Koike, H., Ikeuchi, M., Hiyama, T., Mamada, K., & Inoue, Y. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 351–354, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Metz, J. G., Nixon, P. J., Rögner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.
Nanba, O., & Satoh, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 109–112.
Nilsson, F., Simpson, D. J., Andersson, B., & Jansson, C. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 299–302, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Nixon, P. J., Metz, J. G., Rögner, M., & Diner, B. A. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 471–474, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Nixon, P. J., Chisholm, D., & Diner, B. A. (1991) in *Plant Protein Engineering* (Gutteridge, S., & Shewry, P., Eds.) Edward Arnold, Baltimore, MD (in press).
Petersen, J., Dekker, J. P., Bowlby, N. R., Ghanotakis, D. F., Yocum, C. F., & Babcock, G. T. (1990) *Biochemistry 29*, 3226–3231.
Rochaix, J.-D., & Erickson, J. (1988) *Trends Biochem. Sci. (Pers. Ed.) 13*, 56–59.
Rochaix, J.-D., Kuchka, M., Mayfield, S. Schirmer-Rahire, M., Girard-Bascou, J., & Bennoun, P. (1989) *EMBO J. 8*, 1013–1021.
Rögner, M. (1990) *J. Chromatogr. 512*, 219–226.
Rögner, M., Dekker, J. P., Boekema, E. J., & Witt, H. T. (1987) *FEBS Lett. 219*, 207–211.
Rögner, M., Nixon, P. J., & Diner, B. A. (1990) *J. Biol. Chem. 265*, 6189–6196.
Schatz, G. H., & van Gorkom, H. J. (1985) *Biochim. Biophys. Acta 810*, 283–294.
Takahashi, Y., & Katoh, S. (1986) *Biochim. Biophys. Acta 848*, 183–192.
Taylor, L. A., & Rose, R. E. (1988) *Nucleic Acids Res. 16*, 358.
Vermaas, W. F. J., Williams, J. G. K., & Arntzen, C. J. (1987) *Plant Mol. Biol. 8*, 317–326.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Vermaas, W. F. J., Ikeuchi, M., & Inoue, Y. (1988b) *Photosynth. Res. 17*, 97–113.
Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.
Williams, J. G. K., & Chisholm, D. A. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. IV, pp 809–812, Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
Yamagishi, A., & Katoh, S. (1985) *Biochim. Biophys. Acta 807*, 74–80.
Yamaguchi, N., Takahashi, Y., & Satoh, K. (1988) *Plant Cell Physiol. 29*, 123–129.
Yu, J., & Vermaas, W. F. J. (1990) *Plant Cell 2*, 315–322.

Conformational Changes in Sensory Rhodopsin I: Similarities and Differences with Bacteriorhodopsin, Halorhodopsin, and Rhodopsin†

Olaf Bousché,‡ Elena N. Spudich,§ John L. Spudich,§ and Kenneth J. Rothschild*,‡

Physics Department and Program in Cellular Biophysics, Boston University, Boston, Massachusetts 02215, and Department of Structural Biology and Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461

Received October 22, 1990; Revised Manuscript Received March 8, 1991

ABSTRACT: FTIR difference spectra have been obtained for the $sR_{587} \rightarrow S_{373}$ phototransition of sensory rhodopsin I (sR-I), a signal-transducing protein of *Halobacterium halobium*. The vibrational modes of the sR_{587} chromophore have frequencies close to those of the bacteriorhodopsin bR_{568} chromophore, confirming that the two chromophores have very similar structures and environments. However, the sR-I Schiff base C=N stretch frequency is downshifted relative to bR, consistent with weaker hydrogen bonding with its counterion(s). The carboxyl (COOH) stretch modes of sR-I and halorhodopsin (hR) are at the same frequencies. On the basis of sequence homologies, these bands can be assigned to Asp-106 in helix D and/or Asp-201 in helix G. In contrast, no band was found that could be assigned to the protonation of Asp-76. In bR, the homologous residue Asp-85 serves as the acceptor group for the Schiff base proton. Bands appear in the amide I and II regions at similar frequencies in sR-I, hR, and bR, indicating that despite their different functions they all undergo closely related structural changes. Bands are also detected in the C–H stretch region, possibly due to alterations in the membrane lipids. Similar spectral features are also observed in the lipids of rhodopsin-containing photoreceptor membrane upon light activation.

Sensory rhodopsin I (sR-I)[1] functions as one of two signal-transducing proteins involved in control of phototaxis of *Halobacterium halobium* (Spudich & Bogomolni, 1988). It mediates both an attractant response and a repellent response due to photoreactions of sR-I's resting state (sR_{587}) and its long-lived photoproduct (S_{373}), respectively (Spudich & Bogomolni, 1984). Like rhodopsin, the light receptor in vision, sR-I has seven transmembrane helical segments, as deduced

†Supported by grants from the NSF (DMB-8806007), NIH (EY05499), and ONR (N00014-88-K-0464) to K.J.R. and the NIH (GM27750) and ONR (N-00014-89-J-1629) to J.L.S.

