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A Bacteriorhodopsin Analog Reconstituted with a Nonisomerizable 13-*trans* Retinal Derivative Displays Light Insensitivity*

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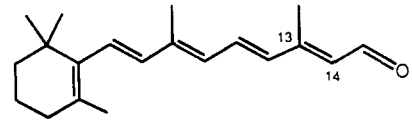
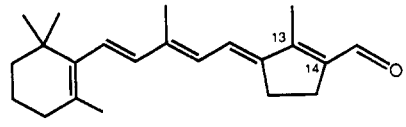
With the aim of preparing a light-insensitive bacteriorhodopsin-like pigment, bacterio-opsin expressed in *Escherichia coli* was treated in phospholipid-detergent micelles with the retinal analog II, in which the C_{13}-C_{14} *trans*-double bond cannot isomerize due to inclusion in a cyclopentene ring. The formation of a complex with a fine structure (λ_{max}, 439 nm) was first observed. This partially converted over a period of 12 days to a bacteriorhodopsin-like chromophore (ebR-II) with λ_{max}, 555 nm. An identical behavior has been observed previously upon reconstitution of bleached purple membrane with the analog II. Purification by gel filtration gave pure ebR-II with λ_{max}, 558 nm, similar to that of light-adapted bacterio-opsin reconstituted with all-*trans* retinal (ebR-I). Spectrophotometric titration of ebR-II as a function of pH showed that the purple to blue transition of bacteriorhodopsin at acidic pH was altered, and the apparent pK_a of Schiff base deprotonation at alkaline pH was lowered by 2.4 units, relative to that of ebR-I. ebR-II showed no light-dark adaptation, no proton pumping, and no intermediates characteristic of the bacteriorhodopsin photocycle. In addition, the rates of reaction with hydroxylamine in the dark and in the light were similar. These results show, as expected, that isomerization of the C_{13}-C_{14} double bond is required for bacteriorhodopsin function and that prevention of this isomerization confers light insensitivity.

Bacteriorhodopsin (bR)[1] is a light-driven proton pump in the purple membrane (PM) patches of *Halobacterium halobium* (1). The protein consists of a single chain of 248 amino acids which form seven α-helical transmembrane segments. The chromophore consists of one molecule of all-*trans* retinal (I, Fig. 1) covalently attached to Lys-216 in the form of a protonated Schiff base. Absorption of light causes isomerization of the chromophore from the all-*trans* to the 13-*cis* configuration, initiating a photocycle during which protons are released on the extracellular side and subsequently taken up on the cytoplasmic side of the membrane. A structural model of bR based on electron microscopic studies has recently been proposed by Henderson *et al.* (2).



I



II

FIG. 1. **Structures of all-*trans* retinal, I, and the cyclopentatrienylidene retinal analog, II.**

A variety of chemical, biochemical, and biophysical studies have been carried out on bR. These studies have used site-specific mutagenesis and spectroscopy to investigate chromophore-protein interactions and the mechanism of proton translocation. The availability of a pure, light-insensitive analog of bR would undoubtedly offer new opportunities for structural studies. In particular, the light-insensitive structure could be used to investigate helix-helix interactions, protein folding, and interactions between the surfaces of the helical bundle and the phospholipids. In addition, a monomeric bR analog with a single isomeric state of the chromophore should be particularly useful in crystallization experiments as dark-adapted bR contains a mixture of both 13-*cis* and all-*trans* retinals (3).

One approach to the preparation of a monomeric, photoinsensitive bR analog is to use an all-*trans* retinal analog which cannot undergo isomerization to the 13-*cis* configuration. The use of such an analog would require that it promote efficient regeneration of the chromophore from the apoprotein in the established micellar reconstitution system for bR (4, 5).

Nakanishi and co-workers (6–8) pioneered the design and synthesis of retinal analogs in which isomerization around specific double bonds are prevented by including the double bonds in carbocyclic rings. In particular, they synthesized an analog of all-*trans* retinal in which the C_{13}-C_{14} *trans*-double bond was included within a cyclopentene ring (II, Fig. 1).

