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Sensory rhodopsin I photocycle intermediate SRI_{380} contains 13-*cis* retinal bound via an unprotonated Schiff base

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Abstract Sensory rhodopsin I (SRI), the mutated derivative SRI-D76N and the complex of SRI with its transducer HtrI were overexpressed in *Halobacterium salinarium* and analyzed by resonance Raman spectroscopy. In the initial state SRI contains all-*trans* retinal bound via a protonated Schiff base as confirmed by retinal extraction which yields 95 ± 3% all-*trans* retinal. The photocycle intermediate absorbing maximally at 380 nm (SRI_{380}) contains a Schiff base linkage between the protein and 13-*cis* retinal. Extraction of illuminated SRI yields up to 93% 13-*cis* retinal. Neither the mutation D76N nor HtrI changed the vibrational pattern of the chromophore.

Key words: Sensory rhodopsin; Resonance Raman; Isomerization

1. Introduction

Sensory rhodopsin I (SRI) is one of four retinal proteins found in the plasma membrane of the halophilic Archeon *Halobacterium salinarium* mediating phototactic behaviour [1] while bacteriorhodopsin (BR) and halorhodopsin (HR) are light-driven ion pumps.

SRI is expected to have seven transmembrane helices [2] and is associated with its tranducer HtrI which has two putative transmembrane helices and a large cytoplasmic loop [3]. This complex represents the functional unit that triggers the signal transduction cascade [4].

Light absorption by the dark-state, absorbing maximally at 590 nm (SRI_{590}), leads to a thermoreversible photocycle. The intermediate SRI_{380} accumulates under photostationary conditions due to its long lifetime of about 800 ms [1]. In the HtrI-SRI complex the photocycle rates are independent of pH in the range of 3 to 8. In contrast, the decay time of the SRI_{380} intermediate of uncomplexed SRI shows an exponential increase with increasing pH [5] accompanied by a 40 nm blue shift of the initial state absorption maximum to 550 nm at alkaline pH [4,6].

BR-M, absorbing maximally at 410 nm, is a key intermediate in the photocycle of BR and the retinal was shown by resonance Raman measurements to be bound to the protein via a deprotonated Schiff base in its 13-*cis*, 14s-*trans*, 15-*anti* configuration [7,8]. Continuous illumination of HR samples in the presence of azide leads to accumulation of HR^{L}_{410} [9] which shows a Resonance Raman spectrum [10] similar to BR-M.

In SRI_{590} all-*trans* retinal and lysine-206 in helix G form a protonated Schiff base in *anti* configuration as has been established by resonance Raman experiments [11]. Light-induced all-*trans* to 13-*cis* isomerization is a prerequisite for the functioning of SRI [12]. Indeed, 13-*cis* retinal could be extracted from SRI_{380}-containing samples [13].

From FTIR studies of HtrI-free SRI and its site-directed mutant D76N it was concluded that the residue Asp-76 (corresponding to Asp-85 in BR) is protonated in SRI_{590} as well as in SRI_{380}, undergoing only a change in environment [14,15]. In the case of BR the Schiff base proton is believed to be accepted by Asp-85 during the L to M transition [16] raising the question of the fate of the proton in the SRI_{590}-to-SRI_{380} transition. It was suggested [2] that the Schiff base might be hydrolyzed in SRI_{380} to the free aldehyde and a protonated lysine residue as found for vertebrate rhodopsins. To clarify the question wether SRI resembles vertebrate rhodopsins not only in function but also in mechanism, i.e. hydrolysis of the Schiff base, we obtained spectra of the intermediate SRI_{380}. Measurements have been done on overproduced wild-type SRI [17], the mutant SRI-D76N as well as on SRI overproduced together with its transducer HtrI. For comparison spectra of BR-M and HR^{L}_{410} were recorded under similar conditions. We also obtained spectra of the unphotolyzed state SRI_{590}. Our results show unambiguously that the intermediate SRI_{380} contains a deprotonated Schiff base of retinal in the 13-*cis* configuration.

2. Materials and methods

2.1. Sample preparation

BR was isolated from *Halobacterium salinarium* strain S9 as described [18]. The isolation procedure for overexpressed HR [19] and SRI (Haupts et al., manuscript in preparation) follows that of BR with minor changes. Membrane preparations containing the HtrI-SRI complex were isolated as described for SRI from a strain overproducing both proteins by a factor of about 5 compared to wild-type level. This overproduction was achieved by transforming the BR, HR-negative strain M417 with a suicide plasmid constructed by PCR and carrying the HtrI-SRI operon under the control of the bop promotor. A more detailed description will be published elsewere.

