SYNTHETIC PIGMENT ANALOGUES OF THE PURPLE MEMBRANE PROTEIN

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ABSTRACT Nonphysiological analogues of retinal have been shown to form pigments in reactions with the apoprotein of the purple membrane of *Halobacterium halobium*. Both the all-*trans* and 13-*cis* isomers of a retinal analogue, having an elongated chain with an extra double bond, formed pigments. Unlike the native all-*trans* and 13-*cis* retinal$_1$-based pigments, the new pigments were not interconvertible with each other and were unstable against hydroxylamine. When incorporated into phospholipid vesicles, they showed no proton pumping activity upon illumination. The ability of the extended-length retinal to form pigments contrasts with its nonreactivity with opsin (apoprotein of rhodopsin), suggesting a less stringent binding site for the purple membrane chromophore. All-*trans* retinal$_2$ also combined with bleached purple membrane to form a blue pigment absorbing at ca. 590 nm. Like the native purple membrane, the blue membrane showed proton pumping activity upon illumination in phospholipid vesicles.

INTRODUCTION

Light can be utilized as an energy source by the purple membrane of *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1971). The pigment involved in the initial steps of this transformation, the purple membrane protein, exists in two forms: a dark-adapted one (λ_{max} = 558 nm; prosthetic group: all-*trans*/13-*cis* retinal), which can be photoconverted to a light-adapted pigment (λ_{max} = 568 nm; all-*trans* retinal) (Oesterhelt and Stoeckenius, 1971; Oesterhelt et al., 1973). The *Halobacterium* pigment has often been called bacteriorhodopsin because of its possible analogies to the visual pigments. Similarities between the two proteins include having the same chromophore, retinal, bound in the same manner, as a protonated Schiff base (Lewis et al., 1974). This leads to many spectroscopic similarities between the two pigments (Honig et al., 1976). In addition, the primary photochemical event in the two systems may be quite similar (Rosenfeld et al., 1977). Differences in the two pigments include different isomeric specificities, such protein properties as the molecular weight (Bridgen and Walker, 1976), the function of the two photoevents (Lozier et al., 1975), and the nature and organization of the membranes containing the pigments (Henderson and Unwin, 1975; Ebrey and Honig, 1975).

The binding site and spectroscopic properties of visual pigments have been studied with nonphysiologically occurring isomers of retinal, with modified retinals (see review

by Honig and Ebrey, 1974), and with competitive inhibitors of retinal binding (Matsumoto and Yoshizawa, 1975). Here we report on the binding of modified retinals to the purple membrane apoprotein to form synthetic pigments. We have found that analogues of retinal with an extended-length side chain and retinal$_2$ bind purple membrane apoprotein and make artificial pigments.

METHODS

H. halobium was cultured and purple membrane was prepared according to standard methods (Becher and Cassim, 1975). The chromophore was removed from its binding site as retinal oxime by irradiating with intense (400 W projector) orange light in the presence of 1 M hydroxylamine, pH 7 (Oesterhelt et al., 1974). Free hydroxylamine was then washed out with water and the bleached purple membrane suspended in distilled water. This bleached purple membrane (Fig. 1 *a*, curve 1) has absorption peaks at 362 nm (retinal-oxime) and 278 nm (the protein) and a broad shoulder above 360 nm due to scattering and residual carotenoids. This membrane preparation can form purple membrane upon incubation with 13-*cis* or all-*trans* retinal.

The retinal$_2$ was a gift of Hoffmann-LaRoche, Inc., (Nutley, N.J.) and was purified by high-pressure liquid chromatography. The extended side chain retinal derivative with an extra double bond (C_{22}) was prepared via a Wittig reaction from the corresponding 13-*cis* or all-*trans* retinal and triethylphosphonacetate. After reduction (diisobutyl aluminum hydroxide) and oxidation (MnO_2), the C_{22} aldehydes were purified by high-pressure liquid chromatography [mass spectra M^+ = 311, λ_{max} (EtOH) of 13-*cis* = 396 nm and λ_{max} (EtOH) of all-*trans* = 392 nm].