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[1] The abbreviations used are: bR, bacteriorhodopsin; PM, purple membrane; bO, bacterio-opsin in bleached purple membrane; ebO, bacterio-opsin produced from the expression of a synthetic wild-type gene in *E. coli*; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; SDS, sodium dodecyl sulfate; DA, dark-adapted; LA, light-adapted; OG, octyl glucoside; HPSEC, high performance size exclusion chromatography; HPLC, high performance liquid chromatography. The standard three letter abbreviations are used for amino acids.

This was used to reconstitute bacterio-opsin in bleached PM (bO) to the bR analog (6). In this paper, we report on the reconstitution and characterization of a monomeric bR-like pigment prepared from bacterio-opsin expressed in *Escherichia coli* (ebO) and the retinal analog **II**. Reconstitution proceeded with a $t_{1/2}$ of 34 h and resulted in the formation of a pigment (ebR-**II**) with λ_{max} at 555 nm. The absorption spectrum displayed an additional fine structure peak with λ_{max} at 439 nm, which formed early during the regeneration process and represents noncovalently linked analog **II**. The nonregenerated protein and fine structure chromophoric species were separated from ebR-**II** using a two-step gel filtration procedure. The purple to blue transition at acidic pH was altered, and the apparent pK_a of the retinyl Schiff base at alkaline pH was lowered by 2.4 units, relative to ebO reconstituted with **I** (ebR-**I**). Upon illumination, ebR-**II** did not translocate protons and did not show any photocycle. Finally, the rates of reaction of ebR-**II** with hydroxylamine in the dark and in the light were nearly similar, indicating that ebR-**II** does not undergo light-induced changes.

EXPERIMENTAL PROCEDURES

Materials—L-α-1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids. CHAPS and *n*-octyl-β-D-glycopyranoside (OG) were from Boehringer Mannheim. The purity of OG was assessed by thin layer chromatography following purification by the published procedure (9). All-*trans* retinal was from Kodak, and hydroxylamine hydrochloride was from Aldrich. Acetone-washed soybean lipids (Sigma) were prepared by the method of Kagawa and Racker (10).

Expression and Purification of ebO—The wild-type apoprotein (ebO) was expressed from a synthetic gene in *E. coli* (11) and purified by a two-step method previously published (12).

Synthesis of Retinal Analog II—The retinal analog II (3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4-ethano-nonatetra-all-*E*-2,4,6,8-enal) was synthesized with minor modifications of the synthetic scheme of Nakanishi and co-workers (6).

Chromophore Formation and UV/Visible Absorption Spectroscopy—The bacterio-opsin apoprotein (ebO, 0.4 mg/ml) in 0.2% (w/v) SDS was added to a micellar mixture of 30 mM NaH_2PO_4, pH 6.2, containing 1% (w/v) DMPC, 1% (w/v) CHAPS, 0.0025% (w/v) sodium azide, and ~1.5-fold molar excess of all-*trans* retinal, **I**, or analog **II**. The regeneration mixture was kept in the dark at room temperature. Regeneration with **I** was complete after 12 h whereas the reconstitution mixture containing analog **II** was kept for ~12 days to allow maximal regeneration. The time course of regeneration of ebR-**I** and ebR-**II** was followed by UV/visible absorption spectroscopy at 20 °C with a Perkin-Elmer λ7 spectrophotometer. All visible absorption maxima are within ±3 nm. The effect of pH on the chromophore was studied in DMPC/CHAPS/SDS mixed micelles as previously described (13).

High Performance Size Exclusion Chromatography (HPSEC)—Excess retinal analog **II**, DMPC, and nonregenerated protein were separated from ebR-**II** by minor modifications of a published procedure (14). Briefly, the method involved size-exclusion chromatography on HPLC columns in two successive steps. In the first step, ebR-**II** was eluted isocratically with a buffer system containing 1% (w/v) CHAPS. After the first step, the protein fraction was essentially free of DMPC and unbound retinal analog **II**. However, the sample still contained nonregenerated protein. To separate the latter, fractions containing 555 nm absorbing material were concentrated and rechromatographed at 4 °C on the same column using 1% (w/v) octyl glucoside as detergent. Upon elution, DMPC was added directly prior to concentration of the gel-filtered fraction.

