ISO-HALORHODOPSIN: A STABLE, 9-*CIS* RETINAL CONTAINING PHOTOPRODUCT OF HALORHODOPSIN

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ABSTRACT Dark-adapted halorhodopsin is a mixture of 13-*cis* and all-*trans* retinal chromophoric species. It is known that illumination with blue light increases the all-*trans* content, and this is reversed partially by brief red illumination. We now find that extended red-light illumination produces a third spectroscopic form. Analysis of composite absorption spectra recorded during various illumination regimes yielded the spectrum for the new species, whose absorption is shifted ~100 nm to the blue. The isomeric composition of retinal extracted from the illuminated pigment indicates that this form contains 9-*cis* retinal. This species, which we name iso-halorhodopsin, is stable in the dark at room temperature for at least a day, but can be quantitatively reconverted into a mixture of all-*trans* and 13-*cis* halorhodopsin by blue-light illumination. A kinetic scheme for the isomeric interconversions was drawn up, where iso-halorhodopsin is produced from either all-*trans* halorhodopsin only, or both 13-*cis* and all-*trans* forms. This kind of scheme is supported by the finding that red illumination of halo-opsin reconstituted with 13-*trans*–locked retinal will generate iso-halorhodopsin. A similar experiment with 13-*cis*–locked retinal could not be done because reconstitution with this retinal analogue was not possible. The photoreaction that leads to iso-halorhodopsin can be readily demonstrated in detergent-solubilized halorhodopsin or in halorhodopsin in liposomes made from phosphatidylcholine plus phosphatidylethanolamine, but only to much reduced extent in cell envelope vesicles and in halorhodopsin incorporated into liposomes made from halobacterial polar lipids.

INTRODUCTION

The transient photocycle intermediates of bacteriorhodopsin (Lanyi, 1984*a*; Stoeckenius and Bogomolni, 1982) and halorhodopsin (Lanyi, 1986*a*) are produced with high yield by thermal decay of the primary photoproducts. Other pathways for the thermal relaxation of the photoproducts, with lower yields, are known, however. Thus, several minutes of illumination of dark-adapted bacteriorhodopsin, a mixture of all-*trans* and 13-*cis* chromophores, will produce the light-adapted pigment, which contains only the all-*trans* chromophore (Sperling et al., 1977). The latter will accumulate in spite of the slow rate of its production, because its reisomerization at room temperature is over periods as long as several hours. Similarly, illumination of dark-adapted halorhodopsin with red or blue light increases the all-*trans* content (Lanyi, 1986*b*), which is thermally stable for several days, although in this system the blue-light–adapted pigment can be driven back to a mixture containing more of the 13-*cis* species with red-light illumination (Lanyi, 1986*b*; Smith et al., 1984). Another such photoproduct of bacteriorhodopsin is observed when the cation-depleted protein, produced either at acid pH (Fischer et al, 1981; Maeda et al., 1980) or by deionization (Chang et al., 1987), is illuminated with red light for several hours. In this case the chromophore is slowly converted to the stable 9-*cis* isomeric form, which can be reisomerized by brief illumination with blue light.

We now report on a photoreaction of halorhodopsin analogous to that of cation-depleted bacteriorhodopsin, which produces the 9-*cis* chromophore we name iso-halorhodopsin, and describe the light-induced interconversions of all-*trans*, 13-*cis*, and 9-*cis* halorhodopsin during illumination at different wavelengths. The photoproduction of iso-halorhodopsin is observed mainly in the absence of halobacterial lipids, e.g., after red-light illumination of halorhodopsin reconstituted with extraneous lipids or detergent-solubilized halorhodopsin.

MATERIALS AND METHODS

Halorhodopsin was purified from *Halobacterium halobium* strain OD-2W (provided to us by J.L. Spudich, Albert Einstein Medical College, Bronx, NY). The preparations were stored at 4°C in 4 M NaCl, 25 mM Tris.Cl, pH 7.2, 0.5% octylglucoside, in the dark. Unless mentioned otherwise, the halorhodopsin was converted to a lipoprotein complex as follows. Lipid of the indicated kind, dissolved in chloroform (1–5 mg), was evaporated under nitrogen, redissolved in a few drops of diethyl ether, and re-evaporated twice, and suspended with sonication in a small volume of water. Addition of one-third volume of 10% (wt/vol) octylglucoside produced a clear solution, which was added in the desired amount to the octylglucoside-solubilized halorhodopsin. The solution was dialyzed against 2 × 100–200 vol of 0.4 M NaCl, 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] pH 6.0, and then against 100–200 vol of 4 M

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[1] *Abbreviations used in this paper:* MES, 2-(*N*-morpholino)ethanesulfonic acid; octylglucoside, *n*-octyl-beta-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

NaCl, 25 mM MES, pH 6.0. Unless otherwise mentioned, all experiments were done in this buffer. Protein-free liposomes, for the determination of light-scattering baselines for absolute absorption spectra, were prepared similarly, but without the halorhodopsin.