2.2. Resonance Raman spectroscopy

The SRI-containing membranes were suspended in high salt buffer (4 M NaCl, 10 mM Na-phosphate, pH 6.0 or 4 M NaCl, 10 mM Tris pH 8.6) to an optical density at λ_{max} of 0.5–1. For recording the BR-M spectrum the pH of the purple membrane suspension in 4 M NaCl, 10 mM Tris was adjusted to 9.5. HR^{L}_{410} was produced in the presence of 50 mM NaN_3.

The Raman spectra were measured with a set-up as described [20]. Wavelength and intensity of the pump and probe beam are indicated in the legends. The spectral resolution of the setup is 2.6 cm^{-1} (3600 lines/mm gratings, 406 nm probe beam) and 4 cm^{-1} (1800 lines/mm

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gratings, 514 nm probe beam). Accuracy of peak locations is ± 1 cm^{-1}. Spectra were recorded from 700 cm^{-1} to 1700 cm^{-1} with a step width of 1 cm^{-1} and a dwell time of 1s at each position. Up to 150 scans were averaged.

2.3. Absorption measurements

Sample compositions were the same as described for the resonance Raman experiments. Due to the long lifetime of SRI_{380} at alkaline pH it was possible to record the difference spectra of the SRI_{590}-to-SRI_{380} transition on a conventional spectrometer (Aminco DW 2A, connected to a personal computer) after the actinic light had been turned off (150 W Xe-lamp, cut-off filter OG530, Schott). The difference spectrum of the Htr-SRI_{590} Htr-SRI_{380} transitions was measured with a flash photolysis spectrometer [21] using light from a dye laser (585 nm, repetition rate 50 Hz) as actinic light.

2.4. Retinal extraction

The protein was denatured by vortexing 300 μl of a SRI membrane suspension ($OD_{590} \approx 0.5$) with an equal volume of isopropanol, followed by extraction of the retinal with 2 × 1 ml of hexane. The combined organic phases were dried over Na_2SO_4. Hexane was evaporated with a stream of nitrogen and the residue was redissolved in 100 μl hexane. HPLC analysis was performed using a column filled with Lichrosorb Si60 5 μm (Bischoff, Leonberg, Germany) and a mixture of hexane and ethylacetate (95:5) as solvent.

Samples were dark-adapted over-night at room temperature. Illumination for SRI_{380} accumulation was performed with light from a 150 W slide projector filtered through a 515 nm cut-off filter (OG_{515} Schott, Mainz). The isopropanol was added to the illuminated sample.

3. Results

3.1. Spectra of the initial states

Wild-type SRI, the mutant SRI-D76N and the HtrI-SRI complex are called collectively 'SRI samples' in the following to avoid lengthy expressions. In Fig. 1 resonance Raman spectra of the three SRI_{590} samples at pH 6.0 in H_2O and D_2O are presented. Due to the lower expression level of the HtrI-SRI complex the signal-to-noise ratio is much lower compared to the spectra of SRI and SRI-D76N. The spectra of all SRI samples are almost identical. Interestingly, neither the mutation at position 76 nor the presence of the transducer HtrI significantly alter the vibrational features of the chromophore at pH 6. For comparison the spectra of overproduced HR_{578} and BR_{568} were recorded under similar conditions (data not shown).

The C = N stretching motion ν(C = NH) and the C = N-H bending motion γ(C = N-H) (Table 1) are coupled in H_2O but decoupled in D_2O as described for protonated Schiff bases of retinal and retinal proteins [22]. Thus the ν(C = ND) represents an almost 'pure' C = N vibration. The ν(C = NH) bands are

Table 1
Summary of peak positions (given in cm^{-1}) of the ν(C = N) mode in H_2O and D_2O, the γ(C-N-D) and the $\nu(C = C)^+$ vibrations

Vibration	SRI_{590}	SRI-D76N	HtrI-SRI	SRI [11]	BR	HR_{578}	HR [10]
ν(C = NH)	1634	1635	1635	1628	1642	1633	1632
ν(C = ND)	1616	1617	1618	1620	1624	1624	1621
difference	18	18	17	8	18	9	11
γ(C-N-D)	974	974	972	960	978	970	970
ν(C = C)	1520	1519	1522	1519	1529	1526	1526

For comparison the results from earlier works on SRI_{590} [11] and HR_{578} [10] are included.

