Halorhodopsin and sensory rhodopsin contain a C_6—C_7 s-*trans* retinal chromophore

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ABSTRACT Halorhodopsin (HR) and sensory rhodopsin (SR) have been regenerated with retinal analogues that are covalently locked in the 6-s-*cis* or 6-s-*trans* conformations. Both pigments regenerate more completely with the locked 6-s-*trans* retinal and produce analogue pigments with absorption maxima (577 nm for HR and 592 nm for SR) nearly identical to those of the native pigments (577 and 587 nm). This indicates that HR and SR bind retinal in the 6-s-*trans* conformation. The opsin shift for the locked 6-s-*trans* analogue in HR is 1,200 cm^{-1} less than that for the native chromophore (5,400 cm^{-1}). The opsin shift for the 6-s-*trans* analogue in SR is 1,100 cm^{-1} less than that for native retinal (5,700 cm^{-1}). This demonstrates that ~20% of the opsin shift in these pigments arises from a protein-induced change in the chromophore conformation from twisted 6-s-*cis* in solution to planar 6-s-*trans* in the protein. The reduced opsin shift observed for the locked 6-s-*cis* analogue pigments compared with the locked 6-s-*trans* pigments may be due to a positive electrostatic perturbation near C_7.

INTRODUCTION

The retinal proteins bacteriorhodopsin (BR), halorhodopsin (HR), and sensory rhodopsin (SR) are responsible for light-driven proton transport, light-driven chloride ion transport, and phototaxis in halophilic bacteria (Stoeckenius and Bogomolni, 1982; Lanyi, 1986; Spudich and Bogomolni, 1984, 1988). The absorption maximum of the retinal protonated Schiff base prosthetic group in these pigments is strongly red shifted compared to the protonated retinal Schiff base in solution, and this red shift is termed the "opsin shift" (Nakanishi et al., 1980). The determination of the in situ structure of the prosthetic group is prerequisite for understanding the mechanism of the opsin shift. Solid-state NMR and locked retinal analogue experiments have recently been used to show that bacteriorhodopsin binds the 6-s-*trans* conformation of the retinal chromophore (Harbison et al., 1985; van der Steen et al., 1986). The conversion of the twisted 6-s-*cis* form found in solution to the planar 6-s-*trans* form in the protein accounts for ~1,300 cm^{-1} of the 5,100 cm^{-1} opsin shift in BR (Lugtenburg et al., 1986; Spudich et al., 1986). The C_6—C_7 bond conformation of the retinal chromophore in HR and SR is unknown, but it is evident that protein-induced changes in the C_6—C_7 conformation could contribute significantly to the opsin shift. To determine the C_6—C_7 bond conformation in HR and SR, we have studied the regeneration of these pigments with retinal analogues locked in the 6-s-*trans* and 6-s-*cis* conformations.

MATERIALS AND METHODS

Purified halorhodopsin was isolated from *H. halobium* strain JW-12 according to previously published procedures (Taylor et al., 1983). The protein was bleached in 1.0 M hydroxylamine pH 7.3 at 37°C in the dark for 20 h following a procedure developed for SR and BR (Weber et al., 1983; Fodor et al., 1988). The excess hydroxylamine was then removed by extensive dialysis (10 mM Hepes, 3.0 M NaCl, pH 7.0). This bleaching procedure produced HR opsin samples that consistently exhibited ~70% regeneration yields. Membranes containing SR opsin were obtained from a BR, HR, and retinal deficient mutant of *H. halobium* (Flux 3R) and were used directly for regeneration (Spudich and Bogomolni, 1983).

Locked 6-s-*cis* and 6-s-*trans* retinal analogues were synthesized according to van der Steen et al. (1986). The chromophores were dissolved in ethanol and added to the bacterial opsin suspensions. The HR regenerations were allowed to proceed at 25°C for 1–2 d. SR membranes regenerate faster so the experiment was complete within 1 h. Regenerations were monitored with an Aminco (Urbana, IL) DW2-C UV-visible spectrometer; samples were referenced against opsin solutions containing no added retinal. Addition of the ethanol solution to the SR membranes caused time-dependent changes in light scattering and in the absorption of contaminating cytochromes (λ_{max} = 428, 561 nm). The sharp cytochrome bands were removed from the SR data by subtracting a reference spectrum of Flux 3R cytochromes. This procedure did not affect the λ_{max} of the regenerated pigments.