Purple membrane or membrane containing each synthetic pigment analogue was mixed with egg phosphatidylcholine (15 mg lipid/mg protein) in 150 mM KCl and sonicated under nitrogen for 10 min. The vesicle preparation was centrifuged at 30,000 rpm (Beckman type 40 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 hr, and the supernatant was used for the proton pumping experiments. Yellow light (Corning CS3-71 filter, Corning Glass Works, Corning, N.Y.) was used to irradiate the samples. The pH change was measured by a ceramic junction combination pH electrode (Beckman type 39030).

RESULTS

Soon after addition of all-*trans* C_{22} to the bleached purple membrane suspension (Fig. 1 *a*, curve 2), the absorbance near 425 nm decreases and the absorbance at longer wavelengths increases. This change is complete after about 2 hr (Fig. 1 *a*, curve 3). The difference spectra of the spectral change has a λ_{max} near 520 nm with a broad shoulder on the long wavelength side. These spectra indicate that all-*trans* C_{22} combines with bleached purple membrane protein to make a pigment(s). The binding site of all-*trans* C_{22} retinal in this pigment is almost surely the same lysine residue to which the retinal originally was bound, because when this site is blocked (by retinal) in the native membrane, incubation with all-*trans* C_{22} leads to no increase in absorption near 560 nm. That the absorption is not due to nonspecific binding is also strongly suggested by the inability of some retinal isomers (9-*cis*, 11-*cis*, and 9,13-*dicis*) to form pigments (Oesterhelt and Schuhmann, 1974; our unpublished observations).

Unlike either the native or retinal$_2$ regenerated purple membrane, the all-*trans* C_{22} pigment(s) is sensitive to hydroxylamine in the dark. Upon addition of hydroxylamine, the absorbance near 560 nm at first decreased (Fig. 1 *a*, curve 4); on further