*Address correspondence to this author.
‡Physics Department and Program in Cellular Biophysics, Boston University.
§Department of Structural Biology and Department of Physiology and Biophysics, Albert Einstein College of Medicine.

[1] Abbreviations: PM, purple membrane; sR-I, sensory rhodopsin I; bR, bacteriorhodopsin; hR, halorhodopsin; FTIR, Fourier transform infrared; au, absorbance units; λ_{max}, wavelength of maximum visible absorption; MPP-I, methyl-accepting phototaxis protein I; sR_{587}, the thermally stable attractant form of sR-I with λ_{max} = 587 nm; S_{373}, the photointermediate (repellent form) of sR-I with λ_{max} = 373 nm (Spudich & Bogomolni, 1984).

0006-2960/91/0430-5395$02.50/0

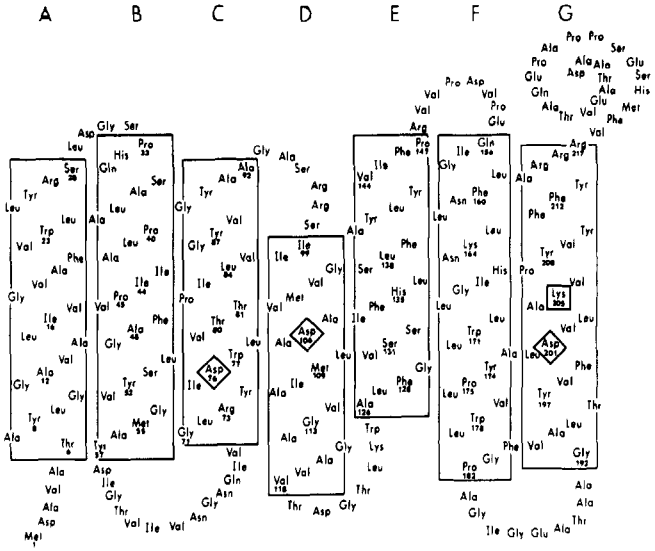

FIGURE 1: Amino acid sequence and proposed two-dimensional folding model of sR-I redrawn from Henderson et al. (1990) showing the location of Asp-76, Asp-106, and Asp-201. Lys-205, which forms the Schiff base with retinal, is shown in a square.

from a hydropathicity analysis of the primary structure (Blanck et al., 1989), and a retinylidene chromophore linked through a lysine Schiff base (Spudich & Bogomolni, 1988; Fodor et al., 1989). Its photocycle appears to activate a 97-kDa methyl-accepting protein (MPP-I) in the membrane (Spudich et al., 1989). In analogy to transducin in vision, MPP-I may function as an intermediary in the control of flagellar motor movement. Thus, a key goal is to understand the molecular mechanism by which sR-I activates MPP-I.

Two other retinylidene membrane proteins in *H. halobium*, bR, a light-driven proton pump and hR, a light-driven chloride pump, have been extensively studied (Stoeckenius, 1985; Lanyi, 1986). Although sR-I is not a transport protein, it exhibits regions of homology to both bR and hR (Blanck et al., 1989; Henderson et al., 1990). In particular, all of the key residues postulated to be involved in the bR proton pump mechanism (Braiman et al., 1988a; Rothschild et al., 1989; Henderson et al., 1990) are present in sR-I, with the exception of Asp-96 in helix C of bR, which appears to be involved in proton uptake from the cytoplasm and in Schiff base reprotonation (Figure 1) (Braiman et al., 1988a; Otto et al., 1989; Holz et al., 1989; Butt et al., 1989; Tittor et al., 1989).

FTIR difference spectroscopy has provided information about structural changes that occur in the chromophore and the protein of bR, hR, and rhodopsin [cf. Braiman and Rothschild (1988) and Rothschild (1988) for reviews]. Bands in the difference spectra can be assigned to specific chemical groups in the molecule on the basis of comparison with resonance Raman spectroscopy (Rothschild et al., 1981, 1984a), isotopic labeling (Engelhard et al., 1985; Dollinger et al., 1986; Rothschild et al., 1986), and site-directed mutagenesis (Braiman et al., 1988a,b; Rothschild et al., 1989, 1990; Gerwert et al., 1989). This information along with other spectroscopic studies has led to a proposal for the bR structure in the retinal-binding pocket and the proton pump mechanism (Braiman et al., 1988a; Rothschild et al., 1989). We report here the first FTIR difference measurements on sR-I. New details emerge about the mechanism of signal transduction in sR-I by comparing the results with those obtained from bR, hR, and rhodopsin.

