# CHARACTERIZATION OF THE CHROMOPHORE OF THE THIRD RHODOPSIN-LIKE PIGMENT OF *HALOBACTERIUM HALOBIUM* AND ITS PHOTOPRODUCT

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ABSTRACT *Halobacterium halobium* contains at least three retinal-containing pigments: bacteriorhodopsin, halorhodopsin, and a third rhodopsin-like pigment (tR) absorbing at ~590 nm, $tR_{590}$. Illumination of $tR_{590}$ gives rise to a very long-lived blue absorbing photoproduct, $tR_{370}$. Using high-performance liquid chromatography we show that the chromophore of $tR_{590}$ is primarily all-*trans* retinal and its conversion by light to $tR_{370}$ causes the chromophore to isomerize primarily to the 13-*cis* conformation. Irradiation of the $tR_{370}$ gives rise to a transient photoproduct absorbing at ~520 nm that decays back to the initial pigment $tR_{590}$. In addition to all-*trans* retinal, the apomembrane of tR can also combine with 13-*cis* retinal but not with the 9- or 11-*cis* isomers.

## INTRODUCTION

Three pigments having retinal as their chromophore have been found in *Halobacterium halobium* (*H. halobium*). Two of these, bacteriorhodopsin (1, 2) and halorhodopsin (3), function as light-driven electrogenic proton and chloride pumps, respectively. The third rhodopsin-like pigment, tR (also called slow rhodopsin, sR), has been shown to be nonelectrogenic, and its function is unknown (4, 5, 14–16). Recently, Bogomolni and Spudich (5) proposed that the third pigment is the photoreceptor for the phototactic behavior of these bacteria, because a mutant containing only the third pigment retains the same phototactic response as the wild type. Moreover, they suggested that its blue photoproduct might be the photoreceptor of the negative phototactic response. Because of its long lifetime (~0.8 s) (4, 5), the blue photoproduct accumulates in significant amounts under physiological light conditions (5). Although the absorption maximum, 590 nm, of tR does not seem to correspond exactly to the wavelength of maximum sensitivity of the photoattractant response, 565 nm (6), its blue photoproduct (4, 5) has its absorption maximum, ~370 nm, very close to the maximum sensitivity of the repellent phototactic response, 370 nm (6).

Retinal photoisomerization plays a key role in the excitation of visual cells in the retina and in proton pumping by the purple membrane of *H. halobium*. Thus, it is important to determine the isomeric form of the chromophore in initial pigment $tR_{590}$ and its photoproduct $tR_{370}$.

## MATERIALS AND METHODS

The *H. halobium* strain Flx 3 lc ($bR^-hR^-tR^+$ low carotenoid) (7) was kindly provided by Dr. J. C. Spudich. The pigment we refer to as tR in this strain has been called sR by Bogomolni and Spudich (5). The culture conditions and preparation of tR-containing membrane vesicles have already been described (4). tR was partially purified as follows: Membrane vesicles prepared from the cells of a 15-l culture were suspended in 40 ml of 4 M NaCl (10 mM HEPES, pH 7.0) and mixed with an equal volume of 5% tween-20 (polyoxyethylene sorbitan monolaurate; Sigma Chemical Co., St. Louis, MO) and stirred for 30 min at 4°C. The heavy membrane fragments were removed by pelleting by low-speed centrifugation (8,000 *g*, 10 min) and the remaining membranes collected by high-speed centrifugation (60,000 *g*, 45 min). After repeating the tween-20 wash, the membranes were washed four times in 4 M NaCl (10 mM HEPES, pH 7.0) to remove the tween-20 and then stored in 4 M NaCl (10 mM HEPES, pH 7.0) at −15°C until use. The photochemical properties of the native membranes are identical with those of the purified membranes.

