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Carotenoid response to retinal excitation and photoisomerization dynamics in xanthorhodopsin

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Abstract

We present a comparative study of xanthorhodopsin, a proton pump with the carotenoid salinixanthin serving as an antenna, and the closely related bacteriorhodopsin. Upon excitation of retinal, xanthorhodopsin exhibits a wavy transient absorption pattern in the region between 470 and 540 nm. We interpret this signal as due to electrochromic effect of the transient electric field of excited retinal on salinixanthin. The spectral shift decreases during the retinal dynamics through the ultrafast part of the photocycle. Differences in dynamics of bacteriorhodopsin and xanthorhodopsin are discussed.

Introduction

Xanthorhodopsin (XR) is a light-activated proton pump found in an extremely halophilic bacterium *Salinibacter ruber*[1]. Related to the archaeal bacteriorhodopsin (BR) and even more to the bacterial proteorhodopsin[2], XR also contains retinal as a chromophore whose all-*trans* to 13-*cis* photoisomerization triggers conformational changes in the protein, finally leading to proton translocation across the membrane. Unlike most proton pumps studied so far, XR non-covalently binds a carotenoid, salinixanthin (SX), serving as an antenna. The crystal structure of XR, at 1.9 Å resolution[2], reveals an 11.7 Å centre-to-centre distance between SX and the retinal. The angle between the conjugated systems of the two chromophores is 46°, providing an arrangement suitable for efficient energy transfer. Indeed, SX-to-retinal energy transfer occurs with an efficiency of 40% as demonstrated by action spectra for oxygen uptake in respiring cells [1], fluorescence excitation spectra in XR-containing cell membranes [3], and by direct measurements of energy transfer by femtosecond transient absorption [4]. Thus, the single carotenoid serving as a light-harvesting pigment makes XR the simplest known antenna system.

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Energy transfer between SX and retinal occurs exclusively from the S_2 state because the dark S_1 state lies significantly below the acceptor state of retinal [4]. Comparison of the S_2 lifetimes of SX in NaBH_4 -treated XR, which has unperturbed SX binding site, but prevents SX-to-retinal energy transfer [3], allowed to determine the energy transfer rate. The S_2 lifetime of 110 fs observed in NaBH_4 -treated XR was shortened to 66 fs in untreated XR, leading to a calculated energy transfer time of 165 fs and an energy transfer efficiency of 40% [4]. The obtained energy transfer rate corresponded to a Coulombic interaction between SX and retinal of about 200 cm^{-1} , which is comparable to other antenna systems employing the carotenoid S_2 state as energy donor [6]. Moreover, this value of SX-retinal interaction was reproduced by transition density cube calculations based on the XR structure [7]. Recent time-resolved experiments using sub-20-fs pulses indicated that the S_2 lifetime of SX in XR could be even shorter [5], challenging the nature of SX-retinal interaction. Therefore, it is important to use different approaches to obtain information about interaction between the two pigments in XR.

The ultrafast part of the BR photocycle is described by four states, H-I-J-K. H is the initial excited state in the Franck-Condon region, the H-I process ($<200 \text{ fs}$) reflects a skeletal change corresponding to a large twisting about the C13-C14 bond of retinal [8], while I-J is the isomerisation that takes place within $\sim 0.5 \text{ ps}$ [9], followed by vibrational relaxation of the J-intermediate, forming the K-state within $\sim 3 \text{ ps}$ [11].

Here we present a study of XR and BR after direct excitation of the retinal chromophore, aiming for better understanding the SX-retinal interaction, and a comparison of BR and XR initial dynamics. We make use of the electrochromic shift of carotenoid absorption bands upon change of an electric field in their surroundings. Contrary to the ‘classical’ version of the experiment using an externally applied electric field, we follow an approach employing excited molecules as sources of electric field changes [13]. In our case, excited retinal in XR induces the electrochromic shift of SX.