Materials and Methods

Sample Preparation. Ten liters of Flx5R cells were grown to stationary phase in peptone medium, washed with basal salts, and pelleted and resuspended in 200 mL of basal salts, 10 mM Hepes, pH 7.0. All-*trans*-retinal (500 μL of a 1 mM ethanolic solution) was added to generate sR-I and the culture agitated for 2 h at 37 °C. A total of 10 mg of DNase was added and the suspension dialyzed against 8 L of 200 mM NaCl at 4 °C with 2 changes and spun at 6000*g* for 20 min. The supernatant was centrifuged at 60000*g* at 4 °C for 1 h and the membrane resuspended in 200 mM NaCl, 500 mM sucrose, and 10 mM Hepes, pH 7. High sucrose concentration prevents the loss of sR-I photoactivity caused by low salt. After being resuspended with a homogenizer, lysolecithin was added to 5 mM and the preparation was incubated at room temperature for 30 min and spun at 15 °C at 10000*g* for 2 h, resuspended in 10 mL of the NaCl/sucrose/Hepes buffer, and 2 mL was loaded onto a 20-mL sucrose gradient (equal volumes at 18%, 20%, 36%, 40%, and 50% sucrose). After a 24-h centrifugation at 60000*g*, the sharp blue band containing sR-I that forms near the center of the gradient was collected, dialyzed against NaCl/sucrose/Hepes buffer overnight, and centrifuged at 100000*g* for 2 h. The wet pellet was applied as a film on AgCl windows for FTIR measurements. This sample preparation method [similar to that described in Manor (1988)] enriches sR-I 6-fold with respect to total protein compared to its concentration in the sonicated envelope vesicles used in previous sR-I studies (Spudich & Bogomolni, 1988).

Visible Absorption Spectroscopy. A film of sR-I deposited on a AgCl window and prepared for FTIR spectroscopy (see below) was illuminated at 300 K for 10 min with yellow light and a 510-nm long-pass filter in front of a 500-W tungsten/halogen lamp. Absorption difference spectra were measured at room temperature and with the sample exposed to room humidity on a UV-2101PC Shimadzu absorption spectrometer with integrating sphere at various times after illumination. Absorption difference spectra in the near ultraviolet were measured under similar conditions on an Aminco DW2000 spectrophotometer (SLM Instruments, Urbana) by using a polyacetate microscope cover slip as a substrate. Illumination for photostationary measurements was for 10 min with a 100-W tungsten/halogen lamp through a 600 ± 20 nm interference filter.

FTIR Spectroscopy. sR-I films were prepared by drying approximately 25–50 μL of a suspension of sR-I membranes in 1 M NaCl onto AgCl windows. Drying was accomplished by using a stream of dry air. Samples were rehumidified by cooling the back side of the AgCl window with a stream of cold N_2 gas until water condensed on the film. The sample was then immediately inserted into a specially constructed cell that mounts in a HeliTran cryostat (Air Products, Allentown, PA). sR-I films were cooled in the dark to 270 K and FTIR difference spectra recorded on a Nicolet 740 spectrometer (Madison, WI) at 2-cm^{-1} resolution by using a protocol similar to that reported for bR (Roepe et al., 1987). Over 2000 interferometer scans (about 15 min) were averaged for each spectrum. Fourier transformation was performed by using a triangular apodization. Spectra were recorded in 15-min periods of dark followed by 15 min of illumination with a ~510-nm long-pass filter. Absorption differences for successive light/dark cycles were calculated and over 25 differences averaged.

Results

Visible Absorption Changes in sR-I Films. Illumination of an sR-I film prepared on a AgCl window with the identical procedure as used for the FTIR measurements shows the

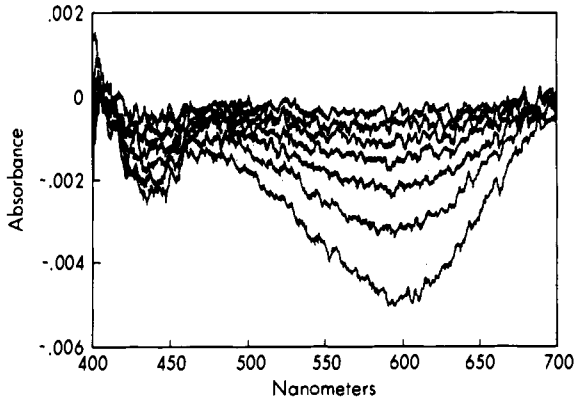

FIGURE 2: Time-resolved visible difference spectra at 300 K of an sR-I film deposited on a AgCl window and partially dehydrated. The spectrum with greatest depletion was measured starting 10 s after illumination, and subsequent spectra were recorded repetitively in the dark with a complete scan taking 4.25 min.

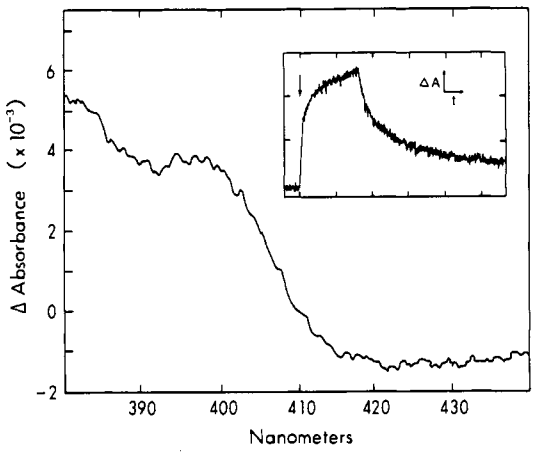

FIGURE 3: Photostationary state difference spectrum at 300 K of an sR-I film deposited on a polyacetate microsocope cover slip and partially dehydrated. Illumination was for 10 min at 600 ● 20 nm. (Inset) Time course of the absorption change at 400 nm with 1.6-min illumination initiated at the arrow, followed by dark. Scale: $\Delta A = 5 \times 10^{-4}$ au, $t = 0.5$ min.

