Removal of Methyl Groups from Retinal Controls the Activity of Bacteriorhodopsin†

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ABSTRACT: The 5-, 9-, and 13-demethylretinal and 9,13-didemethylretinal analogues were synthesized and stereochemically characterized by 400-MHz ^{1}H NMR spectroscopy. When mixed with bovine opsin, the 9-cis and 11-cis isomers of all analogues formed rhodopsins, which were bleached upon illumination. When mixed with bacterioopsin, the 13-cis and all-trans isomers of the analogues formed bacteriorhodopsins. Surprisingly, a chromoprotein also formed when 7-*cis*- or 11-*cis*-13-demethylretinal interacted with bacterioopsin. The activation parameters of the regeneration reactions were measured, and a correlation between the rates and the entropy of the activation was found. Like bacteriorhodopsin the 5- and 9-demethylretinal chromoproteins underwent light–dark adaptation, showing a red shift in their absorbance spectra with a concomitant increase in the amount of the all-trans isomer. The 13-demethylretinal chromoprotein did not undergo light–dark adaptation. The presence of predominantly the 13-cis isomer was revealed by extraction of the retinal analogue and high-performance liquid chromatography analysis. Upon flash illumination the 13-demethylretinal chromoprotein underwent a transient spectral change in which a small conversion to a shorter wavelength intermediate similar to the "M" species of bacteriorhodopsin took place; this transient complex, however, had a lifetime of several seconds. The major conversion was to a 620-nm species, which formed within milliseconds. In contrast, when 5- and 9-demethylretinal chromoproteins were subjected to the same experiment, the formation of an "M" species with a decay time of about 10 ms as in bacteriorhodopsin was the main event. For examination of the bioenergetic function of these bacteriorhodopsin analogues, the demethylretinals were incorporated into the bacterioopsin of *Halobacterium halobium* cells, which were deficient in retinal synthesis. Cells containing 5- or 9-demethylretinal showed photophosphorylation which was comparable in rate and extent to that of cells containing retinal. In contrast, cells containing 13-demethyl- or 9,13-didemethylretinal showed a rate of photophosphorylation decreased by a factor of 100. These results were confirmed by the measurement of light-induced pH changes in cell vesicle preparations containing bacteriorhodopsin analogues: lack of a 13-methyl group corresponded to a very low activity. It is concluded that the lack of a methyl group at position 13 of the retinal molecule, but not at position 5 or 9, results in the entrapment of the retinal moiety in the 13-cis configuration, which mediates a futile photochemical cycle.

Rhodopsin and bacteriorhodopsin (BR)[1] are retinal-containing proteins of molecular weights of 36 000 and 26 000, respectively. The former is an intrinsic membrane glycoprotein responsible for night vision in many animals, and the latter is found in the cell membrane of Halobacteria and acts as a light-driven proton pump. Retinal plays a key role in the function of both proteins. In rhodopsin 11-*cis*-retinal is converted to the all-trans isomer upon illumination and in vivo leads to initiation of a neuronal response (Wald, 1968). In BR light induces a thermoreversible all-trans to 13-cis isomerization, to which a proton transport across the cell membrane is connected. This establishes a protonmotive force which can be utilized for ATP synthesis and transport of metabolites [for a review see Lanyi (1978) and Stoeckenius et al. (1979)]. In both proteins retinal is bound in a 1:1 ratio via a protonated Schiff's base on an ϵ-amino group of a lysine residue. This structure partially accounts for the red shift which retinal experiences upon combination with an opsin. A further red shift is expected to occur if counterions to the positively charged nitrogen are placed in the protein structure at a longer distance than that within a standard salt bridge (Blatz et al., 1972; Fischer & Oesterhelt, 1979; Honig et al., 1979; Nakanishi et al., 1980).

Only selected retinal isomers bind to opsin (9-cis and 11-cis) and to BO (13-cis and all-trans) with a concomitant large red shift of their absorption maxima. Retinal analogue structures which interact with BO can be divided into three classes: first, compounds which do not form red-shifted chromophores and do not mediate catalytic activity (Towner et al., 1981); second, compounds which form chromophores nearly identical with those formed by retinal but do not mediate function; third, compounds which form chromophores and mediate function (Oesterhelt & Christoffel, 1976; Tokunaga et al., 1977; Towner et al., 1980). The second class is of particular interest, because the retinal–protein interaction in the ground state seems to be unchanged, but upon illumination no translocation of protons is found. The structure of such compounds might lead to conclusions about the changes that take place during the photochemical cycle.

BR, in its dark-adapted form, contains equivalent amounts of *all-trans*- and 13-*cis*-retinal whereas the light-adapted form contains only the all-trans isomer (Oesterhelt et al., 1973;

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[1] Abbreviations: BR, bacteriorhodopsin; BO, bacterioopsin; dm, demethyl; dmr, demethylretinal; dmr-BR, demethylretinal-containing bacteriorhodopsin; DA, dark adapted; LA, light adapted; HPLC, high-performance liquid chromatography; ROS, rod outer segments; SB, Schiff's base; λ_{max}, absorption maximum; TPP^+, tetraphenylphosphonium cation.

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Dencher et al., 1976). Light causes BR to undergo a photochemical cycle, which starts with the all-trans isomer of retinal and consists of a sequence of several intermediates. A long-lived species, known as the "M" intermediate, can be trapped at low temperature and has been shown to contain solely 13-*cis*-retinal as a deprotonated SB (Pettei et al., 1977; Tsuda et al., 1980; Aton et al., 1977; Stockburger et al., 1979). Thus, active proton translocation seems to involve an all-trans to 13-cis isomerization of retinal and a reversible deprotonation of the SB linkage.

The 13-cis form of BR obtained in pure form from BO and 13-*cis*-retinal at low temperature in the dark is also photochemically active and undergoes a distinct photocycle (Dencher et al., 1976). However, there is experimental indication that active proton translocation is not connected with this cis cycle (Fahr & Bamberg, 1982).

