SPECTROSCOPIC DISCRIMINATION OF THE THREE RHODOPSINLIKE PIGMENTS IN *HALOBACTERIUM HALOBIUM* MEMBRANES

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ABSTRACT Membranes of *Halobacterium halobium* contain two photochemically reactive retinal pigments in addition to the proton pump bacteriorhodopsin. One, halorhodopsin, is also an electrogenic ion pump with a fast (on a scale of milliseconds) photoreaction cycle. The other, s-rhodopsin, is active in the same spectral region, but has a much slower photoreaction cycle (on a scale of seconds). s-rhodopsin is not an electrogenic ion pump and its properties suggest it functions as the receptor pigment for phototaxis. All three pigments have very similar absorption spectra. The recent isolation of mutants deficient in both bacteriorhodopsin and halorhodopsin and in retinal synthesis has allowed us to resolve the absorption spectra of s-rhodopsin and halorhodopsin. At neutral pH s-rhodopsin has an absorption maximum at 587 ± 2 nm and halorhodopsin at 578 ± 2 nm. At pH 10.8, λ_{max} for s-rhodopsin is shifted to 552 nm and extinction decreases slightly (15%) while halorhodopsin loses all extinction above 500 nm. Both effects are fully reversible and allow determination of the amounts of s-rhodopsin and halorhodopsin in membrane preparations containing both pigments. Both pigments were present in earlier studies of *H. halobium* membranes, and in view of these findings, several observations must be reinterpreted.

INTRODUCTION

Three photochemically reactive pigments have been identified in the membranes of *Halobacterium halobium*. Bacteriorhodopsin (BR), which functions as a light-driven proton pump, was recognized first (1, 2). It drives energy-requiring processes such as ATP synthesis, amino-acid uptake, and locomotion (for review see reference 3). A second light-driven electrogenic ion pump, halorhodopsin (hR), also has a retinal chromophore with an absorption spectrum and photocycle kinetics similar to those of BR (4–8). The transported ion was first thought to be Na^+ (4, 9, 10), but on the basis of recent data hR appears to be a Cl^- pump (11). Flash spectroscopy of membranes from mutants lacking both BR and hR (12) revealed the presence of the third retinal pigment, named s-rhodopsin (sR) (13). Its photoreaction cycle requires seconds for completion, compared with milliseconds for BR and hR, and it does not appear to function as an electrogenic ion pump. The mutants containing only sR still show the phototaxis responses of the wild-type (12) and the properties of sR suggest that it is the photoreceptor for these responses (13).

Wild-type cells contain all three retinal pigments (13). By analyzing mutant membranes we are able to examine hR and sR separately and define their spectral and chemical properties. We derive here the absorption spectra of hR and sR for neutral and alkaline conditions and characterize some of their photoreactions. The results provide criteria for distinquishing the pigments and show that earlier observations attributed only to hR reactions need reinterpretation.

MATERIALS AND METHODS

Bacterial Strains

Phenotypes of H. halobium Strains. F1x3 is $BR^-hR^-sR^+$ carotenoid$^+$ and its isolation is described (12); F1x3R is a retinal-deficient derivative of F1x3, selected by screening for white (i.e., carotenoid$^-$) colonies, which often show loss of retinal synthesis. L33 is $BR^-hR^+sR^+$carotenoid$^-$ and was isolated by Lanyi and co-workers (14). F1x37 is an hR-deficient derivative of L33 selected on the basis of its loss of halorhodopsin function (12). Culture conditions, preparation of membrane vesicles, light-induced proton flux (12) and phototaxis response (15) measurements have been described.

Spectroscopy

Absorption spectra were obtained on an Aminco DW-2a spectrophotometer (American Instrument Co., Silver Spring, MD) and the spectra stored and processed with a Nicolet Instrument Corp., Madison, WI, model 1074 data acquisition system (16). Wavelength calibration was against the absorption lines of the spectrophotometer holmium oxide filter. Spectral resolution was 2 nm. Flash spectrometry was carried out as described (13).

RESULTS AND DISCUSSION

Identification of sR Absorption Spectrum

Ion transport assays (12) and flash spectroscopy (13) have shown that Flx3 membranes lack BR and hR but contain sR. By screening for white variants of Flx3 we isolated Flx3R, which is apparently retinal deficient because it lacks the retinal-dependent phototaxis responses (15, 17, 18) and because phototaxis is restored by addition of exogenous retinal (measured as described in reference 15).

