Acetylenic Retinals Form Functional Bacteriorhodopsins but Do Not Form Bovine Rhodopsins†

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Abstract: Three acetylenic retinals bearing a triple bond between the carbon atoms 7 and 8, and carrying no, two, or three methyl groups on the cyclohexene ring, were synthesized and various isomers obtained by illumination and subsequent chromatographic separation. The introduction of the acetylenic bond leads to a different geometrical arrangement of the cyclohexene ring with respect to the polyene side chain. Attempts to reconstitute rhodopsins and isorhodopsins with any of these compounds failed. Bacteriorhodopsins formed with the 13-cis and the all-trans isomers of all three acetylenic retinals showing absorption maxima between 490 and 530 nm. The increasing number of methyl groups on the ring correlated with an increasing red shift of the absorption maximum of the chromoproteins. This same correlation was found for the proton-pumping activity of the acetylenic bacteriorhodopsins. The results are in agreement with the fact that in rhodopsin a covalent binding at the carbonyl group (Schiff's base formation) and also a fixation at the cyclohexene ring of retinal occurs. The changed geometry of the acetylenic retinals, therefore, prevents binding. In bacteriorhodopsin, which lacks a specific cyclohexene ring binding site, the acetylenic retinals form active molecules. However, the contribution of the ring and its substituents to the function of bacteriorhodopsin is significant.

Retinal (**1**) is the prosthetic group in two types of light-energy converting proteins, (i) the rhodopsins and (ii) several membrane proteins of *Halobacterium halobium*. In both types of proteins, incident light causes an isomerization of the double bonds of the protein-bound retinal molecule, which in the case of the rhodopsins leads to a neuronal response.[1] In bacteriorhodopsin (BR), the dominant membrane protein in *H. halobium*, light energy drives a photochemical cycle, which establishes a proton gradient across the cell membrane.[2] The electrochemically stored energy is then used for physiological processes, e.g., synthesis of ATP or transport of amino acids and metabolites.[3,4]

Only specific isomers of retinal are accepted in the binding site of these proteins; vertebrate rhodopsin is regenerable with 7-, 9-, or 11-*cis*-retinal.[5] The reconstitution from several di- and tricis isomers of retinal, added to an opsin suspension, was also reported.[6-8] Bacterioopsin (BO) will accept 13-*cis*- and *all-trans*-retinal[9] and to a lesser extent the 7-cis isomer.[10] In bacteriorhodopsin and all rhodopsins the retinal is covalently bound via a protonated Schiff's base (SBH^+) to the ϵ-amino group of a lysine residue; absorption measurements of the protonated retinal–butylamine compound gave a wavelength of maximum absorption, λ_{max}, value of 440 nm in ethanol. Therefore, additional noncovalent interactions between the amino acid side chains in the binding site and the retinal moiety were proposed to explain the additional red shift in the absorption maximum of the retinal–protein complexes (λ_{max} of bovine rhodopsin 498 nm, λ_{max} = BR 568 nm) and to maintain the functionality of the chromoprotein.[11-14]

Analogue retinal structures have been used to analyze those parts of the retinal molecule, that are important for binding to the protein, the red shift of absorption, and functionality. It was found that a specific binding at the cyclohexene ring occurs in opsin;[15-17] recently it was shown that this binding site also binds retinoids with partially removed ring structures, e.g., compound **16** (Figure 2b).[18,19] A similar binding site was not found in bacterioopsin.[17] This protein interacts nonspecifically with the ring but shows a very defined interaction with the side chain of retinal around position 13.[12,20,21]

We synthesized three retinals that bear a triple bond in place of the 7,8-double bond (**2–4**), whereas the rest of the side chain is identical with retinal (see Figure 1), and tried to reconstitute rhodopsins and bacteriorhodopsins. As can be seen from the formulas, the introduction of the 7,8-triple bond results in a slightly altered arrangement of the ring and the side chain. This would prevent proper binding in a protein like opsin, which has two sites of precise attachment, one for the ring and the other for the carbonyl group of retinal, but would not prevent binding in a protein like BO, which has a specific site only for the side chain.

The results obtained confirm this basic concept, but also demonstrate an influence of the cyclohexene ring of retinal on the function of BR.

Experimental Section

Materials. Chemical Syntheses. Cyclohexanone, 2-methylcyclohexanone, triethyl phosphite, and chloroacetonitrile were from Aldrich; 3,3-dimethylacrylonitrile was purchased from Fluka. 1-ethynylcyclohexanol (**15**) and 7,8-dehydroretinoic acid (**5**) were from BASF. All

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Table I. ^1H NMR Data of Cis and Trans Isomers of Acetylenic Retinals **2–4**, Measured in $CDCl_3$

