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The photochemical reaction cycle of retinal reconstituted bacteriorhodopsin

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Abstract

The function of three types of bacteriorhodopsins was compared: the wild-type, the bleached and retinal reconstituted and retinal deficient bacteriorhodopsin after retinal addition. The apparent pK_a of the proton acceptor group for the bleached BR and retinal deficient BR shifted toward higher pH values compared to the wild-type BR. Fitting the photocycle model to the absorption kinetic signals for all three proteins showed the existence of the same intermediates, but the time-dependent concentration of the intermediates was different. Although measurements were made at pH 7, the absorption kinetics and photoelectric signals in both retinal reconstituted samples acted as wild-type bacteriorhodopsin at significantly higher pH. Below pH 3 the retinal deficient and reconstituted sample bleached. These results suggested that the added retinal was not able to rebind in the same position in the protein as in native bacteriorhodopsin. This points out that care should be taken, when bleached bacteriorhodopsin is reconstituted with different retinal analogs.

Keywords: Retinal protein; Photocycle; Absorption kinetics; Retinal reconstitution

1. Introduction

The retinal protein bacteriorhodopsin (BR) acts as a light-driven proton pump in the cell membrane of *Halobacterium salinarum* [1–4].

The structure of bacteriorhodopsin is known with 1.55 Å resolution [5]. Retinal is bound to the lysine 216 through a protonated Schiff-base. The dark-adapted BR contains a mixture of all-*trans*, 15-*anti* and 13-*cis*, 15-*syn* retinal in thermal equilibrium, while the light-adapted BR contains only all-*trans*, 15-*anti* retinal. After light excitation, both retinal conformations exhibit a photocycle, but only the all-*trans* retinal containing BR has proton transporting activity [6] at a pH above the pK_a of the proton acceptor Asp 85. This transporting photocycle is formed from a succession of intermediates (noted by K, L, M, N and O). Each intermediate has a well determined absorption spectrum [7], electrogenicity [8] and structure [9].

After absorbing the light, a charge separation along the retinal chain occurs in the fs time domain [10], followed by an all-*trans* to 13-*cis* isomerization in several ps, reaching the state K. In K to L translation, a local rearrangement around the retinal occurs in less than 10 μs. In L to M_1 transition, about 100 μs after the excitation, the Schiff-base of the retinal deprotonates by transferring its proton to the acceptor Asp 85 and a proton is released from the release group on the surface of the membrane close to the external medium [11]. Important step is the M_1 to M_2 transition, when the protein switches between the extracellular and cytoplasmic conformation in about 100 μs [12]. The accessibility of the Schiff-base changes from the extracellular to the cytoplasmic side [13]. The Schiff-base is reprotonated by the proton donor Asp 96 on the cytoplasmic side, reflected by the appearance of intermediate N. In the N to O to BR transition, the retinal reisomerizes to its original all-*trans* form, a proton is taken up from the

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cytoplasmic side and the proton from the acceptor is transferred to the proton release group in ms time domain [14,15]. At low pH, below the pK_a of the proton acceptor group, the photocycle does not translocate the proton across the membrane and has only red shifted intermediates [16].

The electric signals from BR were first measured on oriented samples obtained by incorporating BR into a bilayer lipid membrane [17,18]. Another method for electric signal measurement is the oriented attachment of purple membranes to a lipid-impregnated filter [19] or onto a thin teflon sheet [20]. These techniques are very sensitive to the charge motions inside the protein, but the sample having a small optical density makes the absorption kinetic measurements rather difficult. A possibility to eliminate this problem is to apply an external electric field to the purple membrane suspension, orienting the membrane fragments by their permanent dipole moment [21,22]. When the membranes are incorporated in acrylamide gel and an external electric field is applied during the polymerization of the gel, the orientation can be fixed [23]. On an oriented gel sample, both the absorption kinetic and electric signal measurements can be done. The disadvantage of the technique is the low resistivity of the sample at high salt concentration.