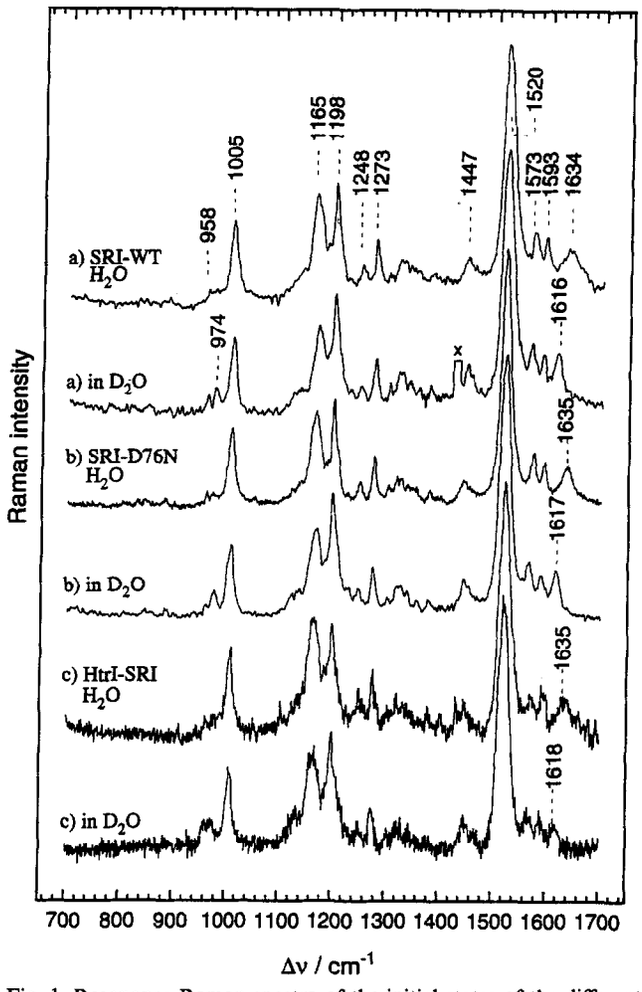

Fig. 1. Resonance Raman spectra of the initial states of the different SRI samples in H_2O at pH 6.0 and D_2O. Probe beam: 514-nm line from an Ar^+ laser, 10 mW intensity. The cuvette was rotating with 50 Hz (a) in D_2O: the feature marked × is due to a laser artifact).

broader in HR_{578} and the SRI_{590} than in BR with halfwidths of about 20 cm^{-1} for HR_{578} and 35 cm^{-1} for SRI. The bands are narrowed down in D_2O to 15 cm^{-1} and 18 cm^{-1}, respectively and the new γ(C = N-D) band appears at 974 cm^{-1}.

The most intense band in the region from 1500 cm^{-1} to 1600 cm^{-1} is the symmetric stretch $\nu(C = C)^+$ lying at 1520 cm^{-1} for SRI_{590} (Table 1) with a larger halfwidth than the corresponding band in BR_{568}. Bands at 1573 cm^{-1} and 1593 cm^{-1} represent more localized ν(C = C) modes which are 9 cm^{-1} and 6 cm^{-1} down-shifted compared to BR [23].

Small differences between the three retinal proteins are seen in the fingerprint region from 1100 cm^{-1} to 1250 cm^{-1} which is indicative for the geometry of the chromophore. Two major bands are located at 1198 cm^{-1} and 1165 cm^{-1} in SRI_{590}. They are down-shifted by 4 cm^{-1} and 5 cm^{-1} compared to BR_{568} and HR_{578}. The band at 1165 cm^{-1} is much broader and more intense than the corresponding band in BR_{568}. A C(8)-C(9) stretch vibration is resolved at 1215 cm^{-1} in BR_{568} [23] and seen as a shoulder in HR_{578} but not in SRI_{590}. Despite those minor differences this pattern is indicative for an all-*trans* retinal as has been proven to exist in BR_{568} [7]. Retinal extraction of