Addition of retinal to Flx3R membranes generates absorbance in the orange region of the spectrum (Fig. 1). A depletion of the retinal peak in the near UV occurs concomitantly with the appearance of long wavelength absorbance generating an isosbestic point near 440 nm. The sR visible absorption spectrum so obtained has a λ_{max} at 587 ± 2 nm.

The sR photocycle is absent in Flx3R but is restored by addition of exogenous retinal to whole cells or membrane vesicles. We determined this by both flash spectroscopy and by photo–steady state absorbance measurements (Fig. 2). Illumination of sR causes a characteristic mixture of sR and its short wavelength photocycle intermediate (13). As shown previously (13), the photo–steady state difference spectrum is identical to the flash-induced difference spectrum and can be used to diagnose the presence of sR. No photo–steady state absorbance difference or flash-induced absorbance changes (Fig. 2, inset) occur in nonreconstituted Flx3R membranes, whereas both occur after retinal

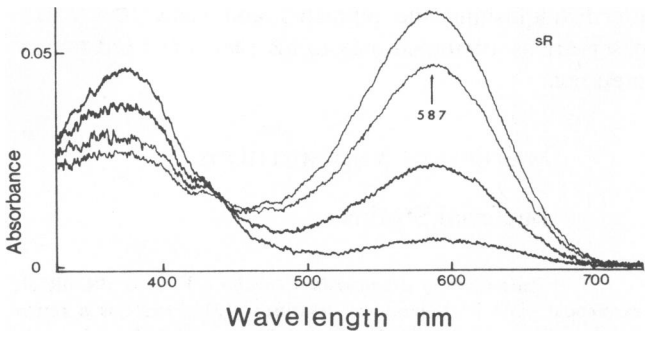

FIGURE 1 Absorbance generated by addition of retinal to Flx3R membrane vesicles. The traces correspond from low to high absorbance at 587 nm at 3, 15, and 30 min, and 1 h after addition of 0.5 μl of 1 mM all-*trans* retinal in 100% ethanol to the sample cuvette and 0.5 μl of ethanol to a matched sample in the reference cuvette. Pathlength 1 cm, 23°C, pH 6.5, 6.9 mg protein/ml.

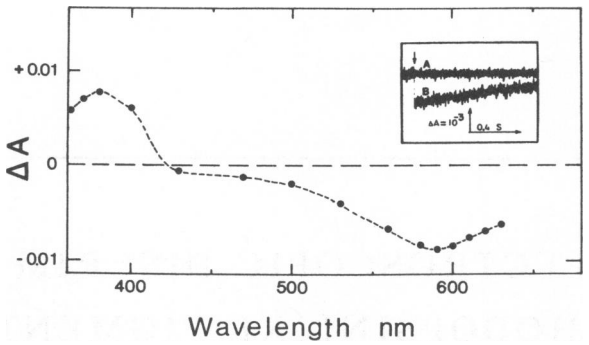

FIGURE 2 Light-induced absorbance changes of Flx3R membrane vesicles. Absorbance changes were recorded 2 h after addition of retinal to the vesicles as in Fig. 1. Actinic light was 5×10^5 ergs/cm^{2}s, 630–700 nm, 30 s duration. *Inset:* flash-induced absorbance changes at 580 nm of the same sample before (*A*) and 2 h after (*B*) addition of retinal; actinic light was ~0.5 mJ, 520 nm, 7 ns.

reconstitution (Fig. 2). Retinal-reconstituted Flx3R membranes are like those of the $BR^-hR^-sR^+$ parent Flx3 in that BR and hR are not detectable by light-induced proton influx assay or flash spectroscopy (Fig. 2, inset); the only detectable retinal pigment in Flx3R after retinal reconstitution is sR.