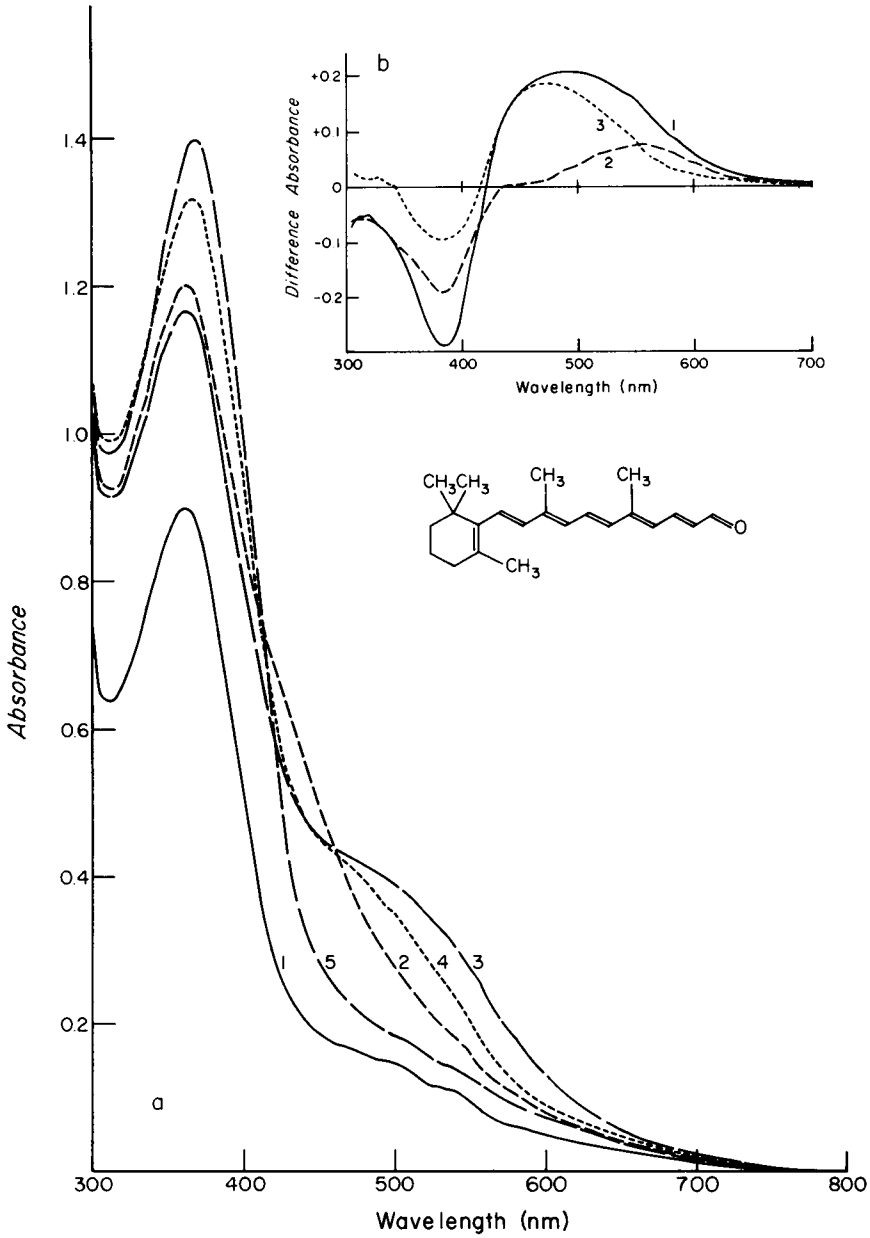

FIGURE 1 Formation of pigment analogues from all-*trans* C_{22} and bleached purple membrane and its destruction by hydroxylamine. *a:* Curve 1, bleached purple membrane (total volume of 0.20 ml). Curve 2, after addition of 1 μl of all-*trans* C_{22} dissolved in ethanol at a concentration of 128 $OD_{392\,nm}$ U/ml. Curve 3, after 2 h incubation. Curve 4, then after addition of 1 ul of hydroxylamine (4 M), pH 7.0, final concentration of 20 mM. Curve 5, finally after incubation for 2 h. *b:* Curve 1, the difference spectrum of the total change observed with the addition of hydroxylamine (curves 3–5 in Fig. 1 *a*). Curve 2, the difference spectrum of the initial (within 5 min) effect of hydroxylamine on all-*trans* C_{22} pigment(s) (curves 3–4 in Fig. 1 *a*). Curve 3, the difference spectrum before and after further incubation for 2 h with hydroxylamine (curves 4–5 in Fig. 1 *a*).