Proton Pumping Assays—Light-dependent proton translocation by ebR-**I** and ebR-**II** was measured in the following manner. An aliquot (10 μl) of the regeneration mixture containing 50 pmol of ebR-**I** or ebR-**II** was reconstituted into 45 μl of 2% soybean lipid vesicles and 4.7 μl of 0.5 M octyl glucoside, and the resulting mixture was incubated on ice under argon for 1 h in the dark. This sample was injected into 1 ml of 2 M NaCl in a jacketed reaction vessel with ports for a pH electrode. The pH was adjusted to 7.0 with aliquots of 1 mM NaOH or HCl, and the baseline was allowed to stabilize. The sample was irradiated with a 100-watt projector lamp equipped with a heat-absorbing and a >495 nm long pass filter. This light intensity was saturating for proton pumping with ebR-**I**. After each measurement, the contents of the vessel were calibrated by the addition of 2 μl of 1 mM HCl.

Photocycle Measurements—Photocycle data were obtained with a homemade flash-photolysis spectrometer of 100-ns resolution. The photocycle measurements were performed similar to a method previously described (15). To obtain data for the light-adapted state, the sample was illuminated for 20 s before each flash by light from a Schott KL 150 lamp equipped with a >510 nm long pass filter. Flash-induced absorbance changes were examined with excitation wavelength at 590 nm and measuring wavelengths at 410, 450, 490, 530, 570, 610, and 650 nm.

Reaction of ebR-I and ebR-II with Hydroxylamine—Micellar solutions of ebR-**I** or ebR-**II** were added to a reaction mixture of 1 mM sodium phosphate, pH 6.3, containing 0.8% (w/v) CHAPS, 0.8% (w/v) DMPC, 0.16% (w/v) SDS, and 100 mM hydroxylamine. The samples were illuminated with a 100-watt light source through a >495 nm long pass filter at 20 °C. The hydroxylamine-induced bleaching of the chromophore was also followed in the dark. The rate of chromophore bleaching was monitored by recording the time-dependent absorbance changes of the protein solutions in mixed micelles. Kinetic parameters for the rate of reactions were obtained from single exponential fits of plots of absorbance at 555 nm *versus* time.

RESULTS

ebR-II

Regeneration of ebO with Retinal Analog II—The absorption spectrum of retinal analog **II** in DMPC/CHAPS/SDS (mixed micelles) is shown in Fig. 2 (*inset*). The λ_{max}, 397 nm, is 13 nm red-shifted relative to all-*trans* retinal, **I**. The time course of the spectral changes observed on addition of analog **II** to ebO in mixed micelles is shown in Fig. 2. A fine structure peak at 439 nm, with pronounced shoulders near 419 and 470 nm, was immediately observed. With time, the absorbance of the fine structure peak diminished with the concomitant formation of a bR-like chromophore (λ_{max}, 555 nm). The interconversion of the two peaks is characterized by a single isosbestic point (480 nm). The formation of the bR-like chromophore with analog **II** was extremely slow, the $t_{1/2}$ of regeneration at 20 °C (34 h) being increased by more than 3 orders of magnitude compared with that of ebR-**I** formation with **I** ($t_{1/2}$ = 1 min). The maximum extent of regeneration of ebR-**II** was 55% after 12 days.

Incubation of ebO in mixed micelles at 20 °C for 12 days prior to the addition of **I** or analog **II** showed only a minor effect on the yield of chromophore formation (decreased by <10%). Thus, the lower extent of chromophore formation with analog **II**, relative to **I**, was not due to denaturation of

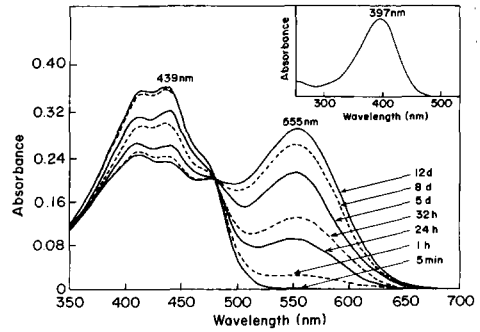

FIG. 2. **Time course of formation of ebR-II in the dark.** The kinetics of chromophore formation with **II** was followed in 30 mM NaH_2PO_4, pH 6.2, containing 1% DMPC, 1% CHAPS, and 0.2% SDS at 20 °C as described under "Experimental Procedures." Note the existence of a single isosbestic point (480 nm). *Inset*, UV/visible absorption spectrum of **II** in 30 mM NaH_2PO_4, pH 6.2, containing 1% DMPC, 1% CHAPS, and 0.2% SDS at 20 °C.

the apoprotein during the extended time period required for maximal regeneration.

