TRANS/13-*CIS* ISOMERIZATION IS ESSENTIAL FOR BOTH THE PHOTOCYCLE AND PROTON PUMPING OF BACTERIORHODOPSIN

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ABSTRACT We studied an analogue of bacteriorhodopsin whose chromophore is based on all-*trans* retinal. A five-membered ring was built around the 13–14 double bond so as to prohibit *trans* to 13-*cis* isomerization. No light-induced photochemical changes were seen, other than those due to a small amount (~5%) of unbleached bacteriorhodopsin remaining in the apomembrane used for regeneration. The techniques used included flash photolysis at room and liquid nitrogen temperatures and Fourier-transform infrared difference spectroscopy. When the *trans*-fixed pigment was incorporated into phospholipid vesicles, no evidence of light-initiated proton pumping could be found. The results indicate that *trans* to 13-*cis* isomerization is essential for the photochemical transformation and function of bacteriorhodopsin.

INTRODUCTION

Bacteriorhodopsin (bR) is the only protein found in the purple membrane of the halophilic microorganism *Halobacterium halobium* (*H. halobium*) (1, 2). Light absorbed by the chromophore of light-adapted bR (bR^{LA}) initiates a photochemical cycle through a set of spectroscopically defined intermediates K, L, M, and O; during the photocycle protons are pumped out of the bacterial cell. However, the relation of the photocycle to proton pumping is not well understood.

The isomeric composition of the chromophore in bR^{LA} is all-*trans*. Photochemical and chromophore extraction studies indicate that a *trans*- to 13-*cis* isomerization occurs in going to M from bR^{LA} (reviewed in references 1 and 2). Furthermore, the vibrational spectra of the fingerprint region of K obtained by either Raman (3) or infrared spectroscopy (4, 5) are substantially different from that of bR supporting the idea that chromophore isomerization is the primary photochemical event in bR. Here, we present direct evidence indicating that the *trans*/13-*cis* photoisomerization is essential for both the photocycle and proton pumping. The evidence is based on studies of a synthetic pigment analogue, *trans*-fixed bR, in which the *trans*/13-*cis* isomerization is blocked by incorporating the 13–14 double bond into a 5-membered ring (Fig. 1, inset). A report on the synthesis and properties of the *trans*-fixed chromophore and its pigment (λ_{max} = 576 nm) has already been presented (6).

MATERIALS AND METHODS

The *trans*-fixed retinal was synthesized by Fang et al. (6). The bleached apomembrane for pigment regeneration was prepared from *H. halobium* purple membrane by the method of Tokunaga and Ebrey (7). For flash-photolysis experiments membrane sheets were suspended in 0.4 M KCl pH = 6.72 (Fig. 1), or 60% glycerol, 0.1 M phosphate buffer, pH = 7 (Fig. 2), or incorporated into egg phosphatidylcholine (PC) vesicles by sonciation (8) in 0.5 M KCl. The sample for the Fourier-transform infrared (FTIR) measurements was prepared by the method of Bagley et al. (4). The FTIR spectra were measured in a Nicolet 7199 FTIR spectrometer, equipped with a Hg-Cd-Te detector.

A single-beam kinetic spectrophotometer was used to measure the M intermediate (8). The light-induced transmission changes were initiated by an orange flash ($\lambda > 530$ nm, obtained from a photoflash with 200 μs half-bandwidth plus a glass cut-off filter [CS 3-67; Corning Glass Works, Corning, NY]). The light-induced absorbance changes from μs to second time scale were monitored with the flash photolysis system described in reference 9. A frequency-doubled 30 ns Q-switched Nd glass laser (530 nm) was used to excite the samples. The photomultiplier signal was digitized continuously from 2 μs to 100 s after the laser flash with a logarithmic time-base digitizer. All samples were light-adapted before spectroscopic measurements.

The light-induced pH changes for purple membrane and pigment analogue membrane sheets, or egg phosphatidylcholine vesicles incorporating these membranes, were monitored by measuring the absorbance changes of a pH indicator dye, *p*-nitrophenol, as previously described (8).

RESULTS

Flash Photolysis

Upon illumination, bR^{LA} undergoes a cyclic photoreaction through a set of intermediates. Since each intermediate has

its own spectral characteristics, the formation and decay of various intermediates can be followed by monitoring the absorbance changes at suitable wavelengths over appropriate time scales.

Fig. 1 shows the absorbance change at 400 nm induced by a photoflash in bR, *trans*-fixed bR, and the apomembrane used to regenerate the pigment. For bR the positive absorbance change is due to the formation and decay of its short wavelength M intermediate. Both the amplitude and kinetics of the short wavelength absorbance change in the *trans*-fixed bR sample are equal to the absorbance change in the apomembrane. Furthermore, the spectrum of the light-induced absorbance change in the *trans*-fixed bR sample, measured from 350 to 460 nm, is the same as the spectrum of a bR sample, with a maximum at 410 nm and crossover point at 460 nm (not shown). Fig. 2 shows a log-log plot of the absorbance changes in both bR and the *trans*-fixed bR over a more rapid time scale than Fig. 1. These changes were initiated by a 30-ns pulse from a frequency-doubled Ng-glass laser (530 nm). The light-induced absorbance changes in *trans*-fixed bR are much smaller than those in bR, but the kinetics are similar. The kinetics of the first photoproduct, the K intermediate, were also investigated by flash photolysis studies at 100 K. The light-induced absorbance change at 610 nm due to K had the same kinetics in the *trans*-fixed bR sample as in the native bR sample, but was ~5–10% as large (data not shown).

