Functional characterization of sensory rhodopsin II from *Halobacterium salinarum* expressed in *Escherichia coli*

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Abstract Sensory rhodopsin II (SRII) from *Halobacterium salinarum* is heterologously expressed in *Escherichia coli* with a yield of 3–4 mg of purified SRII per liter cell culture. UV/Vis absorption spectroscopy display bands characteristic for native SRII. The resonance Raman spectrum provides evidence for a strongly hydrogen-bonded Schiff base like in mammalian rhodopsin but unlike to the homologous pSRII from *Natronobacterium pharaonis*. Laser flash spectroscopy indicates that SRII in detergent as well as after reconstitution into polar lipids shows its typical photochemical properties with prolonged photocycle kinetics. The first functional heterologous expression of SRII from *H. salinarum* provides the basis for studies with its cognate transducer HtrII to investigate the molecular processes involved in phototransduction as well as in chemotransduction.

Keywords: Photosensory receptor; Archaeal phototaxis; Photocycle; Membrane protein; UV–Vis spectroscopy; Resonance Raman spectroscopy

1. Introduction

Four different retinal-containing photoactive proteins have been found in the membrane of the archaeon *Halobacterium salinarum*. Bacteriorhodopsin (BR) and halorhodopsin (HR) are light-driven ion pumps which transport protons or anions, respectively, across the membrane. Sensory rhodopsin I and II (SRI and SRII) are phototaxis receptors causing repellent and/or attractant responses of the cell to light. SRI supports HR and BR to find the optimal solarisation under anoxic conditions by triggering attractant responses to orange light but showing repellent responses to harmful UV light. SRII works exclusively as a repellent receptor to blue light which enables *Halobacteria* to seek the dark when respiration and nutrients are plentiful [1].

The four halobacterial rhodopsins are structurally very similar. The single polypeptide strand folds into seven membrane-spanning helices. The chromophore, *all-trans* retinal, is covalently bound via a protonated Schiff base to a highly conserved Lys residue of the last helix (helix G). The absorption maxima of the ground state of BR, HR and SRI are 568, 578, and 587 nm, respectively, whereas the absorption maximum of SRII is blue-shifted to 487 nm with a shoulder at 460 nm. The latter is discussed as vibrational fine structure [2,3]. The spectrum of the equivalent receptor from *Natronobacterium pharaonis* pSRII shows an absorption maximum at 497 nm with vibronic side bands at 460 and 420 nm [4].

Upon light excitation, the archaeal rhodopsins undergo characteristic and specific photoreaction cycles. Absorption of a photon leads to isomerization of the *all-trans* retinal and 13-*cis* retinal is formed. The conformational changes of the chromophore lead to a series of spectrally distinguishable intermediates which are finally reset to the initial ground state. In analogy to the nomenclature of BR, the photocycle of SRII is characterized by the intermediates K (SRII\(_{530}\)), M (SRII\(_{360}\)), and O (SRII\(_{540}\)). The latter two are slow decaying species which represent the signaling states in the repellent reaction [5–7].

SRII and SRI are complexed with their cognate transducers proteins HtrII and HtrI, respectively. The function of these accessory proteins is to transmit the photosignal from the receptor to proteins in the cytoplasm which influence the action of the flagellar motor [1]. In addition, HtrII from *H. salinarum* acts as a chemotransducer being able to sense serine as substrate via its large periplasmic ligand-binding domain [8]. The equivalent transducer pHtrII from *N. pharaonis* has no chemosensing properties. To our knowledge, HtrII is the only archaeal transducer which accepts signals from two different sources.

The binding of SRII to HtrII influences the photocycle kinetics of the receptor. In the presence of HtrII, the M intermediate (SRII\(_{530}\)) forms more rapidly and the O decay is accelerated [9]. The photochemical properties of SRI are also strongly modulated by the presence of HtrI. The lifetime of the M state in the absence of HtrI has been demonstrated to be very sensitive to external pH, whereas it is almost independent in the presence of the transducer [10,11].

Initial investigations of archaeal sensory rhodopsins were limited to spectroscopic and physiological properties since

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**Abbreviations:** SRI and SRII, sensory rhodopsin I and II from *Halobacterium salinarum*; HtrI and HtrII, corresponding transducer of SRI and SRII from *Halobacterium salinarum*; pSRII, sensory rhodopsin from *Natronobacterium pharaonis*; pHtrII, corresponding transducer of pSRII from *Natronobacterium pharaonis*; BR, bacteriorhodopsin; HR, halorhodopsin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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the homologous expression in *H. salinarum* and the multi step purification did not provide sufficient material for applications that require milligrams of protein [12,13]. In 1995, the homologous expression system as well as the purification procedure has been improved for SRI [14]. Subsequently, the heterologous expression of functional pSRII from *N. pharaonidis* has been achieved in *E. coli* [15] followed by expression of BR, HR and SRI from *H. salinarum* and purification by one step metal affinity chromatography [16,17]. Functional expression of pSRII made it possible to obtain sufficient protein for structural investigation of the receptor alone [18,19] as well as in complex with its cognate transducer [20].