Identification of hR Absorption Spectrum

The absorption difference spectrum of an hR-containing strain and an hR-deficient mutant derived from it yields the hR absorption spectrum provided the mutant contains all other pigments in the same amounts as its parent. Such strains are available through the mutant selection scheme directed specifically against hR (12). L33 and Flx37 constitute such a pair, as shown by ion flux (12) and photocycle assays (13). Using the flash-induced absorbance change of sR at 580 nm, we normalized membrane vesicle preparations of L33 and Flx37 to within 5% for equal sR content and recorded the difference spectrum (Fig. 3). The hR absorption spectrum thus obtained has λ_{max} at 578 ± 2 nm.

Photocycle Difference Spectra

Difference spectra for the photoreaction cycle intermediates of the pigments can be established in essentially the same way (Fig. 4). Flx3R regenerated with retinal directly yields the spectra for sR, whereas differences in matched samples of L33 and Flx37 can be used to extract spectra for hR. Alternatively, differences in the susceptibility of the pigments to bleaching and regeneration may be used. For details see the legend to Fig. 4.

Comparison of Absorption and Photoreaction Spectra

Absorption spectra of each of the three known retinal pigments in *H. halobium* have now been established (Fig. 3) and can be compared with their flash-induced photoreaction cycle spectra (Fig. 4). The photocycle depletion

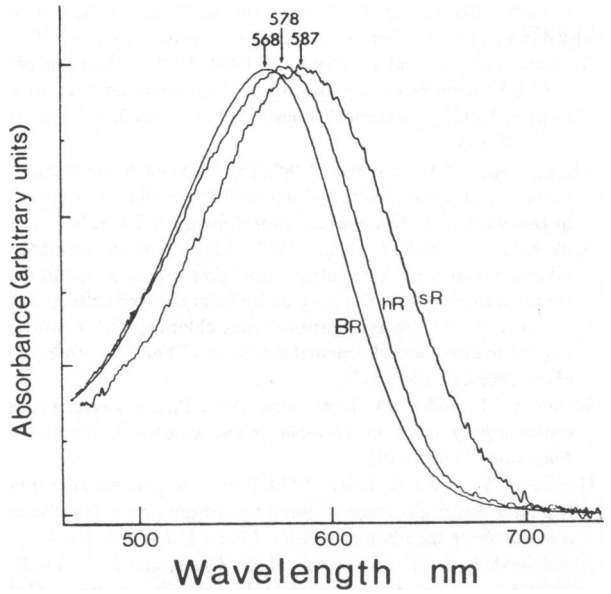

FIGURE 3 Absorption spectra of the three retinal-containing pigments of *H. halobium*. The *BR* spectrum (λ_{max} = 568 nm) is for an aqueous suspension of purple membrane in 100 nM NaCl, pH 6.5, OD_{568} = 0.5, light-adapted (4 min, 2×10^5 ergs/cm^{2}s, 550–700 nm, 1 cm pathlength, 20°C). The hR spectrum (λ_{max} = 578 nm) is the difference between L33 and Flx37 membrane suspensions (6.0 mg protein/ml) in 4M NaCl, pH 6.5, matched for sR photochemical activity at 580 nm as described in the text. The sR spectrum (λ_{max} = 587 nm) is that shown for 1 h in Fig. 1. Spectra were normalized to arbitrary units for comparison.