pigment is photoactive under these conditions. A light-induced depletion in absorption is observed with a minimum near 590 nm, characteristic of sR-I (Figure 2). The band shape of the depletion is also characteristic of sR-I, except for the smaller negative peak near 440 nm, which may result from cytochrome perturbation due to heating of the sample. Such heating should not occur in the FTIR experiments, where the sample temperature was regulated. Since the AgCl window did not allow measurements below 400 nm due to its high absorbance, we also show (Figure 3) the visible absorption difference spectrum of an sR-I film deposited on a plastic substrate that transmitted below 400 nm. This spectrum indicates the formation of a species with a λ_{max} less than or equal to 380 nm and a 400 nm shoulder characteristic of the S_{373} photoproduct (Bogomolni & Spudich, 1987). The inset to Figure 3 shows the rise and decay of the absorbance at 400 nm for this species. The half-time of decay of the absorption differences is on the order of minutes (Figures 2 and 3), indicating a much reduced rate of sR-I photocycling compared to native membranes at room temperature (800 ms), most likely due to the dehydrated conditions.

The photostationary state produced by constant illumination of rehydrated films of sR-I at 270 K used for the FTIR measurement should consist mainly of the S_{373} intermediate of the sR-I photocycle. In support of this, we have observed formation of a structured intermediate with absorption maxima

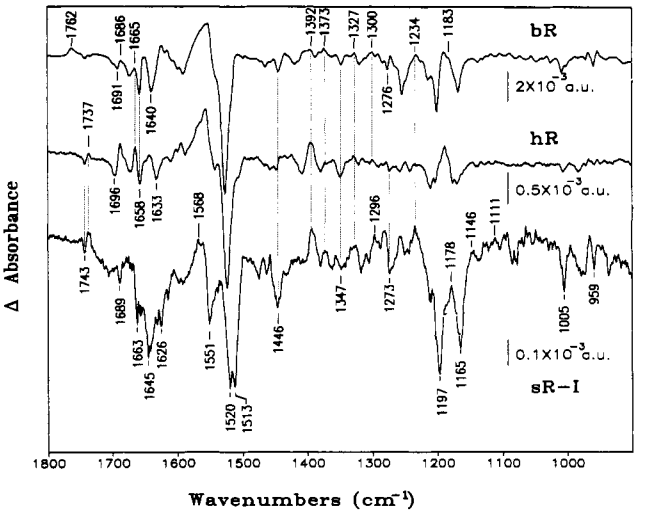

FIGURE 4: FTIR difference spectrum of $sR_{587} \rightarrow S_{373}$ transition recorded at 270 K on a hydrated film of sR-I (see Materials and Methods). The difference spectra of bR and hR reflect the bR → M and hR → hR_{550} transitions and are from Roepe et al. (1987) and Rothschild et al. (1988). Spectra shown were recorded at 2-cm^{-1} resolution. An artifact at 1206 cm^{-1} in the sR-I spectrum due to the spectrometer was removed.

and band shape characteristic of S_{373} with similar yields in films at 280 K under conditions similar to those used for the FTIR measurements (data not shown). In addition, the formation of S_{373} has been observed even at temperatures as low as 213 K (Ariki et al., 1987).

Chromophore. Many of the negative bands in the $sR_{587} \rightarrow S_{373}$ difference spectrum can be assigned to vibrations of the sR_{587} chromophore on the basis of resonance Raman spectroscopy (Fodor et al., 1989) (Figure 4). These include the ethylenic (C=C) stretch at 1520 cm^{-1}, C–C stretch modes at 1197 and 1165 cm^{-1}, and the methyl rock at 1005 cm^{-1}. The Schiff base C=N stretch is found at 1626 cm^{-1} (Fodor et al., 1989). In addition, the band at 959 cm^{-1} is due to a hydrogen-out-of-plane (HOOP) mode of the chromophore.

These chromophore bands also appear in the FTIR difference spectrum (Roepe et al., 1987; Rothschild et al., 1988) and resonance Raman spectrum (Smith et al., 1985; Fodor et al., 1987) of bR_{568} and hR_{578}, although with small shifts in frequency. This most likely reflects a change in the degree of deconjugation of the chromophore and the related shift in the visible λ_{max} (Aton et al., 1977). For example, the C=C stretch frequency of hR_{578} is at 1525 cm^{-1} compared to 1529 cm^{-1} for bR_{568}. This corresponds to a 0.4 cm^{-1}/nm shift leading to a predicted λ_{max} of 590 nm for sR-I (based on a C=C stretch frequency of 1520 cm^{-1}), very close to the measured λ_{max}.