Compound	Chemical shifts and coupling constants (Hz) of position: 1,1-dimethyl	H_{C_2}	H_{C_3}	H_{C_4}	5-methyl	9-methyl	13-methyl	$H_{C_{10}}$	$H_{C_{11}}$	$H_{C_{12}}$	$H_{C_{14}}$	$H_{C_{15}}$	Additional shifts/ coupling constants
all-trans-(2)	1.12	1.50	1.61	2.05	1.91	2.07	2.32	6.48 $^3J_{10,11}$:11.5	7.00	6.37 $^3J_{11,12}$:15.1	5.98 $^3J_{14,15}$:8.2	10.11	$^4J_{9\text{-me},10}$:1.3 $^4J_{13\text{-me},14}$:1.1
13-cis-(2)	1.13	1.49	1.62	2.04	1.91	2.07	2.13	6.51 $^3J_{10,11}$:11.7	6.90	7.28 $^3J_{11,12}$:15.1	5.88 $^3J_{14,15}$:8.0	10.20	$^4J_{9\text{-me},10}$:1.3 $^4J_{13\text{-me},14}$:1.0
9,13-dicis-(2)	1.16	1.51	1.63	2.08	1.95	2.06	2.11	6.36 $^3J_{10,11}$:11.0	7.21	7.27 $^3J_{11,12}$:15.3	5.86 $^3J_{14,15}$:7.9	10.19	$^4J_{13\text{-me},14}$:0.9
9-cis-(2)	1.17	1.51	1.64	2.09	1.95	2.05	2.29	6.32 $^3J_{10,11}$:11.1	7.31	6.36 $^3J_{11,12}$:15.5	5.97 $^3J_{14,15}$:8.2	10.10	$^4J_{13\text{-me},14}$:1.0
11-cis-(2)	1.14	not detected (n.d.)			1.92	2.07	2.34	6.68 $^3J_{10,11}$:12.1	6.84	5.93 $^3J_{11,12}$:11.8	6.05 $^3J_{14,15}$:8.0	10.08	$^4J_{13\text{-me},14}$:1.0
all-trans-(3)	1.12	1.53	1.64	2.08	-	2.03	2.30	6.46 $^3J_{10,11}$:11.3	6.97	6.35 $^3J_{11,12}$:15.1	5.97 $^3J_{14,15}$:8.1	10.10	H_5:6.08 $^3J_{4,5}$:4.3 $^4J_{9\text{-me},10}$:1.2 $^4J_{13\text{-me},14}$:0.9
13-cis-(3)	1.06	1.47	1.61	2.07	-	n.d.	2.29	6.49 $^3J_{10,11}$:11.7	6.87	7.22 $^3J_{11,12}$:15.0	5.87 $^3J_{14,15}$:7.9	10.18	
9,13-dicis-(3)	1.16	1.53	1.64	2.09	-	2.02	2.11	6.35 $^3J_{10,11}$:10.8	7.15	7.25 $^3J_{11,12}$:15.3	5.85 $^3J_{14,15}$:7.9	10.17	H_5:6.12 $^3J_{4,5}$:4.3 $^4J_{9\text{-me},10}$:1.3 $^4J_{13\text{-me},14}$:1.2
9-cis-(3)	1.17	1.53	1.64	2.11	-	2.01	2.29	6.31 $^3J_{10,11}$:11.2	7.25	6.34 $^3J_{11,12}$:15.5	5.96 $^3J_{14,15}$:0.1	10.09	H_5:6.12 $^3J_{4,5}$:4.1 $^4J_{13\text{-me},14}$:0.9 $^3J_{3,4}$:6.0
all-trans-(4)	-	1.59 - 1.68		2.14	-	2.01	2.29	6.46 $^3J_{10,11}$:11.4	6.96	6.34 $^3J_{11,12}$:15.3	5.97 $^3J_{14,15}$:8.2	10.10	H_5:6.15 $^4J_{9\text{-me},10}$:1.0 $^4J_{13\text{-me},14}$:1.3
13-cis-(4)	-	n.d	n.d.	n.d.	-	2.01	2.11	6.49 $^3J_{10,11}$:11.7	6.86	7.25 $^3J_{11,12}$:14.9	5.87 $^3J_{14,15}$:7.9	10.17	
9,13-dicis-(4)	-	1.57 - 1.69		2.17	-	1.99	2.12	6.34 $^3J_{10,11}$:10.9	7.09	7.24 $^3J_{11,12}$:15.3	5.85 $^3J_{14,15}$:7.9	10.17	H_5:6.18 $^4J_{9\text{-me},10}$:1.2
9-cis-(4)	-	1.58 - 1.69		2.17	-	1.98	2.29	6.30 $^3J_{10,11}$:10.5	7.18	6.35 $^3J_{11,12}$:15.0	5.95 $^3J_{14,15}$:8.2	10.09	H_5:6.18

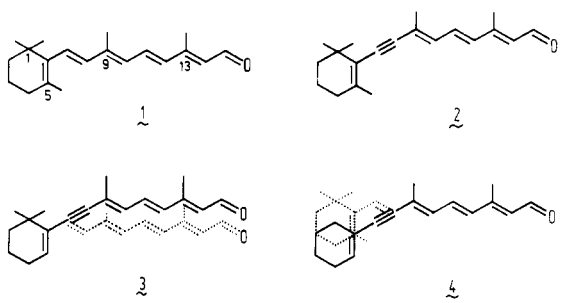

Figure 1. Structures of the all-trans isomers of retinal (**1**), 7,8-dehydroretinal (**2**), 5-desmethyl-7,8-dehydroretinal (**3**), and 1,1,5-tridesmethyl-7,8-dehydroretinal (**4**). The formula of retinal was superimposed (dashed) on the formulas of **3** and **4** to demonstrate the changes in shape upon the introduction of the acetylenic bond. Two possible arrangements of the acetylenic retinals result. Either the cyclohexene ring (demonstrated with compound **3**) or the carbonyl end group (demonstrated with compound **4**) are fixed in the same position as in retinal.

other reagents were of analytical grade; solvents were dried and purified according to standard procedures.