Theoretical calculations have produced a model of the photochemical trans cycle in BR that quantitatively accounts for the observed kinetic constants and the trans to cis isomerization with concomitant deprotonation of the SB (Schulten & Tavan, 1978; Orlandi & Schulten, 1979). The model describes a rotation of the 14-s-trans to 14-s-cis single bond in connection with the isomerization of the 13/14 double bond, thereby changing the acid–base properties of the nitrogen atom. A steric hindrance by the protein was assumed to prevent full rotation of the 14/15 single bond around 180°. This would facilitate the release of a proton from the nitrogen atom. Therefore one could except the C_{13}-methyl group of retinal to be crucial for this isomerization process.

The experiments described in this paper support this model since they demonstrate that removal of the C_{13}-methyl group leads to a chromoprotein nearly identical with BR in absorption but having drastically diminished efficiency as a proton pump.

Materials and Methods

Preparation of Opsins and Formation of Chromoproteins. BR was isolated from *Halobacterium halobium* strain R_1M_1 (Oesterhelt & Stoeckenius, 1974) and converted to BO (Oesterhelt et al., 1974). ROS containing rhodopsin were prepared from fresh bovine retina and converted to opsin by treatment with NH_2OH in light (Towner et al., 1981). Rhodopsin and BR analogues were prepared by addition of equimolar amounts of analogue isomers to a 1-mL suspension of opsin at 20 °C or to a BO suspension at 5 °C at concentrations of about 10 μM in 70 mM potassium phosphate, pH 6.5. Spectra were recorded continuously in the wavelength range 360–700 nm with an Aminco DW 2 spectrophotometer. Activation energies of the reactions were derived by determination of the initial rates of regeneration at different temperatures. With opsin this required a 5-fold excess of analogue to obtain a first-order reaction whereas BO required a 1.6-fold excess.

Incorporation of Retinal Analogues into the BO of H. halobium Cells. A mutant of *H. halobium* (AO 151) deficient in retinal synthesis but possessing BO was grown and dmr incorporated as previously described (Towner et al., 1980). A more useful strain (W296) with similar properties but overproducing BO was also used. In this strain 200 nmol of dmr, administered on the fourth day of a 5-day growth cycle to 700-mL cell suspensions, yielded up to 100 nmol of BR analogue. Cells were harvested by centrifugation and resuspended to a total volume of 20 mL in basal salts.

Two milliliters of this suspension was diluted to 10 mL with basal salts and used in the photophosphorylation test. The remainder was diluted with water in order to lyse the cells, and the membranes containing the BR analogue were isolated as described (Towner et al., 1980). A suspension of the membranes in water was illuminated with the light from a slide projector or a Xe high-pressure Schoeffel lamp (both 150 W) for 5 min, using a 500-nm cutoff filter. Then the absorbance spectrum was recorded. The sample was left in darkness for several hours and a second spectrum taken.

Measurement of Photophosphorylation. H. halobium cells containing analogue chromoproteins were suspended in basal salts at 2 mg of protein/mL [see Hartmann & Oesterhelt (1977)] in a thermostated (20 °C) cylindrical glass cuvette and flushed with a stream of N_2. Light was focused onto the stirred suspension for several minutes and then turned off. Samples (0.05 mL) were removed and quickly lysed in 2.5 mL of 10 mM sodium phosphate buffer, and their ATP content was measured (Hartmann & Oesterhelt, 1977). When the ATP level had decreased to less than 1 nmol/mg of protein, the sample was illuminated for several minutes, the light turned off, and the regime repeated.

Measurement of Proton Translocation in Cell Vesicles. Preparation of vesicles from W296 cells and reconstitution of BR analogues by addition of limiting amounts of dmr is described elsewhere (Oesterhelt, 1982). The activity of the various BR analogues as a function of light intensity was measured by the use of a series of neutral density filters (Schott; 1, 5, 10, 25, 50, and 70% transmission); probes were compared for the same light intensities within the linear part of the plots.

Measurement of Retinylidene–Protein Formation during the Regeneration Reaction. To 15 mL of a BO suspension (5 μM) at 4 °C was added 120 nmol of *all-trans*-retinal as a 1 mM solution in 2-propanol. The reconstitution was followed by continuous recording of spectra of a 1-mL aliquot of the mixture in the wavelength range from 340 to 700 nm. At various times 1-mL aliquots of the reconstituting probe were mixed with 150 μL of a 0.1 M solution of cetyltrimethylammonium bromide containing 3% $NaBH_4$. After addition of 1 mL of ethanol, the samples were extracted 4 times with 1-mL portions of petroleum ether (bp 40–60 °C), and the protein precipitate of the aqueous phase was collected by centrifugation. Covalently bound retinyl residues were determined spectroscopically after the precipitate was dissolved in 1 mL of chloroethanol.

Extraction and Analysis of Retinal Analogue Isomers from Chromoproteins. Samples of chromoproteins (25 nmol) isolated from cells were suspended in 1 mL of water and denatured with an equal volume of 2-propanol and then left in darkness for 30 min at 10 °C and extracted twice with 2 mL of *n*-hexane. By this method 60% (±20%) of the analogue could be routinely extracted. Control experiments showed that this procedure did not cause facile isomerization of any isomer. The combined extracts were dried over Na_2SO_4, evaporated, and taken into 25 μL of 2-propanol, which was then injected into a HPLC system consisting of an Altex Model 110-A pump connected to a Kontron Uvicon LCD 725 detector. The column used was a Knauer 250 × 4.6 mm filled with Lichrosorb (Si 60; 7 μm). Relative peak areas of isomers were determined on a Hewlett-Packard 3380-A integrator, no corrections were made for individual isomer responses, and all peaks were detected at 380 nm.