Properties of the Species with the Fine Structure—Acid denaturation of the fine structure species immediately after its formation resulted in the appearance of a symmetrical absorption band with a λ_{max} at 397 nm (Fig. 3*A*), identical with that of analog **II**. This observation demonstrates that retinal **II** and ebO were not linked in a Schiff base type of covalent linkage. In alkali, the fine structure chromophore also formed a single broad absorption maximum (395 nm), indicating the pH-dependent nature of the species.

When the ebO mutant Lys-216 → Cys was incubated with **I** or **II**, the formation of fine structure species with maxima at 435 or 440 nm, respectively, was observed (data not shown). Acid denaturation converted these species to free retinal with λ_{max} at 381 and 397 nm, respectively. These findings demonstrate that neither **I** nor **II** forms a Schiff base linkage with Lys-216 in order to generate a fine structure species.

Further evidence for the noncovalent linkage between **II** and ebO in the fine structure species is indicated by the fact that they can be separated from regenerated ebR-**II** by gel filtration chromatography (see below).

Purification of ebr-II—A two-step size exclusion procedure was used to purify ebR-**II** (14). In the first step, noncovalently linked analog **II** and DMPC were separated from ebO and ebR-**II** using 1% CHAPS. The second step, which was carried out in 1% octyl glucoside (OG), separated ebO from ebR-**II**. Fig. 4 shows the resulting absorption spectrum of purified ebR-**II** in DMPC/CHAPS. The visible absorbance maximum was 558 nm, and the absorbance ratio (A_{280}/A_{558}) was 1.7. Acid denaturation of purified ebR-**II** formed a species with λ_{max}, 468 nm (Fig. 3*B*), demonstrating that analog **II** is linked to the protein via a protonated Schiff base. Since the purified ebR-**II** preparation was unstable in OG, replacement of OG by DMPC/CHAPS was performed. In DMPC/CHAPS mixed micelles, ebr-**II** is stable for several weeks.

pH Dependence of the Absorption Spectrum of ebR-II—Spectrophotometric titrations were carried out for ebR-**I** and ebR-**II** in mixed micelles. In Fig. 5*A*, the visible absorption maximum is plotted as a function of pH. In the acidic range, where ebR-**I** displayed a purple (λ_{max}, 551 nm) to blue (λ_{max}, 588 nm) transition near pH 3.5 (13, 16), ebR-**II** showed a smaller shift from 558 to 573 nm near pH 3. Further acidification caused denaturation of the protein in both cases, resulting in the formation of a free protonated Schiff base with λ_{max}, 442 or 468 nm, respectively. Fig. 5*B* shows the absorbance increase *versus* pH at 442 nm for ebR-**I** and at 468 nm for ebR-**II**. Analysis of the titration curves yielded pK_a values of 2.20 (n = 2.6) and 1.98 (n = 2.5), respectively, for the cooperative transitions. In the alkaline region, a deprotonated Schiff base with λ_{max} at 365 nm for ebR-**I** and at 380 nm for ebR-**II** was formed. Evaluation of the titration data (Fig. 5*C*) revealed a significantly decreased Schiff base pK_a of 8.90 (n = 1.7) for ebR-**II**, as compared to a Schiff base pK_a of 11.26 (n = 2.5) for ebR-**I** in mixed micelles (13).