RESULTS AND DISCUSSION

Fig. 1 presents the regeneration of halorhodopsin with the locked 6-s-*cis* and 6-s-*trans* analogues. The 6-s-*trans* analogue regenerates more completely than does the

0006-3495/89/01/193/04 $2.00

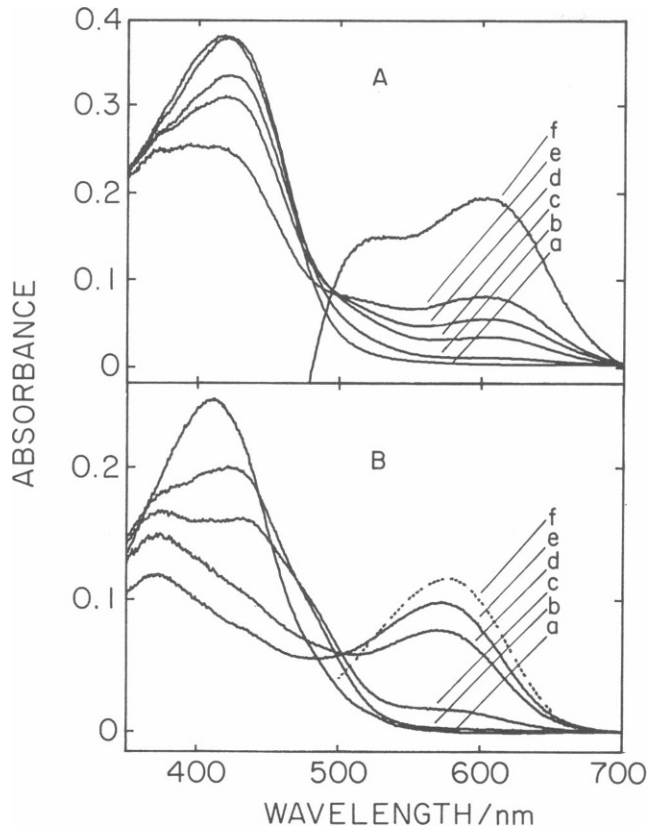

FIGURE 1 Regeneration of halorhodopsin with locked retinal analogues. (*A*) 2 μl of 1.8 mM 6-s-*cis* retinal in ethanol was added to 0.4 ml of a 4 μM HR suspension (25 mM Hepes, pH 7, 3.0 M NaCl). The regeneration times were 1 min (*a*), 15 min (*b*), 2 h (*c*), 24 h (*d*), and 48 h (*e*). Spectrum *f* is the difference between 48 h and 1 min (*e–a*) multiplied by 2.5. (*B*) 6 μl of 0.6 mM 6-s-*trans* retinal in ethanol was added to 0.4 ml of 4 μM HR suspension. The regeneration times were 1 min (*a*), 15 min (*b*), 2 h (*c*), 24 h (*d*), and 48 h (*e*). Spectrum *f* was obtained by using a combination of blue and green light to fully light-adapt the sample (Taylor et al., 1983).

6-s-*cis* analogue. In the 6-s-*cis* regeneration, two pigment forms are produced. Difference spectra show that a 522-nm pigment appears at early times followed by the formation of the 603-nm pigment. This is very similar to what was observed for BR (van der Steen et al., 1986). The λ_{max} values for the 6-s-*cis* and 6-s-*trans* analogues in HR are 603 and 577 nm, respectively, and their opsin shifts are given in Table 1. The halftimes for bleaching recovery were 40 and 50 ms for the 6-s-*trans* and 6-s-*cis* pigments, respectively. The recovery time for the regenerated native pigment under our conditions was 20 ms.

Fig. 2 presents the regeneration of sensory rhodopsin with the locked analogues. The 6-s-*cis* analogue does not regenerate fully and the λ_{max} (604 nm) was determined from the difference spectrum. The 6-s-*trans* analogue, on the other hand, regenerates rapidly and completely. The same absorption maximum (592 nm) was determined from either the direct absorption spectrum or the difference spectrum. The λ_{max} values and the opsin shifts are summarized in Table I. The halftimes for bleaching recovery were 1.1 and 1.2 s for the 6-s-*trans* and 6-s-*cis* pigments, respectively. The recovery time for the regenerated native pigment was 0.9 s.

