PHYSIOLOGICAL AND STRUCTURAL INVESTIGATIONS OF BACTERIORHODOPSIN ANALOGS*

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Received August 8,1977

SUMMARY: Retinal in bacteriorhodopsin was replaced by several analogs. Changes in absorption and Raman spectra and in the capacity to function as a proton pump after reconstitution into liposomes, were recorded. We conclude from these studies that considerable alterations in chemical structure, particularly in the ring of retinal, can be tolerated with retention of some functional activity.

Several analogs of retinal have been incorporated into rhodopsin (1-6). These studies were limited by the fact that there is no biological in vitro assay for the function of the photoreceptor. Bacteriorhodopsin on the other hand has a well-defined role in catalyzing light-driven proton translocation that can be analyzed in a reconstituted in vitro assay (7,8). It was furthermore shown that in the presence of light and hydroxylamine, bacteriorhodopsin is bleached and can be reactivated by addition of retinal (9,10). In a preliminary report it was recently shown (11) that 1,3-dehydroretinal replaced retinal in its biological function, but 5,6-epoxiretinal did not.

In this communication we show that several retinal analogs retain the proton pumping ability of bacteriorhodopsin. We have compared this physiological information on relative proton pumping efficiency with data obtained by absorption and Raman spectroscopy. In addition, we use bacteriorhodopsin grown in deuterated media to test for isotopic effects on active site function.

MATERIALS AND METHODS

Retinal analogs I and II (see Figure 1) were generously donated by Dr. Beverly A. Pawson of Hoffman-La Roche Inc. and 9-dm all-trans retinal by Dr. Allan Kropf. All-trans retinal was purchased from Eastman Kodak Company and used without further purification. All-trans 3-dehydro retinal was a gift from Drs. U. Gloor and F. Weber of Hoffman-La Roche Inc.

*Supported by NIH Grant EY01377 and NSF Grant BMS-75-17887
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ISSN 0006-291X

Table I

Proton Translocation Catalyzed by Reconstituted Vesicles Using Bacteriorhodopsin With Retinal Replaced by Analogs

Rhodopsin used in Reconstitution	ng H^+/mg Protein	% Activity
Bacteriorhodopsin	172 (207)	100
Bleached bacteriorhodopsin	0 (0)	0
Bleached bacteriorhodopsin + retinal	174	100
" + 3-dehydroretinal	123	71
" + 9-dm retinal	118	68
" + sulfur analog (I)	54 (99)	31 (48)
" + methoxy analog (II)	25 (60)	15 (29)
Deuterated bacteriorhodopsin	165	100
Bleached deuterated opsin + retinal	158	97
Bleached deuterated opsin + 3-dehydroretinal	112	68

Growth of Halobacterium halobium S_9, isolation of bacteriorhodopsin (12) and bleaching in the presence of hydroxylamine (10) were carried out as previously described. With the exception of 9-dm retinal all other retinal analogs were incorporated by suspending in the dark 1.5×10^{-5}M bleached and washed membranes in a volume of 2 ml in distilled water together with 0.2 µl of 15 mM retinal or analog. After 50 minutes, absorption spectra were taken and more retinal (or analog) was added until maximal absorption intensity was reached. In the case of 9-dm retinal, because of limited availability, 3.0 µg of the compound were added to 1 ml of membrane suspension.

Reconstitution into liposomes was performed essentially as reported earlier (8). Soybean phospholipids were first sonicated to clarity at 40 mg per ml in 0.15 M KCl. To 0.1 ml of this suspension 0.1 to 0.3 ml of the various bacteriorhodopsin preparations (0.5 to 2×10^{-5}M) were added. The final volume was 0.4 ml and the final KCl concentration was 0.15 M. After sonication for 20 min., a 50 µl volume of the suspension was tested for proton pumping at pH 5.8 in 0.15 M KCl as described previously (7).

Fully deuterated bacteriorhodopsin was grown at Argonne National Laboratories using previously published procedures (13).

