Similarity of bacteriorhodopsin structural changes triggered by chromophore removal and light-driven proton transport

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Abstract Bacteriorhodopsin (bR) is the light-driven proton pump found in the purple membrane of *Halobacterium salinarium*. A series of conformational changes occur during the bR photocycle which involve alterations in buried-helical structure as well as in the protonation state of Asp residues which are part of the proton transport pathway. Here we report evidence that similar conformational changes occur upon removal of the retinylidene chromophore of bacteriorhodopsin to form the apoprotein bacterioopsin (bO). This suggests a simple ligand-binding model of proton transport in bacteriorhodopsin which may have relevance to other transport and signal transducing membrane proteins including the visual photoreceptor rhodopsin.

Key words: Bacteriorhodopsin; Rhodopsin; Infrared; Proton transport; Bioenergetics

1. Introduction

Bacteriorhodopsin (bR), the light-driven proton pump from the purple membrane of *Halobacterium salinarium* [1–4], is of current interest both as a model of ionic transport in biomembranes and as a photonic material for biotechnological applications [5,6]. It has a close sequence homology with other membrane proteins from *H. salinarium* including sensory rhodopsins I and II, light receptors for control of phototaxis [7] and halorhodopsin, which functions as a light-driven chloride pump [8]. It also shares the 7 α-helix motif of all G-protein coupled receptors including rhodopsin [9], which like bR contains a retinylidene chromophore linked through a Schiff base to the ε-amino group of a lysine residue on the G-helix (helix 7) [3].

FTIR difference spectroscopy [10–13] reveals that the initial light-triggered isomerization of the retinylidene chromophore from an all-*trans*→13-*cis* configuration [14,15] results in a series of protein conformational changes. These structural changes culminate in formation of the N intermediate (bR→K→L→M→N) and are then reversed during the completion of the photocycle (N→O→bR). A major conformational change is detected by FTIR difference spectroscopy between the M and N intermediates in wild-type bR [16–18] which in some mutants is observed prior to N formation in the M_N intermediate [19]. This conformational change involves membrane buried α-helical structure and helix F [20] consistent with changes observed by both electron and x-ray diffraction [21,22].

We report here evidence that similar conformational changes occur in bacteriorhodopsin upon removal of the retinylidene chromophore to form the apoprotein, bacteriorhodopsin (bO), and retinal. This suggests that the all-*trans* retinal chromophore of bacteriorhodopsin serves as an activating ligand which when either removed or isomerized to the 13-*cis*/C=N *anti* configuration allows bacteriorhodopsin to relax into a non-active conformation. A similar role may be played by all-*trans* retinal in the Meta II intermediate of rhodopsin, the signaling state for visual transduction.

2. Materials and methods

ATR-FTIR difference spectroscopy on purple membrane films was performed as described elsewhere [20]. Approximately 100–200 μg of purple membrane was dried on a 45° aperture angle 50×20×2 mm^3 Ge internal reflection element (IRE) under a stream of argon gas. The IRE was mounted in a temperature controlled attenuated total reflectance (ATR) cell (Harrick Scientific Co., Ossining, NY) with a quartz window, maintained at a constant temperature of 270 K. The cell was injected with 25 mM phosphate buffer prepared in D_2O at pD 9. ATR-FTIR spectra were recorded on a Nicolet 510P FTIR spectrometer (Nicolet Analytical Instruments, Madison, WI) at 2 cm^{-1} resolution. Membrane swelling which occurs due to hydration and pH changes [23] was monitored from the intensity of the amide I band and the D/H exchange of peptide groups was monitored from the downshift of the amide II (1545 cm^{-1}) band to the amide II′ (1460 cm^{-1}) band. When both swelling and D/H exchange had stabilized a bR spectrum was recorded and then a solution of 25 mM phosphate buffer and 0.8 M NH_2OH·HCl titrated to pD 9 was injected. The sample was light adapted and spectra were taken with the sample in the dark for 1 h. The sample was then illuminated with a 100 W tungsten lamp filtered with a 505 nm long pass filter and optical fiber (Dolan-Jenner Industries, Inc., Lawrence, MA) in 5 min intervals interspersed with 5 min in the dark. Spectra of 300–500 co-added scans were taken in each light and each dark period, allowing for the monitoring of the bR→N difference spectrum [20]. When the envelope of the bR→N signal (peak-to-peak in the 1700–1500 cm^{-1} region) fell below 10^{-5} OD (a minimum of a 100-fold decrease), the sample was considered to be completely bleached. bO spectra of at least 6000 scans were then taken. An interactive subtraction between the bO and bR spectra was performed, scaled to the amide II′ band. Protein viability after the bleaching process was tested by regenerating bO with all-*trans* retinal and measuring the properties (light/dark adaptation and bR→N FTIR difference spectrum) of the resulting bR. To verify that the interactive subtraction does not create spectral artifacts, control experiments without adding hydroxylamine were performed in which membrane swelling was induced both by waiting long periods of time between spectra and by inducing small changes in pD. None of these control experiments show changes consistent with those seen during hydroxylamine bleaching.