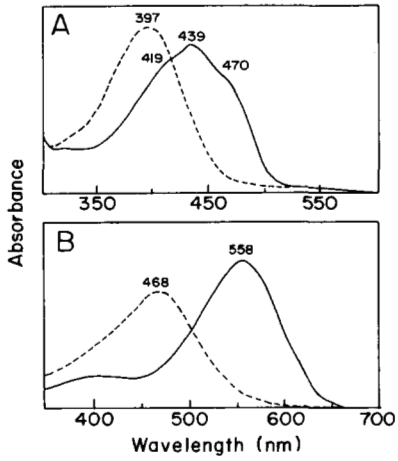

FIG. 3. **Acid denaturation of ebR-II chromophores.** *A*, acid denaturation of the fine structure species formed after mixing ebO with **II** in mixed micelles (DMPC/CHAPS/SDS) for 3 min. *B*, acid denaturation of the HPSEC-purified ebR-**II** (λ_{max}, 558 nm) in mixed micelles (DMPC/CHAPS/SDS). The pigments were denatured in the dark by addition of 1 M HCl to a final pH of 1.5. The resulting absorption spectra are shown by *broken lines*.

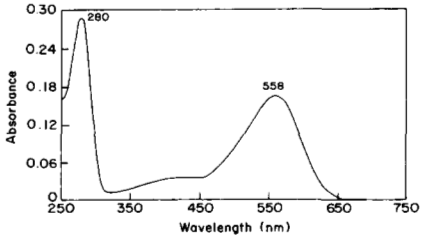

FIG. 4. **UV/visible absorption spectrum of HPSEC-purified ebR-II.** ebO was reconstituted with **II** in mixed micelles (DMPC/CHAPS/SDS) and purified by a two-step gel filtration HPLC procedure as described under "Experimental Procedures." The resulting spectrum was recorded at 20 °C in the dark.

Photochemical Behavior of ebR-II

Dark-Light Adaptation—Light adaptation of ebR-**I** in mixed micelles results in a shift of the λ_{max} from 551 nm to 561 nm and an increase in extinction due to conversion of the 13-*cis*/all-*trans* chromophore into essentially 100% all-*trans* retinal (17). Fig. 6 shows the light minus dark difference spectrum for this reaction. The spectrum was characterized by a large positive peak at 581 nm and a negative peak at 497 nm. Superimposed in Fig. 6 is the light minus dark difference spectrum for an ebR-**II** sample of identical chromophore absorbance. Essentially no change in absorbance was observed upon illumination in the visible wavelength region, indicating that dark adaptation in ebR-**II** was abolished. Thus, ebR-**II** resembles light-adapted ebR-**I**.

Proton Pumping Activity—Following reconstitution into asolectin vesicles, ebR-**I** displayed proton translocation ability

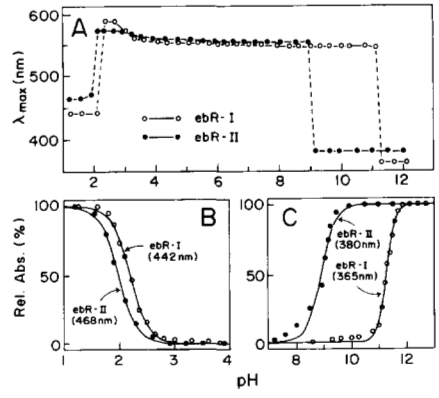

FIG. 5. **pH dependence of the absorption spectra of ebR-I and ebR-II in mixed micelles.** *A*, the wavelength of maximal absorbance in the visible range is plotted *versus* the pH. *B*, titration curves for the transition of ebR-**I** and ebR-**II** at acidic pH. *C*, titration curves for the transition to a deprotonated Schiff base at alkaline pH. The data points in *B* and *C* have been normalized with respect to the total absorbance change at the indicated wavelength. The titration curves were calculated as described in Ref. 13.

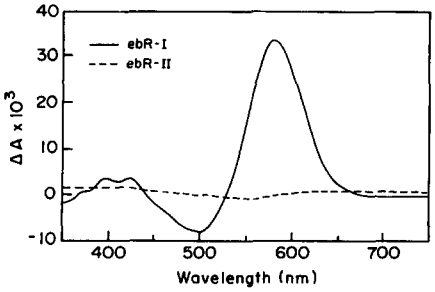

FIG. 6. **Light minus dark difference spectra of ebR-I and ebR-II.** The spectra recorded before and after 5 min of continuous illumination of ebR-II (*broken line*) and ebR-I (*continuous line*) were subtracted. The chromophore absorbance of ebR-I and ebR-II prior to illumination was identical.