A negative band appears at 1513 cm^{-1}, which may be due to the ethylenic stretch from a second form of sR-I that is present under these conditions. This frequency would correspond to a species with a λ_{max} at approximately 605 nm. Alternatively, the ethylenic stretch mode of sR_{587} might be split, such as in the L_{550} intermediate of bacteriorhodopsin (Argade & Rothschild, 1983). However, this is unlikely since such a splitting was not observed in the resonance Raman spectrum of sR_{587} (Fodor et al., 1989). A third possibility is that this band arises from a nonchromophoric vibration such as from tyrosine, for example, which has a strong IR active mode near this frequency (Parker, 1983) and was detected in the bR → K transition of bR (Rothschild et al., 1986; Dollinger et al., 1986). It would be difficult, however, without the use of tyrosine isotope labels, which were used in the case

of bR (Rothschild et al., 1986), to make a positive assignment of this band.

The progressive downshift of the C=N stretch for bR (1640 cm^{-1}), hR (1633 cm^{-1}), and sR (1626 cm^{-1}) agrees with a previously observed correlation for this mode of approximately 0.5 cm^{-1}/nm (Rothschild et al., 1984b). The downshift can be explained by a progressively weakened electrostatic interaction between a counterion and the Schiff base as discussed previously (Fodor et al., 1989; Rothschild et al., 1984b). The pattern of deuterium-induced shifts observed for these pigments supports this prediction (Fodor et al., 1989). *Overall, these results indicate that the chromophore structures in bR, hR, and sR-I are very similar, except for a progressive weakening of the counterion influence on the Schiff base.*

In the case of the S_{373} chromophore, an intense positive peak is seen at 1568 cm^{-1}, the same frequency found for the ethylenic stretch of the M intermediate in bR (Figure 4). This suggests that the chromophore of S_{373} forms an unprotonated Schiff base with a similar environment as the M intermediate. In support of this possibility, positive features near 1178 and 1237 cm^{-1} are found close to the M chromophore C–C stretch modes at 1183 and 1235 cm^{-1} (Braiman & Mathies, 1980). However, it is difficult to conclusively assign the S_{373} chromophore vibrations without using isotopic labeling of the retinal.

Protein. Protonation and hydrogen-bonding changes in Asp and Glu residues give rise to bands in the 1700–1800-cm^{-1} region, where the carboxyl (COOH) stretch mode of these residues appears (Rothschild et al., 1981). In the case of bR, bands in this region have recently been assigned by site-directed mutagenesis to changes of all four buried Asp residues: Asp-96, Asp-85, Asp-115, and Asp-212 (Braiman et al., 1988a; Gerwert et al., 1989). As seen in Figure 4, the $sR_{587} \rightarrow S_{373}$ difference spectrum differs significantly from the bR → M difference spectrum in this region. Most outstandingly, the 1762-cm^{-1} band previously assigned to the Schiff base proton acceptor, Asp-85, (Braiman et al., 1988a) is absent in sR-I. This would suggest, although not establish conclusively, that the homologous residue in sR-I, Asp-76, is not undergoing a protonation change during the $sR_{587} \rightarrow S_{373}$ photoreaction. In contrast, there is a striking similarity in this region between the $sR_{587} \rightarrow S_{373}$ and the $hR_{578} \rightarrow hR_{520}$ difference spectra (Rothschild et al., 1988). In both cases a negative/positive feature at 1743/1737 cm^{-1} appears (Figure 4). There are only two homologous buried Asp residues found in both sR-I and hR in their proposed folded structures based on their analogy with bR (Henderson et al., 1990). These are Asp-106 (Asp-140 in hR) located in helix D and positioned near the β-ionone ring and Asp-201 (Asp-238 in hR), one turn below the retinal-binding residue, Lys-205 (Figure 1). These results thus indicate that one or both of these residues are giving rise to the 1743/1737-cm^{-1} feature in both sR-I and hR. In the case of hR, we have suggested that these bands are due to Asp-140, since they appear as early as the hR → hK transition, similar to a feature appearing in the bR → K difference spectrum and assigned to the homologous bR residue, Asp-115 (Braiman et al., 1988a).

The frequencies of bands in the amide I (1620–1690 cm^{-1}) and amide II (1530–1560 cm^{-1}) regions are sensitive to protein secondary structure (Parker, 1983). Since bands appear at similar frequencies in these regions of the bR → M and $hR_{578} \rightarrow hR_{520}$ difference spectra, it was concluded that the secondary structural changes of bR and hR are similar. In the case of sR-I, the amide I region is somewhat obscured due to a broad negative band centered near 1640 cm^{-1}, most likely due to changes in the H_2O absorption. However, strong negative bands are still observed at 1689 and 1662 cm^{-1}, close to the frequency of bands found in the hR and bR difference spectra. A strong negative band is also observed in the amide II region at 1551 cm^{-1} in the $sR_{587} \rightarrow S_{373}$ difference spectrum. Similar features also appear in the bR and hR difference spectra as shoulders. These bands are partially obscured compared to sR-I due to the higher frequency of the ethylenic stretch modes of hR and bR. Thus, there are indications that the structural changes involving peptide bonds are similar in sR-I, hR, and bR.