The chromophore structure of $tR_{590}$ and its blue intermediate $tR_{370}$ were determined by chemical extraction (9–12). 1 ml of membranes in 4 M NaCl (10 mM HEPES, pH 7.0) was mixed with 4 ml of a denaturing emulsion (hexane, dichloromethane, 4 M NaCl [10 mM HEPES, pH 7.0], glycerol; 0.06:0.85:1:2) in the dark or in the light ($\lambda > 580$ nm, Corning CS 2-63 filter; Corning Glass Works, Corning Science Products, Corning, NY) and then mixed with 0.5 ml of 2.0 M hydroxylamine to form retinaloximes. The retinaloximes were extracted by methanol-hexane (1:2) and the isomers analyzed by an HPLC system consisting of Waters Associates solvent delivery system (M6000A; Waters Associates, Millipore Corp., Milford, MA), U6K injection system, and μporasil column (3.0 × 300 mm). Detection was carried out at 360 nm with a Schoeffel Spectroflow Monitor (SF 770; Schoeffel Instruments Div., Westwood, NY) connected to a Waters Associates 730 integrating Data Module (Waters Associates, Millipore Corp.). Hexane/isopentyl alcohol

(98:2 by volume) served as the eluent. The resolved retinaloxime peaks were identified by a comparison of their retention times to those of authentic samples. The retinaloxime method leads to efficient extraction (>90%) of the chromophores of rhodopsin (9, 12) and bacteriorhodopsin (Nelson, B., and M. Tsuda, unpublished results). With the tR samples, more uncertainty is involved because of the uncertainty of the extinction coefficients of tR (16) and 13-*cis* retinaloxime, the poor optical properties of the tR samples, and the small amount of tR in the samples. Nevertheless, we find the retinaloxime method also efficiently extracts the chromophore of tR; our estimate of this efficiency is 60 ± 30%.

A single-beam kinetic spectrophotometer was used to measure the light-induced absorbance changes in the millisecond time scale (13). Each kinetic trace is an average of 16 flashes. The phototransients from $tR_{590}$ were initiated with light of wavelengths above 600 nm by placing a cut-off filter (CS 2-62; Corning Glass Works, Corning Science Products) in front of a photoflash lamp. The phototransient from the blue-absorbing photoproduct, $tR_{370}$ was observed by exciting a sample that was under constant red illumination ($\lambda > 600$ nm, Corning CS 2-62 cut-off filter), with a blue actinic flash ($\lambda_{max} \cong 380$ nm) (CS 7-51 filter; Corning Glass Works, Corning Science Products). The constant background illumination created a high concentration of $tR_{370}$.

To prepare the apomembrane of tR, a pellet of the membranes was suspended in 10 ml of 0.2 M $NH_2OH$ in 4 M NaCl (10 mM HEPES, pH 7.0) and illuminated for 10 h with orange light (CS 3-69; Corning Glass Works, Corning Science Products) from a projector (150 W) placed 10 cm from the stirred, thermostatted sample chamber (0°C). After bleaching, the hydroxlamine was removed by washing the membranes repeatedly with 4 M NaCl. Small amounts of retinal isomers in ethanol were added to test pigment regeneration. The absorption spectra were measured with a Cary 118 spectrophotometer (Varian Associates, Inc., Palo Alto, CA) interfaced to a Minc 11 computer (Digital Equipment Corp., Maynard, MA).

## RESULTS AND DISCUSSION

As shown previously (4, 5, 14–16) the $bR^-$ mutant of *H. halobium* has two photocycles, a fast photocycle with a phototransient at 490 nm ($t_{1/2} \cong 10$ ms), and a slow photocycle with a phototransient at 370 nm ($t_{1/2} \cong 0.8$ s). Studies of the photochemistry of envelope vesicles under various conditions have shown that the fast photocycle belongs to halorhodopsin (hR) and the slow photocycle to tR. Recently Spudich and Spudich (7) isolated a mutant (Flx 3) containing only the third pigment. The effect of pressure on the rate and amplitude of tR recovery after a flash was identical for both the $bR^-$ mutant and Flx 3 (Tsuda, M., manuscript in preparation) indicating that the tR in these two strains is identical.

Fig. 1 (curve *a*) is a difference spectrum showing that within 5 ms after a red flash ($\lambda > 600$ nm) a blue photoproduct ($tR_{370}$) is formed from $tR_{590}$. This intermediate has already been described (4, 5). Since the half-life for the decay of $tR_{370}$ is ~0.8 s, it accumulates to a significant degree when a $tR_{590}$ sample is exposed to constant red light ($\lambda > 600$ nm).