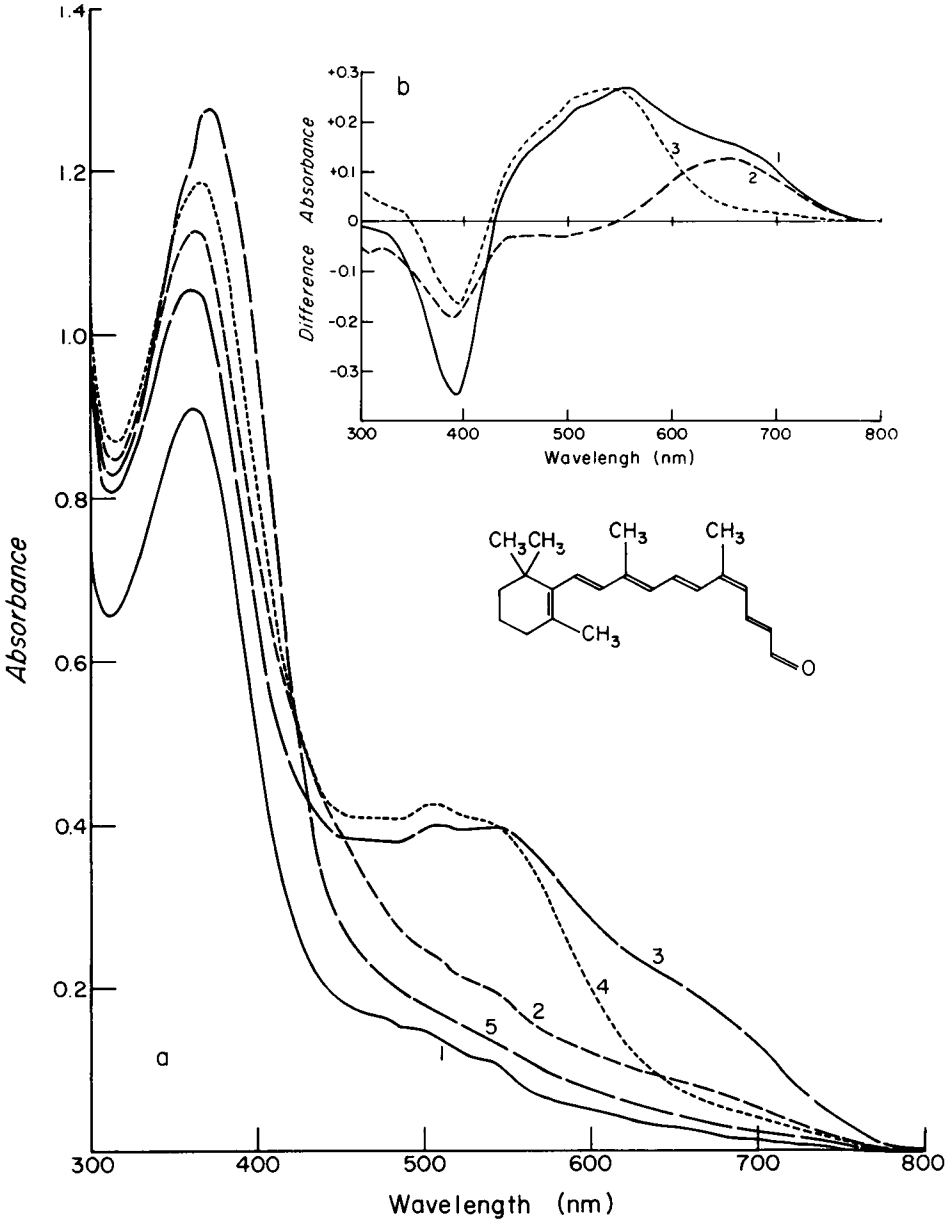

FIGURE 2 Formation of pigment analogues from 13-*cis* C_{22} and bleached purple membrane and its destruction by hydroxylamine. *a:* Curve 1, bleached purple membrane (total volume of 0.20 ml). Curve 2, after addition of 1 ul of 13-*cis* C_{22} dissolved in ethanol at a concentration of 111 $OD_{396\,nm}$ U/ml. Curve 3, after 2 h incubation. Curve 4, after addition of 1 ul hydroxylamine (4 M), pH 7.0, final concentration of 20 mM (within 5 min). Curve 5, finally after incubation with hydroxylamine for 2 h. *b:* Curve 1, difference spectrum before and after 2 hr incubation of 13-*cis* C_{22} pigment(s) with hydroxylamine (curves 3–5 in Fig. 2 *a*). Curve 2, difference spectrum before and after addition of hydroxylamine to the pigments (curve 3–4 in Fig. 2 *a*). Curve 3, the difference spectrum before and after further incubation for 2 h with hydroxylamine (curves 4–5 in Fig. 2 *a*).

incubation, the entire long wavelength spectrum disappeared (Fig. 1 *a*, curve 5). The difference spectra of the immediate (Fig. 1 *b*, curve 2) and subsequent (Fig. 1 *b*, curve 3) spectral changes are quite different. This stepwise destruction of the all-*trans* C_{22} pigments suggests that the product consists of a mixture containing at least two distinct species, with the 560 nm pigment being more sensitive to hydroxylamine than the 480 nm pigment.

When 13-*cis* C_{22} was added to the bleached purple membrane suspension (Fig. 2 *a*, curve 2) and the mixture incubated, the absorbance in the longer wavelength region increased (Fig. 2 *a*, curve 3), indicating that 13-*cis* C_{22} also combined with the bleached purple membrane to form a pigment(s). The absorbance extended to much longer wavelengths than in the case of all-*trans* C_{22}. The difference spectrum of this change is very broad, with a λ_{max} near 560 nm and a shoulder on the longer wavelength side. The addition of hydroxylamine rapidly decreased the absorbance at longer wavelengths (Fig. 2 *a*, curve 4). The difference spectrum has a λ_{max} at 650 nm (Fig. 2 *b*, curve 2). Further incubation in the dark led to a decrease in absorbance near 540 nm (Fig. 2 *b*, curve 3). This stepwise destruction of the product indicates that it consists of at least two species sensitive to hydroxylamine. In both regeneration experiments, after the final destruction of the pigments by hydroxylamine (Fig. 2 *a*, curve 5), some absorbance in the longer wavelength region remained, possibly due to increased turbidity.