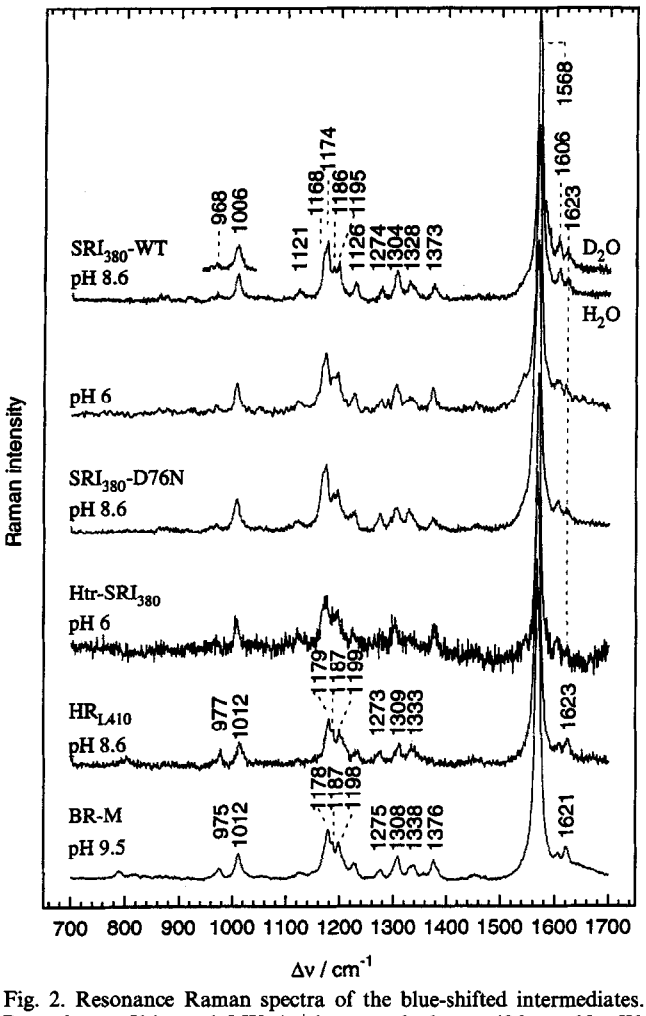

Fig. 2. Resonance Raman spectra of the blue-shifted intermediates. Pump beam: 514-nm, 1.5 W, Ar^+ laser; probe beam: 406-nm, 10 mW, Kr^+ laser. The cuvette was rotating with 10 Hz.

dark-adapted SRI samples yielded 95 ± 3% all-*trans* retinal (Table 2) thus confirming the spectroscopic result.

The C(14)-C(15) stretch mode at 1165 cm^{-1} can be used as a marker band for the configuration of the C = N bond due to the different sensitivity to Schiff base deuteration in *syn* and *anti* configuration [24]. Deuteration of the SRI_{590} samples, HR_{578} and BR_{568} does not lead to a significant up-shift of the mode, which is expected for a *syn* configuration, indicating an *anti* geometry in all cases.

While several hydrogen-out-of-plane modes (HOOPs), located below 1000 cm^{-1}, are resolved in BR_{568} and HR_{578}, almost no features are found in the SRI_{590} samples. This implies that the polyene chain is less twisted [25] in SRI_{590} than in either BR_{568} or HR_{578}.

3.2. The blue shifted intermediates

The absorption difference spectra in Fig. 3 show the accumulation of SRI_{380} under continuous illumination. A well structured band with maxima at 397, 379 and 362 nm is seen (not resolved for HtrI-SRI_{380}).

Resonance Raman spectra of SRI_{380} are shown in Fig. 2 in comparison with BR-M and HR^L_{410}. The contributions of a cytochrome contamination in the spectra of SRI_{380} and HR^L_{410} and a background were eliminated by subtracting a probe-only spectrum.

The C = N stretch mode is located at 1621 cm^{-1} in BR-M and at 1623 cm^{-1} in all other species. No shift of this band is observed in D_2O (shown for SRI_{380}, Fig. 2) and no additional band at about 970 cm^{-1} appears. This proves that in SRI_{380} retinal is covalently linked to the protein via a deprotonated Schiff base as it is in BR-M and HR^L_{410}.

Even though the absorption maximum of SRI_{380} is blue-shifted by 30 nm compared to BR-M and HR^L_{410} the strong in-phase C = C stretch mode peaks at 1568 cm^{-1} in either case.

