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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 31, Issue of November 5, pp. 18280-18283, 1989

Structure of the Retinal Chromophore in Sensory Rhodopsin I from Resonance Raman Spectroscopy*

(Received for publication, June 9, 1989)

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Sensory rhodopsin I (SR-I) is a retinal-containing pigment which functions as a phototaxis receptor in *Halobacterium halobium*. We have obtained resonance Raman vibrational spectra of the native membrane-bound form of SR_{587} and used these data to determine the structure of its retinal prosthetic group. The similar frequencies and intensities of the skeletal fingerprint modes in SR_{587}, bacteriorhodopsin (BR_{568}), and halorhodopsin (HR_{578}) as well as the position of the dideuterio rocking mode when SR-I is regenerated with 12,14-D_2 retinal (915 cm^{-1}) demonstrate that the retinal chromophore has an all-*trans* configuration. The shift of the C=N stretching mode from 1628 cm^{-1} in H_2O to 1620 cm^{-1} in D_2O demonstrates that the chromophore in SR_{587} is bound to the protein by a protonated Schiff base linkage. The small shift of the 1195 cm^{-1} C_{14}–C_{15} stretching mode in D_2O establishes that the protonated Schiff base bond has an *anti* configuration. The low value of the Schiff base stretching frequency together with its small 8 cm^{-1} shift in D_2O indicates that the Schiff base proton is weakly hydrogen bonded to its protein counterion. This suggests that the red shift in the absorption maximum of SR-I (587 nm) compared with HR (578 nm) and BR (568 nm) is due to a reduction of the electrostatic interaction between the protonated Schiff base group and its protein counterion.

Sensory rhodopsin I (SR-I),[1] halorhodopsin (HR), and bacteriorhodopsin (BR) are retinal proteins found in the cytoplasmic membrane of the bacterium *Halobacterium halobium* (Lanyi, 1986; Spudich and Bogomolni, 1988; Stoeckenius and Bogomolni, 1982). Although these proteins have similar molecular masses (~26,000 daltons) and photocycles, their physiological roles are very different. BR uses light energy to translocate protons out of the cell, HR is an inwardly directed light-driven chloride ion pump, and SR-I is a receptor for bacterial phototaxis. While the structure of the retinal chromophore in all of bacteriorhodopsin's intermediates (Ames *et al.*, 1989; Fodor *et al.*, 1988; Mathies *et al.*, 1987) and many of halorhodopsin's intermediates are known (Fodor *et al.*, 1987; Rothschild *et al.*, 1988; Pande *et al.*, 1989; Smith *et al.*, 1984a), little is known about the chromophore structure in SR-I.

Absorption and phototaxis action spectra have provided most of the available information about the structure and function of SR-I. The photocycle begins when the SR_{587} form absorbs a photon producing the red-shifted S_{610} species (Fig. 1). The decay of S_{610} proceeds through S_{560} and S_{373} before returning thermally to SR_{587} in ~750 ms (Bogomolni and Spudich, 1987). This cyclic one-photon reaction generates the photoattractant response. The S_{373} intermediate can also be photochemically driven back to SR_{587} through S^b_{510} producing the photorepellant response (Bogomolni and Spudich, 1984). Regeneration of SR-I with dihydroretinal analogues has indicated that the mechanism of the opsin shift is similar to that in BR (Spudich *et al.*, 1986), and regeneration with C_6–C_7 locked analogues has shown that SR-I binds the chromophore in the 6-*S*-*trans* conformation (Baselt *et al.*, 1989).

To more completely define the structure of the chromophore in SR_{587} we have obtained resonance Raman spectra of SR-I in native membranes. Raman spectra of SR-I were obtained in H_2O and D_2O buffer and after regenerating the pigment with 12,14-D_2-labeled retinal. These results show that the chromophore in SR_{587} is bound to the protein by a protonated Schiff base linkage and that it adopts a 13-*trans* and C=N *anti* configuration. The Schiff base vibrational properties further suggest that the Schiff base proton is weakly hydrogen-bonded to its protein counterion. This result is critical for understanding the factors which dictate the position of the absorption maximum in this light-sensing pigment.