Cell envelope vesicles were prepared from the same *Halobacterium* strain, as described elsewhere (Lanyi and MacDonald, 1979), and stored in 4 M NaCl. Other rhodopsins (i.e., the sensory pigments present in the OD-2W strain) were removed by bleaching for 4 h with 0.2 M NH_2OH adjusted to pH 6, at 40°C in the dark (Weber et al., 1983), followed by extensive washing with 4 M NaCl, 25 mM MES pH 6.0 to remove the hydroxylamine.

Illumination was with a 250 watt tungsten-halogen lamp (Sylvania Consumer Lighting, Danvers, MA), operated at 40% power, through 5 cm of water and one of the following filters: for red illumination a 610-nm high-pass filter, for blue illumination a color filter with transmission below 492 nm (wavelengths of 50% transmission), and for green illumination a 40-nm bandwidth interference filter centered at 505 nm. The light was focused on a 0.4 × 1 cm quartz cuvette (1 cm pathlength), thermostatted at 15°C to prevent heating, but no attempts were made to keep the sample cooled after the illuminations. Blue-light adaptation was for 10–15 min, after which no further spectroscopic changes were observed. Dark-adaptation was at room temperature for at least 24 h. All samples contained 15 mM NaN_3, and the spectra were recorded after 5 min dark periods following the illuminations, so as to eliminate the light-induced accumulation of the deprotonated Schiff-base form of halorhodopsin, which absorbs near 410 nm (Lanyi and Schobert, 1983; Steiner et al., 1984). The spectra were recorded on a (double monochromator) spectrophotometer, (model UV250; Shimadzu Corp., Columbia, MD) connected to an XPC computer which acquired the data with software supplied by Shimadzu Corp. The data were analyzed with Lotus 123 software. Properly normalized light-scattering spectra were subtracted from the absolute spectra where indicated, in order to obtain flat baselines. The criterion for such subtractions was that the absorption ratio of UV and visible peak be ~1.7, the value measured for the optically clear detergent-solubilized halorhodopsin.

Retinal extractions were adapted from the "formaldehyde method," designed to keep isomerization to a minimum (Suzuki et al., 1986). This procedure was carried out under dim light. To 1 ml halorhodopsin suspension 1 ml 6 M formaldehyde in 0.1 M phosphate (pH 7.5) was added, and the mixture incubated 2 min at 30°C. Addition of 2 ml dichloromethane and mixing 10 times in a Potter-Elvehjem homogenizer was followed by 10 min incubation at 30°C and centrifugation at 3,000 *g* for 5 min. The lower phase was collected and the dichloromethane addition and subsequent steps repeated. The combined organic phase was evaporated under nitrogen, dissolved in 2 ml hexane, and washed with 3 ml water. The hexane phase was recovered by centrifugation as above, dried with anh. $CaCl_2$, evaporated, and the retinals redissolved in 200 μl hexane. High-performance liquid chromatography (HPLC) analysis was as described before (Lanyi, 1986*b*), except that a Zorbax SIL (E.I. DuPont de Nemours, Inc., Wilmington, DE) column was used, and the hexane-diethyl ether (8%) used as eluting solvent was saturated with water. The use of standards and quantitation was as described before (Lanyi, 1986*b*).

Halorhodopsin with 13-*trans*–locked retinal as chromophore was prepared according to a reconstitution method we have recently developed (Lanyi et al., 1988) as follows. First, proteoliposomes were made with cardiolipin at a lipid/protein ratio of 1:3, as described above. The halorhodopsin was bleached for 18 h at 10°C with intense light from two 250-W lamps (ENH, operated at 40% power) through 530-nm high-pass filters, in the presence of 0.2 M NH_2OH at pH 6. Spectroscopic examination showed that all but a few percent of the pigment was removed by this procedure. The product was dialyzed against 3 × 100 vol of 4 M NaCl, 25 mM MES (pH 6.0), and reconstituted with excess 13-*trans*–locked retinal (a generous gift of K. Nakanishi, Dept. of Chemistry, Columbia University, New York). The extent of reconstitution was ~35% of the original absorption of the chromophore. The excess retinal analogue was removed by adding Na cholate to 2% concentration, and recovering the pigment by centrifugation for 10 min at 12,000 *g*, and repeating the cholate washing once more, followed by washing with the NaCl buffer.

Polar lipids from halobacteria were prepared by acetone precipitation of a Bligh-Dyer extract of membranes (Kates, 1978), and stored at −20°C dissolved in chloroform. Phosphatidylethanolamine (PE) (from *E. coli*, Type V-A), cardiolipin (from bovine heart), and phosphatidylcholine (PC) (from egg yolk, Type V-EA) were from Sigma Chemical Co., St. Louis, MO.