The light activated bleaching of the purple membrane in the presence of hydroxylamine can be observed [24,25]. In this process, the retinal reacts with the hydroxylamine forming retinaloxime, which is released from the protein and can be separated from the membrane suspension. Adding back the retinal to the apomembrane suspension, obtained from hydroxylamine bleaching, or the retinal deficient *H. salinarum* strain, the purple color of the membranes can be reconstituted [26,27]. This technique is widely used to introduce artificial retinal analogs into the retinal binding pocket of the BR [28,29], but no study has been conducted on the changes in the protein function introduced by the retinal rebinding.

This study aims to describe the changes occurring in the photocycle and charge motions after the all-*trans* retinal is bound to the protein. We compared reconstituted retinal deficient membrane suspension or, reconstituted bleached membranes with wild-type BR purple membranes.

2. Materials and methods

Purple membranes were isolated from *H. salinarum* strain S9 according to a standard procedure [30]. The retinal deficient BR membranes were isolated from the strain JW2N. The bleached BR membranes were obtained from wild-type BR, illuminated for 20 hours in 1 M hydroxylamine solution at pH 7, as described [24] and washed extensively by multiple centrifugations in distilled water. To the retinal deficient and the bleached BR samples, all-*trans* retinal was added [25]. Three purple colored samples were obtained: the unmodified purple membrane suspension (wild type BR), the reconstituted bleached BR and the retinal reconstituted retinal deficient BR membranes.

The spectral titration and absorption kinetic measurements were carried out on BR polymerized in acrylamide gel, following the procedure described elsewhere [31]. The thoroughly washed gels were soaked overnight in 100 mM NaCl, 20 mM MES (2-[*N*-morpholino]ethanesulfonic acid), 20 mM TRIS and 20 mM acetic acid buffers at the desired pH. For electric signal measurements, oriented gel samples were prepared [23]. The gels were equilibrated with a bathing solution containing 100 mM NaCl and buffers, 25 mM MES or 25 mM TRIS.

During the experiments, the sample was in a 4x10 mm cuvette in a temperature controlled sample holder. The photocycle was initiated by a laser flash from a frequency-doubled Nd-Yag laser (Surelite I-10, Continuum, Santa Clara, Ca). The measuring light was provided by a 55 W halogen lamp with heat filter. Measurements were taken at five wavelengths (410, 500, 570, 610 and 650 nm) selected by a monochromator placed between the sample and the photomultiplier. By these the measuring wavelength did not affect the light intensity going through the sample. Signals were recorded using a transient recorder card, having 16 Mb memories and 50 ns time resolution (NI-DAQ PCI-5102, National Instruments, Austin, TX). Each measurement was the average of 100 signals. At the end of the measurements, the linear time base was converted to logarithmic one by averaging in the time interval between logarithmically equidistant points. The signals were fitted to a photocycle model by using the RATE program [32]. Electric signals were measured on oriented gel samples on the setup described earlier [8].

3. Results and discussion

The absorption spectra of the three samples at pH 7 were indistinguishable (Fig. 1). By spectral titration, the pK_a of the proton acceptor Asp 85 in the wild-type, bleached and retinal deficient BR samples were determined. To observe the minor changes in the spectra, the difference spectra (Fig. 2a.) were obtained after subtracting the spectrum measured at pH 7. The relative amplitude change calculated at two wavelengths 560 and 640 nm was plotted

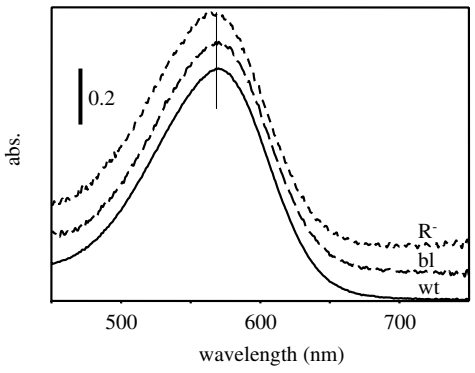

Fig. 1. The spectrum of the wild-type, bleached and retinal deficient BR measured at pH 7 in 100 mM NaCl, 20 mM MES. No characteristic difference can be observed.