Resonance Raman spectra were obtained on all samples using a Spex 1401 double monochromator, a thermoelectrically cooled RCA C31034 photomultiplier tube and home-built photon counting electronics (14).

RESULTS AND DISCUSSION

Proton translocation. As shown in Table I virtually full proton translocation activity was recovered when all-trans retinal was added to bleached bacteriorhodopsin membranes. With 3-dehydroretinal and 9-dm retinal, about 70% pumping activity was achieved. With the sulfur analog (II) and the methoxy analog (I),

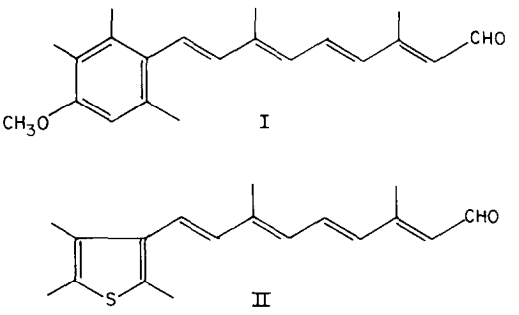

Figure 1. The structures of [(I)] 9-(4-Methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenal and [(II)] (E,E,E,E)-3,7-Dimethyl-9-(2,4,5-trimethyl-3-thienyl)-2,4,6,8-nonatetraenal.

31% and 15% proton translocation activity was observed, respectively, when a yellow light filter was used. It should be noted, however, that with white light (values given in parenthesis) considerably larger percent reactivation with some analogs was observed, a finding in line with the shifts in absorption spectra described below.

In all cases tested the proton movements were abolished by addition of nigericin showing that transmembranous proton translocation has taken place.

Absorption spectra and light-dark adaptation. 3-Dehydrobacteriorhodopsin was red shifted by 25 nm when compared to the original retinal complex. This red shift is analogous to changes in the absorption spectrum recorded for porphyropsin formed with vertebrate opsin (5). The alteration in the absorption maximum can be understood in terms of extending the conjugation in the π electron system by the presence of an extra double bond. Compound I and II (see Figure 1) when incorporated into bleached bacteriorhodopsin, exhibited absorption maxima at 480 and 510 nm, respectively. The 9-dm retinal complex had an absorption maximum of about 530 nm in the dark. This blue shift is also similar to that observed when this isomer is incorporated into vertebrate opsin (1).

To estimate the % incorporation of the various analogs into bleached membranes we assumed, as is observed in photoreceptor rhodopsins (1.5), that the extinction ratio of free analog to bacteriorhodopsin analog was the same as the