Characteristic bands of the BR-M and HR^L_{410} spectra are located at 1198 cm^{-1} and 1178 cm^{-1} with a small shoulder at 1187 cm^{-1}. In the case of SRI_{380} there is a downshift to 1195 cm^{-1} and 1174 cm^{-1} of the corresponding bands. A small shoulder at 1168 cm^{-1} is resolved which is not present in either the BR-M or HR^L_{410} spectra. The overall pattern clearly shows that the retinal is in the 13-*cis* configuration also confirmed by the retinal extraction data (Table 2). The larger content of 13-*cis* retinal at alkaline pH is due to the prolonged lifetime of SRI_{380} at high pH.

There are well resolved HOOP modes in the BR-M and HR^L_{410} spectra at 975 cm^{-1} and 789 cm^{-1}, but only very small features are seen below 1000 cm^{-1} for SRI_{380}, again indicating less torsional strain in SRI_{380}.

4. Discussion

The resonance Raman spectra of the initial states absorbing maximally at 590 nm are identical within the limits of the experimental accuracy. This is especially interesting because the presence of HtrI up-shifts the apparent p*K* of the SRI_{590}-to-SRI_{550} *t*ransition by about 1 unit to 8.5. In SRI_{590} Asp-76 is protonated at low pH [15]. Thus the SRI_{590}-to-SRI_{550} transition should be due to a deprotonation of Asp-76 since this transition is not seen in the D76N mutant. The p*K* shift induced by HtrI therefore implies a physical interaction of HtrI and SRI resulting in a change of the microenvironment of Asp-76. A stable complex of the two proteins was also inferred from kinetic data [4].

Substitution of Asp-76 in SRI does not shift ν(C = ND) which is expected to be influenced by surrounding charges. This supports the assumption that Asp-76 is protonated in SRI_{590} because the mutation apparently does not change the charge pattern in the vicinity of the Schiff base. Preliminary results on SRI_{550} in H_2O suggest an up-shift of ν(C = NH) to a value >1640 cm^{-1} comparable with BR_{568}. This could reflect similar situations in BR_{568} and in SRI_{550} both having a deprotonated aspartate counterion.

Table 2
All-*trans*/13-*cis* composition of retinal extracted from the SRI samples in either dark-adapted (d.a.) or illuminated (ill.) state

	SRI_{590}		SRI-D76N		Htr-SRI	
Sample	all-*trans*	13-*cis*	all-*trans*	13-*cis*	all-*trans*	13-*cis*
d.a. pH 6.0	93%	7%	96%	4%	97%	3%
d.a. pH 8.5	92%	8%	94%	6%	98%	2%
ill. pH 6.0	43%	54%	20%	79%	18%	81%
ill. pH 8.5	34%	63%	7%	93%	12%	88%

Deviations of the sum from 100% are due to minor amounts of other isomers.

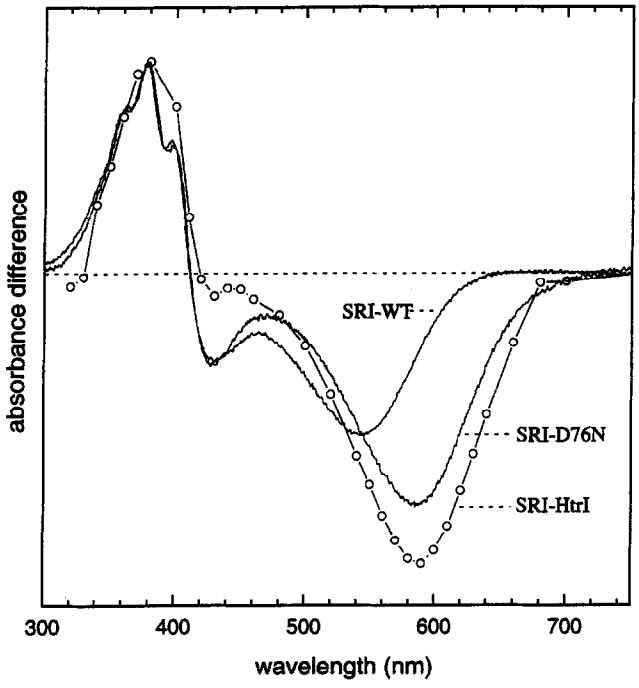

Fig. 3. Difference spectra of SRI initial states and SRI_{380}. The amplitudes of the spectra are normalized at 380 nm and correspond to 0.01 OD for HtrI-SRI_{380} and 0.1 OD for SRI_{380} and SRI-$D76N_{380}$ (linear scale).