Materials & Methods

Sample preparation

Salinibacter ruber strain M31 was grown in the medium described previously [18]. Cells were collected by centrifugation and exposed to overnight dialysis against water. Cell membranes were then washed in 100 mM NaCl and in distilled water. Finally, membrane fractions containing XR were collected by centrifugation [19]. Purple membranes containing BR were obtained as described previously [20]. pH was adjusted to 7.8 and 8.0 for XR and BR, respectively.

Femtosecond pump-probe spectroscopy

For measurements in the visible region, pulses were obtained from a femtosecond laser system (Integra-I, Quantronix) with a repetition rate of 1 kHz , producing pulses with average energy of $\sim 1.9 \text{ mJ/pulse}$ and central wavelength $\sim 785 \text{ nm}$. The output beam was divided into excitation (pump) and probe beams. Pump pulses of desired wavelength were generated in an optical parametric amplifier (TOPAS, Light Conversion), and their intensity was kept below $3 \cdot 10^{15} \text{ photons.pulse}^{-1} \cdot \text{cm}^{-2}$. A fraction of the amplifier output used to generate probe pulses was sent through computer-controlled delay line and then focused to a 2-mm sapphire plate to produce a white light continuum. Before reaching the sample, the probe was divided into two beams, the former overlapping with the excitation beam in the sample and the latter serving as a reference. The instrument response function was $\sim 130 \text{ fs}$ as determined by fitting the instantaneous rise of a bleaching signal from a laser dye. Both beams were then dispersed onto a dual photodiode array detection system (ExciPro, CDP

Systems). A rotating quartz cuvette with a 1-mm path length, spinning at a rate ensuring that each pulse hits a fresh sample, was used for all measurements. The mutual orientation of the excitation and probe beams was set to the magic angle (54.7°). Kinetics in the near-IR region were recorded with a low-noise femtosecond transient absorption spectrometer with 40-fs time resolution described in detail earlier [4]. Data from the visible region were fitted globally (DAFit, Pascher Instruments) to a sum of exponentials using a sequential kinetic scheme with increasing lifetimes [21].

Results

Absorption spectra of XR, BR, and SX in n-hexane are compared in Fig. 1. The absorption spectrum of BR consists of a single featureless band due to the retinal chromophore with a maximum at 570 nm, while the spectrum of XR is dominated by SX bands below 550 nm with retinal absorption appearing as a shoulder around 565 nm. The vibrational bands of the S_0 - S_2 transition of SX in XR are well resolved, with maxima at 521 nm (0-0), 486 nm (0-1), 456 nm (0-2), and 428 nm (0-3). The improved resolution of these bands in XR, as compared to SX in solution, results from locking SX in the XR binding site [2], which isolates its keto-ring by rotating it about 82° out of the plane determined by the main conjugated system of SX (Fig. 1). Thus the conformational disorder, which is induced in solution by distribution of dihedral angles of the terminal rings, is significantly reduced for SX in XR.

Transient absorption spectra of BR and XR for different time delays recorded after excitation at 580 nm are shown in Fig. 2. The transient absorption spectrum of BR consists of three distinct spectral features[22]: (1) excited state absorption (ESA) in the blue part of the spectrum (the I state), (2) ground state bleaching (GSB) near 555 nm, and (3) a positive signal around 635 nm that appears at later times and reflects formation of the J and K states. Because of the spectral overlap, the J-K transition is distinguishable only as a shift of the band around 630 nm to the blue with time. The same spectral features are identified also in XR, with an additional wavy pattern modulating the ESA below 545 nm. Since SX is not excited at 580 nm (Fig. 1), these bands cannot be assigned to SX bleaching. Instead, as addressed in detail in Discussion, we explain these bands as due to electrochromic shift of the S_0 - S_2 transition of SX induced by the excited retinal chromophore.

Fig. 3 further addresses differences in the dynamics of BR and XR. It compares kinetics of XR and BR at wavelengths corresponding to ESA (477 nm), GSB (555 nm) and J/K (635 nm) signals. The blue-most part of the ESA signal was chosen to minimize the influence of GSB of retinal. In addition, kinetics recorded in the near-IR region at 900 nm, monitoring decay of retinal stimulated emission (SE) [23], are depicted in Fig. 3D. The qualitative comparison of the kinetics unequivocally demonstrates that while no significant differences are found in the kinetics that monitor the GSB and J/K states, the excited state of retinal decays slower in XR than in BR.