3. Results

Fig. 1 compares the ATR-FTIR difference spectrum of the

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bR → N transition (Fig. 1a) with that of bR → bO conversion (Fig. 1b) obtained by the light-induced bleaching of bacteriorhodopsin in the presence of hydroxylamine. A negative band in both spectra at 1528 cm^{-1} can be assigned to the C=C ethylenic stretch of the retinylidene chromophore of light-adapted bacteriorhodopsin (bR_{570}). This reflects a change in the visible absorption of the chromophore due to formation of the N intermediate (Fig. 1a) or the loss of the chromophore due to hydroxylamine bleaching (Fig. 1b). Bands at 1254, 1201 and 1167 cm^{-1} (C–C stretch modes of the chromophore), which are present in the bR → N spectrum are not seen in the bR → bO spectrum due to the overlap of the bulk D_2O band at 1200 cm^{-1}, whose relative intensity changes upon bleaching due to membrane swelling. However, similar bands are observed when bleaching is performed in the presence of H_2O (data not shown). Note that a by-product of bleaching, retinaloxime, remains bound to the membrane but does not contribute significantly to the spectrum due to the weak infrared absorbance of vibrations of a deprotonated retinal Schiff base molecule [24].

Spectral agreement is also found in the amide I region, reflecting the C=O vibrations of amide carbonyl groups. Bands near 1651 cm^{-1} (positive), 1671 cm^{-1} and 1692 cm^{-1} (negative) appear during the M → N transition of the wild-type photocycle [16,19,25] with the two lower frequency bands assigned to the amide I mode of α-helical structure [17]. That all of these bands are also observed in the bR → bO difference spectrum indicates that removal of the retinylidene chromophore causes a similar conformational change. Note that the absolute sizes of the difference bands in this region are small (~1 mOD) relative to the absolute absorption (data not shown). This is consistent with an earlier study that compared the absolute infrared absorption of bleached and unbleached bacteriorhodopsin and concluded that bleaching produces only small changes in overall protein secondary structure [26].

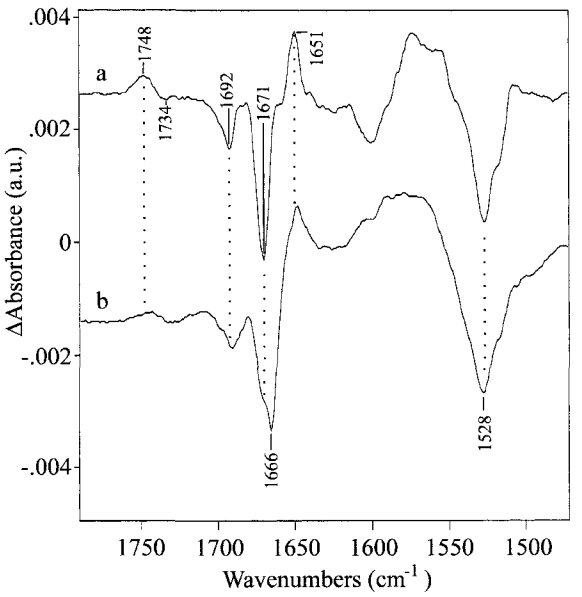

Fig. 1. Comparison of ATR-FTIR difference spectra obtained at 270 K in D_2O solution for: (a) the bR → N transition of the bacteriorhodopsin photocycle [20] and (b) bR → bO conversion due to light-induced bleaching in the presence of hydroxylamine at pD 9 (see Section 2). Y-axis shown is for trace a. Trace b is magnified 2.6× relative to trace a.

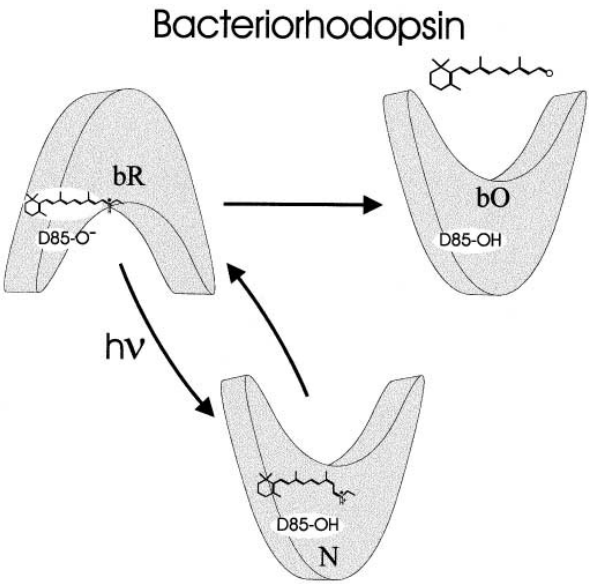

Fig. 2. Schematic drawing showing the conformational changes which occur in bacteriorhodopsin during its photocycle and during conversion to bacterioopsin. Note that the major structural change may actually occur in the bR photocycle during a transition between the M and M_N intermediate. The activating all-*trans* retinylidene ligand is enclosed in white.