RESULTS

Illumination-induced Spectroscopic Changes

Dark-adapted and blue-illuminated halorhodopsin, reconstituted with PE plus PC (1:1) at a 1:1 (wt/wt) lipid/protein ratio, were illuminated with red light and difference spectra were recorded at various times, from a few seconds to several hours (Fig. 1, *A* and *B*). Illumination of dark-adapted halorhodopsin (Fig. 1 *A*) during the first minutes produced absorption increases around 607 and 450 nm, and a decrease around 530 nm, but at later times a dominant negative peak at 567 nm and a positive peak at 469 nm appeared. Red-light illumination of blue-light–adapted halorhodopsin (Fig. 1 *B*) produced such negative and positive peaks from the beginning, but the positions of these shifted from the early values of ~597 and 490 nm to 586 and 473 nm, respectively. Both sets of difference spectra suggest that at least two photoreactions occur under these conditions, and these are separated by the difference in their rates. Thus, while at shorter illumination times the changes in the dark-adapted pigment are consistent with the earlier described, reversible light-induced 13-*cis* ↔ all-*trans* equilibration (Lanyi, 1986*b*), at longer illuminations a new and more extensive photoreaction, resulting in a product with a considerably blue-shifted absorption spectrum, must be postulated. Blue-light or green-light illumination of extensively red-illuminated samples, initially either dark-adapted or blue-light adapted, restored the spectrum of blue-light–adapted halo-rhodopsin (not shown). Repeated illumination cycles and/or dark adaptation reproduced this pattern exactly, indicating that no loss of chromophore had taken place.

The occurrence of two photoreactions with different rates was confirmed by comparing the initial red-light–illuminated vs. blue-light–adapted difference spectra from Fig. 1 *B*, up to 4 min of illumination, to one another (Fig. 2 *A*), and similarly comparing the rest of the data up to 3 h of illumination, represented as illuminated vs. 4-min-illuminated difference spectra (Fig. 2 *B*). Each of these sets of difference spectra was scaled to the largest of the set. The rationale was that interconversion of only two spectroscopic species always gives the same difference spectrum, and only the amplitude should vary, depending on the ratio of the two components. Indeed, as shown in Fig. 2 *A*, during the first 4 min the process can be described by interconversion of two components only. After 16 min,

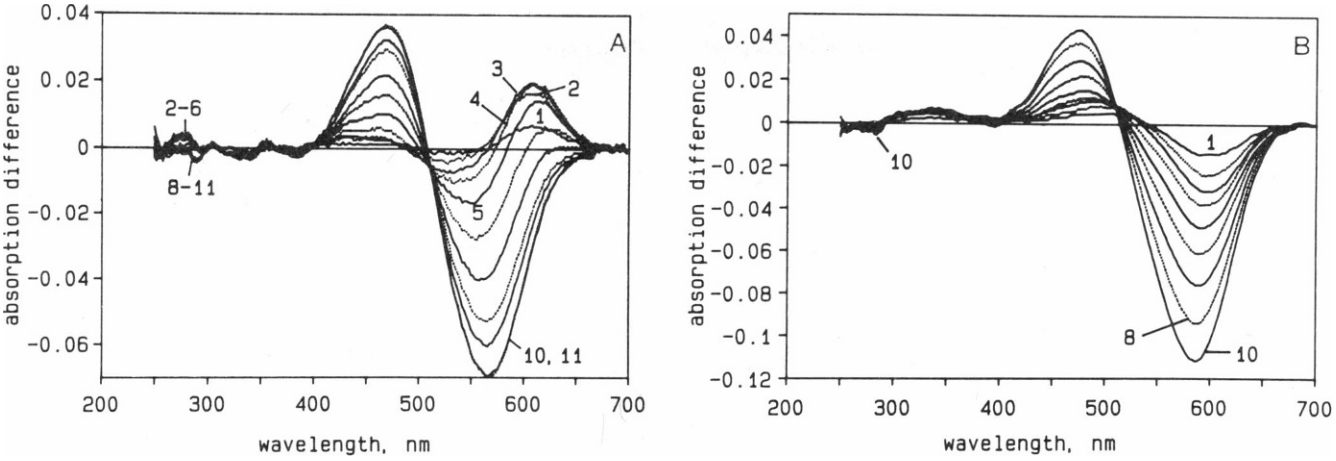

FIGURE 1 Spectroscopic changes during red-light illumination of dark-adapted (*A*) and blue-light adapted (*B*) halorhodopsin in PC/PE liposomes (lipid/protein = 1:1). The changes are shown as illuminated vs. dark-adapted (*A*) or vs. blue-light adapted (*B*) difference spectra. Red-light illumination periods in the two experiments, starting from the curves labeled as 1: 20 s, 1 min, 2 min, 4 min, 8 min, 16 min, 30 min, 1 h, 2 h (only *A*), 3 h, and 5 h (only *A*). The absorption of the dark-adapted halorhodopsin at 570 nm, after subtracting light scattering, was 0.27.