Flash Photometry of Analogue Chromoproteins. Samples of analogue chromoproteins isolated from cells were suspended in water at pH 6 and pipetted into a glass cuvette of 2.2 mL volume with a path length of 28 mm, and the optical density of the solutions was adjusted to 1.0 at their λ_{max}. A beam of light used for measuring purposes was obtained by focusing

Table I: Regeneration Parameters of dmr-BR's

compounds	λ_{max} (nm)	k (25 °C) (s^{-1})	AE (kcal·mol^{-1})	$\Delta H^{\ddagger}$ (25 °C) (kcal·mol^{-1})	$\Delta G^{\ddagger}$ (25 °C) (kcal·mol^{-1})	$\Delta S^{\ddagger}$ (25 °C) (cal·mol^{-1}·grad^{-1})
cis compounds						
5-dmr (13-cis)	533	16.0×10^{-2}	16.0	15.4	18.9	−11.7
retinal (13-cis)	558	6.1×10^{-2}	15.1	14.5	19.8	−17.8
9-dmr (13-cis)	532	0.4×10^{-2}	15.2	14.6	22.6	−26.7
13-dmr (13-cis)	565	0.2×10^{-2}	12.1	11.5	23.3	−39.5
13-dmr (11-cis)	565	0.03×10^{-2}	13.5	12.9	25.3	−41.6
all-trans isomers						
5-dmr	550	3.6×10^{-2}	16.5	15.9	20.4	−15.0
retinal	570	1.4×10^{-2}	13.6	13.0	21.3	−28.0
9-dmr	548	0.6×10^{-2}	17.8	17.2	22.2	−16.8
13-dmr	565	0.12×10^{-4}	20.1	19.5	28.4	−30.0

the light from a 150-W projector bulb onto the sample. The beam travelled through the sample and then fell onto the opening of a monochromator where the intensity of light at any wavelength in the 360–700-nm region was measured. Two air-filled (50 torr) flash lights were positioned on each side of the cuvette with 485-nm cutoff filters, and changes in absorbance of the sample were plotted on an XY display. The duration times of the flashes were 15 μs. The apparatus used consisted of a Hamamatsu R 928 photomultiplier, a Data Lab DL 905 transient recorder, and a Canberra Elektronik 8603-D XY display. Time intervals between successive flashes were at least 30 s.

Synthesis of Retinal Analogues. The synthesis of 9- and 13-dmr and 9,13-di-dmr was performed accordingly to the procedure of v. d. Tempel & Huismann (1966). The synthesis of 13-dmr was modified as previously described (Gärtner et al., 1980). 9-dmr was synthesized by condensing cyclocitral with methyl 4-(diethylphosphonato)crotonate followed by a second condensing reaction with methyl 4-(diethylphosphonato)senecioate (senecioate = 3,3-dimethylacrylate) with subsequent reduction to the corresponding alcohols and reoxidation to aldehydes by MnO_2 in both steps. 9,13-di-dmr was obtained by similar procedures. Synthesis of 5-dmr was achieved by condensing 2,2-dimethylcyclohexanone with sodium acetylide followed by elimination of water. After conversion to the anion, the resulting compound was allowed to react with acetic anhydride to yield 5-dm-7,8-didehydro-β-ionone, which after treatment with lithium aluminum hydride followed by reoxidation gave 5-dm-β-ionone. Elongation of the polyene chain to form 5-dmr was as described for the 9-dm compound with (diethylphosphonato)acetonitrile and methyl 4-(diethylphosphonato)senecioate as condensation reagents.

Isomers of the analogues were prepared by irradiation of 10^{-3} M solutions in 2-propanol or acetonitrile for several minutes with light from a Schoeffel lamp (150 W) with a 420-nm cutoff filter. The isomers were then purified by HPLC and characterized by 400- and 500-MHz ^{1}H NMR spectroscopy (Towner & Gärtner, 1982).

All chemicals used were obtained from Ega-Chemie or Merck and were of analytical grade. 13-dm-14-methylretinal was a gift of Dr. A. Nürrenbach (BASF).

Results

Reconstitution of BR. The reconstitution of BR by the reaction of BO with either *all-trans-* or 13-*cis*-retinal was formerly studied in detail by Schreckenbach et al. (1978) and can be described by the following reaction sequence:

$$\text{retinal} + \text{BO} \xrightarrow{a} \text{400-nm chromophore} \xrightarrow{b} \text{430–460-nm chromophore} \xrightarrow{c} \text{purple complex}$$

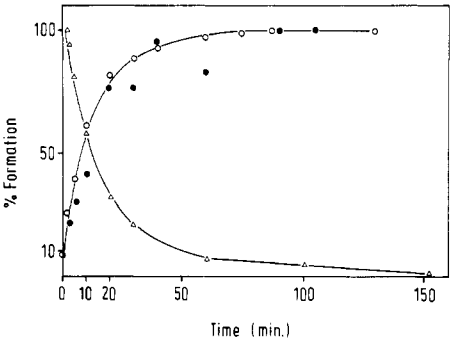


FIGURE 1: Covalent binding parallels purple complex formation. A continuous series of spectra after addition of *all-trans*-retinal to a 5 μM suspension of BO was recorded, and the time course of 430–460 nm chromophore decay (Δ) and purple complex formation (○) were evaluated from the spectra. Retinyl–protein formation (●) was determined as described under Materials and Methods.

The 400-nm complex still contains free retinal, but it was unclear whether covalent bond formation between retinal and the protein occurs during step b or c. The experiment shown in Figure 1 gives strong indication that the SB indeed is formed in the last step, c, because on reduction under denaturing conditions retinyl–protein formation parallels purple complex formation. This last step is very sensitive to the methyl group pattern of the retinal used for the reconstitution. As an example, 13-dm-14-methylretinal reacted with BO to a red-shifted complex (λ_{max} 430 nm) with a fine structured absorption spectrum but did not bind covalently to the protein.

For the 13-cis and for the all-trans isomers step c is rate limiting in the reaction with BO, and the reaction of the 13-cis isomers is usually faster than that of the all trans form. This was found for retinal and for most of the analogues used (see Table I).

Figure 2 shows the reaction between 9-dmr and BO. In the case of the all-trans isomer a fast formation (step b) of an intermediate with λ_{max} around 430 nm is followed by a slow conversion (step c) into the BR analogue (Figure 2a). When the 13-cis isomer of 9-dmr is mixed with BO, the final product was formed from a steady-state mixture of the intermediates mentioned above (Figure 2b). This indicates that the relative rates of steps a and b depend on the 13-cis or all-trans configuration of the retinals, but in all cases step c is rate limiting [see also Schreckenbach et al. (1978)]. A similar behavior was also found with *all-trans-* and with 13-*cis*-5-dmr. In the case of 13-dmr besides the all-trans and the 13-cis isomers chromoproteins were also formed, when the 7-cis and the 11-cis isomers were used (see below).