Methods. Chemical Procedures. (1) Synthesis of Acetylenic Retinals. Only the synthesis of the acetylenic retinal **3** as the most complex one will be discussed in detail. The synthesis of acetylenic retinal **2**, starting with the corresponding retinoic acid, is shortly described in the results. The synthesis of acetylenic retinal **4** followed that of acetylenic retinal **3**, which will be described in the next paragraph. The only exception was the use of the commercially available ring component (**15**) as starting material. An overview on the synthetic route chosen for acetylenic retinal **3** is given in Figure 2a. The physical and spectroscopic properties of such intermediates of the synthesis, which were already formerly described, were in accordance with the previously published data. Newly produced compounds were characterized by ^1H NMR, IR, and mass spectroscopy; the spectroscopic data were in full accordance with the expected structures. The ^1H NMR data of the isolated isomers of the acetylenic compounds **2** to **4** are given in Table I.

2,2-Dimethylcyclohexanone (8). Sodium (9.1 g, 0.4 mol) was dissolved in 500 mL of liquid ammonia to form sodium amide. After complete evaporation of the ammonia, 200 mL of diethyl ether were added under nitrogen. After the mixture was cooled to 0–5 °C, 22.4 g (0.2 mol) of

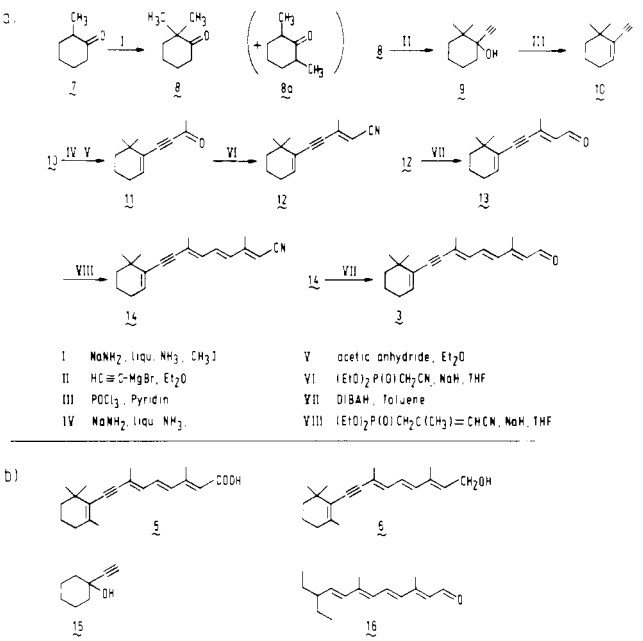

Figure 2. (a) Scheme of the synthesis of retinal **3**. (b) Structures of compounds mentioned in the text.

2-methylcyclohexanone (**7**) dissolved in diethyl ether was added dropwise to the stirred suspension. The reaction mixture was allowed to warm up to room temperature and was then refluxed for 30 min. The mixture was again cooled to room temperature, and 28.4 g (0.2 mol) of methyl iodide were slowly added. Refluxing for 1 h completed the reaction. The workup procedure according to ref 22, followed by fractionated distillation, yielded 18.1 g of 2,2-dimethylcyclohexanone (**8**, 74%, $bp_{13/14}$ 56–58 °C).

1-Ethynyl-2,2-dimethylcyclohexene (10). Into a suspension of sodium amide in 500 mL of liquid nitrogen (made from 3.3 g = 0.14 mol of sodium) containing ca. 100 mg of $Fe(NO_3)_3$, acetylene was introduced during 15 min using triphenylmethane as indicator for complete formation of sodium acetylide.[23] To this suspension was added 16 g (0.13 mol)

of 2,2-dimethylcyclohexanone (**8**) in diethyl ether during 90 min, and the mixture was allowed to react for an additional 50 min. After evaporation of the ammonia, the organic layer was washed with water. Distillation of the crude reaction mixture after evaporation of the diethyl ether yielded 17.3 g (0.11 mol = 85%) of alcohol **9**.

Alcohol **9** (17 g) was dissolved in 15 mL of pyridine and heated to 100 °C. $POCl_3$ (16 mL), dissolved in 15 mL pyridine, was added dropwise. The reaction mixture was stirred at that temperature for 2 h and, after cooling to room temperature, very carefully hydrolyzed on ice. The aqueous layer was extracted with diethyl ether, and the combined organic phases were washed with diluted HCl, a saturated solution of sodium hydrogen carbonate, and water. Fractionated distillation of the crude reaction products yielded 6.4 g (0.05 mol = 45%) of compound **10**.