Fig. 1 (curve *b*) shows the difference spectrum induced by a blue flash ($\lambda_{max} \cong 380$ nm) in a membrane vesicle sample containing a high concentration of $tR_{370}$ formed by continuous red illumination. The maximum in the difference spectrum measured 1 ms after the flash is ~520 nm. We call this phototransient, which appears within 1 ms and decays within 500 ms back to $tR_{590}$, $tR_{520}$. A similar intermediate has been reported by Tomioka et al. (17) and J. Spudich (personal communication).

The isomeric form of the chromophore of $tR_{590}$ was determined by chemical extraction as described in Materials and Methods. Trace *a* in Fig. 2 shows that dark-adapted tR contains almost entirely one retinal isomer,

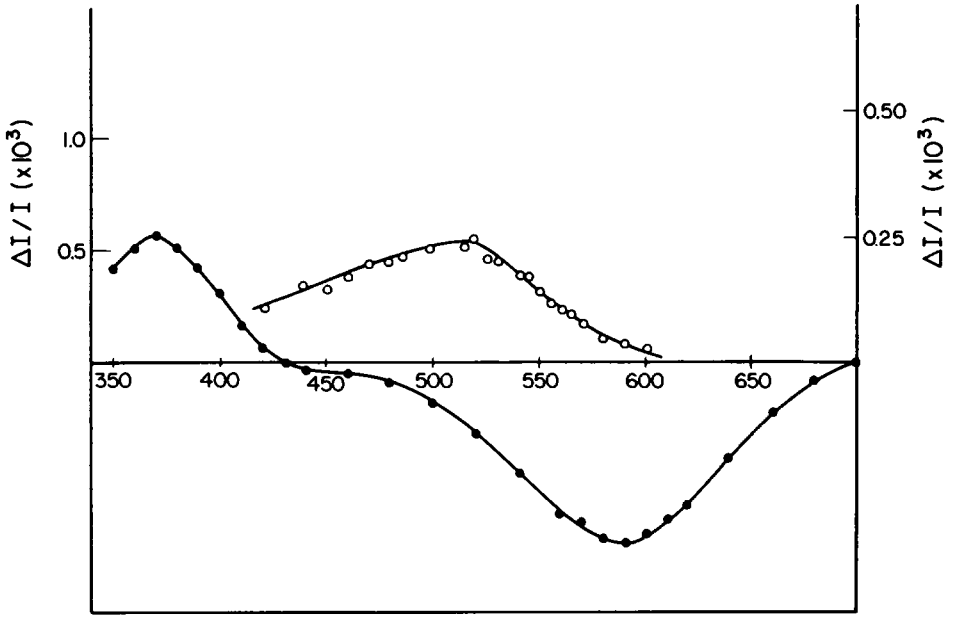


FIGURE 1 Curve *a*: Flash-induced difference spectrum of $tR_{590}$ containing purified membrane in 4 M NaCl and 10 mM HEPES; pH 7; temperature 25°C; λ actinic > 500 nm (obtained from photoflash plus Corning glass cut-off filter CS 2-62; Corning Glass Works, Corning Science Products). Curve *b*: Flash-induced difference spectrum of the phototransient species, $tR_{520}$, produced by irradiating $tR_{370}$. Conditions as above except λ actinic ≅ 380 nm (photoflash plus Corning CS 7-51 filter; Corning Glass Works, Corning Science Products); background illumination λ > 600 nm obtained from a 150-W projector plus Corning CS 2-62 filter (Corning Glass Works, Corning Science Products).