Fitting the kinetics of retinal SE in BR at 900 nm yields time constants of 0.4 ps (91%) and 2.1 ps (9%). On the other hand, time constants extracted from SE kinetics of XR are 0.7 (70%) and 3.3 ps (30%), thus the overall decay is slower in XR. Based on number of previous reports, for global fitting of BR data in the visible region we fixed the components to 0.5 (excited-state lifetime) and 3 ps (J-K transition)[24]. The resulting fit provided amplitudes of these components of 92% and 8% at 477 nm (ESA region), respectively, matching the results obtained for decay of SE signal in near-IR. This confirms that the blue-most part of BR transient absorption spectra reflects the decay of the excited state of retinal (the I state). Since the time constants of SE decay of XR are comparable to those of BR (see above), we have used the same time constants of 0.5 and 3 ps for global fitting of XR data

aiming at unequivocal comparison of amplitudes of ESA decay. The amplitudes of the time constants characterizing the ESA decay in XR were 58% and 42%, clearly demonstrating the longer lifetime of retinal excited-state in XR. Essentially the same result was obtained when the fast time constant was fixed to 0.7 ps obtained from fitting the near-IR kinetics of XR. We emphasize that no signal from J or K intermediates is present either in the region around 480 nm, or in the near-IR in BR[24], so the slower decay of the excited state in XR is not caused by product formation.

Discussion

Electrochromic shift in XR

We first address the origin of the wavy pattern in the region below 545 nm in XR, which we assigned to electrochromic shift of SX S_0 - S_2 transition. It is known that electrochromic shifts can be modelled by shifting the absorption spectrum of the particular pigment and constructing a difference absorption ‘shifted minus normal’ [25], which is then compared with a transient absorption signal. To apply this approach, we chose the signal recorded at 150 fs after retinal excitation where its amplitude is the largest. In order to extract a pure SX signal in XR, the BR signal was scaled to the GSB at 555 nm and subtracted from the XR transient absorption spectrum. The resulting transient absorption spectrum is best reproduced by a difference spectrum constructed as ‘325-cm⁻¹ blue shifted minus normal’ XR absorption spectrum (Fig. 4). Such shift of the S_0 - S_2 transition reproduces peak positions in transient absorption spectrum at 150 fs, which justifies our conclusion that electrochromic shift of absorption spectrum of SX is responsible for spectral patterns in the high-energy part of transient absorption spectra.

Having established the origin of the wavy pattern in the transient absorption spectra of XR as due to electrochromic shift, we may now proceed to a more quantitative analysis. We adopt here the equation for the Stark shift of transition energy given by [26]:

$$\Delta\tilde{\nu} = -\frac{1}{hc} \left(\Delta\mu E_T + \Delta\alpha E_{int} E_T + \frac{1}{2} \Delta\alpha E_T^2 \right) \quad (1)$$

where $\Delta\mu$ and $\Delta\alpha$ stand for changes in dipole moment and polarizability between the initial and final state, respectively, E_T is the transient electric field induced by excited retinal and E_{int} is the local stationary electric field experienced by SX. Although quantities in the right side of Eq. 1 are generally vectors ($\Delta\alpha$ is even tensor), we will first treat them as scalars for simplicity. Our aim is to calculate E_{int} from Eq. 1 and use this value to calculate the shift of carotenoid S_0 - S_2 transition induced solely by its protein environment. $\Delta\tilde{\nu}$ is 325 cm⁻¹ (see above), $\Delta\mu$ and $\Delta\alpha$ are not known for SX, so we apply those determined for a similar carotenoid astaxanthin [27]: $\Delta\mu \approx 7$ D, $\Delta\alpha \approx 1350$ Å³. In order to estimate E_T , we use the following formula:

$$E_T = \frac{1}{4\pi\epsilon_0} \frac{\Delta\mu_{RET}}{r^3} \quad (2)$$

where r is the center-to-center distance between retinal and SX, and $\Delta\mu_{RET}$ is a difference between dipole moments of ground and excited states of retinal in XR. Dielectric constant ϵ_r is omitted in Eq. 2 because it not only appears with ϵ_0 , but also affects $\Delta\mu_{RET}$. Thus, if we use the usual value of $\epsilon_r = 2$, the correction due to ϵ_r would not exceed 10% [28]. The value of $\Delta\mu_{RET}$ is not known for XR, but it has been determined by various techniques for BR