In this study, we demonstrate the heterologous expression of *H. salinarum* SRII in *E. coli*. High yield purification is achieved by introducing a C-terminal histidine tag. The UV/Vis spectroscopic properties of the purified protein essentially agree with those described for SRII isolated from *H. salinarum*. However, prolonged photocycle kinetics of the heterologously expressed protein in detergent as well as in polar lipids are reported. The resonance Raman spectrum argues for a strongly hydrogen-bonded retinal Schiff base and indicates the presence of significant amounts of 13-cis retinal.

2. Materials and methods

2.1. Plasmids and strains

*E. coli* strain Top10 (Invitrogen) has been used for manipulations of the DNA and the strain BL21-CodonPlus (DE3)-RP (Stratagene) was employed for gene expression. The gene *sop*II was amplified by PCR from a total extract of *H. salinarum* L33 cells. The 5′-primer introduced a NdeI site upstream to the start codon and the 3′-primer added a coding region for six histidines upstream to the stop codon and a HindIII site downstream to the stop codon. The resulting PCR fragment was cloned in corresponding sites of the vector pET 27b (Novagen). Positive clones were identified by restriction analysis and further confirmed by sequencing.

2.2. Expression and purification

His-tagged SRII protein has been expressed in *E. coli* essentially as described [15–17]. For production of SRII transformed BL21-CodonPlus (DE3)-RP (Stratagene) was employed for gene expression. The gene *sop*II was amplified by PCR from a total extract of *H. salinarum* L33 cells. The 5′-primer introduced a NdeI site upstream to the start codon and the 3′-primer added a coding region for six histidines upstream to the stop codon and a HindIII site downstream to the stop codon. The resulting PCR fragment was cloned in corresponding sites of the vector pET 27b (Novagen). Positive clones were identified by restriction analysis and further confirmed by sequencing.

2.3. Reconstitution of SRII into lipid membranes

Purified SRII solubilized in 4 M NaCl 0.05% DDM, 50 mM MES (pH 6.0), and halobacterial polar lipids from purple membranes [21] (in 50 mM MES, pH 6.0) were mixed in a 1:30 molar ratio and incubated for 1 h at 4 °C. After adding BioBeads SM2 (BioRad), the sample was incubated for 14–16 h at 4 °C. A thin glass capillary was used to remove the BioBeads. The lipid reconstituted SRII was separated from detergent solubilized protein by centrifugation (5200 × g, 10 min, 4 °C). Finally, the pellet was resuspended in 4 M NaCl, 50 mM MES, pH 6.0.

2.4. Time-resolved UV/Vis spectroscopy

Absorption spectra of SRII were recorded on a UV-2401 PC (Shimadzu, Japan) spectrophotometer. SRII solubilized in detergent was measured in a quartz cuvette with 1 mm pathlength.

2.5. Resonance Raman spectroscopy

To avoid any accumulation of photoproducts, the resonance Raman spectrum of ground-state SRII was obtained under pre-resonant conditions with near infrared excitation from the 752 nm line of a Krypton laser (Innova 90K, Coherent, Dieburg, Germany). The laser beam was steered into a LabRam spectrometer (Jobin Yvon, Bensheim, Germany). Passing a microscope objective (Olympus), the laser emission is focussed to a spot size of 20 μm with a power of 30 mW at the sample. Photons from the sample are collected in back-scattering configuration. Rayleigh scattering is rejected by a holographic notch filter. The inelastically scattered photons are dispersed by a grating (1800 lines/mm) and detected by a Peltier-cooled CCD camera. The resulting data points of the Raman spectrum (recording time about 15 min) are spaced by 0.5 cm⁻¹, and the spectral resolution was 2 cm⁻¹. About 0.1 mg of reconstituted SRII in 10 mM sodium phosphate (pH 6.7) was dried on the inner surface of a quartz cuvette. The resulting film with a pathlength of 1 cm were used and filled with samples of a concentration of 1 mg/mL. SRII reconstituted in lipids was dried to a film and then immersed in an aqueous solution of 4 M NaCl, 50 mM MES (pH 6.0). Flash-induced absorption changes were recorded on a home-built flash-photolysis system. Monochromatic light from the continuous emission of a Xe light source was selected by placing an appropriate interference filter (half-width less than 5 nm) in front of the Xe lamp. The time-dependent intensity changes at the photomultiplier tube (R3788, Hamamatsu) were recorded with a digital oscilloscope (Hewlett-Packard 54510A, 250 MHz analogue bandwidth, 8000 data points). Data were acquired in the time-interval between 10 μs and 400 s. Data acquisition is controlled by procedures written under Visual Basic 7.0 (National Instruments Corp., Austin, USA). Pulsed sample excitation was performed with the frequency-doubled emission of a Nd-YAG laser (GCR 125, Spectra Physics, Germany; emission at 532 nm, 8 ns pulse duration, 3 mJ/cm² energy density). A holographic notch filter (OD 6 at 532 nm, Kaiser Optical Systems) was placed in front of the photomultiplier to block scattered laser light. The transient intensity changes were converted into absorbance changes by taking the logarithm of the kinetics.