Evidence for similarities between the bR → N and bR → bO transition are also found in the carboxylic acid C=O stretch region (1700–1780 cm^{-1}), which reflects protonation and hydrogen-bonding changes of Asp/Glu carboxylic acid groups [27,28]. Most outstanding is a band appearing near 1748 cm^{-1} in both the bR → N and bR → bO difference spectra (Fig. 1a and b, respectively). This band, assigned to the C=O stretch mode of Asp-85 in the bR → N and bR → M_N difference spectra [19,25], is downshifted approximately 6 cm^{-1} due to D/H exchange. In the case of the bR photocycle it arises due to the transfer of a proton from the Schiff base to Asp-85 during the L → M transition [27]. In the case of the bR → bO difference spectrum it is most likely also due to Asp-85 protonation, although a definitive assignment will require further experiments using site-directed isotope labeling and site-directed mutagenesis. A negative band, most likely due to Asp-96 deprotonation, which appears in the bR → N difference spectrum at 1734 cm^{-1}, was not detected in the bR → bO difference spectrum (Fig. 2b). However, we cannot exclude the possibility that this is due to overlap with the positive band at 1748 cm^{-1} along with baseline drift over long time periods.

4. Discussion

Our results are summarized in Fig. 2. We find that hydrolysis of the all-*trans* retinylidene chromophore of bacteriorhodopsin to form opsin and all-*trans* retinal (or a retinal oxime) involves conformational changes which are similar to those occurring during the bR photocycle. For example, we deduce a protonation of Asp-85 which in the bR photocycle is associated with the transfer of a proton from the Schiff base to Asp-85 during the L → M step [27]. This event is coupled to ejection of a proton into the extracellular medium, possibly through the concerted deprotonation of Glu-204 [29]. We also observe structural changes of α-helices which are similar to

those detected by FTIR during the M → N step of the photocycle. Based on measurements on bR mutants such as D96N which exhibit a slow M decay, these changes may actually occur earlier than formation of the N intermediate during the M → M_N transition [19] where the Schiff base is still deprotonated. Importantly, these structural rearrangements are likely to play a critical role in switching the Schiff base accessibility from a proton pathway leading to the extracellular medium through Asp-85 (proton ejection) to one which is accessible to Asp-96, thereby facilitating Schiff base reprotonation [30].

The similarity between the structural changes which occur during bR → bO conversion and the bR photocycle can be viewed from the perspective of receptor-ligand interactions. Our findings show that like most receptor-ligand interactions, the binding of all-*trans* retinal to bacterioopsin triggers a set of conformational changes which results in formation of the active state of the protein, i.e. the light-adapted form of bacteriorhodopsin which is able to pump protons. Photoisomerization of the all-*trans* retinylidene chromophore to the 13-*cis*/C=N *anti* form during the bR photocycle can then be viewed as a process which inactivates the ligand by altering its structure, thus resulting in a set of conformational changes similar to those observed in bR → bO conversion. It is interesting to note that other configurations of the retinylidene chromophore may also trigger similar conformational changes but still not activate the protein. One example is the 13-*cis*/C=N *syn* isomer of retinal which is the chromophore for the non-proton pumping component of dark-adapted bacteriorhodopsin. The FTIR difference spectrum of dark adaptation contains bands reflecting the all-*trans*/C=N *anti* to 13-*cis*/C=N *syn* isomerization of the chromophore but not the characteristic protein conformational changes observed during M → N or bR → bO [31]. The existence of a protonated Schiff base may also not be necessary to trigger secondary protein changes since bacteriorhodopsin can still pump protons in the normal direction if the chromophore consists of an all-*trans* retinal with a deprotonated Schiff base or even in the absence of a covalent Schiff base linkage to Lys-216 [32–34].

Evidence exists that all-*trans* retinal also serves as an activating ligand in rhodopsin, the photoreceptor in vision. The resting (non-signaling) state of rhodopsin contains an 11-*cis* retinylidene chromophore. Activation occurs when light triggers a femtosecond isomerization of this chromophore to an all-*trans* configuration [35,36]. This is followed by a series of rapid thermal dark reactions [37,38] involving structural changes [39–45] which facilitate binding and activation of the G-protein, transducin [46]. Removal of all-*trans* retinal from the binding pocket, which occurs during Meta II decay to Meta III or to opsin plus all-*trans* retinal, results in the refolding of the protein to a conformation similar rhodopsin [47,48]. Hence, like bacteriorhodopsin, absence of the activating ligand either due to removal from the binding pocket or due to its presence in an 11-*cis* configuration results in inactivation. Similarities may also be revealed between bacteriorhodopsin and other G-protein coupled receptors, such as the β-adrenergic receptor, once the details of the conformational changes and receptor-ligand interactions which trigger these changes are better understood.

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