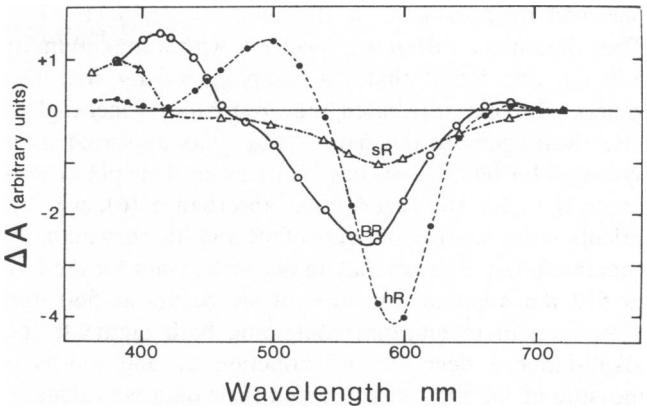

FIGURE 4 Flash-induced difference spectra 1 ms after photoexcitation. *sR:* regenerated sample of Flx3R shown in Fig. 2. *BR:* suspension of membranes of the BR-producing strain S9-P (12). *hR:* L33 membrane suspension incubated with 0.2 N hydroxylamine at pH 8 under illumination (2×10^6 ergs/cm^{2}s, 530–700 nm, 35°C) for 45 min. This treatment reduced the slow absorbance change at 580 nm (sR) to ≤ 3% of its initial value while the hR photosignal was ≥85% of its initial value. The Flx3R and hydroxylamine-bleached L33 absorbance changes superimpose well (maximum discrepancy ≤5%) on the kinetically resolved changes attributed to sR and hR in L33 membranes in reference 13. The absorbance changes of S9-P are essentially those of BR which greatly exceeds the contributions of hR and sR, which are also present in the membrane (the molar ratios BR/hR/sR are approximately 100:3:1.5).

maximum of sR coincides precisely with the sR absorption maximum. On the other hand, the BR and hR depletion maxima are shifted to longer wavelengths with respect to the maxima of their visible absorbance. We interpret this red shift as due to the transient accumulation of photocycle intermediates absorbing to the blue of BR and of hR. The near UV-absorbing species of sR (S_{373}, see reference 13) is evidently too distant from sR_{587} to influence the sR depletion maximum.

Recently Tsuda et al. (19) resolved slow and fast photoreactions in a BR-deficient strain. Although their data alone do not allow one to assign the slow and fast components to separate pigments, our previous work (13) clearly shows that the slow reactions they observe belong to sR and the fast reactions to hR. Their tentative interpretation that the fast cycling species (hR) absorbs at longer wavelengths than the slow cycling species (sR) is only in apparent conflict with our results. It derives from their assumption that the depletion maxima occur at the same wavelength as the maxima of the absorption spectra, which is not true for hR as shown here.

The Alkali-induced Transitions of sR and hR

Fig. 4 shows that the sR absorption maximum shifts from 587 to 552 nm with a slight (15%) loss in extinction when the membranes are taken from neutral pH to pH 10.8. This alkaline transition is completely reversible (Fig. 5). It provides a method for distinquishing sR from hR on absorption critera, because hR loses all extinction above 500 nm when shifted to pH 10.8, with production of absorbance in the UV/blue region (data not shown). The alkaline transition of hR is also completely reversible.

This observation leads us to a reinterpretation of earlier data. Lanyi and Weber (6) measured the absorbance generated by addition of retinal to hydroxylamine-

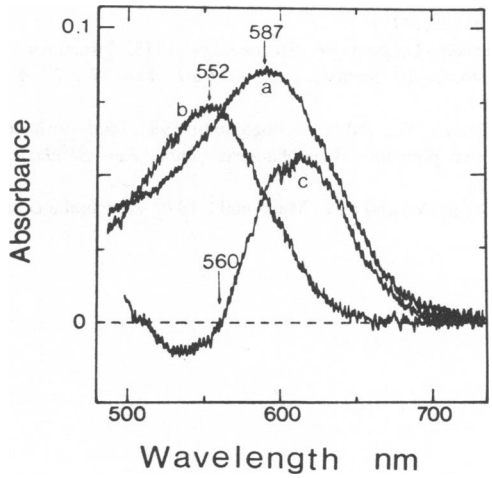

FIGURE 5 Alkali-induced transition in sR absorbance. Trace *a*, sR absorbance at pH 6.5 in Flx3R membranes reconstituted with retinal as in Fig. 2. Trace *b*, absorbance after addition of 5 N NaOH to give pH 10.8. After readjustment to pH 6.5 by HCl addition, the spectrum returns to the 587 nm maximum (shown superimposed on trace *a*). Trace $c = a - b$.

bleached membranes of a BR-deficient strain (ET-15). They obtained a difference spectrum with a maximum at 588 nm and found that ion-pumping activity was also generated. They attributed the absorbance generated to hR. (Subsequently the name "P_{588}" has appeared as a synonym for halorhodopsin [3, 8].) From their pH dependence data for the regenerated absorbance (6), we can calculate the relative amounts of sR and hR they actually generated. Fig. 5 shows that an isosbestic point for the 587 to 552 nm alkaline transition of sR occurs at 560 nm. Therefore, in membranes containing both pigments the alkali-induced decrease in extinction at 560 nm is a measure of the hR content. From their data we calculate that P_{588} actually consists of 68% sR and 32% hR. We applied their procedure to L33 vesicles and found a similar molar ratio in the regenerated membranes.