4-(2,2-Dimethylcyclohexen-1-yl)-3-butyn-2-one (11). To a solution of ethylmagnesium bromide (prepared from 1.35 g of Mg and 4.1 mL of ethyl bromide)[23] in diethyl ether was added 6 g (45 mmol) of compound **10**, dissolved in diethyl ether. This solution was added dropwise at −60 °C to a solution of 7.5 g of acetic anhydride in diethyl ether. The reaction mixture was allowed to warmup to −15 to −5 °C within 3 h and was then hydrolyzed on ice. Extraction, drying, and evaporation of the organic phase was followed by fractionated distillation and yielded 3.3 g of ketone **11** (19 mmol = 40%).

3-Methyl-5-(2,2-dimethylcyclohexen-1-yl)-2-penten-4-ynenitrile (12). The ylide of diethyl (cyanomethyl)phosphonate in tetrahydrofuran (formed from 4 g of phosphonate and 0.6 g of sodium hydride) was allowed to react with 3 g (17 mmol) of ketone **11** for 4 h at room temperature. Distillation of the reaction mixture after the workup procedure described for the formation of compound **10** yielded 2.6 g (13 mmol = 76%) of nitrile **12** ($bp_{0.001}$ 80–82 °C).

C_{14} Aldehyde 13. Nitrile **12** (2.4 g, 12 mmol) in toluene was allowed to react with 2.8 mL of DIBAH (diisobutylaluminum hydride) (14 mmol) for 30 min at room temperature. After distillation (Kugelrohr) 0.5 g (2.5 mmol = 21%) of aldehyde **13** were obtained ($bp_{0.001}$ 110 °C).

5-Desmethyl-7,8-dehydroretinal (3). The condensation reaction between the aldehyde **13** (0.4 g = 2 mmol) and the ylide of diethyl (3-cyano-2-methyl-2-*trans*-propenyl)phosphonate (formed from 0.66 g of phosphonate and 0.09 g of sodium hydride) was identical with the reaction that produced nitrile **12** and yielded 0.4 g (1.6 mmol = 80%) of nitrile **14** after purification by preparative HPLC (high performance liquid chromatography) (column dimensions were 1.6 × 25 cm, filled with 7 μm Si 60, the flow was set at 6–7 mL per min, the solvent used was cyclohexane/ether 80/20 (v/v)). The reduction of the obtained 0.4 g of nitrile **14** with 0.4 mL of DIBAH (2 mmol) in toluene, according to the procedure described for the aldehyde **13**, produced the acetylenic retinal **3**, 0.24 g (0.9 mmol = 56%).

(2) Purification and Characterization of Reaction Products. Purification of reaction products and photoisomerization of compounds **2–4** in organic solvents were performed as previously described,[12,24] except that a 360-nm cutoff filter was applied for illumination. The reaction mixtures were purified by TLC (thin-layer chromatography) or HPLC.[24] The purified products of the photoisomerization were characterized by 400-MHz ^{1}H NMR, IR, UV, and MS.

Biochemical Procedures. Preparation of ROS (rod outer segments), bleaching with hydroxylamine, and reconstitution reactions followed the methods described in ref 17. Isolation of purple membranes, preparation of AM (apomembrane), and regeneration of BR analogues from AM and acetylenic retinals and also by the addition of the acetylenic retinals to retinal-deficient cells grown in the dark were carried out as described previously.[12,25] The assays of light-induced ATP synthesis in intact cells and light-dependent proton transport in cell vesicles were performed according to published procedures,[12,26,27] except that the regeneration of BR analogues in cell vesicles was performed in 2 M salt (150 μL of vesible suspension containing 14.5 nmol of BO). After regeneration was complete, the vesicle suspension was brought to 6 mL with basal salts, and then proton translocation was measured as published.[26] Flash experiments were performed as described in ref 12.

Results

Synthesis and Photoisomerization of Compounds 2–4. (a) Synthesis of 7,8-Dehydroretinal (2). Acetylenic retinal **2** was formed by reduction of 7,8-dehydroretinoic acid **5** (gift from BASF) with lithium aluminum hydride, yielding the alcohol **6**. This compound, which was already described,[28] was oxidized with manganese dioxide in methylene chloride to the acetylenic aldehyde **2** in an overall yield of 58%.

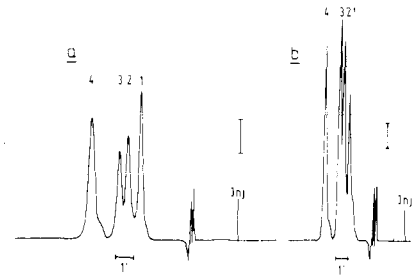

Figure 3. Analytical HPLC traces of the illuminated all-trans isomer of acetylenic retinal **2** in isopropyl alcohol, detected at 360 nm. (a) After 15 s of illumination. (b) After 5 min of illumination. 1 = 13-cis, 2 = 9,13-dicis, 3 = 9-cis, and 4 = all-trans isomer. The vertical bar corresponds to 0.02 OD units. The polarity of the solvent increased slightly from the injection shown in (a) to that shown in (b), leading to a faster elution of all isomers. For conditions of illumination and of HPLC see Experimental Section. The identification of the isomers was performed by 400-MHz ^{1}H NMR (see Table I).