[29]. Here we use the consensual value of $\Delta\mu_{RET} = 15\text{D}$ used also in [17], which yields $E_T = 2.8\text{ MV.cm}^{-1}$.

Now we can calculate the static local electric field experienced by SX, E_{int} , from Eq. 1. Since there are two possible orientations of $\Delta\mu$ in respect to E_T , we obtain two values of E_{int} , $E_{int,1} \approx -4.5\text{ MV.cm}^{-1}$ and $E_{int,2} \approx -1.4\text{ MV.cm}^{-1}$. Knowing the value of E_{int} , we can adapt Eq. 1 to calculate shift of SX S_0 - S_2 transition in protein compared to solution ($\Delta\nu_{P-S}$). To do this, we replace E_T by E_{int} in Eq.1 and disregard the cross term $\Delta\alpha E_{int} E_T$ since we examine only effect of E_{int} :

$$\Delta\tilde{\nu}_{P-S} = -\frac{1}{hc}(\Delta\mu E_{int} + \frac{1}{2}\Delta\alpha E_{int}^2) \quad (3)$$

We obtain $\Delta\tilde{\nu}_{P-S} \approx -240\text{ cm}^{-1}$ for both values of E_{int} . This result is close to the observed red shift of $\Delta\nu_{P-S} \approx -290\text{ cm}^{-1}$ of SX absorption in XR compared to that in n-hexane (Fig. 1). Thus most of the SX red shift can be explained by local electrostatic interaction, which is in agreement with previous results obtained for purple bacterial antenna LH2[14].

We can further refine our results if we take into account the vector nature of $\Delta\mu$ and E_T . We make use of Eq.3 from which we calculate magnitude of E_{int} , leading to the experimentally observed red shift of 290 cm^{-1} . The resulting values are: $E_{int,1}' \approx \pm 1.6\text{ MV.cm}^{-1}$ and $E_{int,2}' \approx \pm 4.7\text{ MV.cm}^{-1}$. Then, the vector nature of $\Delta\mu$ and E_T is accounted for in Eq. 1 by introducing an angle β between these quantities, leading to

$$\Delta\tilde{\nu} = -\frac{1}{hc} \left(\Delta\mu E_T \cos\beta + \Delta\alpha E_{int}' E_T + \frac{1}{2}\Delta\alpha E_T^2 \right) \quad (4)$$

We can calculate β for various combinations of $\pm E_{int,1,2}'$ and $\pm\Delta\mu$. Since only one such combination, $E_{int,1}' = -1.6\text{ MV.cm}^{-1}$ and $\Delta\mu = -7\text{ D}$, results in $|\cos\beta| < 1$, we obtain a single value of $\beta = 32^\circ$.

This result can be corroborated by Fig. 5 showing the field induced by $\Delta\mu_{RET}$. It is obvious that although part of the SX conjugated system (and thus $\Delta\mu$) is parallel to E_T , some other parts are almost perpendicular to it. Thus the calculated angle of 32° , which represents a 'mean' angle between $\Delta\mu$ and E_T , is a reasonable estimate. Most importantly, however, while treating the $\Delta\mu$ and E_T as scalars gave two possible values of E_{int} (see above), treating these quantities as vectors allowed to single out the value of $|E_{int}'| \approx 1.6\text{ MV.cm}^{-1}$. Finding the unique values of E_{int}' and $\Delta\mu$ also allows an evaluation of the contributions of the three terms in Eq. 4 to the observed blue shift. They are 274 cm^{-1} , 342 cm^{-1} , and -292 cm^{-1} , resulting in the observed electrochromic shift of 325 cm^{-1} . Fig. 5 also provides information about the direction of $\Delta\mu$ of SX. Since $\Delta\mu_{RET}$ points from the Schiff base to the retinal ring [17] and since $\Delta\mu$ of SX is negative (see above), it must be opposite to the direction of force lines, and thus have the direction as depicted in Fig.5.