The fully regenerated dmr-containing chromoproteins were characterized by their λ_{max} values (Table I), light–dark adaptation, reactivity toward hydroxylamine, and acid–base

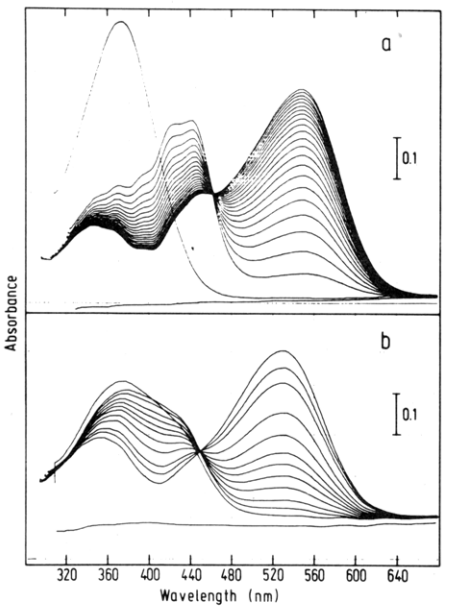


FIGURE 2: Regeneration of 9-dmr-BR. A 1-mL sample of BO (5 nmol) at pH 7 was placed in a cuvette at 15 °C, and 5 nmol of (a) *all-trans*- or (b) 13-*cis*-9-dmr in 2-propanol was added. Spectra were recorded for (a) every 90 s and for (b) every 60 s for the first nine consecutive spectra, and spectra 10 and 11 of (b) were taken 20 and 30 min later. Panel a also shows the absorption of free *all-trans*-9-dmr (λ_{max} at 380 nm).

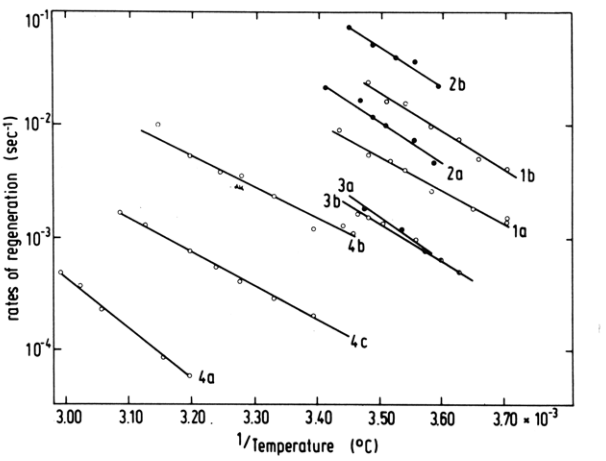


FIGURE 3: Rate of regeneration of dmr-BR's at different temperatures. Regeneration was started by the rapid addition of a 1.6 M excess of dmr in 10 μL of 2-propanol to 5 nmol of BO in 1 mL of water. Increase in absorbance at the λ_{max} of the chromoprotein was followed. Rates of regeneration in the temperature range 0–40 °C, which obey pseudo first order under the conditions used (Towner et al., 1981), were plotted semilogarithmically against the reciprocal temperatures. Slopes of the lines were used to calculate the activation energies (AE) according to the Arrhenius equation. 1 = retinal; 2 = 5-dmr; 3 = 9-dmr; 4 = 13-dmr; a = all-trans isomers; b = 13-cis isomers; 4c = 11-cis isomer of 13-dmr.

titration. Apart from 9,13-di- and 13-dmr-BR the chromoproteins underwent light–dark adaptation in which the all-trans and 13-cis regeneration products became indistinguishable. The 13-dmr-BR was not more susceptible to hydroxylamine than BR. However, in the dark the 5- and 9-dmr and 9,13-di-dmr chromoproteins were bleached in the presence of 0.2 M NH_2OH at 20 °C with half-times of 100 min for 5-dmr-BR and of 40 min for 9-dmr-BR and 9,13-di-dmr-BR. These reactions were accelerated substantially upon illumination to half-times of about 10 min. At pH values below 2.5, BR and 13-dmr-BR were both red shifted to a 605-nm species. Similar treatment of 5- and 9-dmr-BR caused a broadening of their absorbance spectra with no observable red shift.

The first-order rate constants of step c were derived from the initial velocities and from half-time measurements of the reconstitution reaction. The temperature dependence of the initial rate of the reaction between the analogues and BO is shown in Figure 3. In the temperature range used (0–40 °C) the relationship was linear; however, higher and lower temperatures caused tailing off due to denaturation of the protein of immobilization of the reactants.

The slope of the lines in Figure 3 was used to calculate the activation energy (AE) of the reactions according to the Arrhenius equation. Values were in the range of 12.1–20.1 kcal·mol^{-1} and are listed in Table I. The rates of regeneration, when extrapolated to 25 °C, varied by a factor of up to 10^4 (compare *all-trans*-13-dmr with 13-*cis*-5-dmr). By treatment of the data on AE and the rates with the standard equation of the transition theory, values for enthalpy, free energy, and entropy of activation were obtained (Table I). For both 13-cis and all-trans isomers, the rate constants decrease in the order of 5-dmr, retinal, 9-dmr, and 13-dmr. This means that the lack of the 5-methyl group facilitates reconstition while lack of the 9- or 13-methyl group decreases the rate constants of reconstitution. The 5-methyl group interacts sterically with the hydrogen at C_8, preventing the cyclohexene ring from being coplanar with the side chain of retinal in organic solvents. The binding site of BO on the other hand enforces the retinal to become coplanar during association (Schreckenbach et al., 1977). Therefore the enhanced reaction rate of 5-dmr compared to retinal can be understood as a lack of steric hindrance. Comparison of the activation parameters for the 13-cis isomers shows that upon removal of the 9- or 13-methyl groups the entropy of activation has the largest negative value for the reaction of 13-dmr. We interpret this as a reaction pathway in which the 13-methyl group has a sterically directing effect which is more pronounced than that by the 9-methyl group. This result is confirmed by inhibitor studies with retinals containing shortened side chains: BO, in contrast to opsin, recognizes the polyene chain of retinal stereospecifically, with a specific interaction around position 13 (Towner et al., 1981).