The striking agreement between the absorption maxima of the locked 6-s-*trans* analogue pigments and those of the native pigments (see Table I) argues that both HR and SR bind the 6-s-*trans* conformation of the retinal chromophore. The similar bleaching recovery kinetics for the native and analogue pigments suggest that the chromophores are bound in the same way to the native chromophore-binding site. Attempts to understand the spectroscopic properties of these pigments and to determine their mode of action must take into account the fact that the in situ chromophore conformation is 6-s-*trans*. In this respect the structure of retinal in HR and SR is analogous to BR which is known to contain a 6-s-*trans* chromophore (van der Steen et al., 1986; Harbison et al., 1985). This suggests that the chromophore binding sites in these pigments are similar. The remarkable agreement between the Raman spectra of HR_{578} and BR_{568} (Smith et

TABLE 1 Absorption maxima and opsin shifts for retinal analogues in BR, HR, and SR*

Analogue	PSB λ_{max}	BR		HR		SR	
		λ_{max}	Opsin shift	λ_{max}	Opsin shift	λ_{max}	Opsin shift
All-*trans* retinal	440‡	568 ± 2	5,100 ± 50	577 ± 2	5,400 ± 50	587 ± 2	5,700 ± 50
Locked 6-s-*cis*	485‡	596 ± 2	3,850 ± 50	603 ± 4	4,050 ± 50	604 ± 7	4,050 ± 200
Locked 6-s-*trans*	465‡	572 ± 2§	4,000 ± 50	577 ± 3	4,200 ± 100	592 ± 5	4,600 ± 150

*The opsin shift is the difference (in wavenumbers) between the absorption maximum of the chloride salt of the *n*-butylamine protonated Schiff base (PSB) in methanol and that of the light-adapted pigment.
‡van der Steen et al., 1986.
§This value is for the light-adapted pigment. If the sample is partially dark-adapted the λ_{max} is 564 nm (van der Steen et al., 1986).

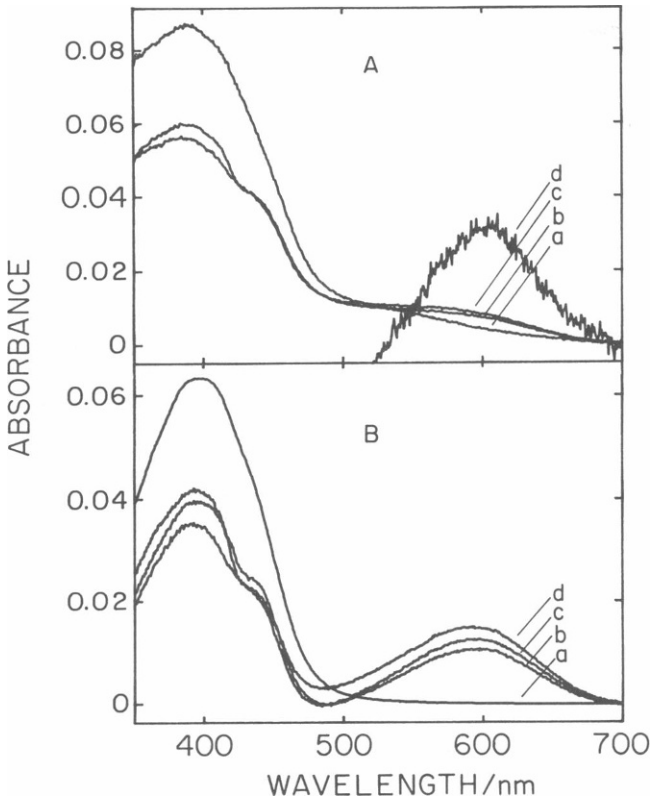

FIGURE 2 Regeneration of sensory rhodopsin with locked retinal analogues. (*A*) 3 μl of 0.33 mM 6-s-*cis* retinal in ethanol was added to 0.5 ml of a 12 mg/ml suspension of Flux 3R membranes (pH 6, 4.0 M NaCl). The regeneration times were 1 min (*a*), 15 min (*b*), and 45 min (*c*). Spectrum *d* is the difference between 45 min and 1 min (*c*–*a*) multiplied by 10. (*B*) 3 μl of a 0.25 mM 6-s-*trans* retinal solution was added to 0.5 ml of a 12 mg/ml suspension of Flux 3R membranes. The regeneration times were 1 min (*a*), 15 min (*b*), 30 min (*c*), and 1 h (*d*).

al., 1984; Fodor et al., 1987) is consistent with this conclusion. The similarity of the BR and SR binding sites is supported by the retinal analogue studies of Spudich et al. (1986).