MATERIALS AND METHODS

Sensory rhodopsin was isolated from a BR, HR, and retinal deficient strain of *H. halobium* (Flx5R) (Spudich and Spudich, 1982). The SR membranes were isolated as previously described (Spudich and Bogomolni, 1983) and washed by repeated high speed centrifugation (200,000 x *g*). The samples were regenerated to ~95% completion with either all-*trans* retinal or 12,14-D_2 all-*trans* retinal (Pardoen *et al.*, 1986). The membranes were pelleted and resuspended in ~6–7 ml of pH 7.0 buffer (3 M NaCl, 25 mM Tris). Isolation, bleaching, and regeneration of halorhodopsin was carried out as previously described (Baselt *et al.*, 1989; Taylor *et al.*, 1983).

To obtain Raman spectra of SR_{587}, 6 ml of membrane suspension were placed in a reservoir and circulated by a peristaltic pump through a 1.5-mm diameter glass capillary which served as the Raman cell. The flow speed (40 cm/s) and laser power (<1 milliwatt at 514.5 or 568 nm) were adjusted so that the photoalteration parameter was kept below 0.1 (Mathies *et al.*, 1976). The recirculation time (~8 s) was sufficient to ensure recovery of the photolyzed SR. The sample temperature was maintained at ~18 °C. The Raman spectra of BR and HR were obtained as previously described (Fodor *et al.*, 1987).

Raman spectra were acquired using a Spex 1401 double monochromator with photon counting detection interfaced to a PDP 11/23 computer. The monochromator step size was 2 cm^{-1} with a dwell time of 6–10 s, and the spectral bandpass was ~6 cm^{-1}. Typically 10–20

* This work was supported by Grants CHE 86-15093 and DMB 87-17913 from the National Science Foundation and by Grants GM 27057, GM 11266, and GM 34219 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SR, sensory rhodopsin; HR, halorhodopsin; BR, bacteriorhodopsin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

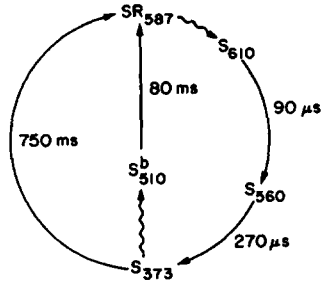

FIG. 1. **Sensory rhodopsin I photocycle adapted from Bogomolni and Spudich (1987).** Photolysis of SR_{587} produces the red-shifted S_{610} photoproduct which thermally decays back to the parent SR_{587} through S_{560} and S_{373}. This process yields the photoattractant response. Secondary photolysis of the long-lived S_{373} intermediate by blue light returns the pigment to SR_{587} by an alternate path through S^b_{510} which produces the photorepellent response.

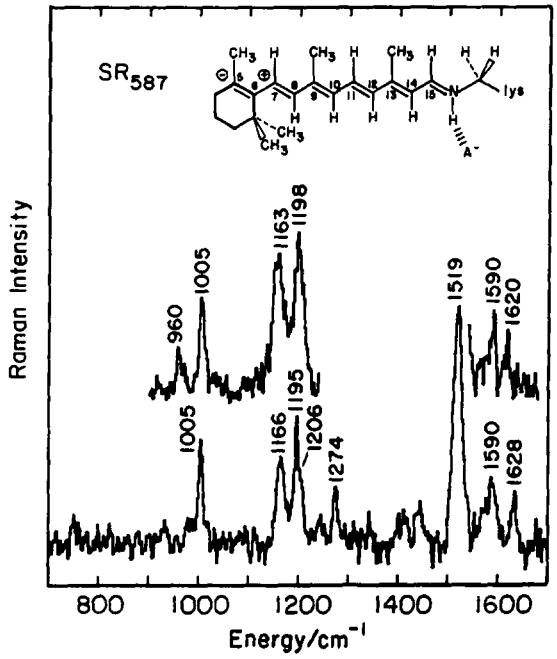

FIG. 2. **Resonance Raman spectra of SR_{587} in 3 M NaCl, 25 mM Tris buffer at pH 7.0.** Spectra were obtained with either 568 nm excitation in H_2O (bottom) or with 514.5 nm excitation in D_2O (*top*). The indicated Schiff base frequencies were determined by averaging five independent measurements in H_2O and four in D_2O.

scans were digitally summed. The backgrounds were removed using a cubic spline fitting routine.