Isomerization of Retinal Analogues within the Binding Site of BO. It is well-known that upon regeneration of BR from 13-*cis*- or *all-trans*-retinal and BO, a slow thermal isomerization takes place. Depending on the relative rates of both processes for a given retinal structure this results in the presence of both isomers and changes in λ_{max}.

Complete regeneration of BR analogue from either *all-trans*- or 13-*cis*-5-dmr, 9-dmr, or retinal required about 20 min. Extraction and HPLC analysis of the samples gave only the isomer used for regeneration, indicating a negligible isomerization during the reconstitution. When the BR analogues were illuminated, a red shift occurred. In the dark a blue-shifted species appeared. This process, known for BR as light and dark adaptation, involves trans to 13-cis isomerization of the retinal moiety (Oesterhelt et al., 1973; Pettei et al., 1977). Extensive light–dark adaptation of BR analogues was only observed with freshly regenerated preparations; aged BR analogues showed smaller λ_{max} shifts. For demonstration of trans–cis isomerization as the cause of λ_{max} shifts, we extracted the retinal analogues from purple membranes which had been isolated from a retinal-deficient strain grown in the presence of these compounds. The absorbance spectra of these samples are shown in Figure 4. The results of isomeric composition, determined by extraction of light- or dark-adapted samples, are summarized in Table II. Only all-trans and 13-cis isomers could be detected; other isomers were not found. Light adaptation in dmr-BR's caused a maximal red shift of

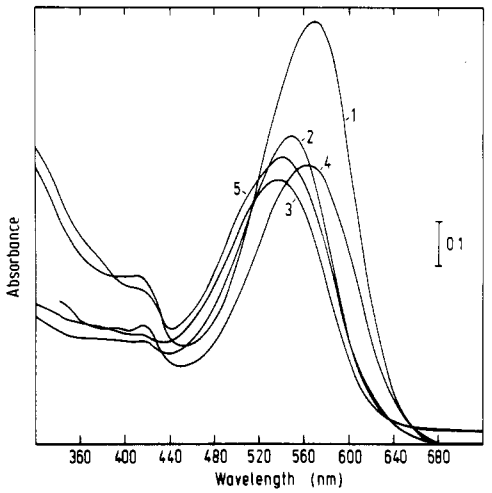


FIGURE 4: Absorbance spectra of dmr-BR's. dmr-BR's were isolated from retinal-deficient mutant cells, to which retinal analogues were administered during growth. Spectra were taken at different concentrations of the various samples. 1 = Br; 2 = 5-dmr-BR; 3 = 9-dmr-BR; 4 = 13-dmr-BR; 5 = 9,13-di-dmr-BR.

Table II: Isomeric Distribution of Retinal Analogues in the Binding Site of dmr-BR's

	compound					
	light-adapted chromoprotein			dark-adapted chromoprotein		
		isomer found			isomer found	
	λ_{max} (nm)	all-trans (%)	13-cis (%)	λ_{max} (nm)	all-trans (%)	13-cis (%)
retinal	570	91	9	558	57	43
5-dmr	551	92	8	546	62	38
9-dmr	548	70	30	540	44	56
13-dmr	565	16	84	565	15	85

8 nm (9-dmr-BR), which correlated with the increase in the amount of the all-trans isomer. Dark adaptation produced increased levels of the 13-cis isomer. The photochemical behavior of 5-dmr-BR was indistinguishable from retinal. After light adaptation of the 9-dmr-BR, a significant amount of the 13-cis isomer remained (30%).

The most interesting result was obtained with 13-dmr-BR, where regardless of light or dark adaptation, the 13-cis isomer prevailed. Correspondingly, light–dark adaptation of 13-dmr-BR did not alter its λ_{max}, and the regeneration product always absorbed at 565 nm whether the regeneration was started from the all-trans, the 11-cis, or the 13-cis isomer of 13-dmr. This indicates that in contrast to retinal and other dmr's, isomerization of the 13-dmr occurs during regeneration as shown in Table III. Because regeneration with 11-cis and with all-trans was considerably slower than that with 13-cis, the result from Table III could be interpreted as an isomerization taking place before regeneration. Consequently, we investigated the isomeric composition of a regenerating sample by using a limiting quantity of 11-*cis*-13-dmr. Figure 5 shows clearly that regeneration is faster than isomerization; for example, after 60 min 80% regeneration had occurred but only 25% isomerization of 11-*cis*-13-dmr. During the course of regeneration the λ_{max} at 565 nm did not change. We attempted the same experiment using *all-trans*-13-dmr and obtained a similar but less convincing result, because regeneration with this isomer took several days and was only slightly faster than the isomerization. These results show the 13-cis configuration of 13-dmr to be thermodynamically the most stable isomer in the binding site. We also found that 7-*cis*-13-dmr regenerated virtually to completion, with BO giving a stable 495-nm chromoprotein in which the 7-cis isomer remained. Illumination (495-nm interference filter) of this species instantly converted it to the 565-nm chromophore with concomitant appearance of the 13-cis isomer. The 9-cis isomer of 13-dmr did not undergo a red shift with BO. However, illumination during a reconstitution experiment (405-nm interference filter) yielded a 565-nm species containing 80% 13-*cis*-13-dmr.

Table III: Isomeric Distribution of 13-dmr in the Binding Site of 13-dmr-BR

isomer of 13-dmr used for reconstitution	isomer of 13-dmr (%) extracted from the binding site[a]				
	all-trans	7-cis	9-cis	13-cis	11-cis
all-trans	21	0	8	69	2
11-cis	10	0	0	80	10
13-cis	8	0	0	92	0

[a] Determined after complete reconstitution (no further increase at λ_{max}) and extraction with 2-propanol/hexane by HPLC.