Dynamics of the electrochromic shift

We will now focus on the dynamics of SX transient signal at 477 nm by searching for a possible correlation of its dynamics with kinetics of other spectral features in the transient absorption spectra of XR. Normalized kinetics recorded at various wavelengths are shown in Fig.6. It is obvious that kinetic taken at 477 nm matches that measured in the near-IR region at 900 nm . Keeping in mind that the SX transient signal is superimposed on the background ESA of retinal, one would expect a modification of the decay at 477 nm if the dynamics of

the electrochromic shift were different from those of retinal ESA. Consequently, identity of kinetics at 477 and 900 nm suggests that the electrochromic shift decays with the same rate as the excited state of retinal.

Closer inspection of transient absorption spectra, however, indicates that other intermediates may also contribute to an electrochromic shift. Although the main pattern due to electrochromic response of SX is nearly gone at ~10 ps, it is clear that a weak wavy signal remains even at this delay time (Fig. 2B). Comparing the maxima of the electrochromic response at different delay times, it is clear that the peaks red-shift with time. Since at 10 ps after excitation the excited state of retinal decayed to form photoproducts, it is likely that electrochromic response at this delay time is rather due to the 13-*cis* isomer of retinal in the photoproducts J/K. This assumption could be tested by subtracting the kinetic at 509 nm (initial position of the peak) from that measured at 515 nm (red-shifted peak). Such procedure should reveal 'pure' dynamics of the 515-nm band. Indeed, comparison of the difference kinetic and dynamics of the 635-nm band monitoring a formation of the J/K states reveals a reasonable match (Fig. 7). Consequently, we conclude that the J and K states, corresponding to the ground state configuration of retinal 13-*cis* isomer as revealed for a closely related BR [10], induce an electrochromic shift of SX. It is in agreement with previous finding that the K intermediate trapped at low temperature causes a small (~2 nm) blue shift of SX bands [1].

Comparison of BR and XR dynamics

Comparison of kinetics depicted in Fig. 3 clearly shows that retinal excited state decays slower in XR, while the dynamics of GSB (555 nm) and J/K states (635 nm) are similar in BR and XR. This suggests that while the excited-state processes are slower in XR, the dynamics of photoproduct formation is the same in BR and XR. It must be noted, however, that fitting the SE kinetics of BR and XR revealed similar time constants. Time constants for SE of retinal in BR have been reported to be 0.5 ps (95%) and 2 ps (5%) [24], while values of 0.7 ps (64%) and 3.3 ps (36%) have been determined for XR [4]. Our single-wavelength fitting analysis showed corresponding time constants: 0.4 ps (91%) and 2.1 ps (9%) for BR; 0.7 (70%) and 3.3 ps (30%) for XR. Thus, the slower overall excited-state decay in XR is almost solely due to increase of amplitude of the slower, ~3-ps component. The ~2-ps component has been attributed to a 13-*cis*, 15-*syn* retinal conformation in BR [24] and alternatively to conformational heterogeneity of retinal chromophore environment causing multicomponent decay of the excited state [30]. It is thus possible that either a fraction of the retinal chromophores with longer excited state lifetime in XR is larger than in BR, or that branching occurs in the excited state and a larger fraction evolves through a slower path. One would expect that the difference in excited state decay, clearly seen at 477 nm and 900 nm, could be detected at wavelengths that test mainly ground state recovery (555 nm) and photoproduct formation (635 nm). However, no large differences in kinetics between BR and XR were detected at these wavelengths (Fig. 3). The likely reason is that the two processes (slow decay of the excited state and the J to K transition) occur with similar time constants thus the J does not accumulate and consequently does not contribute to the signal. In addition, the extinction changes during photoproduct formation might be less for XR than for BR at these wavelengths.