(b) Synthesis of 5-Desmethyl-7,8-dehydroretinal (3). The synthesis is described in detail in the Experimental Section and is summarized in Figure 2a. The synthesis began with the conversion of 2-methylcyclohexanone (**7**) into 2,2-dimethylcyclohexanone (**8**), which was let to react with sodium acetylide to produce the alcohol **9**. After elimination of water this compound was converted into its anion and formed upon reaction with acetic anhydride the ketone **11**. Chain elongation by Wittig–Horner condensation reaction, followed by reduction of the formed nitrile **12**, yielded the aldehyde **13**, which in a subsequent C_5-chain elongation, followed by reduction, was converted into acetylenic retinal **3**.

(c) Synthesis of 1,1,5-Tridesmethyl-7,8-dehydroretinal (4). The synthesis of **4** was as described for compound **3**, except that the starting material was the commercially available 1-ethynylcyclohexanol (**15**, see Figure 2b) instead of alcohol **9** in Figure 2a. The overall yield of compound **4** from alcohol **15** was 11%.

(d) Purification of the Acetylenic Retinals 2–4 and their photoisomerization. Compounds **2–4** were formed as a mixture of mainly the all-trans isomer and some cis isomers. The photoisomerization experiment of the purified all-trans isomers of **2–4** was performed in isopropyl alcohol. This solvent was chosen on the basis of our previously published results on photoisomerization of retinal analogues.[24] Illumination produced a similar distribution of mainly three cis isomers for all three acetylenic all-trans isomers, which after separation by HPLC were identified by ^{1}H NMR (see Table I). For retinal **2** the following ratios were determined: 13-cis, 22%; 9,13-dicis, 24%; 9-cis, 26%; the remaining all-trans isomer was 28%. The isomers are listed by increasing retention time. The formation of these isomers is shown in Figure 3 for two illumination periods. The purification of the formed cis isomers was performed with a preparative column and a solvent of lower polarity. The photoequilibrium was reached after 2 min. Only in the case of acetylenic retinal **2**, a small amount of the 11-cis isomer could be detected, coeluting with the 9-cis compound (<10%).

Formation of Chromoproteins. When mixed with freshly prepared bovine opsin none of the isomers of compounds **2–4** formed a chromoprotein, even when added in a 6-fold excess. This is shown for compound **4** in Figure 4. After incubation of opsin with the analogue retinals for several hours without chromoprotein formation, the opsin was still active, since addition of 11-*cis*-retinal (**1**) to the reaction mixture caused an increase in absorbance at 500 nm, corresponding to nearly 100% regeneration of rhodopsin (curve 5 in Figure 4). When the acetylenic retinals **2–4** were

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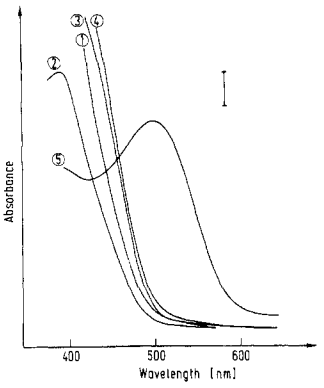

Figure 4. Incubation of opsin (15 nmol in 1 mL of 50 mM phosphate buffer, pH 7.0) with the 13-cis (6 nmol, curve 1), 9,13-dicis (40 nmol, curve 2), 9-cis (86 nmol, curve 3), and all-trans isomer (9 nmol, curve 4) of retinal **4**. Curve 5 shows the regeneration of opsin upon addition of 40 nmol of 11-*cis*-retinal (**1**) to the incubation mixture. Full scales were 0.2 for curves 1 and 4, 2.0 for curves 2 and 3, and 1.0 for curve 5. The bar corresponds to 0.1 OD units at a full scale of 1.0.

Tabel II. Spectroscopic and Functional Properties of BR Analogues Containing the Acetylenic Retinals **2–4**, in Comparison to BR

Compound	Absorption maximum (nm) of chromoprotein containing the all-trans- or	the 13-cis-isomer	Relative activity (%) (Proton translocation in cell vesicles under irradiance, see Figure 5)
Retinal (1)	570	548	100
Retinal (2)	532	515	45
Retinal 3)	518	500	24
Retinal (4)	504	486	7.5

mixed with bacterioopsin, in all three cases the 13-cis and the all-trans isomers formed chromoproteins as exemplified for all-*trans*-**3** in Figure 5a. With compound **2** the all-trans isomer gave rise to a BR analogue with λ_{max} at 532 nm, whereas the 13-cis isomer yielded a 515-nm species (see also Table II). With compound **3** the all-trans configuration produced a 518-nm species, and with 13-*cis*-**3** a chromoprotein absorbing at 500 nm was formed. The corresponding values for retinal **4** were found to be 504 nm for the all-trans form and 486 nm in the case of the 13-cis structure. The nonreacting 9-cis and 9,13-dicis isomers of acetylenic retinals **2–4** showed a slow thermal isomerization into active isomers, which then reacted to form the red-shifted chromoproteins. This conversion could be accelerated by illumination.