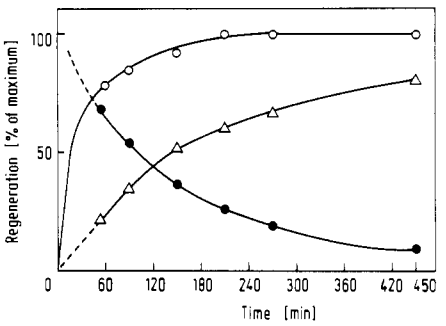


FIGURE 5: Isomerization of 11-*cis*-13-dmr during reaction with BO. A 6-mL sample of BO (150 nmol) at 35 °C was mixed with an equal amount of 11-*cis*-13-dmr in 2-propanol and the regeneration monitored. Samples (1 mL) were denatured with 2-propanol after various times of incubation, and 13-dmr was extracted and subjected to HPLC (see Materials and Methods). The open circles represent the increase in OD at 560 nm. The amounts of the 11-cis (●) and the 13-cis isomer (△) of 13-dmr show a change in the isomer composition during regeneration. Samples which were extracted after 2 h of regeneration also contained 5–10% of the all-trans isomer.

Interaction of Retinol Analogues with BO. When retinol is mixed with BO, the absorption maximum (325 nm) is red shifted by 31 nm, and the absorption peak shows a fine structure. This absorption at 356 nm is indistinguishable from that of retroretinol and has been interpreted as a planarization of the retinol molecule in the binding site (Schreckenbach et al., 1977). When this experiment was repeated with *all-trans*-13-dm- and 9-dm-retinol, the 30-nm red shift together with a fine structure in the absorbance band occurred. However, when 5-dm-retinol was used, the fine-structured, three-peaked spectrum which appeared was shifted to a longer wavelength by only 11 nm. The fine-structured absorption band indicates clearly that immobilization of the cyclohexene ring, but no planarization, takes place for 5-dmr. In a control experiment the retrostructures of 5-, 9-, and 13-dm-retinols were synthesized as described by Goesswein (1976) and were shown to have λ_{max} values in organic solvents similar to that of retroretinol.

Comparison of the excitation energies for retinol and dm-retinols in 2-propanol and in the binding site, respectively, which were evaluated from the measured λ_{max} shifts, showed that this energy is lowered by 7.4 kcal·mol^{-1} for retinol and 9- and 13-dm-retinol but only by 2.8 kcal·mol^{-1} for 5-dm-retinol in the protein-binding site. With the assumption of the same conformation of all retinols in the excited state, extra

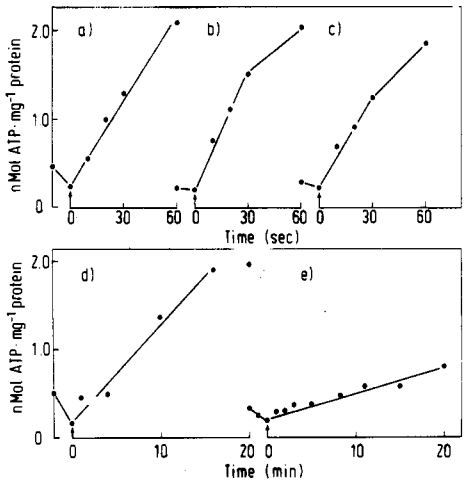


FIGURE 6: Photophosphorylation in halobacterial cells containing various BR analogues. Retinal analogues were incorporated into the BO of whole cells during growth as described under Materials and Methods. The cells were held in darkness in a cuvette at 20 °C under N_2 until the ATP content reached a basal level. The onset of photophosphorylation which was repeated several times is indicated by the arrows on the graphs. The amounts of BR's (nmol) were determined spectroscopically after lysis of the cells. In the 10-mL suspensions used, the amounts of BR's were the following: (a) 6.4 nmol; (b) 5-dmr-BR, 8.0 nmol; (c) 9-dmr-BR, 10 nmol; (d) 13-dmr-BR, 37 nmol; (e) 9,13-di-dmr-BR, 13 nmol. The rate of photophosphorylation as nmol of ATP·(mg of protein)$^{-1}$·(nmol of BR)$^{-1}$·min^{-1} was determined from the slopes of the curves according to Hartmann & Oesterhelt (1977). Note the different time scales for (a)–(c) and (d) and (e).

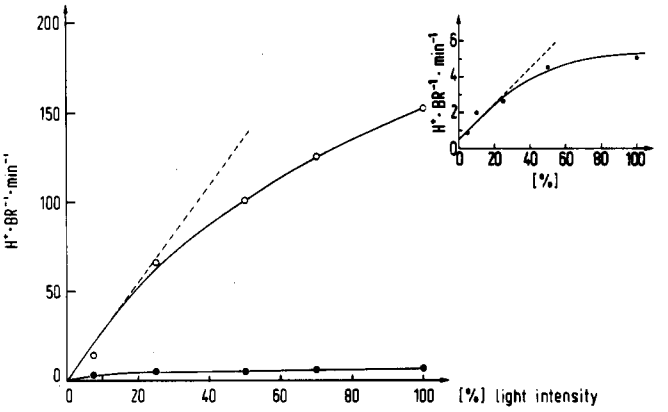


FIGURE 7: Rates of proton translocation of 13-dmr-BR and BR in cell vesicles as a function of light intensity. Cell vesicle preparations (25 nmol of BO) in 8 mL of basal salts were incubated in a thermostated cuvette (25 °C, N_2, magnetic stirrer) with 14.6 (2.5, two experiments) nmol of 13-dmr (closed circles) and with 17.4 (4.3) nmol of retinal (open circles), until no further increase in absorbance at 560 nm was found. To the reconstituted vesicles was added 20 μL of 100 mM TPP^+ in ethanol. The rate of protons translocated per BR per minute was determined after calibration with known amounts of acid and base. Light intensity was reduced by neutral density filters (70, 50, 25, 10, 5, and 1% transmission). Inset shows proton translocation of 13-dmr-BR with expanded scale.

energy of 4.6 kcal·mol^{-1} in the case of 5-dm-retinol is needed for planarization of the ring. Thus, we suggest a planar conformation for the excited state. This is in full accordance with the observation that photochemical conversion of retinol to retroretinol with high efficiency is only possible in the binding site (Schreckenbach et al., 1978).

