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LIGHT-DARK ADAPTATION OF BACTERIORHODOPSIN IN TRITON-TREATED PURPLE MEMBRANE

R. CASADIO, H. GUTOWITZ, P. MOWERY, M. TAYLOR and W. STOECKENIUS

Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco, CA 94143 (U.S.A.)

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Summary

Solubilization of purple membrane with Triton X-100 yields Triton micelles containing bacteriorhodopsin monomers. The absorption maximum of dark-adapted solubilized bacteriorhodopsin is blue-shifted to 549 nm. Light adaptation increases the absorbance by 4% and shifts the absorption maximum to 553 nm, i.e., the extent of light adaptation is considerably less than in intact purple membrane. Extraction of dark-adapted bacteriorhodopsin in Triton yields a 13-*cis*- to all-*trans*-retinal ratio of 58 : 42 which changes after light adaptation to 38 : 62. It has been shown by Sperling et al. (Sperling, W., Carl, P., Rafferty, Ch.N. and Dencher, N.A. (1977) Biophys. Struct. Mech. 3, 79—94) that light adaptation in intact purple membrane occurs through a branching of the 13-*cis* photoreaction cycle, so that part of the pigment during each cycle crosses over into the all-*trans* photoreaction cycle. We explain the decreased extent of light adaptation in solubilized bacteriorhodopsin by assuming a significant back reaction from the all-*trans* to the 13-*cis* cycle. This assumption predicts a wavelength dependence of the extent of light adaptation, which is born out by experiment.

Introduction

Bacteriorhodopsin, the photoreceptor protein of halobacteria [1] forms patches of a planar hexagonal lattice in the cell membrane [2]; they are known as the purple membrane. Its chromophore is a molecule of retinal, bound via

Abbreviations: BR^{LA}, light-adapted bacteriorhodopsin; BR^{DA}, dark-adapted bacteriorhodopsin.

a Schiff base to a protein moiety of 26 000 molecular weight and the retinal absorption band is red-shifted into the green region of the spectrum. The purple membrane generates an electrochemical proton gradient when it absorbs light [3]. Bacteriorhodopsin, both in intact cells and in isolated purple membrane, exists in two interconvertible forms [4]. When kept in the dark, it shows an absorption maximum at 560 nm and is called dark-adapted bacteriorhodopsin (BR_{560}^{DA}). Illumination causes a 10 nm red shift of the absorption maximum and an increase in absorbance of approx. 14%; this form is termed light-adapted bacteriorhodopsin (BR_{570}^{LA}). Extraction of the chromophore from BR_{560}^{DA} yields a mixture of 50% 13-*cis*- and 50% all-*trans*-retinal [5,6]; only all-*trans*-retinal is extracted from BR_{570}^{LA}. Light apparently shifts the equilibrium of the retinal isomers towards the all-*trans* configuration and during dark adaptation the chromophore thermally relaxes to the original 1 : 1 ratio of isomers. It is difficult to imagine any simple relation between the 1 : 1 ratio of chromophore forms in BR_{560}^{DA} and the 3-fold symmetry of the lattice; however, the change to all-*trans*-retinal upon light-adaption could be influenced by the lattice. Triton X-100 solubilizes purple membrane and under appropriate conditions yields a homogeneous population of bacteriorhodopsin monomers, leaving the spectroscopic properties essentially intact [7]. It should, therefore, be of interest to compare light-dark adaptation of bacteriorhodopsin in intact and solubilized purple membrane; at slightly acid pH the solubilized preparations are stable for many hours at room temperature and for weeks in the refrigerator. We have, therefore, used this detergent for our light-dark adaptation studies. The results suggest that the native structure of the purple membrane stabilizes the all-*trans* configuration of the retinal.

Materials and Methods

Halobacterium halobium R_1 was grown and purple membrane was isolated as described [8]. Solubilization was routinely carried out at room temperature in the dark for 48 h with a protein : detergent ratio of 1 : 35 (w/w), in 0.1 M sodium acetate buffer, pH 5. These stock solutions, which had a protein concentration of 0.5 mg BR · ml^{-1} were kept at 4° C for up to two weeks. No change in their properties was noted. Triton X-100 was purchased from Sigma and used without further purification.

Some of the absorbance spectra were obtained with a Cary 14 spectrophotometer modified to operate in a single-beam mode, and equipped with an integrating sphere. Usually the Cary 14 was used as a double-beam instrument and equipped with the scattered transmission accessory and a thermostated cuvette holder. When necessary spectra were corrected for light scattering based on the ratio between the 550 nm and the 390 nm absorbance measured with the integrating sphere. All the experiments were done at 23° C unless specified. The concentration of purple membrane was determined from the absorbance (A) at 560 nm using an extinction coefficient for BR_{560}^{DA} of 51 000 (Bogomolni, R., personal communication). Spectra were stored on magnetic tape and calculations carried out with a Nicolet 1180 computer.