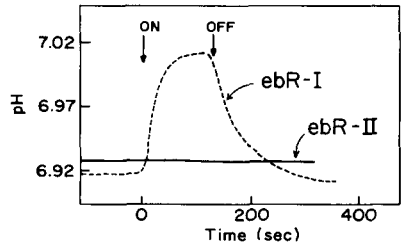

FIG. 7. **Light-induced proton pumping capacities of ebR-I and ebR-II.** Proton translocation by ebR-I (*broken line*) and by ebR-II (*continuous line*) was performed as described under "Experimental Procedures." The samples were irradiated with light of >495 nm at 20 °C. The *arrows* indicate the start and the end of irradiation.

(Fig. 7). The initial rate of pumping for ebR-I at pH 6.2 was 3.2 H^+/bR/s, and the steady-state level was 40 H^+/bR. In contrast, purified ebR-II displayed no detectable proton pumping activity (<1% of ebR-I).

Photocycle Measurements—A time course of absorbance changes during the photocycles of ebR-I or ebR-II in mixed micelles is shown in Fig. 8. Whereas ebR-I displayed characteristic amplitude changes near 650 nm (decay of the K intermediate in the microsecond range; rise and decay of the O intermediate in the millisecond range), 410 nm (rise and decay of the M intermediate), and 530 nm (formation and decay of the depletion signal), the corresponding signals for ebR-II were very close to zero at all wavelengths. It should be noted that the chromophore concentration of the two pigments was matched for these experiments. No difference was observed when "dark-adapted" ebR-II was used, as opposed to a sample that had been "light-adapted" prior to each flash. The absence of a negative signal at 530 nm, close to the λ_{max} of the pigment, clearly demonstrates that ebR-II does not undergo a photocycle in the time window of 100 ns to 10 s.

Reaction of ebR-II with Hydroxylamine—The time course of the reaction of ebR-II in mixed micelles at pH 6.3 with hydroxylamine under steady illumination is shown in Fig. 9*A*. Prior to illumination, the absorption spectrum displayed a peak at 375 nm, originating from the immediate reaction of the fine structure species with hydroxylamine. The light-induced conversion of the purple chromophore to the corresponding oxime proceeded through a single isosbestic point at 418 nm. The kinetics of the reaction were determined by following the absorbance decrease at 555 nm. The half-time for the light-induced reaction of ebR-II was 65 min, which is approximately 1.2-fold faster than the corresponding reaction in the dark ($t_{½}$ = 81 min; Fig. 9*B*). Based on the small difference in the rates of reaction, it is evident that light-induced conformational changes are essentially absent in ebR-II. In contrast, the reaction of ebR-I with hydroxylamine in

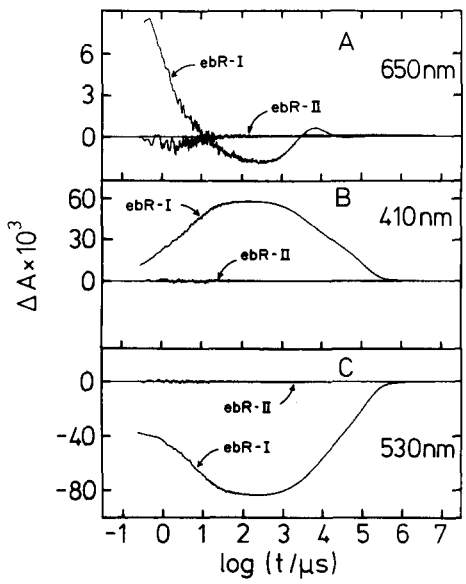

FIG. 8. **Time-resolved photocycle absorbance changes of ebR-I and ebR-II.** Time course of photocycle absorbance changes of ebR-I and ebR-II at 650 (*panel A*), 410 (*panel B*), and 530 nm (*panel C*). The traces are averages of 30 flashes. Both samples, in 30 mM NaH_2PO_4, pH 7, containing 1% DMPC, 1% CHAPS had the same chromophore absorbance. The logarithmic time scale is from 100 ns to 10 s in each panel. Note that the absorbance difference in *panel A* is approximately 10 times smaller compared with *panels B* and *C*.