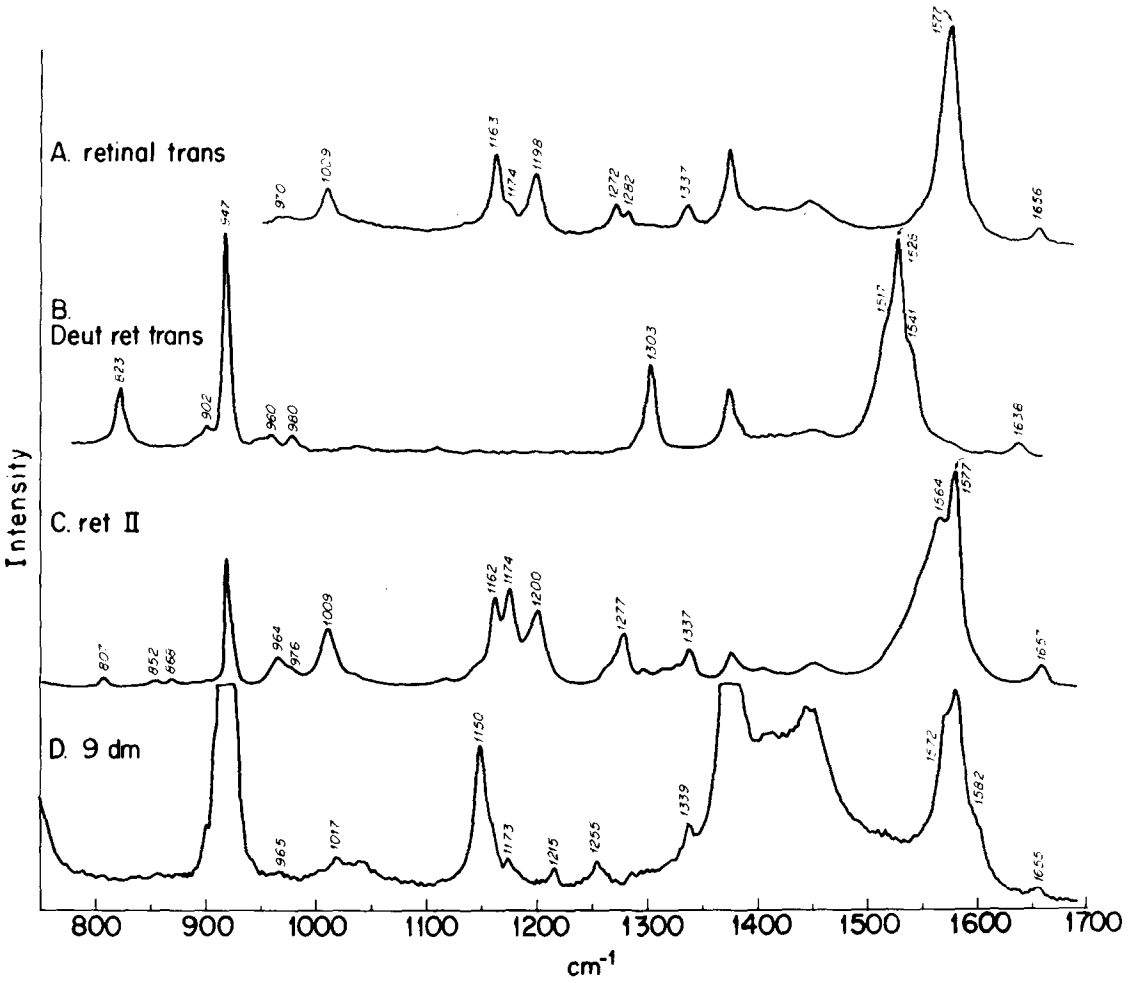

Figure 2. All-trans isomers of (A) Retinal, (B) Deuterated Retinal extracted from fully deuterated bacteria, (C) 3-dehydroretinal, (D) 9-desmethyl-retinal. Spectra were taken at room temperature in CH_3CN solvent (solvent bands visible at 918 cm^{-1}, 1039 cm^{-1}, 1375 cm^{-1}, 1412 cm^{-1} and 1447 cm^{-1}) with 2 cm^{-1} resolution. Laser excitation was 647.1 nm at 40 mW. All labeled bands are accurate to ± 2 cm^{-1} and have been observed in multiple experiments.

all-trans retinal to bacteriorhodopsin extinction ratio. In the reconstitution experiments thus far performed, there was reasonable agreement between % reconstitution and proton translocation activity.

The analogs of bacteriorhodopsins behaved similarly to the native membrane with respect to light-dark adaptation. Dark adapted 3-dehydrobacteriorhodopsin absorbed maximally at 585 nm, and, upon light adaptation, there was an 8 nm red