It should be noted, however, that direct parallels between BR and XR probably can be applied only cautiously due to differences between these two proton pumps. First of all, the presence of a large molecule such as SX in the vicinity of retinal chromophore may alter properties of the retinal binding pocket [2]. Despite functional similarities between BR and XR, these two proteins have only ~20% of identical amino acids [2]. Although five amino acids closest to retinal in BR (Trp⁸⁶, Ser¹⁴¹, Thr¹⁴², Trp¹⁸², and Lys²¹⁶ [17]) are conserved in both BR and XR, two other tryptophans (Trp¹³⁸ and Trp¹⁸⁹) in the proximity of retinal are

in XR replaced by Gly¹⁵⁶ and Tyr²⁰⁷ involved in SX binding[2]. Perhaps the most relevant difference is that in XR the Schiff base counterion is an aspartate-histidine complex rather than an aspartate as in BR [2]. Sharing of a proton between the aspartate and the histidine would lead to an extended lifetime of the excited state in a fraction of XR pool at neutral pH and especially upon lowering the pH [3], as it does neutralization of Asp⁸⁵ in BR [31].

Conclusions

We found that excitation of retinal in XR results in a strong electric field, $E_T = 2.8 \text{ MV.cm}^{-1}$, which causes an electrochromic shift of the SX absorption spectrum that is responsible for a wavy pattern in the blue region of transient absorption spectra. This enabled us to estimate the magnitude of the local electric field strength around SX in XR as 1.6 MV.cm^{-1} . The dynamics of the SX electrochromically shifted spectrum is associated with the decay of excited states of retinal, which is substantially slower than in BR. Additionally, J and K states induce an electrochromic response of SX, though far weaker.

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Highlights

- Ultrafast spectroscopy of xanthorhodopsin (XR) and bacteriorhodopsin (BR).
- Salinixanthin (SX) in XR exhibits an electrochromic shift after retinal excitation.
- Electrochromic shift of SX decays co-ordinately with excited state of retinal.
- Differences in ultrafast dynamics of XR and BR were found.

Figure 1. Absorption spectra of XR, BR, and SX in n-hexane. BR absorbance was scaled to the absorbance of retinal in XR. Excitation wavelength for time-resolved measurements (580 nm) is indicated by a vertical line. In the bottom part are shown chemical structures of SX (top) and retinal (bottom) bound by a Schiff base linkage to lysine of BR and XR.

Figure 2.

Transient absorption spectra of BR (panel A) and XR (panel B) following the excitation at 580 nm. Distinct spectral features are indicated by vertical dashed lines – ESA of retinal (477 nm), GSB of retinal (555 nm), and J/K states of retinal (635 nm).

Figure 3. Kinetics recorded after excitation at 580 nm for XR and BR in spectral regions shown in Fig. 2. Panel D shows the decay of SE of retinal in XR and BR.

Figure 4. Comparison of SX transient signal at 150 fs and difference absorption spectrum of XR. The difference absorption spectrum was simulated as '325-cm⁻¹ blue shifted minus normal' absorption spectrum of XR. SX transient signal represents the 'XR minus BR' transient absorption spectra at 150 fs normalized to the GSB.

Figure 5. Retinal and SX molecules with the field induced by $\Delta\mu_{RET}$ in XR. The colour coding illustrates the strength of the local electric field E_T , while the force lines show its direction. The arrows indicate approximate directions (not magnitudes) of $\Delta\mu_{RET}$ [17] and $\Delta\mu$ of SX. SX acyl chain and glucoside were removed to simplify the picture. Two projections are depicted to better account for positions of chromophores in space.

Figure 6. Normalized kinetics of distinct spectral features of XR. Where necessary, they are inverted and/or translated to enable comparison.

Figure 7.

Comparison of the difference kinetics (515 nm minus 509 nm) with the kinetics reflecting the J/K states in XR. The continuous lines resulted from a smoothing procedure. The data are normalized and vertically shifted.