3. Results and discussion

3.1. Biochemical analysis of purified SRII

The purity of the solubilized protein was analyzed by UV/Vis absorption spectroscopy (Fig. 1) and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2A). The peak ratio of the absorbance of the protein (at 280 nm) and the absorption of the bound chromophore (measured at 487 nm) was determined to ~1.7. The average yield of purified protein was about 3–4 mg of recombinant SRII per liter of cell culture as calculated from the absorption at 487 nm with an extinction coefficient of 48 000 M⁻¹·cm⁻¹ [12]. The yield is about the same as of recombinant SRI but significantly higher than of pSRII (1 mg/l) [16,17]. The UV/Vis spectrum (Fig. 1) shows the typical shoulder at 460 nm and a second shoulder around 420 nm. The latter has been previously observed in SRII preparations from
H. salinarum and was ascribed to the presence of a cytochrome [12]. Preparations of purified pSRII and BR expressed in E. coli also exhibit impurities from cytochromes [16]. A typical characteristic of the Soret band of a cytochrome in this region is the shift to longer wavelength of the reduced form in comparison to the oxidized form. The addition of sodium dithionite (Na₂S₂O₄) to the sample did not induce any changes in the spectrum (data not shown). This result indicates that the absorption at 420 nm is not caused by a cytochrome. It may be interpreted as an additional band due to the vibrational fine structure of the retinal as has been done for pSRII [4]. SRII shows a homogeneity of about 80–90% as derived from SDS–PAGE analysis (Fig. 2A). The protein preparation exhibits two dominant bands corresponding to the mass of the monomeric and dimeric SRII, respectively. Both bands were confirmed in Western blots developed with antibodies against the His-tag of the recombinant SRII (Fig. 2B). Dimer formation may be due to the unique cysteine at position 25 of SRII forming a cystine linkage.

3.2. Resonance Raman spectroscopy

Resonance Raman scattering is a prominent spectroscopic tool to selectively probe the configuration of the chromophore retinal. Pre-resonant excitation conditions (λ_ex = 752 nm) have been chosen in our experiments to avoid the photostationary accumulation of reaction intermediates during the Raman experiment. This is inevitable under resonant conditions because of the long photocycling time of SRII (vide infra). The far-red excitation wavelength ensures that only ground-state SRII is probed under retention of the selective enhancement of the retinal bands. Vibrational contributions from non-resonant Raman scattering of the apo-protein are negligible.

Fig. 3 shows the resonance Raman spectrum of SRII reconstituted into polar lipids (continuous trace). The resonance Raman spectrum of bacteriorhodopsin (BR, dotted trace) is also shown for comparison. Since the vibrational assignment is complete for the latter, it serves as a blueprint for assigning the Raman bands of SRII. The weak band at 1657 cm⁻¹ is due to the C=N–H vibration of the retinal Schiff base. The H–D exchange by immersing the protein in D₂O characteristically downshifts this band by 23 cm⁻¹ ruling out the putative assignment to non-resonant Raman scattering of the amide I vibration of the peptide bond (data not shown). The frequency of the C=N–H stretch is very high as compared to the other halobacterial retinal proteins (1640 cm⁻¹ in bacteriorhodopsin, 1632 cm⁻¹ in halorhodopsin, and 1628 cm⁻¹ in sensory rhodopsin I) [22]. The frequency is almost as high as that in mammalian rhodopsin (1660 cm⁻¹ [23]) and is indicative for very strong hydrogen-bonding of the retinal Schiff base. Very interestingly, pSRII from N. pharaonis exhibits the C=N–H stretching vibration at 1650 cm⁻¹ [24], i.e., 7 cm⁻¹ lower than in SRII from Halobacterium salinarum studied here.