however, this difference spectrum was clearly replaced by another (Fig. 2 *B*), which remained constant during the latter part of the photoconversion, indicating that (*a*) the photoequilibrium ratio of the initial two components, reached during the first minutes, did not change further, and (*b*) the photoreaction at longer times could be described by the decrease in the concentration of both initial species and the production of a third, single spectroscopic entity. The same conclusion could be reached for the red-light photoreaction of dark-adapted halorhodopsin. The longer-times vs. 4-min red-light illumination difference spectra were similar, with exactly the same extrema and isosbestic points, whether the samples were first dark-adapted (not shown) or blue-light–adapted. This indicates that after the first minutes of red-light adaptation the spectral composition of the samples reached a common point, independent of their previous illumination history.

Retinal Isomeric Composition

Analysis of extracted retinal had indicated in an earlier report (Lanyi, 1986*b*) that what we now describe as the initial red-light reaction consists of the photochromic interconversion of 13-*cis* and all-*trans* halorhodopsin. Retinal extractions of the samples in this study, carried out in the presence of formaldehyde to minimize isomerization during the extraction (Suzuki et al., 1986), and HPLC separation of the isomers, confirmed this conclusion (Table I). The isomeric analysis of the extracted retinal also showed (Table I) that after longer times of red-light

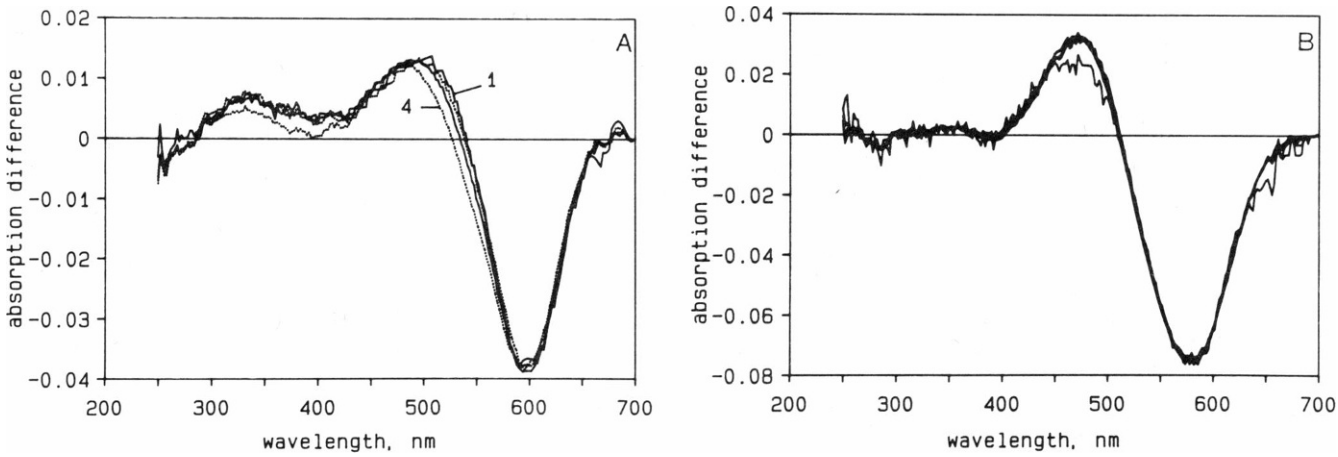

FIGURE 2 (*A*) Difference spectra for halorhodopsin, from Fig. 1 *B*, during the first 4 min of red-light illumination after blue-light adaptation, shown vs. the blue-light–adapted form. Curves 1–4, in sequence from Fig. 1 *B*, were scaled to the amplitude of curve 4. (*B*) Difference spectra for halorhodopsin, from Fig. 1 *B*, during prolonged red-light illumination after blue-light adaptation as in *A*, but shown vs. the 4-min red-light–illuminated form. Curves 5–10, in sequence from Fig. 1 *B*, were subtrated from curve 4, and then scaled to match their amplitudes.