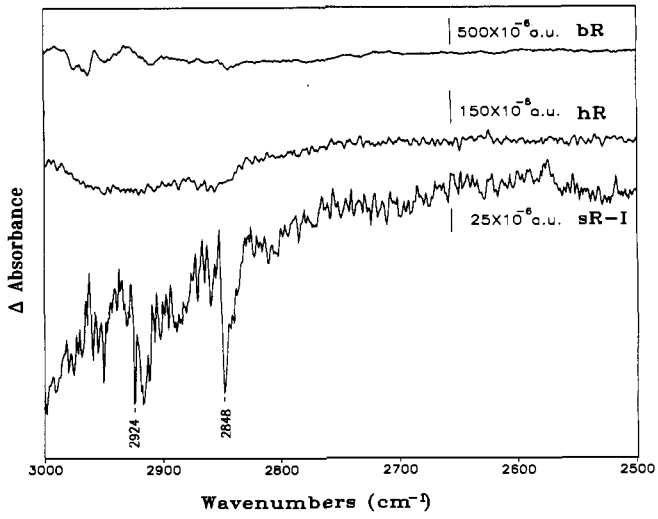

FIGURE 5: Same as Figure 1 in 2500–3000-cm^{-1} region. Spectra are scaled by using the C=C stretch mode at 1520–1530 cm^{-1}.

Lipids. Prominent negative bands are detected at 2848 and 2924 cm^{-1} (Figure 5) that correspond to the frequency of the symmetric and antisymmetric stretch of CH_2 (methylene) groups in lipid acyl chains (Cameron et al., 1980). The bR → M spectrum also exhibits bands in this region, although they are not at similar frequencies and much less intense (Figure 5). It is likely that these bands arise in sR-I from methylene groups on lipids, although we cannot exclude an origin due to protein residues such as leucine or chromophore C–H stretch modes. The negative bands and the smaller positive bands at higher frequency are consistent with an effective increase in the fluidity of the lipids (Cameron et al., 1980), which might be caused by a conformational change of sR-I that disrupts the packing of the acyl chains. Interestingly, a very similar effect is seen during the bleaching of rhodopsin in bovine photoreceptor membrane (DeGrip et al., 1988).

Discussion

The present results confirm the conclusions of earlier investigations by regeneration with retinal analogues (Spudich et al., 1986; Baselt et al., 1989) and resonance Raman spectroscopy (Fodor et al., 1989) that the sR-I, bR, and hR chromophores exist in very similar configurations. A corollary to this conclusion is that retinal-binding pockets and protein–retinal interactions are very similar in all three pigments. Some differences must exist, however, since the Schiff base C=N stretch of sR-I is downshifted almost 15 cm^{-1} relative to bR. This indicates that the sR-I protonated Schiff base has a weaker electrostatic interaction with nearby counterion(s) in the retinal pocket relative to bR. As discussed below, a change in the position, environment, or protonation state of Asp-76 relative to the putative Schiff base counterion, Asp-85 in bR (Braiman et al., 1988a; Subramaniam et al., 1990; Otto et al., 1990), might account for this finding.

The striking similarity between the hR and sR-I difference spectra above 1700 cm^{-1} suggests that the Asp (and Glu) residues of both proteins undergo very similar changes during their respective photocycles. This evidence and sequence homologies point to an involvement of the bR homologues Asp-106 (Asp-115 in bR) and Asp-201 (Asp-212 in bR) but not Asp-76 (Asp-85 in bR). Since Asp-85 appears to be the acceptor group for the Schiff base proton in bR (Braiman et al., 1988a), this result is unexpected considering that the Schiff base of sR-I appears to be deprotonated in the S_{373} state since its λ_{max} is near that of retinal and unprotonated retinylidene Schiff bases. Several possible mechanisms might account for a deprotonation of the sR-I Schiff base without a direct involvement of Asp-76. If the Schiff base of the sR-I chromophore is hydrolyzed upon S_{373} formation, a possibility raised by Blanck et al. (1989), then the Schiff base proton could be donated directly to the lysine ϵ-amino group. The Schiff base might also donate its proton directly to a water molecule in the putative extracellular channel of sR-I (Henderson et al., 1990), consistent with the slow formation of S_{373} (300 μs) relative to that of the M_{412} intermediate in bR (40 μs). The red-shifted λ_{max} and downshifted C=N stretch frequency of sR-I relative to bR might indicate that Asp-76 does not interact as strongly with the Schiff base, explaining why it might not serve as a proton acceptor in sR-I.

Our results indicate that protein structural changes involving peptide groups are similar in hR, bR, and sR-I. This might be expected due to the significant similarity in the primary sequence of these proteins. However, this raises the question of how such similar structural changes result in such different functions. In the case of sR-I, retinal isomerization most likely leads to alterations in surface domains of the protein that interact with subsequent signal-transduction components (putatively MPP-I). In the case of hR and bR, these structural changes are involved in active transport of a proton and chloride ion, respectively.