When either the all-*trans* or 13-*cis* C_{22} pigment(s) was irradiated by red light, no spectral change occurred; therefore, the pigment is either not sensitive to light or its reversion to a dark-adapted form is very fast. Thus, unlike the 13-*cis*/all-*trans* and all-*trans* forms of the original purple membrane protein, (*a*) these C_{22} pigments are not interconvertible by light or dark incubation and (*b*) the separation of the absorption maxima of these pigments is quite large.

All-*trans* $retinal_2$ combined with the bleached purple membrane to form a blue pigment, the "blue membrane." After addition of $retinal_2$ to the bleached purple membrane suspension, the absorbance at longer wavelengths rose (Fig. 3 *a*, curves 2, 3). The difference spectrum had a λ_{max} near 590 nm. This is the λ_{max} of the dark-adapted form. Light adaptation shifted the λ_{max} to ca. 602. The dark adaptation of the blue membrane at room temperature was much faster than the purple membrane regenerated from $retinal_1$ and bleached membrane. Unlike the other synthetic pigments, in the dark the $retinal_2$ pigment was stable to hydroxylamine.

Phospholipid vesicles containing blue membrane showed proton pumping activity (trace 5; Fig. 4) like that of purple membrane vesicles (trace 2; Fig. 4). In contrast, the proton pumping activity of the blank, bleached purple membrane vesicles, was indistinguishable from that of the all-*trans* and 13-*cis* C_{22} aldehyde pigments (traces 1, 3, and 4; Fig. 4). This suggests that the C_{22} pigments cannot pump protons across the vesicle membranes.

DISCUSSION

We have shown that artificial purple membrane pigment analogues can be made from the purple membrane apoprotein and analogues of retinal. $Retinal_2$ formed a pigment