TABLE I

PERCENT RETINAL ISOMERIC COMPOSITION FOR HALORHODOPSIN AFTER DIFFERENT ILLUMINATIONS, AS DETERMINED BY RETINAL EXTRACTION AND BY ANALYZING ABSORPTION SPECTRA (IN PARENTHESES)*

Illumination history	Lipid/protein = 1			Lipid/protein = 5		
	all-*trans*	13-*cis*	9-*cis*	all-*trans*	13-*cis*	9-*cis*
Dark-adapted	42	56	2	46	52	2
Blue-light adapted	59	37	4	79	20	1
Red-light adapted (3 min)	50	45	5	50	45	5
Red-light adapted (3 h)	31	31	38 (43)	26	16	58 (47)

*Halorhodopsin was reconstituted with PE plus PC (1:1) at the lipid/protein ratios indicated, and retinal isomeric composition was determined as described in Methods. The 9-*cis* contents of a few percent or less in some of the samples are within the error of experimental determinations. The calculation of 9-*cis* contents by resolution of composite spectra is described in the text.

illumination large amounts of 9-*cis* retinal had accumulated, in proportions roughly comparable to the predictions of the resolution of composite spectra. The lipid/protein ratio had an influence on the retinal isomeric compositions; both retinal extractions and spectra had indicated, in Table I and in other experiments not shown, that the yield of 9-*cis* chromophore was higher at a lipid/protein ratio of 5:1 than 1:1.

Absolute and Difference Absorption Spectra

Manipulation of absolute and difference spectra for the samples, in order to subtract light-scattering and to decompose the spectra into its components, yielded the curves in Fig. 3. The resulting spectra for dark-adapted, blue-light–adapted, 2-mins, 16-min, and 3-h red-light–adapted halorhodopsin are shown in Fig. 3 *A*, to indicate the extents of illumination-dependent shifts in the absorption maxima and the changes of amplitudes. From these composite spectra, individual spectra for 9-*cis* halorhodopsin and the red-light–adapted mixture of 13-*cis* and all-*trans* halorhodopsin were obtained (Fig. 3 *B*). The criteria for the decomposition of the spectra were that the ratios of the components roughly agree with the retinal extraction data, and that the spectra obtained be similar in shape to those measured for light- or dark-adapted halorhodopsin. The absorption maximum of 9-*cis* halorhodopsin is at 478 nm in the buffer containing 4 M NaCl. Because of the substantial overlap of the absorption bands of dark-adapted and blue-light–adapted halorhodopsin, and because of the limitations in the accuracy of retinal extraction data, reliable spectra for the pure all-*trans* and 13-*cis* chromophores could not be obtained.

The spectra in Figs. 1 and 2 indicate that characteristic changes in the UV range accompany the spectroscopic shift observed in the visible. Although not shown, the spectra of the UV region that contain these changes have

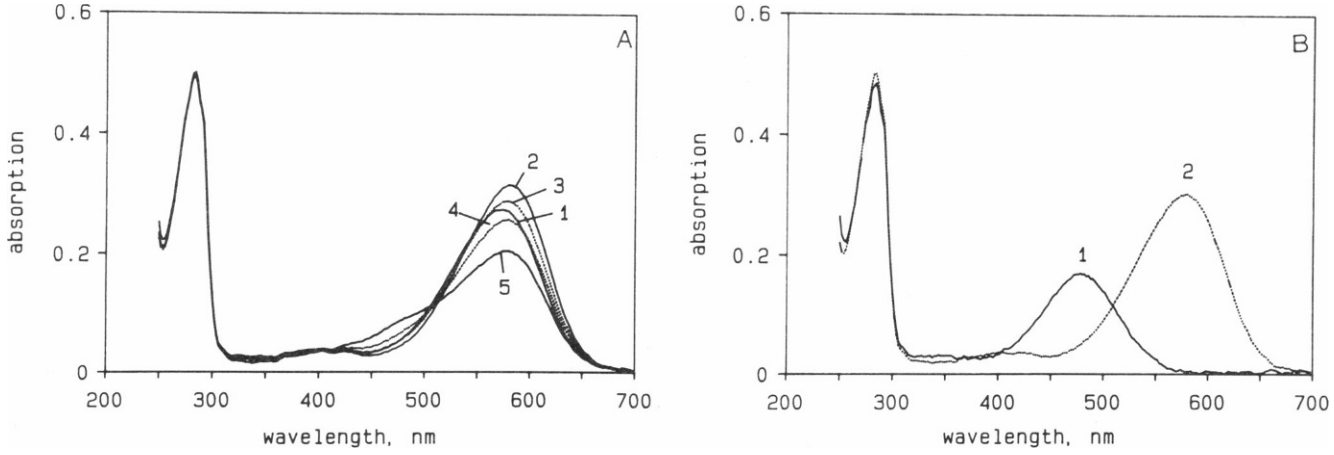

FIGURE 3 (*A*) Absorption spectra of various states of halorhodopsin in PC/PE liposomes (lipid/protein = 1:1), obtained from the difference spectra in Fig. 1 *B*, and by subtraction of light-scattering. Curve 1, dark-adapted; curve 2, blue-light adapted; curve 3, 2 min; curve 4, 16 min; and curve 5, 3 h red-light–illuminated samples, respectively. (*B*) Decomposition of the red-light–illuminated spectrum into the spectrum of the 9-*cis* pigment (curve 1), and that of the red-light–produced all-*trans* + 13-*cis* mixture of halorhodopsin (curve 2). Curve 1 was obtained by subtracting curve 4 multiplied by 0.795 from curve 5 in *A*, and normalizing by the factor of (1-0.795). Curve 2 was constructed by subtracting 0.325 times the 9-*cis* spectrum (curve 1) from curve 5 in *A*, and normalizing by (1-0.325). The rationale for choosing the weighting factors is explained in the text.