The strongest band in the resonance Raman spectrum peaks at 1550 cm⁻¹ and corresponds to the C=C stretching vibration of retinal. The high frequency of this band correlates well to the absorption maximum of 490 nm in the visible range [25]. The side band at 1582 cm⁻¹ may be attributed to a higher energetic C=C mode (presumably C₁₃=C₁₄ stretch [26]). The fingerprint region (~1100–1300 cm⁻¹) where predominantly the C=C stretches absorb is very sensitive to the conformation of the retinal. The band at 1200 cm⁻¹ which has predominantly C₁₄=C₁₅ character, coincides with that of BR. Though the band at 1162 cm⁻¹ (mainly from the C₁₀–C₁₁ stretch [27]) is...
downshifted by 5 cm\(^{-1}\) with respect to BR, these two bands demonstrate the predominance of all-\textit{trans} retinal in the heterologously expressed SRII from \textit{H. salinarum}. The occurrence of the weak band at 1184 cm\(^{-1}\) indicates the presence of low amounts of 13-\textit{cis} retinal in the dark-adapted protein. This finding agrees with results from homologously expressed SRII [12]. H–D exchange reduces the intensity of the 1184 cm\(^{-1}\) band and a frequency up-shift occurs (data not shown). The sensitivity of this band towards deuteration of the Schiff base nitrogen argues for the C=\(\text{N}-\text{H}\) bond in \textit{syn}-configuration in the 13-\textit{cis} isomer [28] which is characteristic for the thermally isomerized 13-\textit{cis} state. Finally, the strong band at 1009 cm\(^{-1}\) can be assigned to the in-plane rocking mode of the two methyl groups of the retinal chain and the weak bands at 882 and 826 cm\(^{-1}\) are due to HOOP (hydrogen-out-of-plane) modes [26].

3.3. Photocycle kinetics

The photoactivity of rhodopsins is considerably affected by the chemical nature of the environment [17]. To approximate the native environment, the heterologously expressed SRII was reconstituted in polar lipids extracted from the purple membrane of \textit{H. salinarum}. Successful exchange of detergent versus lipids was observable as the solubilized and orange colored SRII moves into the pellet fraction and the supernatant becomes colorless. SDS–PAGE analysis revealed that the protein resides exclusively in the pellet (data not shown). About 50\% of SRII was functionally reconstituted into halobacterial lipids as determined by the absorption at 487 nm. The residual 50\% seems to be denatured protein.

The photocycle kinetics of SRII solubilized in detergent (Fig. 4(a)) or reconstituted in lipids (Fig. 4(b)), respectively, was analyzed by recording the transient absorption changes at three different wavelengths: 360, 475, and 525 nm. These wavelengths were selected to follow the rise and decay of the characteristic photocycle intermediates. The recovery kinetics of the ground state SRII has been monitored at 475 nm. The absorption transients most sensitive to the formation of the M intermediate were monitored at 360 nm. In the solubilized protein, the M intermediate rises with a time-constant of 350 \(\mu\)s and decays with 600 ms. SRII reconstituted in polar lipids exhibits a four times slower rise (\(\tau = 1.42\) ms) and two times faster decay of M (\(\tau = 350\) ms). The kinetics of the red-shifted intermediates K and O have been recorded at 525 nm. The K state decays with a time-constant of 50 and 180 \(\mu\)s for SRII in detergent (Fig. 4(a)) and in lipids (Fig. 4(b)), respectively. The formation of O with \(\tau = 0.48\) and 0.31 s for solubilized and reconstituted protein are in good agreement with the respective M decays as monitored at 360 nm indicating that M directly passes to O. The decay of O shows a \(\tau\) of 13.3 and 8.3 s for the solubilized and for the reconstituted protein, respectively.

Although the characteristic intermediates of the photocycle are comparable in both protein preparations and are in agreement with published data [7,12], the kinetic data indicate differences in the time constants depending on the environment of SRII. It is noticeable that the photocycles described here are prolonged. Comparable effects have been reported for the lifetime of the photointermediates especially the deceleration of the O state decay in transducer free SRII [9]. In these investigations the SRII was embedded in its native surrounding. For SRII solubilized in digitonin, the half life times were also slowed down when compared to receptor in membranes [12]. In the latter case, it is likely that the transducer HtrII was co-purified. These two effects add up in the SRII heterologously expressed in \textit{E. coli} without the transducer and solubilized in DDM.

4. Conclusions

Halobacterial SRII expressed in \textit{E. coli} shows photocycle characteristics in agreement with the homologously expressed protein. Yet, the \textit{E. coli} system delivers for the first time sufficient amounts of halobacterial SRII for investigations that require material in the range of milligrams. In combination with heterologously expressed HtrII (unpublished results) which is stimulated by the photoreceptor SRII or, alternatively, by serine, the molecular processes as well as the putative structural changes can be investigated.

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\textit{References}