Most pigment analogues show a slight sensitivity to hydroxylamine in the dark, which is greatly enhanced by the presence of light (10); thus hydroxylamine can be used to preferentially destroy an artificial pigment in the presence of bR, which is not as susceptible. Hydroxylamine (0.05 M, pH = 7) was added to a *trans*-fixed bR suspension in the presence of light. When ~60% of the *trans*-fixed bR was destroyed, a flash photolysis experiment similar to that shown in Fig. 1 showed that the residual light-induced

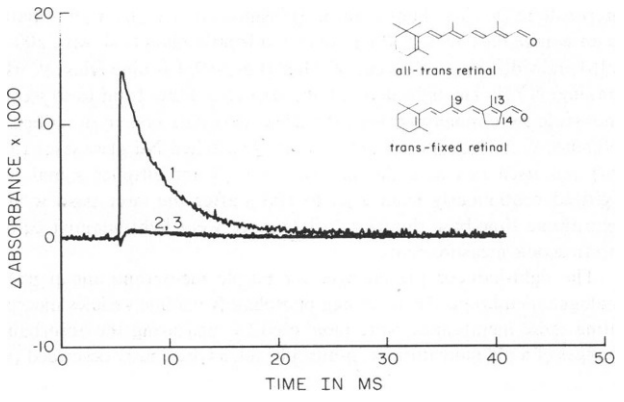

FIGURE 1 Flash-induced ($\lambda > 530$ nm; photoflash plus Corning CS 3-67 cut-off filter; Corning Glass Works) absorbance changes at 400 nm in (*1*) native bR, (*2*) *trans*-fixed bR, and (*3*) apomembrane, all suspended in 0.4 M KCl; pH = 6.72 ± 0.05; temperature = 20°C. Optical density of both pigments was 0.23 at 570 nm and 0.45 at 280 nm for apomembrane. Each trace is an average of four flashes 10 s apart.

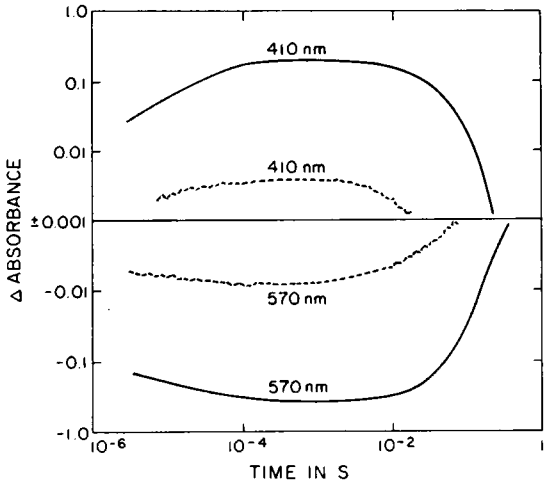

FIGURE 2 Absorbance changes at 410 nm (predominantly formation and decay of M) and 570 nm (predominantly depletion and reformation of bR) induced by a 30-ns pulse from a frequency-doubled Nd-glass laser (530 nm) in native bR (——) and *trans*-fixed bR (---) at 300°K. The optical density at 570 nm was 0.7 for bR and 0.5 for *trans*-fixed bR. The samples were in 60% glycerol, 0.1 M phosphate buffer, pH 7.0. Each trace is the average of three to six flashes.

absorbance change at 410 nm was reduced by only 25%. This suggests that the small light-induced absorbance signals are not due to *trans*-fixed bR.

Taken together, the above results strongly suggest that *trans*-fixed bR does not undergo a photocycle, and that the small signals seen in *trans*-fixed bR are due to residual bR in the apomembrane used for regeneration.

FTIR Difference Spectroscopy

FTIR difference spectroscopy has recently been used to study the conformational changes occurring in bacteriorhodopsin; the chromophoric vibrational modes measured by FTIR difference spectroscopy agree quite well with those studied by resonance Raman spectroscopy (4, 5).

The FTIR difference spectrum between K and bR at 70°K is shown in Fig. 3. The peaks between 1,100 and 1,400 cm^{-1} (fingerprint region) suggest that a change in chromophore conformation takes place in the bR to K transition. The shape of the difference spectrum for the *trans*-fixed bR sample is similar to bR; however, the amplitude is reduced to ~5% of that of bR. Again, this small change can be attributed to the residual bR present in the pigment analog sample.