been manipulated in the same manner as the visible bands, and the results suggest that (*a*) the 13-*cis* → all-*trans* conversion generates a difference spectrum which resembles the dark-adapted vs. light-adapted spectrum of bacteriorhodopsin, as found previously for the photocycle intermediates (Lanyi, 1984*b*; Lanyi and Vodyanoy, 1986), and (*b*) the accumulation of the 9-*cis* chromophore is accompanied by this change and an additional negative band near 290 nm (cf. Fig. 2 *B*), which may originate from protonation change of an aromatic amino acid residue.

A Kinetic Scheme for the Photoreactions

A general model for the analysis of the kinetics of the red-light–induced isomerizations is given in Fig. 4 (*inset*). Independent evidence for the all-*trans* → 9-*cis* photoconversion was obtained by illuminating halorhodopsin containing 13-*trans* locked retinal (data not shown). Unfortunately, a similar experiment could not be done with halorhodopsin containing 13-*cis* locked retinal, since reconstitution with this analogue was unsuccessful. Since a 13-*cis* → 9-*cis* photoconversion might require the formation of 9,13-di*cis* halorhodopsin, this reaction is shown as a tentative pathway only in Fig. 4.

The differential equations corresponding to the scheme in Fig. 4, *inset*, were solved analytically. The primary rate constants designated as k_{AB}, k_{BA}, etc. in Fig. 4 depend on the light intensity, the extinctions of the different forms at the wavelengths of illumination, and the quantum yields. As an example, the model was fitted to the 9-*cis* chromophore concentrations calculated from the sequential spectra in Fig. 1 *A*. The photostationary state ratios of 13-*cis* and all-*trans* isomer concentrations were estimated from the measured absorption maxima of dark-adapted, blue-light–adapted, and red-light–adapted halorhodopsin (572, 580, and 578 nm, respectively), and the ratios of retinal isomer concentrations from the retinal analyses of dark-adapted and blue-light–adapted samples, determined under similar conditions (Table I, lipid/protein = 1). The rate constants which yielded a good fit (Fig. 4) were as follows: (all in s^{-1}), $k_{AB} = 8.0 \times 10^{-3}$; $k_{BA} = 5.5 \times 10^{-3}$; $k_{AC} = k_{BC} = 1.5 \times 10^{-4}$; $k_{CA} = k_{CB} = 2.0 \times 10^{-4}$. A similarly good fit could be obtained when the 13-*cis* ↔ 9-*cis* interconversion was disallowed, with $k_{BC} = 3.0 \times 10^{-4}$ and $k_{CB} = 4.8 \times 10^{-4}\ s^{-1}$. The rates of the forward and reverse processes in the all-*trans* (13-*cis*) ↔ 9-*cis* isomerization are roughly similar, despite the obviously much smaller extinction of the 9-*cis* form (cf. Fig. 3 *B*) at the wavelength of red light. This indicates that the reisomerization reaction must have a many-fold greater quantum yield than the isomerization leading to the 9-*cis* chromophore.

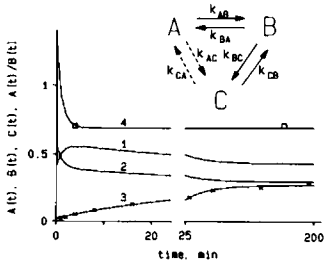

FIGURE 4 Fitting of calculated time-dependent isomeric concentrations to the concentrations estimated from the difference spectra in Fig. 1 *A*, in terms of the reaction scheme in the inset. Curve 1, all-*trans* chromophore /*B*(*t*)/; curve 2, 13-*cis* chromophore /*A*(*t*)/; and curve 3, 9-*cis* chromophore /*C*(*t*)/ concentrations; curve 4, ratio of 13-*cis*/all-*trans* concentration. Symbols: (×) 9-*cis* retinal concentration; (□) 13-*cis*/all-*trans* concentration ratio, calculated from the absorption spectra as described in the text.

Influence of Lipids on the Photoreactions

Illuminations were carried out with three other kinds of halorhodopsin preparations: (*a*) halorhodopsin in cell envelope vesicles, (*b*) halorhodopsin solubilized with octylglucoside, and (*c*) halorhodopsin reconstituted with the polar lipids of *H. halobium* rather than with the PE plus PC mixture. In addition, the lipid/protein ratio was varied, using PE plus PC and the halobacterial lipids.