One interesting hypothesis is that the sR-I mechanism evolved by utilizing the same protein conformational changes that are inherent in the active transport mechanisms of bR and hR. This might be possible if these structural changes are large enough to perturb the surrounding bilayer region of the membrane. A signaling mechanism might thereby evolve that amplifies these changes in order to activate the next component in the transduction pathway. Such a mechanism might account for the signals that we detect in the lipid C–H stretch region. The similarities with rhodopsin are particularly striking in this respect. Lipid-associated perturbations are detected at the meta II stage of bleaching along with protein conformational changes that begin early in the bleaching sequence (DeGrip et al., 1988). In both the case of rhodopsin and sR-I, these lipid perturbations may reflect the extent of the changed conformation of the protein in the membrane. Further studies on the secondary structural changes occurring in bR, hR, and sR-I will be necessary in order to establish how these changes are related to the respective protein functions.

Acknowledgments

We thank Drs. D. Manor and C. A. Hasselbacher for their assistance in the early stages of this work.

References

Argade, P. V., & Rothschild, K. J. (1983) *Biochemistry 22*, 3460–3466.

Ariki, M., Shichida, Y., & Yoshizawa, T. (1987) *FEBS Lett. 225*, 255–258.

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 27*, 6775–6778.

Baselt, D. R., Fodor, S. P. A., van der Steen, R., Lugtenburg, J., Bogomolni, R., & Mathies, R. A. (1989) *Biophys. J. 55*, 193–196.

Blanck, A., Oesterhelt, D., Ferrando, E., Schegk, E. S., & Lottspeich, F. (1989) *EMBO J. 8*, 3963–3971.

Bogomolni, R. A., & Spudich, J. L. (1987) *Biophys. J. 52*, 1071–1075.

Braiman, M. S., & Mathies, R. A. (1980) *Biochemistry 19*, 5421–5428.

Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541–570.

Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., & Rothschild, K. J. (1988a) *Biochemistry 27*, 8516–8520.

Braiman, M. S., Mogi, T., Stern, L. J., Hackett, N. R., Chao, B. H., Khorana, H. G., & Rothschild, K. J. (1988b) *Proteins: Struct., Funct., Genet. 3*, 219–229.

Butt, H. J., Fendler, K., Bamberg, E., Tittor, J., & Oesterhelt, D. (1989) *EMBO J. 8*, 1657–1663.

Cameron, D. G., Casal, H. L., & Mantsch, H. H. (1980) *Biochemistry 19*, 3665–3674.

DeGrip, W. J., Gillespie, J., Gray, D., Bovee, P. H. M., Lugtenburg, J., & Rothschild, K. J. (1988) *Photochem. Photobiol. 48*, 497–504.

Dollinger, G., Eisenstein, L., Lin, S., Nakanishi, K., & Termini, J. (1986) *Biochemistry 25*, 6524–6533.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Fodor, P. A., Bogomolni, R., & Mathies, R. (1987) *Biochemistry 26*, 6775–6778.

Fodor, S. P. A., Gebhard, R., Lugtenburg, J., Bogomolni, R. A., & Mathies, R. A. (1989) *J. Biol. Chem. 264*, 18280–18283.

Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943–4947.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A. D., Heyn, M. P., Skulachev, V. P., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2167–2171.

Lanyi, J. K. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 11–28.

Manor, D. (1988) Ph.D. Disertation. Albert Einstein College of Medicine, Bronx, New York.

Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G., & Heyn, M. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9228–9232.

Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018–1022.

Parker, F. S. (1983) *Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry*, Plenum Press, New York.

Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry 26*, 6696–6707.

Rothschild, K. J. (1988) *Photochem. Photobiol. 47*, 883–887.

Rothschild, K. J., Zagaeski, M., & Cantore, B. (1981) *Biochem. Biophys. Res. Commun. 103*, 483–489.

Rothschild, K. J., Marrero, H., Braiman, M., & Mathies, R. (1984a) *Photochem. Photobiol. 40*, 675–679.

Rothschild, K. J., Roepe, P., Lugtenburg, J., & Pardoen, J. A. (1984b) *Biochemistry 23*, 6103–6109.

Rothschild, K. J., Roepe, P., Earnest, T., & Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347-351.
Rothschild, K. J., Bousché, O., Braiman, M. S., Hasselbacher, C. A., & Spudich, J. L. (1988) *Biochemistry 27*, 2420-2424.
Rothschild, K. J., Braiman, M. S., Mogi, T., Stern, L. J., & Khorana, H. G. (1989) *FEBS Lett. 250*, 448-452.
Rothschild, K. J., Braiman, M. S., He, Y. W., Marti, T., & Khorana, H. G. (1990) *J. Biol. Chem. 265*, 16985-16991.
Smith, S. O., Lugtenburg, J., & Mathies, R. A. (1985) *J. Membr. Biol. 85*, 95-109.
Spudich, J. L., & Bogomolni, R. A. (1984) *Nature 312*, 509-513.
Spudich, J. L., & Bogomolni, R. A. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 193-215.
Spudich, J. L., McCain, D. A., Nakanishi, K., Okabe, M., Shimizu, N., Rodman, H., Honig, B., & Bogomolni, R. A. (1986) *Biophys. J. 49*, 479-483.
Spudich, E. N., Takahashi, T., & Spudich, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7746-7750.
Stoeckenius, W. (1985) *Trends Biochem. Sci. 10*, 483-486.
Subramaniam, S., Marti, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1013-1017.
Tittor, J., Soell, C., Oesterhelt, D., Butt, H., & Bamberg, E. (1989) *EMBO J. 8*, 3477-3482.