Light adaptation was obtained with a slide projector equipped with a 250 W quartz iodine lamp, heat filter and a Corning glass long pass filter No. 368.

Light intensity was measured with a Model 68 Kettering Radiometer (Laboratory Data Control Div. Milton Roy. Co.).

Retinal was extracted from intact and solubilized purple membrane with CH_2Cl_2 as previously reported and the isomer composition was determined by high-pressure liquid chromatography [9]. Absorbance spectra were recorded immediately before extraction.

Results

The solubilization of membrane proteins with Triton X-100 is pH, ionic strength, and temperature dependent [10]. At any given condition the rate of solubilization is also a function of the detergent : protein ratio. We have chosen pH 5 and 0.1 M acetate buffer because some bleaching occurs in the dark at higher pH. Beginning our measurements 1 min after the addition of Triton to dark-adapted purple membrane suspensions, we find a very fast blue shift of the absorption maximum from 559 to 549 nm and a slow 12% decrease of the molar extinction coefficient as measured with the integrating sphere.

At 25°C and at a protein : detergent molar ratio of 1 : 35, the solubilization is complete within 48 h. After this time we can no longer detect any sedimenting material by centrifugation at 200 000 $\times g$ for 2 h. The solubilized purple membrane migrates in the included volume of a BioGel A 1.5 m column equilibrated at pH 5 with 1% Triton X-100.

The Stokes radius of the protein-lipid-detergent complex is approx. 50 Å and the calculated mol. wt. is 120 000, which is in satisfactory agreement with analytical ultracentrifugation data [7]. As already reported by others [11,18], solubilization is accompanied by the disappearance of the negative circular dichroism (CD) band at 600 nm, a large decrease in the amplitude, a 10 nm blue shift of the positive band, and a decrease in the negative band at 320 nm. This observation is consistent with the assumption that the long wavelength negative CD band arises from an exciton interaction between the chromophores in the lattice. The Triton-induced blue shift of the visible absorption band is nearly complete in 10 min while the decrease in absorbance roughly shows the same time dependence as the solubilization process, i.e., it has a half-time of approx. 10 h (Fig. 1). The disappearance of the negative CD band also follows the slow time course [12]. The decrease in absorbance at 549—559 nm during the solubilization is not accompanied by the appearance of a 380 nm band, indicating that it is not due to chromophore destruction.

Light adaptation after only 10 min exposure to Triton X-100, when the blue shift is essentially complete, shows a 9 nm red shift and 8% increase in absorbance, i.e., changes nearly as large as those seen in native purple membrane. Illumination of fully solubilized membrane in the 549 nm absorption band with $2.2 \cdot 10^5$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1} for 10 min causes a 4 nm red shift of the absorbance maximum of the dark-adapted BR and an increase in the absorbance of 3—4%. The extent of light adaptation in fully solubilized purple membrane, therefore, is considerably less than the corresponding changes seen in intact purple membrane (Fig. 2A). The difference spectrum ($BR^{LA} - BR^{DA}$) of solubilized purple membrane shows the same general pattern as the difference spectrum of intact purple membrane, but is slightly blue-shifted (Fig. 2B).