The spectroscopic changes observed with the cell envelope vesicles were complicated by changes in light-scattering, which arose during illumination because of volume increase due to active chloride transport (Schobert and Lanyi, 1982). These were eliminated by appropriate subtraction of a scattering curve. Fig. 5 contains the spectra obtained when dark-adapted envelope vesicles were illuminated with red-light for (*a*) 3 min, (*b*) 7 h, and then (*c*) with blue light. As seen in Fig. 5, red-light illumination for a few minutes produced difference spectra consistent with 13-*cis* → all-*trans* conversion, as described before (Lanyi, 1986*b*). Comparison with the spectra in Fig. 1 *A* indicates, however, that extended red-light illumination of the envelope vesicles produced a photostationary state containing little of the 9-*cis* chromophore. However, the rise of absorption between 450 and 500 nm after the illumination appears to be real and indicates that a small amount (no more than a few percent) of 9-*cis* pigment is indeed present, since additional blue-light illumination caused an absorption decrease in this region (Fig. 5).

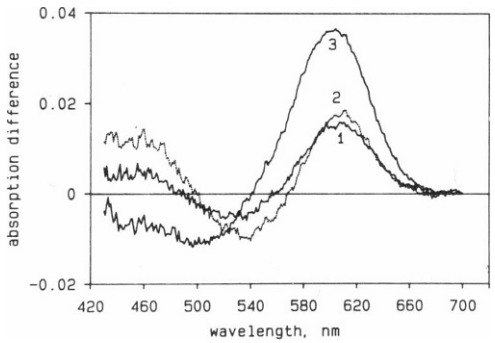

FIGURE 5 Difference spectra of red-light–and blue-light–illuminated cell envelope vesicles vs. dark-adapted vesicles after various illumination regimes. Curve 1, 3 min red-light; curve 2, 7 h red-light; curve 3, 10 min blue-light illumination. Vesicle concentration, 42.5 mg/ml; optical pathlength, 2 mm.

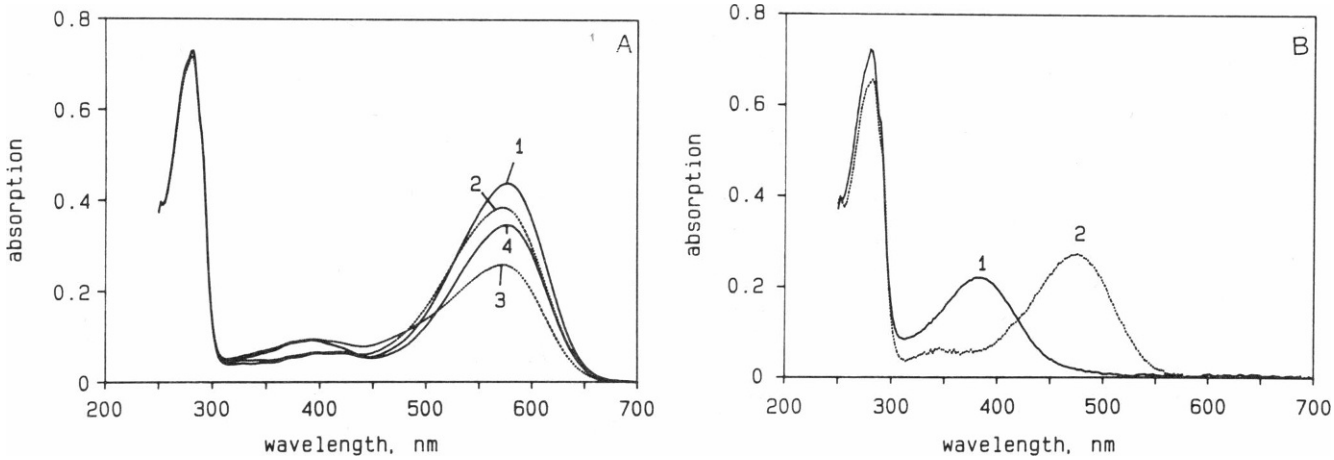

FIGURE 6 (*A*) Absorption spectra of octylglucoside-solubilized halorhodopsin after various illumination regimens. Curve 1, blue-light adapted; curve 2, 3 min; curve 3, 2 h red-light illumination, followed by 10 min green illumination (curve 4). (*B*) Spectrum of the dissociated chromophore (curve 1), calculated by subtracting curve 1 in *A*, multiplied by 0.8, from curve 4 of *A*, normalized by (1-0.8); and the spectrum of the 9-*cis* chromophore, calculated by first removing the dissociated chromophore contribution (20%) from curve 3 of *A*, and then subtracting from this normalized spectrum curve 2 of *A* multiplied by 0.84, and finally normalized by (1-0.84).