Light-induced pH Change

Upon illumination, purple membrane sheets show a monophasic proton release into the medium followed by an uptake (reviewed in references 1 and 2). Fig. 4 *a* shows the light-induced pH change in bR and the much smaller change in *trans*-fixed bR. Since it is conceivable that the *trans*-fixed bR could support light-initiated pumping of protons across the membrane without the proton release and uptake being detected in sheets, we measured the

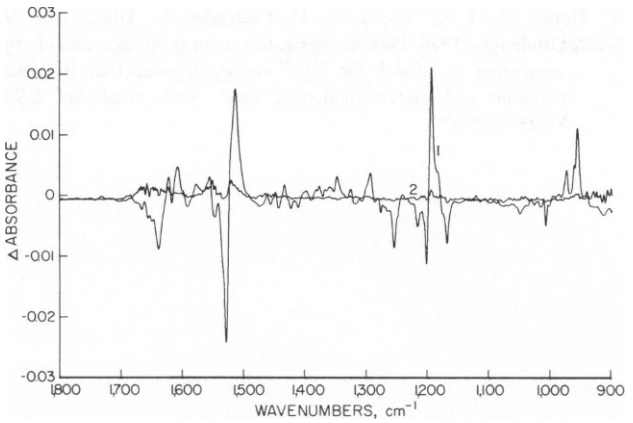

FIGURE 3 Low temperature (70°K) FTIR difference spectra, between K (positive peaks) and bR (negative peaks), of native bR (*1*) and *trans*-fixed bR (*2*). The optical density of both samples was 1.0 at 570 nm. Samples were prepared as in reference 4.

light-induced pH change in egg phosphatidylcholine vesicles containing bR, *trans*-fixed bR, and the apomembrane. The size of the pH change due to pumped protons in the phosphatidylcholine vesicles containing *trans*-fixed bR is the same as that in apomembrane-containing vesicles (Fig. 4 *b*). As before, these small pH changes in the *trans*-fixed bR sheets and vesicles can be attributed to the residual bR present in the pigment analogues. Therefore, we conclude that the *trans*-fixed bR cannot pump protons when illuminated.

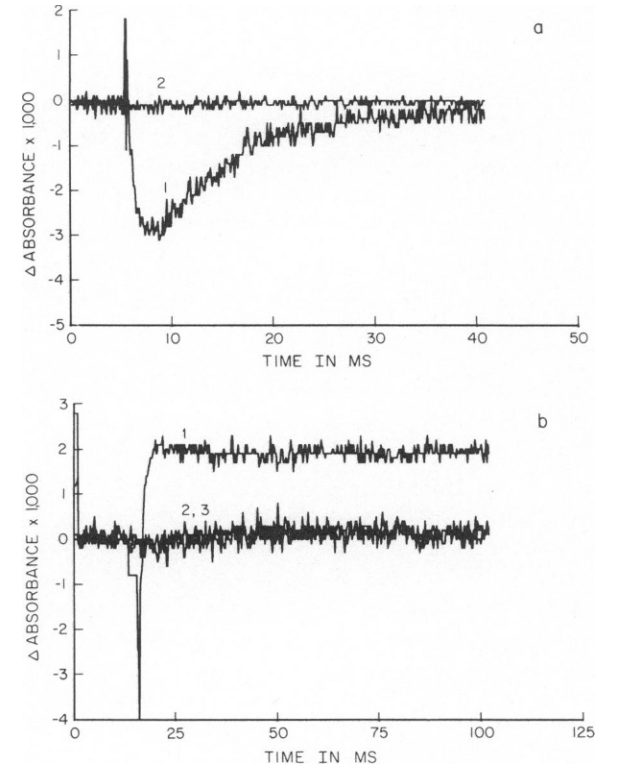

FIGURE 4 Flash-induced absorbance changes of a pH-sensitive dye, *p*-nitrophenol, (*a*) in membrane sheets of (*1*) native bR, and (*2*) *trans*-fixed bR. The optical density of the samples was 0.23 at 570 nm. Conditions same as in Fig. 1; (*b*) in PC vesicles containing (*1*) native bR, (*2*) *trans*-fixed bR, and (*3*) apomembrane. The optical density of both pigments was 0.24 at 570 nm, and 0.5 at 280 nm for the apomembrane in 0.5 M KCl. Conditions same as in Fig. 1. Each trace is an average of 10 flashes, 10 s apart.

DISCUSSION

Photoisomerization of the retinal chromophore from the all-*trans* to the 13-*cis* configuration has been proposed to be the primary photochemical event in bR^{LA} (reviewed in references 1 and 2). It has been suggested that the effect of the photoisomerization is to separate the positive and negative charges of a salt bridge formed by the protonated Schiff base of the chromophore with a negatively charged amino acid residue (11). This would lead to a significant amount of energy stored that could be used to pump protons out of the bacterial cell. The results of flash photolysis and FTIR spectroscopy experiments presented here indicate that when the *trans*/13-*cis* isomerization is blocked, the pigment neither undergoes a photocycle nor pumps protons. We conclude that photoisomerization is required for light-energy transduction in bacteriorhodopsin.

These studies were supported by National Science Foundation grants PCM 82-01924, CHE 81-10505, and PCM 82-09616, Department of Energy grant DEACO2-82 ER12087, and National Institutes of Health grants GM 32455 and GM 18051.

Received for publication 6 August 1984.

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