Population of the Triplet States of Bacteriorhodopsin and of Related Model Compounds by Intramolecular Energy Transfer†

N. Friedman,‡ M. Sheves,*,‡ and M. Ottolenghi*,§
The Weizmann Institute of Science, Rehovot 76100, Israel, and The Hebrew University of Jerusalem, Jerusalem 91904, Israel
Received October 17, 1990; Revised Manuscript Received February 12, 1991

ABSTRACT: In variance with chlorophyll-based photosynthetic pigments, the triplet states of rhodopsins, either visual or photosynthetic, have not been observed experimentally. This is due to the ultrafast crossing from S_1 to S_0, which effectively competes with intersystem crossing to the triplet (T_1) state. In order to populate T_1 indirectly, laser photolysis experiments are performed with model protonated Schiff bases of retinal in solution, in which both inter- and intramolecular energy transfer to the polyene chromophore are carried out from an appropriate triplet energy donor. The experiments are then extended to bacteriorhodopsin (bR) by detaching the native retinal chromophore from the protein-binding site and replacing it by an analogous (synthetic) protonated Schiff base polyene, attached in a nonconjugated way to a naphthone triplet donor. Pulsed laser excitation of the latter moiety led, for the first time, to the observation of the triplet state of a rhodopsin. Possible locations and roles of the T_1 state in bR and in visual pigments are discussed briefly.

Bacteriorhodopsin (bR),[1] the purple membrane pigment in the photosynthetic microorganism *Halobacterium halobium*, as well as visual pigments are both composed of a retinal polyene chromophore bound to the parent protein (opsin) via a protonated Schiff base bound with a lysine residue. It is now well established that in both systems the photocycle is initiated by primary isomerization around a polyene double bond [for reviews see Ottolenghi (1980); Birge (1981, 1989); Packer (1982); Becker (1988); and Ottolenghi and Sheves (1989)]. The subsequent steps initiate the cross-membrane proton pump (in bacteriorhodopsin) or the transduction process (in visual rhodopsins, Rho).

As in the case of chlorophyll-based photosynthetic systems, accumulated evidence suggests that the generation of the primary ground-state photoproducts in the photocycles of bR and Rho, which take place on a subpicosecond time scale, proceeds on an excited singlet potential energy surface with no participation of triplet states. This conclusion is in keeping with the photochemical behavior of model protonated Schiff bases in solution ($RSBH^+$). Thus, although substantial intersystem crossing to the triplet state is observed in the free retinal aldehydes [for a review, see Ottolenghi (1980)], cis ↔ trans isomerization about double bonds is the major detectable photoprocess in $RSBH^+$ (Becker & Freedman, 1985; Becker et al., 1985; Freedman & Becker, 1986). The intersystem crossing yield in $RSBH^+$ is extremely small ($\sim 10^{-4}$) so that triplet-state generation from the singlet manifold can be observed only under the multiple excitation conditions prevailing in high-power laser pulses (Friedman et al., 1989). Apart from the primary photoisomerization, the possible role of the triplet state in visual and photosynthetic rhodopsin has also been discussed in relation to the reverse thermal reaction from primary photoproducts such as bathorhodopsin (in Rho) or K (in bR) back to the parent pigments (Friedman et al., 1989).

In variance with chlorophyll-based photosynthetic pigments, the triplet state of visual or photosynthetic rhodopsins has not been detected experimentally. Our major objective in the present work was to gain direct information on the triplet state of a rhodopsin by populating it selectively via energy transfer. Intermolecular energy transfer has been applied to generate

† This work was supported by the United States-Israel Binational Science Foundation, by the Fund for Basic Research (administered by the Israeli Academy of Sciences and Humanities), by the E. D. Bergman (Hebrew University) Applied Science Foundation, and by the Yeda Fund (Weizmann Institute of Science).
‡ The Weizmann Institute of Science, Rehovot 76100, Israel.
§ The Hebrew University of Jerusalem, Jerusalem 91904, Israel.

[1] Abbreviations: T_1, triplet state; $RSBH^+$, protonated Schiff base; bR, bacteriorhodopsin; Rho, rhodopsin; DIBAH, diisobutylaluminum hydride; $(Bu)_4NF$, tetrabutylammonium fluoride; TCA, trichloroacetic acid; DMAP, dimethylaminopyridine.

0006-2960/91/0430-5400$02.50/0