RESULTS

The resonance Raman spectrum of SR_{587} in native membranes is presented in Fig. 2. The most prominent feature is the ethylenic stretch observed at 1519 cm^{-1}. An approximate linear correlation exists between the absorption maximum of retinal compounds and the ethylenic stretching frequency (Aton *et al.*, 1977). In SR_{587}, the 1519 cm^{-1} frequency of the ethylenic stretch corresponds to an absorption maximum of ~600 nm which is in reasonable agreement with the measured absorption maximum of 587 nm.

The line at 1628 cm^{-1} is near the frequency expected for the C=N stretch of a Schiff base linkage to the protein. This assignment as well as the protonation state of the Schiff base can be determined by the sensitivity of this mode to deuteration. When the SR_{587} membranes are suspended in D_2O (Fig. 2, *inset*) the 1628 cm^{-1} band shifts down to 1620 cm^{-1}, and a new band appears at 960 cm^{-1}. The 1628 cm^{-1} band is assigned as a protonated Schiff base stretch based on this shift, and the 960 cm^{-1} band is the N-D rocking mode.

In retinals the C-C skeletal stretching modes in the 1100–1400 cm^{-1} "fingerprint region" are sensitive indicators of chromophore geometry (Smith *et al.*, 1987a, 1987b). In Fig. 3, the resonance Raman spectra of BR_{568}, HR_{578}, and SR_{587} are compared. The similarity of the SR_{587} fingerprint modes to those of HR_{578} and BR_{568} argues that the chromophore in SR_{587} is all-*trans*. By analogy with the all-*trans* BR_{568} assign-

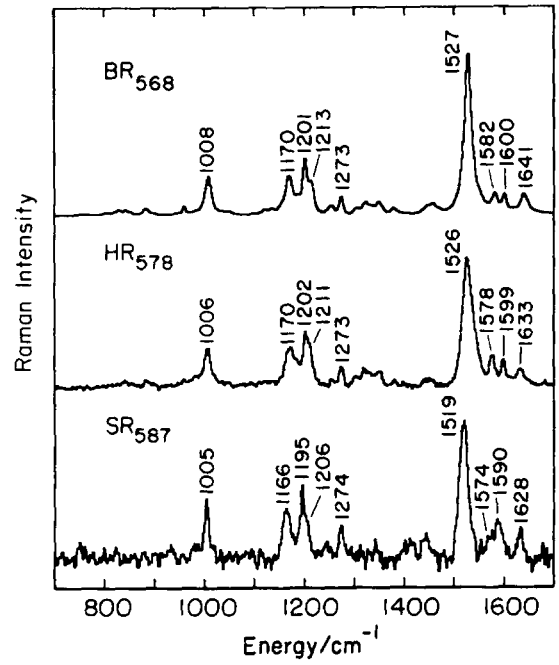

FIG. 3. **Comparison of the resonance Raman spectra of BR_{568}, HR_{578}, and SR_{587}.** The BR_{568} and HR_{578} spectra were obtained with 514.5 nm excitation in 3 M NaCl, 10 mM HEPES buffer at pH 7.3.

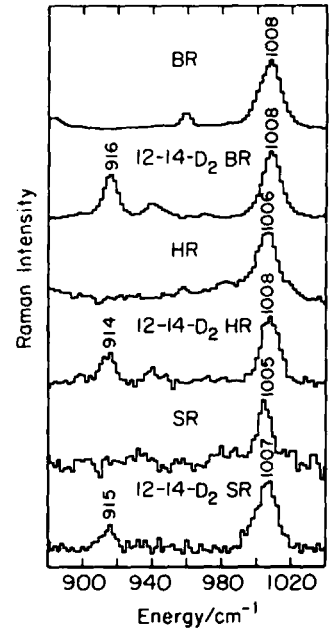

FIG. 4. **Resonance Raman spectra of BR_{568}, HR_{578}, and SR_{587} regenerated with 12,14-D_2 all-*trans* retinal (514.5 nm excitation).** Native spectra of each pigment are shown for comparison.