All photoactive *H. halobium* strains so far investigated contain sR, and other observations reported in the literature will need reinterpretation based on the findings reported here and in reference 13.

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REFERENCES

1. Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* 233:149–152.
2. Oesterhelt, D., and W. Stoeckenius. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. USA* 70:2853–2857.
3. Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 52:587–616.
4. Lindley, E. V., and R. E. Macdonald. 1979. A second mechanism for sodium extrusion in *Halobacterium halobium:* a light-driven sodium pump. *Biochem. Biophys. Res. Commun.* 88:491–499.
5. Matsuno-Yagi, A., and Y. Mukohata. 1980. ATP synthesis linked to light-dependent proton uptake in a red mutant strain of *Halobacterium* lacking bacteriorhodopsin. *Arch. Biochem. Biophys.* 199:297–303.
6. Lanyi, J. K., and H. J. Weber. 1980. Spectrophotometric identification of a pigment associated with light-driven sodium translocation in *Halobacterium halobium*. *J. Biol. Chem.* 255:243–250.
7. Mukohata, Y., and T. Kaji. 1981. Light-induced membrane-potential increase, ATP-synthesis, and proton uptake in *Halobacterium halobium* R_1mR catalyzed by halorhodopsin: effects of *N, N'*-dicyclohexylcarbomide, triphenyltin chloride, and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847). *Arch. Biochem. Biophys.* 206:72–76.
8. Weber, H. J., and R. A. Bogomolni. 1981. P_{588}, a second retinal-containing pigment in *Halobacterium halobium*. *Photochem. Photobiol.* 33:601–608.
9. Greene, R. V., and J. K. Lanyi. 1979. Proton movements in response to a light-driven electrogenic pump for sodium ions in *Halobacterium halobium* membranes. *J. Biol. Chem.* 254:10986–10994.
10. Macdonald, R. E., R. V. Greene, R. D. Clark, and E. V. Lindley. 1979. Characterization of the light-driven sodium pump of *Halobacterium halobium*. *J. Biol. Chem.* 254:11831–11838.
11. Schobert, B., and J. K. Lanyi. 1982. Halorhodopsin is a light-driven chloride pump. *J. Biol. Chem.* 257:10306–10313.
12. Spudich, E. N., and J. L. Spudich. 1982. Control of transmembrane ion fluxes to select halorhodopsin-deficient and other energy-transduction mutants of *Halobacterium halobium*. *Proc. Natl. Acad. Sci. USA* 79:4308–4312.
13. Bogomolni, R. A., and J. L. Spudich. 1982. Identification of a third rhodopsin-like pigment in phototactic *Halobacterium halobium*. *Proc. Natl. Acad. Sci. USA* 79:6250–6254.
14. Wagner, G., D. Oesterhelt, G. Krippahl, and J. K. Lanyi. 1981. Bioenergetic role of halorhodopsin in *Halobacterium halobium* cells. *Febs (Fed. Eur. Biochem. Soc.) Lett.* 131:341–345.
15. Spudich, J. L., and W. Stoeckenius. 1979. Photosensory and chemosensory behavior of *Halobacterium halobium*. *Photobiochem. Photobiophys.* 1:43–53.
16. Bogomolni, R. A., R. A. Baker, R. H. Lozier, and W. Stoeckenius. 1981. Action spectrum and quantum efficiency for proton pumping in *Halobacterium halobium*. *Biochemistry.* 19:2152–2159.
17. Dencher, N. A., and E. Hildebrand. 1979. Sensory transduction in *Halobacterium halobium:* retinal protein pigment controls UV-induced behavioral response. *Z. Naturforsch.* 34:841–847.
18. Sperling, W., and A. Schimz. 1980. Photosensory Retinal Pigments in *Halobacterium halobium*. *Biophys. Struct. Mech.* 6:165–169.
19. Tsuda, M., N., Hazemoto, M. Kondo, N. Kamo, Y. Kobatake, and Y. Terayama. 1982. Two photocycles in *Halobacterium halobium* that lacks bacteriorhodopsin. *Biochem. Biophys. Res. Commun.* 108:970–976.