When the analogue retinals **2–4** were extracted from either light- or dark-adapted BR analogue samples, in all cases the all-trans isomers make up to 70–90% of the retinals found. The predominance of the all-trans structure must be attributed to the acetylenic bond, since other analogues, modified in the cyclohexene ring (α-retinal, 5-desmethylretinal), when extracted from their corresponding dark-adapted chromoproteins yield roughly equal amounts of 13-cis and all-trans isomers, as is found for retinal itself.[12,29]

Bioenergetic Function of BR Analogues. The bioenergetic function of BR analogues containing the acetylenic retinals was determined by the measurement of their light-driven proton translocation capacity. All three acetylenic retinals were allowed to react with BO-containing cell vesicles obtained by sonication of *H. halobium* cells from a retinal-deficient mutant strain (JW 5).[26]

Illumination of the cell vesicles containing the reconstituted chromoproteins (for conditions of reconstitution, see Experimental Section) resulted in an acidification of the medium for all three retinal analogues. Figure 6 shows the specific proton translocation

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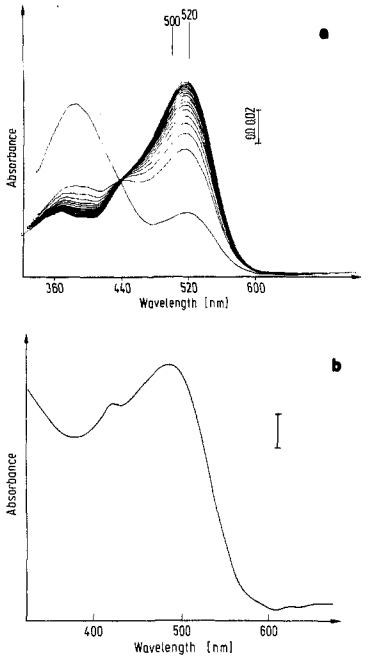


Figure 5. (a) Formation of a BR analogue containing the acetylenic retinal **3**. To a 1-mL suspension of 5 nmol of AM at 10 °C, 3.5 nmol of the all-trans isomer of **3** was added (reference: 5 nmol of AM in 1 mL of water). Spectra were recorded at: 20 (spectrum with the lowest absorption at 520 nm), 40, 80, 120, 170, 220, 260, 310, 350, 390, 440, 490, 530, 580, 620, 660, 760, and 810 s (spectrum with the highest absorption at 520 nm). (b) Spectrum of a BR analogue containing the acetylenic retinal **4**. Retinal-deficient cells were grown in the presence of **4**. The spectrum was taken after the cells were lysed in water. The shoulder at 420 nm is due to contaminating cytochromes (soret band). The sample was measured in a 0.2-cm path length cuvette at a full scale of 2.0. The bar inserted corresponds to 0.2 OD units.

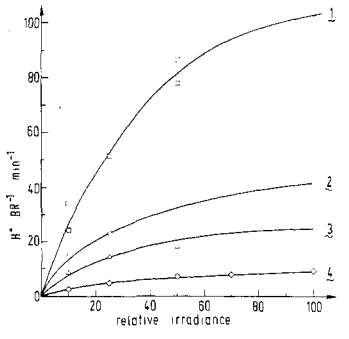
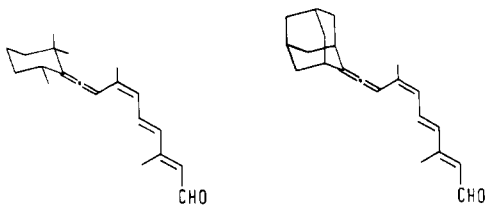


Figure 6. Light-induced proton translocation in cell vesicles containing reconstituted BR's. The BR's were reconstituted with retinal (**1**) (yielding 13.2 nmol of BR, curve 1, 530-nm cutoff filter), with acetylenic retinal **2** (19 nmol, curve 2, 480-nm cutoff filter), with acetylenic retinal **3** (11 nmol, curve 3, 480-nm cutoff filter), and with acetylenic retinal **4** (6.2 nmol, curve 4, 455-nm cutoff filter). Illumination was with light from a 150W slide projector bulb filtered through the respective cutoff filters. For details see ref 26.

activity as a function of irradiance for the various BR analogues. Under conditions of saturating illumination, 42 protons per BR per min (41%) were measured for an acetylenic retinal **2** containing sample, 25 protons (24%) for the retinal compound **3**, 8 protons (7.5%) for the retinal compound **4**, and 103 protons (100%) for retinal **1**. Because some acetylenic retinals are photolabile (see below), the proton translocation activities were also compared after extrapolation to irradiance equal zero, with retinal (**1**) set at 100%. Retinal **2** yielded 54%, retinal **3** 30%, and retinal **4** 10.5% of retinal **1**. The photolability of the acetylenic BR analogues, however, was not the reason for the reduced proton translocation capacity of the acetylenic retinals. The data from the vesicle experiments were found to be fully reproducible in a second, identical set of measurements even after exposure of the sample to maximal



17 18

Figure 7. Structures of allenic retinals **17** and **18** mentioned in the text.[36,37]

irradiation. An explanation may be given, when the lifetimes of the photocycle intermediates are taken into account (data not given, see description in Discussion).

An additional assay to test the biological function of analogue retinals is the photophosphorylating capacity of retinal-deficient cells after addition of analogue retinals to the medium.[12,27] Such experiments were performed with the acetylenic retinals **3** and **4**. As a result, cells containing retinal **3** synthesized 0.6 compared of ATP per min under conditions where retinal (**1**) produced 8.9 nmol of ATP per min. These values are normalized to the amount of chromoprotein in the cells. The chromoprotein-containing retinal **3** could be isolated in only low amounts, 25 nmol per L of cell culture. The absorption maximum was at 518 nm as was found for the in vitro reconstituted sample.