ments (Smith *et al.*, 1987a), the mode at 1166 cm^{-1} is assigned to the C_{10}–C_{11} stretch, the 1195 cm^{-1} band is the C_{14}–C_{15} stretch, and the C_8–C_9 stretch is the shoulder at 1206 cm^{-1}. As a further test of the chromophore configuration, BR, HR, and SR were regenerated with 12,14-D_2 retinal (Fig. 4). In all-*trans* retinal chromophores, the coupled 12,14-D_2 rocking mode appears at ~915 cm^{-1} (Smith *et al.*, 1985a). In 13-*cis* retinals, the 12,14-D_2 rocking mode appears near 945 cm^{-1}. The coupled 12,14-D_2 rocking mode is clearly apparent near 915 cm^{-1} in each pigment. This confirms that both HR_{578} and SR_{587} contain 13-*trans* retinal chromophores.

The configuration of the C=N bond in protonated Schiff base chromophores can be determined by the sensitivity of the C_{14}–C_{15} stretching mode to Schiff base deuteration (Smith *et al.*, 1984b). In C=N *syn* chromophores, the C_{14}–C_{15} stretch near 1200 cm^{-1} and the N–H rocking mode near 1350 cm^{-1} are strongly coupled. Therefore, when the N–H proton is exchanged for a deuteron the C_{14}–C_{15} stretch upshifts by ~50 cm^{-1}. In C=N *anti* chromophores, the coupling between the C_{14}–C_{15} stretch and the N–H rock is negligible, and Schiff base deuteration results in no significant shift of the C_{14}–C_{15} stretching frequency. In the *inset* of Fig. 2 there is only a 3-cm^{-1} upshift of the C_{14}–C_{15} stretching mode in D_2O. This indicates that the C=N configuration in SR_{587} is anti.

DISCUSSION

We have obtained the resonance Raman spectrum of SR_{587} in native Flx5R membranes. The analysis of the vibrational spectra shows that the chromophore in SR_{587} is an all-*trans* C=N *anti* protonated Schiff base. The C–C single bond fingerprint modes are very similar in BR_{568}, HR_{578}, and SR_{587}. This indicates that they have similar retinal chain structures and environments in agreement with recent retinal analog regeneration experiments (Baselt *et al.*, 1989; Lanyi *et al.*, 1988; Spudich *et al.*, 1986). In contrast, the ethylenic frequency is significantly lower in SR_{587} (1519 cm^{-1}) than it is in BR_{568} (1527 cm^{-1}) or HR_{578} (1526 cm^{-1}). This is due to an increase in the electron delocalization of the chromophore consistent with the red-shifted absorption maximum of SR_{587}.

The shift of the chromophore absorption maximum in BR_{568}, HR_{578}, and SR_{587} compared with the protonated Schiff base in solution is known as the opsin shift. The opsin shift in BR is ~5100 cm^{-1}, in HR it is ~5400 cm^{-1}, and in SR-I it is ~5700 cm^{-1}. In all three pigments, isomerization from a twisted 6-*S*-*cis* conformation in solution to a planar 6-*S*-*trans* structure accounts for at least 1200 cm^{-1} of the opsin shift (Baselt *et al.*, 1989; Harbison *et al.*, 1985; van der Steen *et al.*, 1986). In BR, the majority of the residual opsin shift (2500–3900 cm^{-1}) has been attributed to a decrease in the electrostatic interaction between the positively charged Schiff base group and its protein counterion (Lugtenburg *et al.*, 1986; Spudich *et al.*, 1986). The most direct experimental support for this interpretation is provided by the ^{15}N NMR and vibrational data, which demonstrate that the Schiff base is weakly hydrogen-bonded to its protein counterion (Baasov *et al.*, 1987; Lugtenburg *et al.*, 1986; Harbison *et al.*, 1983).