The broadness of the $\nu(C = NH)^+$ band which is removed upon deuteration could be accounted for by assuming an inhomogeneous microenvironment of the Schiff base. Contributions from SRI photocycle intermediates were excluded by recording spectra at different probe beam intensities ranging from 1 to 10 mW which were found to be identical. Retinal extractions of SRI_{590} samples and HPLC analysis yielded 95 ± 3% all-*trans* retinal excluding an inhomogeneous mixture of initial states on the basis of retinal isomers.

It should be noted that our results differ from those of Fodor et al. [11] who reported a $\nu(C = NH)$ at 1628 cm^{-1}, a deuterium shift of only 8 cm^{-1} and a $\gamma(C = N\text{-}D)$ at 960 cm^{-1}. Also Rath et al. [15] and Bousché et al. [14] assigned a negative band at 1627 cm^{-1} as the $\nu(C = NH)$ in FTIR difference spectra.

The band pattern of the SRI_{590} samples in the fingerprint region is very similar to that of BR_{568} and HR_{578} strongly suggesting the same all-*trans* geometry of the retinal chain in all three species, consistent with an earlier work [11]. The insensitivity of the ν[C(14)-C(15)] vibration to deuteration supports an *anti*-configuration of the C = N bond [24]. Similar retinal protein interactions in BR_{568}, HR_{578} and SRI_{590} were also concluded from reconstitution experiments with retinal analogs [12,26]. What remains as a difference is the reduced intensity of HOOP modes which indicates a less twisted polyene chain in SRI_{590}.

Resonance Raman spectra of the blue-shifted intermediates reveal a high degree of similarity between SRI_{380}, BR-M and HR^L_{410}. The appearence of a band at 1623 cm^{-1} in SRI_{380} and its insensitivity to deuteration proves the presence of an unprotonated Schiff base linkage between retinal and the protein. Neither the complexation of SRI by HtrI nor the mutation D76N or the variation of the pH do influence the C = N vibration. The presence of a C = N bond throughout the photocycle would also be concluded from the proton translocation activity found for Htr-free SRI (Haupts et al., manuscript in preparation).

The HOOP region of SRI_{380} does not contain well-resolved features as do the spectra of BR-M and HR^L_{410}. This would indicate a less torsionally strained chromophore as is also the case for the unphotolyzed states.

The fingerprint region from 1100–1400 cm^{-1} shows excellent correspondence between the blue-shifted intermediates of BR, HR and SRI. A striking difference is the absence of the 1373 cm^{-1} band in the HR^L_{410} spectrum as found in an earlier work [10]. For BR-M the chromophore structure represented by this band pattern has been shown to be a 13-*cis*, 14-s-*trans*, 15-*anti* conformation on the basis of resonance Raman measurements [7,8] and solid-state NMR studies [27]. Illumination of SRI_{590} leads to the accumulation of SRI_{380} (Fig. 3) and retinal extraction from illuminated SRI yields an increase of 13-*cis* retinal in agreement with earlier results [13]. From the close correspondence of the resonance Raman spectra and the retinal extraction data we therefore conclude that SRI_{380} contains 13-*cis* retinal.

Most interestingly, the chromophore geometry of SRI_{380} seems not to be influenced by complexation of HtrI (at least at our signal-to-noise ratio). SRI_{380} is the signalling state [28] and stays complexed with HtrI throughout the photocycle [4]. Since the signal must be relayed to HtrI by some kind of interaction one could have expected to find differences between complexed and free SRI_{380}.

The presence of a Schiff base was also inferred for bovine metarhodpsin II [29] but unlike the vertebrate rhodopsins, which finally undergo hydrolysis, SRI_{380} thermally reverts to the initial state. We have demonstrated that SRI_{380} shares this feature with the blue-shifted intermediates BR-M and HR^L_{410}. This represents a fundamental difference in the retinal protein interaction between the vertebrate and the bacterial rhodopsins. Only the latter group is able to catalyze a 13-*cis* to all-*trans* thermal isomerization of retinal by their protein moieties.

In summary we have shown that a striking similarity exists in the basic photochemistry of BR, HR and SRI. The basis for the isomerization of a protonated all-*trans* retinal Schiff base to a deprotonated 13-*cis* Schiff base is provided by almost identical protein-chromophore interactions in the three pigments despite their different biological function. This provides further evidence for the close relatedness of SRI to the bacterial rhodopsins BR and HR although its role as a sensory pigment resembles the vertebrate rhodopsin function.

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