Transient Spectral Changes of the BR Analogues. Because of varying proportions of the 13-cis and the all-trans isomers in the light-adapted dmr-BR's, flash experiments were necessary for an interpretation of the functional capacities described below. In particular, the transient spectral changes in the millisecond range, in which the intermediates M_{412} (deprotonated 13-cis form) and O_{640} are formed, should be different for the various dmr-BR's. The dmr-BR's, which were used in these experiments, had been isolated from cells and proven to be free of material absorbing in the cytochrome region (420 nm). When flashed, changes in transmission of the sample were recorded for 100 ms in the wavelength region from 350 to 700 nm. From these data the times of maximal steady-state concentration of various intermediates were derived. With BR and 5-dmr-BR a maximal increase in absorbance appeared at 1.5 ms at 410 nm due to the "M" intermediate, and a transient absorbance increase at 640 nm occurred at 9 ms due to the "O" intermediate. The 9-dmr chromoprotein converted to a 410- and a 620-nm species simultaneously. We believe that this 620-nm species, which is different from the O intermediate of the trans cycle, arises from the 13-cis form of 9-dmr-BR. This isomer is present to an extent of 30% even in the light-adapted form.

Illumination of 13-dmr-BR produced only a small amount of the M_{410} species. Furthermore, the half-time of decay of this species was about 3 s, whereas the decay of M_{412} in BR is 10 ms. The predominant conversion of 13-dmr-BR was to a 620-nm species within 10 ms, which was also found for 9-dmr-BR. This conversion is consistent with the fact that the 13-cis isomer accounts for at least 80% of the isomers in the binding site of 13-dmr-BR. As a result of these flash spectroscopic experiments, we propose that the increasing 13-cis contents of dmr-BR's and, especially in 13-dmr-BR, the increased half-time for the trans photocycle cumulatively inhibit the photochemical activity of bacteriorhodopsin.

Bioenergetic Function of BR Analogues. Light-induced ATP formation as an assay of bioenergetic function of BR was first carried out with cells deficient in retinal synthesis, which did form BR's by addition of retinals during growth (Towner et al., 1980; Hartmann & Oesterhelt, 1977). The results are shown (Figure 6) as initial rates of photophosphorylation in the various cell cultures. The BR or dmr-BR content of the cells was determined with aliquots of the culture as described under Materials and Methods. The values of photophosphorylation activity were calculated as nanomoles of ATP per minute per mg of protein per nanomole of BR and were as follows: BR (Figure 6a) 6), 0.29; 5-dmr-BR (b), 0.33; 9-dmr-BR (c), 0.18; 13-dmr-BR (d), 0.0032; 9,13-di-dmr-BR (e), 0.0023. This shows that the lack of the methyl group at position 13, but not at 9 or 5, reduces the efficiency of photophosphorylation by a factor of 100.

As a further assay of bioenergetic function, cell vesicle preparations were used. These vesicles containing BO, but no retinal, were complemented with the different analogues. The amount of retinoid added was limiting, and the final concentrations of chromoproteins were identical. Light-induced acidification in the presence of the TPP^+ ion was then measured as described elsewhere (Oesterhelt, 1982). Under these conditions of quenched membrane potential, proton extrusion is a quantitative measure of BR activity. The number of protons pumped per molecule of BR per minute as a function of light intensity is shown in Figure 7 for 13-dmr-BR and for BR containing cell vesicles. Comparison of the slopes within the linear parts of the curves for BR and 13-dmr-BR showed that 13-dmr-BR is less active than BR by a factor of 30 in agreement with the photophosphorylation experiment. The amount of protons maximally translocated (6–9 H^+ per BR per min) can also be correlated to the frequency of the photocycle of 13-dmr-BR (about 5 min^{-1}). In contrast to 13-dmr-BR, the two other dmr's mediated proton translocation as efficiently as retinal (data not shown). These

Table IV: Regeneration Parameters of Rhodopsin Formation

compound	λ_{max} (nm)	k (20 °C) (s^{-1})	AE (kcal·mol^{-1})
11-*cis*-retinal	500	3.3×10^{-2}	19.0
11-*cis*-13-dmr	500	0.3×10^{-2}	17.5
9-*cis*-retinal	485	2.7×10^{-2}	18.1
9-*cis*-13-dmr	488	0.7×10^{-2}	19.2

results imply again that the methyl group at position 13 is essential for the proper function of BR.

Interaction of Retinal Analogues with Opsin. The interaction of 5-dmr and 9-dmr with opsin has been reported previously (Kropf et al., 1973), and we concur with these results. Our own studies concerning 13-dmr are reported in more detail, because the 11-cis isomer of 13-dmr reconstituted a chromoprotein with BO and with opsin. Nelson et al. (1970), however, reported that the 11-cis isomer only weakly interacts with opsin to give an equilibrium mixture between the analogues rhodopsin and opsin and the free retinoid. We find a complete regeneration when pure 11-*cis*-13-dmr is added to opsin. However, the reaction is 10-fold slower than with retinal, as shown in Table IV. A slower regeneration rate is also found with the 9-cis and the 7-cis isomer of 13-dmr. The latter one reacted with opsin to a rhodopsin with a λ_{max} at 460 nm. The 11-*cis*-13-dmr rhodopsin was very labile and bleached when either frozen, dissolved in detergent, or mixed with hydroxylamine in the dark. The activation energies for the regeneration of these analogues are listed in Table IV.

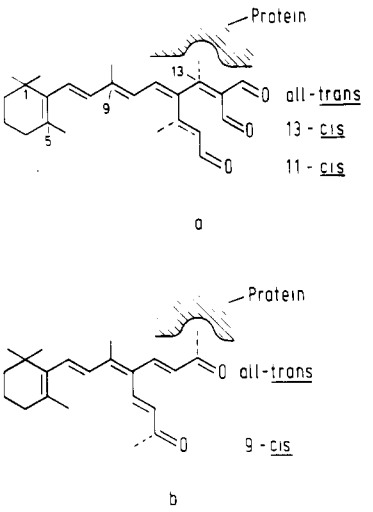
Discussion

In this work we studied the influence of the removal of methyl groups from retinal on the retinal–BO interaction and the function of the BR analogues. We found that the 13-cis and the all-trans isomers of the dmr's form chromoproteins. There was one exception of this rule: When the methyl group at position 13 was removed, the 11-cis isomer was accepted by the binding site of BO to form a chromoprotein. 11-*cis*-13-dmr is the first compound known so far which forms a chromoprotein with BO as well as with opsin. Considering the three configurations of the 13-dmr isomers (see Chart I, structure a), it becomes clear that the methyl group in position 13 prevents chromoprotein formation with BO, if positioned differently than in the 13-cis/all-trans configuration. The same phenomenon was found during inhibitor studies, which revealed that a 9-cis C_{18}-ketone (Chart I, structure b) only inhibited a chromoprotein formation from retinal and BO, if the methyl group in position 13 was removed (=9-cis C_{17}-aldehyde), whereas the all-trans configurations of both compounds (C_{17}-aldehyde and C_{18}-ketone) inhibited equally strongly (Towner et al., 1981).