The resonance Raman spectrum of SR-I provides a great deal of information about its Schiff base hydrogen-bonding environment. There is an empirical correlation between the Schiff base stretching frequency (and its D_2O shift) and the strength of the hydrogen bond[2]. The Schiff base properties for BR, HR, and SR are summarized in Table I along with their opsin shifts. In the protonated Schiff base in solution, there is a strong electrostatic interaction between the Cl^- anion and the positively charged Schiff base which keeps the positive charge in the retinyl cation localized. The absorption maximum is at ~440 nm and is correlated with a high Schiff base frequency and a large 23-cm^{-1} deuterium shift. In the red-absorbing pigments, the Schiff base stretch is found at lower wave numbers, and the deuterium shift is smaller. For example, the opsin shift is 300 cm^{-1} greater in HR_{578} than it is in BR_{568}, and this is correlated with a Schiff base frequency that is 8 cm^{-1} lower and a 4-cm^{-1} decrease in the deuterium shift. In SR_{587}, the opsin shift is 300 cm^{-1} greater than that in HR_{578}, the Schiff base frequency decreases 5 cm^{-1}, and the deuterium shift decreases by another 4 cm^{-1}. The increased opsin shift in SR_{587} is clearly correlated with a weakening of the hydrogen-bonding interaction between the Schiff base and its counterion. *This strongly suggests that a weakening of the electrostatic interaction between the Schiff base group and its protein counterion is responsible for the red shift of the* λ_{max} *in* SR_{587} *and that the opsin shift in these pigments is controlled by this interaction.*

The structure of the retinal chromophore in SR-I is presented in Fig. 2. The C_6–C_7 *S*-*trans* conformation was deduced from pigment regeneration experiments using 6-*S*-*cis* and 6-*S*-*trans* locked analogues (Baselt *et al.*, 1989). Rotation of the C_6–C_7 bond to *S*-*trans* is expected to contribute at least 1200 cm^{-1} to the opsin shift (van der Steen *et al.*, 1986). The difference between the opsin shift of the locked 6-*S*-*cis* and 6-*S*-*trans* analogues provided evidence for a weak interaction of the chromophore with a protein dipole in the C_6 to C_7 vicinity, similar to what has been proposed for BR (Harbison *et al.*, 1985). In BR, the dipolar interaction seems to contribute about 500 cm^{-1} to the opsin shift (Lugtenburg *et al.*, 1986), and the magnitude of this interaction in SR-I is probably similar (Baselt *et al.*, 1989). The *dotted line* in the structure of Fig. 2 indicates a weakened hydrogen-bonding interaction between the Schiff base group and its counterion. Although a quantitative relationship between the Schiff base vibrational properties and the counterion environment is not yet available, it is reasonable to infer that the majority of the opsin shift in SR-I is due to a weak electrostatic interaction between the Schiff base and its counterion as has been found in more detailed studies of BR (Baasov *et al.*, 1987; Lugtenburg *et al.*, 1986; Spudich *et al.*, 1986). Now that the structure of the retinal chromophore in SR-I is known, it will be of interest to study the chromophore and protein structure in SR's intermediates to determine how light absorption is coupled to sensory transduction in this pigment.

Acknowledgements—We thank John and Elena Spudich for providing the Flx5R mutant of *H. halobium*.

TABLE I

Characteristics of the Schiff base mode in BR, HR, and SR

	$\nu_{C=N}$	D_2O shift	Opsin shift
	cm^{-1}		cm^{-1}
PSB[a]	1654[b]	23[b]	0
BR_{568}	1641[c]	17[c]	5100[d]
HR_{578}	1633[e] ± 2	13[e] ± 2	5400[d]
SR_{587}	1628[e] ± 3	8[e] ± 4	5700[d]

[a] Chloride salt of the *N*-butylamine protonated Schiff base.
[b] From Smith *et al.* (1985b).
[c] From Smith *et al.* (1987a).
[d] From Baselt *et al.* (1989).
[e] From this work and Fodor *et al.* (1987).

[2] Coupling between the N–H rock at ~1350 cm^{-1} and the C=N stretch pushes the Schiff base normal mode to higher wavenumbers. Decreasing the strength of the hydrogen bond decreases the magnitude of the N–H rock and C=N stretch coupling (Baasov *et al.*, 1987; Smith *et al.*, 1985b, 1987a; Rodman Gilson *et al.*, 1988). When the Schiff base proton is exchanged for a deuteron this shifts the N–H rocking frequency to ~950 cm^{-1}, and the corresponding shift of the Schiff base mode provides a measure of the rock-stretch coupling strength.

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