Acetylenic retinal **4**, when incorporated under the same conditions, did not mediate photophosphorylation, but the corresponding chromoprotein could be found spectroscopically in lysates of the cells, when prepared under conditions of low irradiance (Figure 5b). It was very unstable under illumination and at high or low pH. Its absorption maximum (494 nm) was 10-nm blue shifted compared with the in vitro reconstituted all-trans sample. The discrepancy between the inactivity of retinal compound **4** in photophosphorylation and its activity in proton translocation might be explained by the influence of the membrane potential ($\Delta\psi$) on the stability of the chromoprotein. In the vesicle system used for the proton translocation assay[26] only small pH changes but no $\Delta\psi$ changes occur in contrast to the intact cells used in the photophosphorylation experiment (see Discussion).

Discussion

The results obtained with the various isomers of the acetylenic retinals **2–4** demonstrate the different interactions between retinal and the binding sites of opsin and bacterioopsin.

The failure of rhodopsin or isorhodopsin formation upon the reaction of the appropriate isomers of acetylenic retinals with opsin, when compared with retinoid-binding experiments already published, allow a more detailed decription of the architecture of the binding site of opsin: Compounds such as cyclocitral or β-ionone competitively inhibit the reconstitution reaction of rhodopsin from 11-*cis*-retinal and opsin, and, therefore, a β-ionone-like shaped binding pocket was imagined.[15-17] This binding site was found to accept also slightly modified retinals, e.g., α-retinal, 3,4-dehydroretinal, 5-desmethyl-, and 9-desmethylretinal and retinoids with alkyl substituents, representing only part of the cyclohexane ring.[18,19,30-34] Decent interactions leading to red-shifted absorption bands were also observed when aromatic retinoids were used for reconstitution experiments with bovine opsin.[35]

Surprisingly, the allenic retinal **17** (Figure 7) was reported to form an isorhodopsin.[36] Because in this compound the cyclohexene ring is tilted by 90° relative to the side chain, one would predict that this compound would not fit into the binding site. If, however, the cyclohexene ring of retinal (**1**) is also turned into the right-angle position by a rotation around the 6,7-single bond, both molecules have identical positions for carbons 7–15 and the cyclohexene ring of the allenic retinal is positioned about 2.7 Å above the plane of the ring of retinal (Figure 8). The results obtained from the adamantyl derivative (**18**) of the allenic retinal[37] and from the acetylenic retinals presented here corroborate our conclusions. The adamantyl derivative has only a slight red shift compared to its SBH^+ and is very labile, whereas the acetylenic compounds do not undergo a red shift at all. If these compounds are superimposed in the 90° tilted position on retinal or the allenic derivative **17**, it becomes obvious that the adamantyl compound **18**, carrying additional carbon atoms above the plane of the cyclohexene ring, would require a much more extended binding site for fixation. In the case of the acetylenic compounds the plane of the ring is placed below that found for retinal itself, and the carbon atom 7 has a different position (Figure 8). Obviously there is no recess of the binding site below the plane of the ring of retinal, thus leading to the nonacceptance of the acetylenic retinals in opsin. Therefore all four types of retinal analogues confirm the existence of a precisely shaped β-ionone binding pocket in rhodopsin.

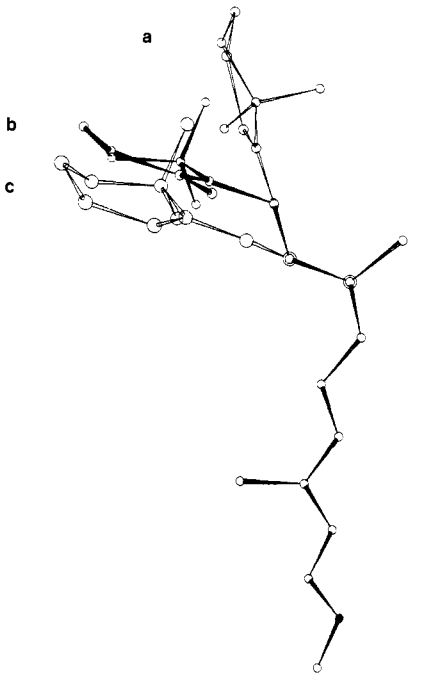


Figure 8. Superposition of the structure of allenic retinal **17** (a) on the structure of retinal **1** (b), which is shown in the 90° tilted conformation of ring and polyene side chain, and on the structure of acetylenic retinal **3** (c), also presented in the 90° tilted ring/side chain conformation. All compounds are shwon in the 9-cis configuration.