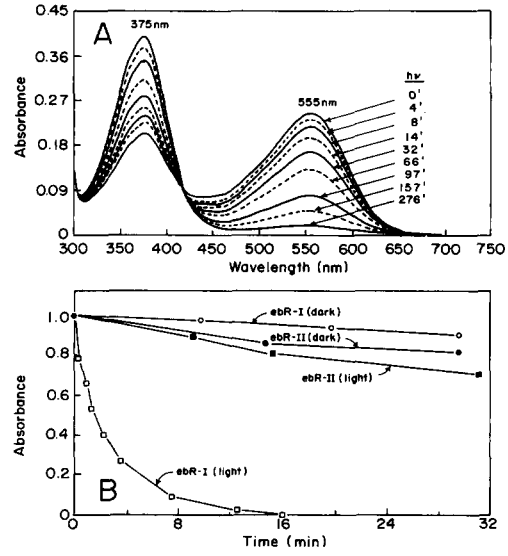

FIG. 9. **Hydroxylamine-induced bleaching of ebR-I and ebR-II.** *A*, time course of the light-induced reaction of ebR-II with hydroxylamine in mixed micelles (DMPC/CHAPS/SDS) at pH 6.3. The sample was illuminated with a >495 nm long pass filter for the indicated time period. *B*, comparison of the reaction rates in the presence and absence of illumination of ebR-I (*open symbols*) and ebR-II (*filled symbols*) with hydroxylamine as measured by changes in absorbance at 555 nm. The absorbance at 555 nm (*ordinate*) have been scaled to equal 1.0 at t = 0. The *abscissa* represents the length of time hydroxylamine was allowed to react with the chromophore in the presence or absence of illumination.

the presence of light ($t_{½}$ = 1.8 min) was increased by more than 2 orders of magnitude relative to the corresponding reaction in the dark ($t_{½}$ = 460 min) (18). The results demonstrate that in ebR-I, all-*trans* → 13-*cis* isomerization is required for the light-induced reaction with hydroxylamine.

DISCUSSION

Retinal analogs have been used in the past in a variety of studies of chromophore-opsin interactions (19–21). The retinal analog **II** (Fig. 1), which cannot undergo all-*trans* to 13-*cis* isomerization, has been used previously in the reconstitution of bO in bleached PM. The preparation evidently showed detectable photocycle and proton pumping (6–8). Retinal analog **II** has also been used in sensory rhodopsin to test the role of C_{13}-C_{14} double bond isomerization in phototaxis (22).

To prepare a light-insensitive bR-like pigment, we have now employed analog **II** to reconstitute bacterio-opsin that was expressed in *E. coli* (ebO) and therefore could not contain any retinal. Analog **II** regenerated a bR-like chromophore (ebR-**II**) in mixed micelles (DMPC/CHAPS/SDS), but the process was complex. A fine structure intermediate was first observed and after some 12 days, about 55% of this was converted to ebR-**II**. Presumably, the additional methylene groups in the cyclopentene ring encompassing the C_{13}-C_{14} double bond caused steric hindrance near the retinal attachment site, possibly due to interference from Trp-86 and/or Thr-89 (2). The absorption bands due to the fine structure intermediate appeared not to be due to multiple species, since a single isosbestic point (Fig. 2) was observed during conversion to the purple chromophore. Although fine structure intermediates can hardly be seen when ebO is reconstituted with **I** at 20 °C, they are evident at 2 °C.[2] Structured intermediates have also been observed during the regeneration of amino acid substitution mutants of ebO in mixed micelles. Thus, Thr-89 substitution mutants displayed fine structures during reconstitution with **I** (17). A number of additional examples are known where during the reaction of retinal or its analogs with bO, species with fine structure are formed. Thus, Schreckenbach *et al.* (23, 24) reported the formation of fine structure species in the reconstitution of the purple pigment from retinal **I** and bleached PM suspensions. Similarly, all-*trans* C_{18}-ketone, a shorter analog of retinal **I**, formed a fine structure intermediate with bO (25). Additionally, retinal **I** and analog **II** form fine structure species with the ebO mutant Lys-216 → Cys, in which the formation of the Schiff base linkage between retinal and the protein cannot be formed. What is the nature of the species with fine structure? Do they represent retinal covalently bound to the protein? Acid denaturation of the complex does not show the presence of a covalent Schiff base linkage (Fig. 3*A*). Therefore, these structures must arise by noncovalent interaction between the apoproteins and the retinal derivatives. Furthermore, ebR-**II** can be separated from the fine structure species by gel filtration. Taken together, the total evidence shows that in the fine structure species there is no covalent linkage between **II** and ebO.