The observation of a 6-s-*trans* chromophore conformation in HR and SR is important for understanding the mechanism of the opsin shift in these pigments. Native retinal in solution adopts a distorted 6-s-*cis* conformation which is twisted by ~40 degrees about the C_6—C_7 bond (Honig et al., 1971). Calculations and locked analogue experiments have shown that conformational isomerization to a planar 6-s-*trans* form red-shifts the absorption by ~1,300 cm^{-1} (van der Steen et al., 1986; Honig et al., 1976). Therefore, if the protein binds the 6-s-*trans* conformation, this will contribute ~1,300 cm^{-1} to the red shift. Conversely, if the C_6—C_7 conformation is locked then the opsin shift should be ~1,300 cm^{-1} less and this is exactly what is observed for both HR and SR.

The opsin shifts for the locked analogues provide a way of examining external protein–chromophore interactions independent of protein-induced chromophore conformational changes. The opsin shift for the locked 6-s-*trans* analogue in HR (4,200 cm^{-1}) is 200 ± 100 cm^{-1} greater than that for BR. It has been shown that the majority of the opsin shift in BR is due to a weakened hydrogen-bonding interaction between the Schiff base proton and its protein counterion (Lugtenburg et al., 1986; Spudich et al., 1986; Harbison et al., 1983; Baasov and Sheves, 1986). Resonance Raman studies of HR_{578} have suggested that the hydrogen-bonding interaction in HR_{578} is even weaker than that in BR_{568} (Smith et al., 1984; Fodor et al., 1987). This strongly suggests that the enhanced opsin shift in HR is due to a further weakening of the hydrogen-bonding interaction between the Schiff base proton and its counterion. The opsin shift for the 6-s-*trans* locked analogue in SR (4,600 cm^{-1}) is 600 ± 200 cm^{-1} greater than that for BR. The enhanced opsin shift in SR relative to BR and HR may be due to a further weakening of the hydrogen bonding of the Schiff base.

The opsin shifts for the 6-s-*cis* analogues in BR, HR, and SR are systematically smaller than those for the 6-s-*trans* analogues. Although these differences are small, they are always larger than the experimental error. This may arise because the non-isomorphous chemical analogues are accommodated differently by the protein binding sites. However, the 150–550 cm^{-1} lower opsin shifts of the 6-s-*cis* analogues in BR, HR, and SR could also be due to interaction of the chromophore with a dipolar pair of charges (or a dipolar residue) near the ionone ring as depicted in Fig. 3 (Harbison et al., 1985). The locked 6-s-*cis* retinyl cation would interact more strongly with the positive electrostatic perturbation near

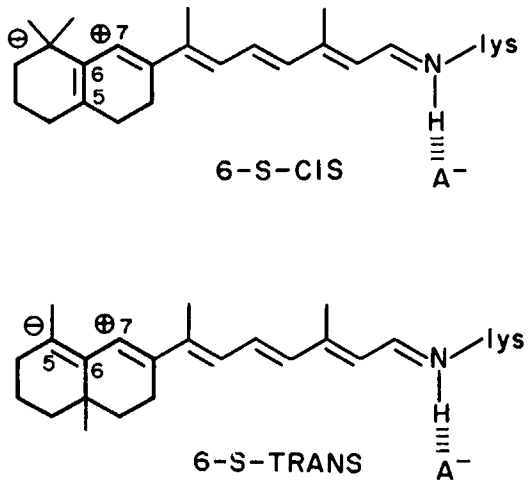

FIGURE 3 Structures of the locked 6-s-*cis* and 6-s-*trans* retinal analogues in BR, HR, and SR.

C_7 than with the negative electrostatic perturbation, producing the lower opsin shift compared to the 6-s-*trans* analogue.

We thank S. Sundberg for measuring the photocycle recovery times of the analogue pigments.

This research was supported by grants from the National Institutes of Health (GM-27057, GM-34219, and GM-11266), the National Science Foundation (DMB 83-16139), the Netherlands Foundation for Chemical Research (SON), and the Netherlands Organization for the Advancement of Pure Research (NWO).

Received for publication 31 May 1988 and in final form 2 September 1988.

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