Chromoproteins readily formed when either the all-trans or the 13-cis isomers of all three acetylenic retinals were mixed with bacterioopsin and also when these analogue retinals were added to the medium of growing cells. The completely ring demethylated compound **4**, however, produced a pigment in living cells, which was very unstable. Addition of two or three methyl groups stabilized the chromoproteins. This result clearly shows the importance of the methyl groups at positions 1 and 5 in acetylenic retinals. All acetylenic BR analogues had blue-shifted absorption maxima compared to BR, but the increasing number of ring methyl groups diminished this blue shift. This was observed for both the all-trans and the 13-cis isomers. The absorption maxima of the compounds **2–4** in organic solvents are similar, therefore the methyl groups in the β-ionone region reduce the excitation

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energy, as is similarly found for retinal.[12]

An increase in the number of methyl groups attached to the ring also correlated with a rise of proton translocation activity (retinal **4** 7.5%, retinal **3** 24%, retinal **2** 45% of the value found for retinal (**1**); the data are taken from the measurements under saturating illumination). An explanation for this strong influence of the methyl groups can be given. Flash photolysis of the BR analogue containing the acetylenic retinal **3** showed the appearance of a blue-shifted intermediate with an absorption around 390 nm, comparable to the M intermediate in BR. This decayed with a $t_{1/2}$ value of ca. 6 ms and gave rise to a red-shifted intermediate around 590 nm, which took 200 ms for full decay. This slower photocycle, compared to that of BR, can lower the proton translocation efficiency.

The increasing lability of chromoproteins with decreasing numbers of methyl groups cannot be used as an explanation for the decreasing activity, because the measurements shown in Figure 6 were reproducible also after application to the highest irradiance. The proton translocation measurements were always carried out with cell vesicles in the presence of tetraphenylphosphonium cations, thus preventing the establishment of a membrane potential. In contrast, the photophosphorylation experiments, which were carried out with living cells, showed an even lower activity (6.7% instead of 24% for retinal **3**. Here, the developing membrane potential could play a role by destabilizing several intermediates of the photocycle of that BR analogue.

By comparison of the experiments performed with opsin to those with BO the following conclusions can be drawn: In opsin two binding sites exist, which in the case of retinal supplement each other. In the case of analogue retinals with different geometric arrangements of ring and side chain, association and thereby covalent binding are complicated or even prevented.

BO interacts predominantly with the carbonyl end group of retinal; competition studies of the BR reconstitution reaction by the use of cyclocitral or β-ionone gave no indication for the existence of a β-ionone binding site comparable to that found in opsin.[17] However, interactions of the cyclohexene ring with BO must exist and are enhanced by the presence of the methyl groups at the positions 1 and 5. These interactions have a considerable influence on the function of BR as a light-driven proton pump.

Acknowledgment. We are greatly indebted to Dr. L. Ernst (GBF Stöckheim) for the measurement of the 400-MHz ^{1}H NMR spectra. We thank Dr. P. Tavan for MNDOC calculations to elucidate the ground-state structures of retinals shown in Figure 8 and Dr. J. Deisenhofer for assistance with the computergraphics of that figure.

Registry No. *all-trans*-**2**, 91365-73-0; 13-*cis*-**2**, 91422-74-1; 9,13-*cis*,*cis*-**2**, 91422-75-2; 9-*cis*-**2**, 91422-76-3; 11-*cis*-**2**, 91422-77-4; *all-trans*-**3**, 91365-74-1; 13-*cis*-**3**, 91365-75-2; 9,13-*cis*,*cis*-**3**, 91365-76-3; 9-*cis*-**3**, 91365-77-4; *all-trans*-**4**, 91365-78-5; 13-*cis*-**4**, 91365-79-6; 9,13-*cis*,*cis*-**4**, 91365-80-9; 9-*cis*-**4**, 91365-81-0; **5**, 74193-14-9; **6**, 91365-82-1; **7**, 583-60-8; **8**, 1193-47-1; **9**, 91365-83-2; **10**, 91365-84-3; **11**, 91365-85-4; **12**, 91365-86-5; **13**, 91365-87-6; **14**, 91365-88-7; **15**, 78-27-3; $(EtO)_2P(O)CH_2CN$, 2537-48-6; $(EtO)_2P(O)CH_2C(CH_3)$=CHCN, 87549-50-6.

Photochemistry and Photophysics of Surfactant *trans*-Stilbenes in Supported Multilayers and Films at the Air–Water Interface[1]

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Received November 21, 1983

Abstract: The synthesis and study of several surfactant *trans*-stilbene derivatives are described. Both compounds containing the *trans*-stilbene at the hydrophobic terminal of a surfactant chain and those containing an intrachain stilbene chromophore have been studied. These molecules form stable films at the air–water interface which can be transferred sequentially to give quartz-supported multilayers. The stilbenes in the multilayer assemblies show very low photoreactivity and specifically little photoisomerization. Strong fluorescence is observed from all of the stilbenes studied; the fluorescence shows similar vibronic structure to the molecular fluorescence of *trans*-stilbene in solution. However, the fluorescence is substantially red-shifted and the fluorescent lifetime is greatly increased over that in solution. The observed changes in absorption and fluorescence can be explained by a "cardpack" exciton model in which the excitation is shared by clusters of closely packed stilbene units and in which considerable migration and delocalization of excitation energy occurs.

Introduction

The properties and reactivity of molecules incorporated into films or attached to solid surfaces have been the subject of numerous recent investigations.[4-15] There has been particular interest in the "interfacial" behavior of such molecules and their interaction with other molecules incorporated into the same film or molecular assembly due to widespread potential applications including the development of photosensitive devices giving reso-

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