The visible absorption maximum of purified ebR-**II** (558 nm) is very close to that of light-adapted ebR-**I** (561 nm) in mixed micelles (17). The opsin shift of ~3600 cm^{-1} suggests that fixation of the C_{13}-C_{14} double bond does not significantly alter the chromophore environment in ebR-**II** relative to the light-adapted ebR-**I** (opsin shift ~4800 cm^{-1}). In mixed micelles, the ebR-**I** chromophore displays a purple (λ_{max}, 551 nm) to blue (λ_{max}, 588 nm) transition near pH 3.5, analogous to the transition observed from PM (26). On the other hand, the absorption maximum of ebR-**II** shows a smaller shift from 558 nm to 573 nm near pH 3 (Fig. 5*A*). Further acidification causes denaturation of the protein in both cases, resulting in the formation of a free protonated Schiff base. This cooperative unfolding, which is presumably triggered by protonation of the counterions Asp-85 and Asp-212 (13), occurs at a lower pH in ebR-**II** than in ebR-**I** (Fig. 5*B*). At alkaline pH, a cooperative transition to a deprotonated Schiff base is observed. The titration data (Fig. 5*C*) reveal a significantly decreased Schiff base pK_a of 8.9 for ebR-**II**, as compared with a Schiff base pK_a of 11.3 for ebR-**I** in mixed micelles (13). In ebR-**II**, the purple to blue transition is weakened presumably because the chromophore is restricted in the all-*trans* configuration. Indeed, it has been reported that such pH-dependent color transition is accompanied with an alteration in the isomer composition in bR (27). The decrease in the pK_a of the unfolding transition as well as the reduced red-shift observed for the "blue form" of ebR-**II** suggest that the pK_a of the counterions is lowered. The difference could originate from an increased accessibility of the Schiff base in ebR-**II** to water as is suggested by the hydroxylamine reaction in the dark. Alternatively, the presence of the cyclopentene ring could change either the proximity or the nature of counterion(s) to the protonated imine. Even a slight distortion in the polyene planarity can bring about a looser structure particularly near the protonated Schiff base end of the chromophore as reflected by enhanced hydroxylamine reactivity toward ebR-**II** over ebR-**I** in the dark. The lowering of the Schiff base pK_a for ebR-**II** could also result from a conformational alteration in the protein while accommodating the cyclopentene ring around the C_{13}-C_{14} double bond of **II**.

As expected, ebR-**II** does not show any response to light. This result confirms that isomerization of the C_{13}-C_{14} double bond of the chromophore is essential for bR function. ebR-**II** does not translocate protons (Fig. 7) nor does it undergo any photocycle (Fig. 8). Previous mutagenesis studies have shown that mutants which are inactive in proton pumping, such as Asp-85 → Asn (16), show photocyles (15) that are only affected at specific steps. However, no photocycle intermediates are observed in ebR-**II**. This result is consistent with a previous report implying that rotations around C_{12}-C_{13} and C_{10}-C_{11} single bonds and isomerizations around C_{11}-C_{12} and C_9-C_{10} double bonds are not sufficient for initiation of a photocycle (28). Further, the absence of any change in the absorption spectrum of ebR-**II** upon illumination (Fig. 6) shows that light-dark adaptation is primarily dependent on isomerization around the C_{13}-C_{14} double bond. Finally, all-*trans* to 13-*cis* isomerization and subsequent photocycling is required for the light-induced reaction with hydroxylamine (Fig. 9). The small enhancement observed in the rate of reaction in the light is not clearly understood.

The preparation of monomeric ebR-**II** now described should be useful in biophysical studies of the light-adapted state of bR. It should also be useful in structural studies of the protein and for the preparation of single crystals. In the following papers, we have adopted a similar approach using a nonisomerizable analog of 11-*cis* retinal for the preparation of a light-stable rhodopsin-like pigment (29, 30).

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