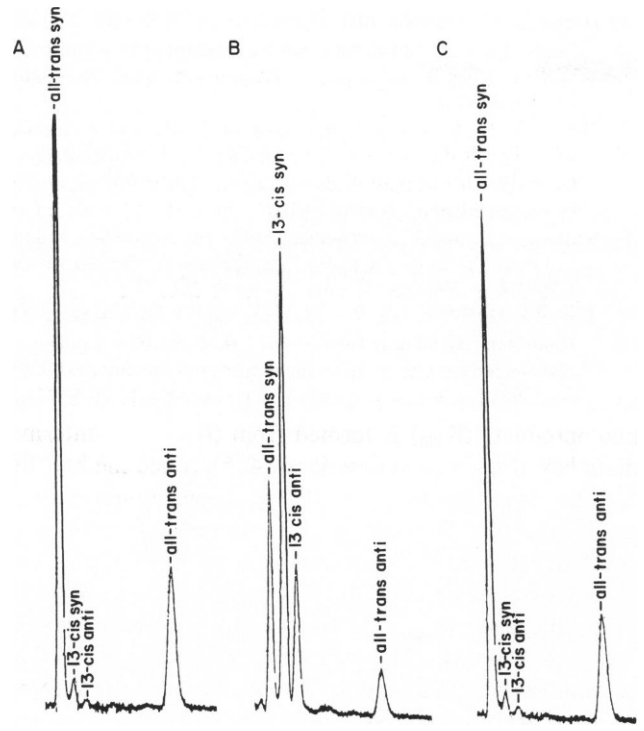


FIGURE 2 Isomeric composition of tR samples determined by HPLC. After denaturation and before extraction, retinals present in the tR samples were quantitively converted to their oximes. During this conversion each isomer of retinal generates a pair of stereoisomers: a syn- and anti-oxime; there are thus two HPLC peaks corresponding to each retinal isomer originally present in the sample. Trace *a*: dark-adapted tR; trace *b*: denaturation and extraction under red illumination; trace *c*: irradiated and kept in the dark for 30 s before denaturation and extraction.

all-*trans*. A minor peak corresponding to 13-*cis* retinal is ~5% that of the all-*trans* peak.

The isomeric composition of $tR_{370}$ was determined by extracting the chromophore under red light illumination ($\lambda > 600$ nm). Red light partially converts $tR_{580}$ to $tR_{370}$ and denaturation by the extraction emulsion occurs within 1 s, which is on the order of the half-life of $tR_{370}$. The results of this extraction are shown in trace *b* of Fig. 2: the amount of all-*trans* decreased by 66%, and 13-*cis* retinal now represented the major component. Chromophores extracted from samples that were irradiated and subsequently kept in the dark for 30 s yielded HPLC traces identical to those of the original dark-adapted samples; they had reverted to the primarily all-*trans* isomer (Fig. 2 *c*). That there was no difference between the isomeric composition of sample denatured 30 s after the end of irradiation and the dark-adapted sample shows that there is no dark-adaptation process taking place on the same time scale as in bacteriorhodopsin. We conclude from these results that the chromophore of $tR_{590}$ is primarily all-*trans* retinal, whereas that of $tR_{370}$ is primarily 13-*cis* retinal.

A referee has suggested that there may be a hitherto undiscovered fourth retinal-based pigment present in the mutant. While there is no evidence for such a pigment, it is also unlikely to be present from our results. The only light-induced optical density changes that can be observed in this mutant (from $10^{-11}$ s to $10^{1}$ s) can be assigned entirely to tR (references 4 and 5, the results leading to Fig. 1, and Kobayashi, T., and M. Tsuda, unpublished observations) and so we estimate that any change due to the fourth pigment must be <10% of what we observe. Since all retinal pigments have roughly the same extinction coefficient (50,000 ± 30,000), then any light-induced changes due to a hypothetical fourth pigment must not make a significant contribution (conservatively <20%) to the light-induced changes in chromophore conformation we observe. Moreover, any hypothetical fourth pigment that has no light-induced absorbance changes must be present at a concentration of <40% that of tR, since when we illuminate the sample we convert over 60% of the all-*trans* chromophore to the 13-*cis* conformation.

The isomeric form of retinal in $tR_{590}$ was found to be all-*trans*; it would be interesting to know whether other retinal isomers can regenerate pigments when combined with the bleached membrane. We tested all-*trans*, 9-*cis*, 11-*cis*, and 13-*cis* retinal. As expected, all-*trans* retinal regenerated pigment from bleached membrane; complete pigment formation required ~ 2 h. Although 9-*cis* and 11-*cis* retinals do not form pigments, 13-*cis* retinal did form a pigment.

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