Octylglucoside-solubilized halorhodopsin was optically the most favorable preparation; the spectra of the blue-light–adapted sample, and the products obtained by illuminations with different lights, are shown in Fig. 6 *A*. The photoreactions greatly resemble those of halorhodopsin reconstituted with the PE plus PC mixture (Fig. 3 *A*), except that during lengthy red-light illumination an additional photoproduct is produced. Green-light illumination of a 2-h red-light–adapted sample, which depleted exclusively the 9-*cis* chromophore, allowed the determination of the spectrum of this fourth spectroscopic form (Fig. 6 *B*). It absorbs at 384 nm, and probably contains a dissociated chromophore, i.e., free retinal, since it could not be photoconverted with blue-light illumination (not shown). After subtracting the contribution of the 384-nm species from the spectrum of the 2-h red-light–illuminated halorhodopsin, the spectrum of the 9-*cis* form produced in this sample was obtained (Fig. 6 *B*) as in the other experiments (cf. Fig. 3 *B*). It is evident from this spectrum that 9-*cis* halorhodopsin is produced in the presence of octylglucoside in large amounts, corresponding to yields about half of what was obtained when PE and PC were present. The absorption maximum of the 9-*cis* chromophore under these conditions was at 472 nm, i.e., noticeably blue-shifted from the maximum seen when lipids were present, as were the absorption maxima of the dark-adapted and blue-light–adapted octylglucoside samples (570 and 576 nm, respectively).

Halorhodopsin reconstituted with the polar lipids of *H. halobium* exhibited similar photoreactions to what was observed with halorhodopsin in cell envelope vesicles (Lanyi, 1986*b*), but considerably less 9-*cis* chromophore was produced even after 3 h of red-light illumination, i.e., about a fourth, than in the presence of PE and PC. As with PE and PC, more 9-*cis* pigment was produced at higher lipid/protein ratios (not shown).

DISCUSSION

Earlier we had found (Lanyi, 1986*b*) that the underlying cause of a photochromic effect in halorhodopsin (Smith et al., 1984) was the interconversion of 13-*cis* ↔ all-*trans* chromophores. The results of this study extend the description of this phenomenon and indicate the occurrence of another kind of hypsochromic effect in this system: extensive red-light illumination of halorhodopsin produces an additional, new chromophoric species, which contains 9-*cis* retinal. By analogy with isorhodopsin (Yoshizawa and Wald, 1963), we name this species iso-halorhodopsin. Since this species is formed also from a 13-*trans*–locked chromophore, it is probably generated near the primary photoreaction of all-*trans* halorhodopsin, i.e., before the isomerization at carbon 13. Iso-halorhodopsin is produced with very low yield by such a branching reaction, but it accumulates in large amounts because of its thermal stability. The photostationary concentrations depend on the presence and kind of lipid, and somewhat on the lipid/protein ratio. In liposomes containing PE and PC the steady-state amount of iso-halorhodopsin is 30–50%. Much smaller amounts are produced, however, in the presence of the halobacterial lipids, either in envelope vesicles or in liposomes. This is the first reported instance of lipid specificity in this system.

Iso-halorhodopsin is thermally slowly converted back to dark-adapted halorhodopsin, which is a mixture of 13-*cis* and all-*trans* forms. Blue-light illumination, on the other hand, causes the rapid reconversion of iso-halorhodopsin to a mixture, which as always after blue-light illumination, contains more all-*trans* than 13-*cis* species.

The spectroscopic changes in halorhodopsin–lipid complexes, and their kinetics during red-light illumination, can be nearly exactly described in terms of three species only, which contain 13-*cis*, all-*trans*, and 9-*cis* retinal. In octyl-

glucoside-solubilized halorhodopsin a fourth species, which probably represents the dissociated chromophore, is also formed. Inasmuch as here, as in a previous study (Lanyi, 1986*b*), we find that halorhodopsin always contains a mixture of retinal isomers, the absorption maxima for the pure 13-*cis* and all-*trans* chromophores are somewhat uncertain and may significantly deviate from accepted values (Spudich and Bogomolni, 1983).

The formation of iso-halorhodopsin during lengthy red-light illumination greatly resembles the behavior of blue bacteriorhodopsin, formed either at low pH (Fischer et al., 1981; Maeda et al., 1980) or by deionization (Chang et al., 1987): in both kinds of bacteriorhodopsin preparation the low quantum yield production of a stable 9-*cis* form has been reported. Analogously also, iso-halorhodopsin exhibits the higher quantum-yield reconversion of 9-*cis* bacteriorhodopsin into 13-*cis* and/or all-*trans* forms by blue light. The physiological significance of these photoreactions, if any, is not clear, since in bacteriorhodopsin the 9-*cis* form is produced from the cation-depleted pigment only, and in halorhodopsin large amounts of this species are produced only in the absence of bilayer containing halobacterial lipids.

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