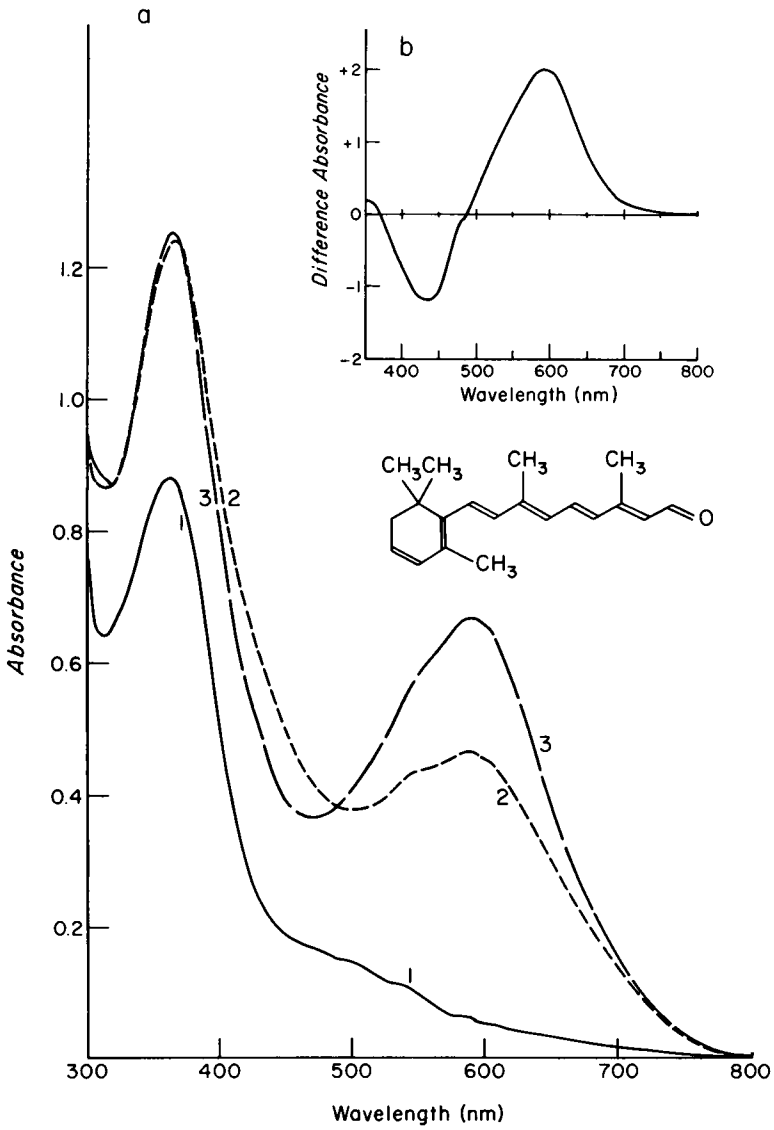

FIGURE 3 Formation of blue membrane from retinal$_2$ and bleached purple membrane. *a:* Curve 1, the bleached purple membrane. Curve 2, after addition of 1 ul of retinal (dissolved in ethanol at a concentration of 3 mM) to 0.20 ml of bleached purple membrane. Curve 3, after incubation for 2 h. *b:* Curve 1, the difference spectrum of blue membrane and bleached purple membrane (curves 3–2 in Fig. 3 *a*).

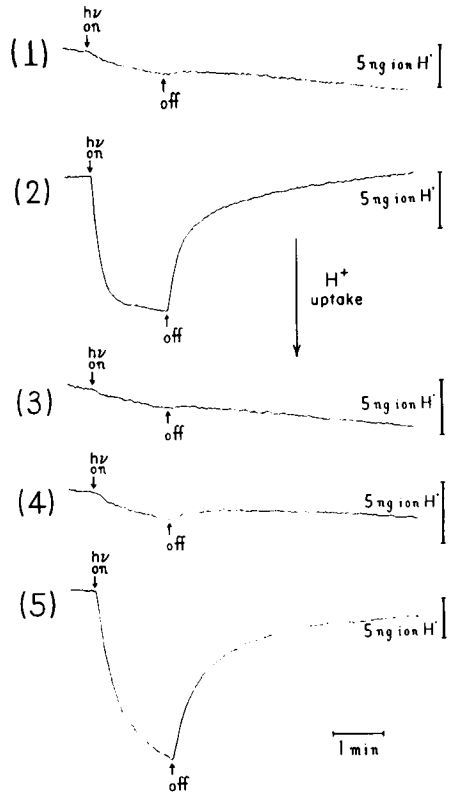

FIGURE 4 Light-induced proton uptake by purple membrane and several synthetic pigment analogues incorporated into egg lecithin vesicles. All vesicles had approximately equal protein concentrations. (1) Bleached purple membrane, initial pH 6.63; (2) Purple membrane ($retinal_1$), initial pH 6.94; (3) All-*trans* C_{22} aldehyde pigment, initial pH 6.49; (4) 13-*cis* C_{22} aldehyde pigment, initial pH 6.65; (5) Blue membrane ($retinal_2$), initial pH 6.73.

(the blue membrane) with an absorption maximum at c. 590 nm. Both the all-*trans* and 13-*cis* isomers of the extended length retinal combine with the apoprotein, but at least two spectrally distinct pigments with different sensitivities to hydroxylamine are formed from each isomer. We are unsure of the origin of the pigments' heterogeneous population. As noted above, it is very unlikely the chromophore is binding other than at the native site. One possible explanation is that the extended chromophore can assume more than one conformation that fits into the binding site and thus shows more than one type of pigment absorption spectrum.

It is quite interesting that while the apoprotein of rhodopsin, opsin, cannot bind any C_{22} isomer (Blatz et al., 1969; our unpublished observations), the purple membrane apoprotein can. This suggests, as does the different isomer specificity of the two apoproteins, that the retinal binding sites of rhodopsin and the purple membrane are not analogous.

The observation that the retinal$_2$-based pigment can pump protons suggests that the 3 and 4 positions in the β-ionone ring play no special role in proton pumping. Moreover, the lack of pH changes when the C_{22} pigment vesicles were illuminated suggests that mere light absorption by a pigment in a vesicle is not sufficient to pump protons.

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