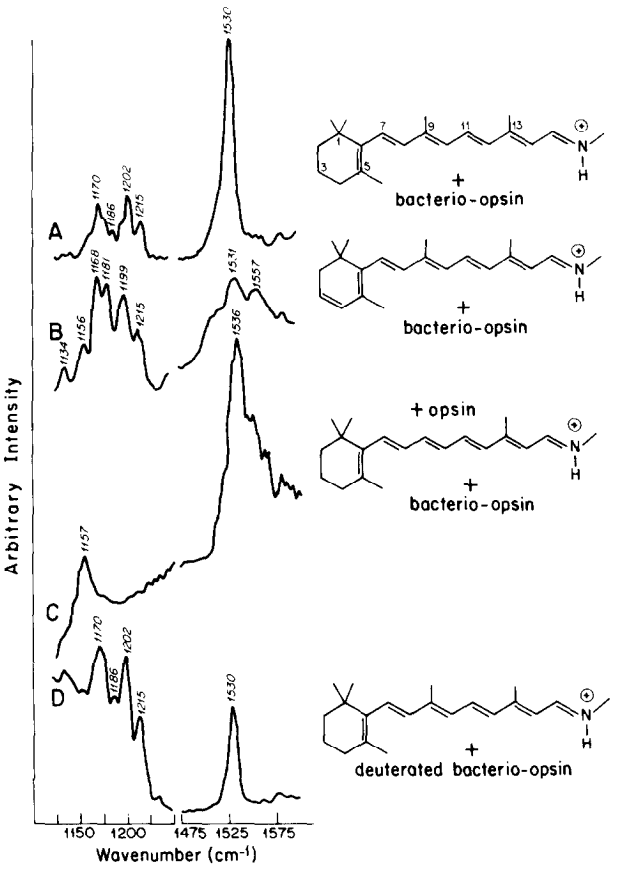

Figure 3. Resonance Raman spectra of reincorporated bacteriorhodopsin complexes and conformation of the retinals used in these experiments. All spectra were taken with 10 mW of 514.5 nm light from a 2W Coherent Radiation argon ion laser. Samples were concentrated to about OD 4 by centrifugation and placed on the tip of a glass rod in a liquid nitrogen immersion cryostat. Spectra were taken in a 90° scattering arrangement utilizing a Spex 1401 double grating monochromator, an RCA C31034 photomultiplier tube and home built photon counting electronics (14). All spectra were recorded with 2 cm^{-1} resolution. (A) All-trans retinal bacterio-opsin complex; (B) 3-dehydrobacteriorhodopsin; (C) 9-dm bacteriorhodopsin; (D) all-trans retinal deuterated bacterio-opsin complex.

shift accompanied by a 5% increase in extinction. In the case of 9-dm bacteriorhodopsin there was a 5-10 nm red shift upon light adaptation but its exact magnitude could not be ascertained due to the small amount of material available.

Resonance Raman Spectroscopy. Figure 2 shows the resonance Raman spectra for all-trans retinal and retinal analogs. A comparison with the resonance Raman spectra of bacteriorhodopsin and bacteriorhodopsin analogs (Figure 3) revealed

a shift to lower frequencies in the C=C stretching region between 1475 and 1600 cm^{-1} (15-19) as well as changes in the 1100-1250 cm^{-1} fingerprint region with the appearance of a band at 1215 cm^{-1} in bacteriorhodopsin. With the exception of the latter, the observed changes are similar to those seen in resonance Raman spectra of crystalline all-trans protonated Schiff-base model compounds (20). Since the 1215 cm^{-1} band is still present in the spectrum of retinal incorporated into fully deuterated bacterio-opsin (Figure 3D), where protein bands would be expected to move down in frequency relative to protonated bands, it seems unlikely that the 1215 cm^{-1} band arises from a protein residue. This finding and the observation that the 1215 cm^{-1} band is present in 3-dehydrobacteriorhodopsin (Figure 3B) suggest that the 1215 cm^{-1} band arises from a bond rotation in a region of the chromophore far from the β-ionone ring and closer to the terminal end of the isoprenoid chain.

Chemical modifications that perturb the active site structure may alter the proton pumping function of this membrane protein. Further experiments along the lines of this communication should test this hypothesis and define the primary interactions which constitute this biological proton pump.

Acknowledgements: We thank Robert Cookingham for obtaining resonance Raman spectra of all-trans retinal, deuterated retinal, 9-desmethyl retinal and 3-dehydroretinal. These spectra will be discussed in detail in a forthcoming work.

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