All formed chromoproteins showed λ_{max} values above 530 nm and similar molar extinction coefficients. The removal of the methyl group from position 13 did not influence the absorption maximum of the analogue BR, whereas for 5- and 9-dmr-BR a blue shift by about 20 nm was found. It is worth noting that in visual pigments only the lack of the methyl group at position 9, but not at position 5, causes a similar blue shift (Blatz et al., 1969; Kropf et al., 1973).

The velocities of chromoprotein formation varied by a factor of 10^4 and the 13-cis isomers of all compounds reacted faster than their all-trans forms except of the 13-cis and the all-trans forms of 9-dmr, which showed the same reaction rate (Table I). Independent of the isomer, the 5-dmr reacted faster than retinal, 9-dmr, or 13-dmr. The lack of steric interaction caused by the removal of the methyl group at position 5 results in a nonplanarization upon association with BO and may be responsible for the enhanced reaction rate. It is more difficult to explain the decreased rates of 9-dmr and of 13-dmr compared to retinal.

Chart I



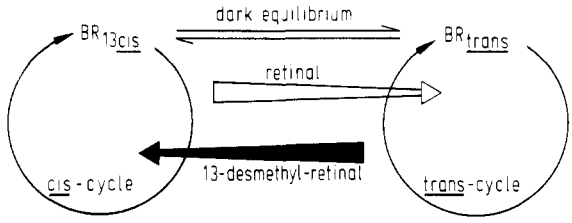
Some conclusions can be drawn from the activation parameters of the reactions. The usual meaning of the heat of activation (ΔH^*) is the energy barrier, which has to be overcome by the reacting molecules, in our case the aldehyde group of retinal and lysine residue 216 of BO. The entropy of activation (ΔS^*) on the other hand includes steric and orientational requirements. Although the reaction between retinal and BO is bimolecular, the change in entropy of activation by the approach of the two reactants may be neglected, because the rate-limiting step of the reaction (step c in the text) occurs, when the retinal molecule is already firmly placed in the active site of the protein. Removal of the methyl group at position 13 increases the ΔH^* value for the all-trans isomers from 13.0 to 19.5 kcal·mol^{-1}. This is explained by the assumption that the methyl group produces a steric hindrance upon association with BO, releasing less binding energy than its demethyl counterpart but, on the other hand, requiring less heat of activation to overcome the energy barrier of the condensation reaction. The opposite holds true for the 13-cis configuration, in which the methyl group is assumed not to cause a comparable steric hindrance. However, the lack of the methyl group leads to the largest contribution of entropy of activation to the rate of the reaction. This means that 13-*cis*-13-dmr encounters the greatest loss in flexibility during the condensation; in other words, in the 13-cis geometry the methyl group at position 13 has a large directing effect on the reaction.

The methyl group at position 13 and much less at position 9 strongly influences not only the kinetics of chromoprotein formation but also the 13-cis/all-trans isomeric equilibrium after the condensation. As clearly demonstrated in Table II, the 13-cis forms of the dmr's dominate in the equilibrium. This is explained by a stabilization of the cis configuration by 1.2 kcal·mol^{-1} for 13-dmr.

The clue, however, to understand why 13-dmr has such a greatly diminished capacity to mediate the function of BR comes from the observation that illumination also preferentially produces the 13-cis isomer in 13-dmr-BR, as shown in Scheme I.

Illumination induces in BR_{cis} as well as in BR_{trans} cyclic reactions as mentioned in the introduction. In the case of retinal, BR_{cis} switches from the cis cycle into the trans cycle

Scheme I



with a yield of almost 100% for BR_{trans} after a short period of illumination. So far it is unknown which intermediates species are involved in this pathway. The opposite reaction, from the trans into the cis cycle, was first observed when α-retinal was used in functional studies on BR (Towner et al., 1980). In this work we present evidence that a switch from the trans to the cis cycle is also found as a consequence of removal of methyl groups from retinal. However, the most striking result is that this effect is specific for position 13 in accordance to the theoretical calculations of Schulten & Tavan (1978). Furthermore, the result that in 13-dmr-BR the proton pumping and the photophosphorylating capacity is less than 5% compared to that of BR demonstrates clearly that the 13-cis cycle is inactive with respect to proton translocation [see also Fahr & Bamberg (1982)], and that the trans cycle is slowed down in its frequency to less than 0.3 s^{-1}. This can be understood on the basis that the ratio of the reconstitution for 13-*cis*-retinal and for 13-*cis*-13-dmr is 30 and that a similar ratio occurs for the regeneration reactions of BR from the M_{412} state.

Finally, we conclude that specifically the methyl group at position 13 of retinal has an important steric function in the BR photocycle by directing a fast reisomerization of the 13-cis form (M_{412}) into the all-trans ground state, and thereby creating an efficient proton pump.

Acknowledgments

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Registry No. 5-dmr, 52152-35-9; 13-*cis*-5-dmr, 17781-11-2; 9-dmr, 24336-19-4; 13-*cis*-9-dmr, 52152-30-4; 13-dmr, 24336-20-7; 13-*cis*-13-dmr, 74311-19-6; 7-*cis*-13-dmr, 69575-84-4; 11-*cis*-13-dmr, 29706-62-5; 9-*cis*-13-dmr, 29706-61-4; 9,13-di-dmr, 24336-21-8; 13-dm-14-methylretinal, 53121-26-9; hydrogen ion, 12408-02-5.

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