Hydroxyl amine reaction with phR and artificial pigments

Experiments with hydroxyl amine were carried out at variable temperatures by stabilizing the temperature of the sample as well as the freshly prepared hydroxyl amine at pH 7. The illumination conditions were similar to that described above for preparation of apoprotein. The reactions rates were monitored at various temperatures both in the dark and light by following the disappearance of the absorption bands of the various pigments. The kinetic data were fitted to the double exponential equation:

$$y = a * e^{-k1*t} + (1-a) * e^{-k2*t}.$$

The obtained reactions rate constants at various temperatures were fitted to the Eyring equation [26],

$$ln\frac{k}{T} = -\frac{\Delta H}{R}\cdot\frac{1}{T} + ln\frac{kB}{h} + \frac{\Delta S}{R}.$$

### 2.5. HPLC analysis

HPLC extractions of the retinal chromophore were carried out using reported protocol [27]. Waters 1525 HPLC equipped with a Waters 2487 Dual $\lambda$ absorbance detector and a Purospher STAR Si-5 μm (LichroCART 250-4, Merck) analytical column was used to analyze the samples. The pigments i.e. the native phR and the artificial pigment with the *trans* locked retinal were treated with hydroxyl amine either in the dark or under 30 s irradiation followed by denaturation with ethanol. The retinal oxime was extracted with hexane and dried over magnesium sulfate. The extraction was performed under red light at room temperature. The extracted chromophore was analyzed in HPLC using 10% ethyl acetate in hexane as eluent. The flow rate was adjusted to 1.0 ml/min.

## 3. Results

### 3.1. Reconstitution of the non-isomerizable artificial pigments *II*, *III* and *IV*

The artificial pigments **II**, **III** and **IV** are generated by reconstituting apo-membrane with the respective synthetic retinals **2**–**4**. The synthetic retinals contain rigid ring structures around $C_{13}$=$C_{14}$ bond to prevent the isomerization process of this double bond. Artificial pigment **II** is locked in *trans* configuration whereas the artificial pigment **III** is locked in the *cis* configuration. Artificial pigment **IV** bears a five membered ring that locks two double bonds (Fig. 1). The absorption spectra of the reconstituted pigments are shown in the Figs. S1–S3 (Supplementary data). The absorption maxima of the artificial pigments **II** and **III** are red shifted relative to native phR (580 nm) to 590 nm while artificial pigment **IV** absorbs at 575 nm.

### 3.2. Light catalysis of the hydroxyl amine reaction with phR and its artificial pigments

The hydroxyl amine reaction cleaves the protonated Schiff base retinal–protein covalent bond. Apart from the preparation of apoprotein the hydroxyl amine reaction is useful for detection of light induced catalysis by the protein since light accelerate the reaction. We have observed that in the over-expressed mutant of halorhodopsin (phR) the hydroxyl amine reaction rate is biphasic and is tenfold faster under illumination relative to the dark reaction (Table 1A–D). Strikingly, similar phenomenon is observed for