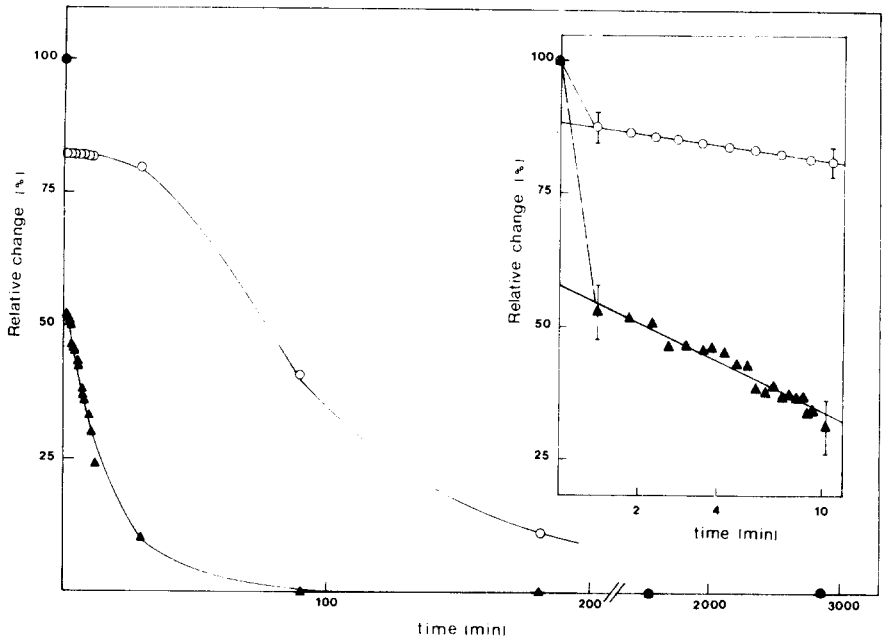

Fig. 1. Time course of the blue shift (▲———▲) and decrease in absorbance (○———○) during solubilization in Triton measured with the integrating sphere. The relative change in absorbance (A) is calculated as $[(At_x - At_b)/(At_a - At_b)] \times 100\%$ and the relative shift of the absorption (λ) as $[(\lambda t_x - \lambda t_b)/(\lambda t_a - \lambda t_b)] \times 100\%$ where t_a indicates the values for A and λ at the time before Triton addition, t_b at the time after complete solubilization (48 h), and t_x at any given time during solubilization. $At_a = 0.765\ A$; $At_b = 0.68\ A$; $\lambda t_a = 559$ nm; $\lambda t_b = 549$ nm. 10% change = 0.008 A and 1.0 nm, respectively. Equal volumes of purple membrane suspension and Triton X-100 solution were mixed at time t_a. The 100% value at time zero (t_a) was obtained from a control mixed with buffer instead of Triton solution. The apparent initial rapid drop in absorbance is small and may at least in part be due to a decrease in scattering resulting from the higher refractive index of the Triton solution. The large blue shift occurring during the first minute must result from a fast interaction of the detergent with the chromophore. Spectra during the first 10 min were scanned only from 570 nm to 540 nm. (At the two time points above 200 min, the ○ and ▲ points coincide.)

As in intact purple membrane the light-adapted state of solubilized BR relaxes thermally to the dark-adapted state. The half-time for this reaction is temperature dependent, and at 26° C is about 30 min, i.e., twice as fast as in intact purple membrane at the same pH (Fig. 3). In the Triton-solubilized material the kinetics of dark adaptation can be approximated by an exponential curve whereas in intact purple membrane the same process shows a more complicated pattern.

Light adaptation of solubilized purple membrane is complicated by bleaching [19]. At higher light intensity or at longer times of illumination, a progessive decrease in absorbance at 553 nm can be detected without any further red shift. Upon dark readaptation a loss of 549 nm-absorbing material becomes obvious. Comparing the light-adapted spectrum to the dark-adapted spectrum after illumination, we find that the total extent of light adaptation is the same as in preparations with shorter light exposure where bleaching is negligible. At the light intensity used in our experiments bleaching begins to occur after 10 min of illumination. Longer exposures cause larger bleaching and a proges-

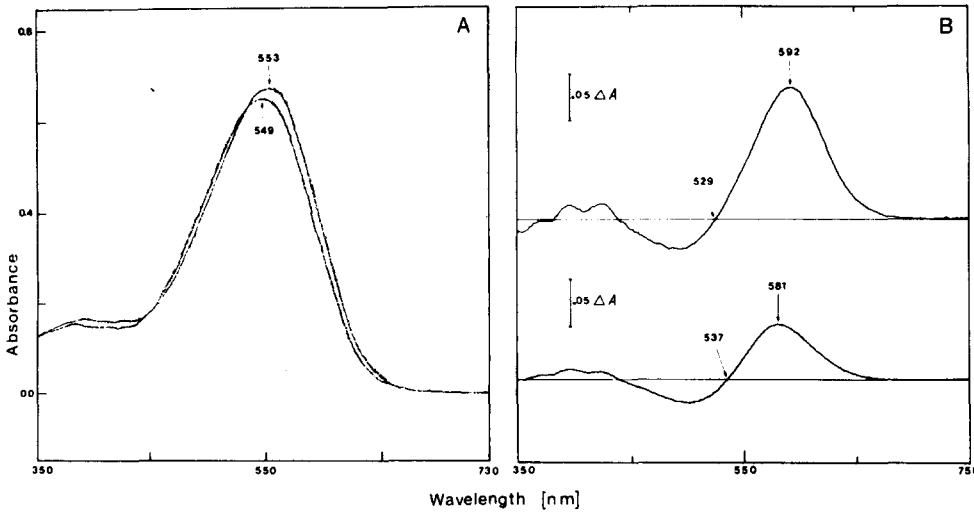

Fig. 2. A. Light adaptation of solubilized membrane. Saturating illumination shifts the absorption maximum from 549 mn to 553 nm and increases the absorbance by 4%. For illumination conditions see Materials and Methods. (B) The upper curve shows the difference spectrum of light-adapted minus dark-adapted intact purple membrane, the lower the difference spectrum after solubilization under standard conditions. The absorbance of the light-adapted intact membrane was 0.86, the absorbance of the light-adapted solubilized membrane was 0.71.

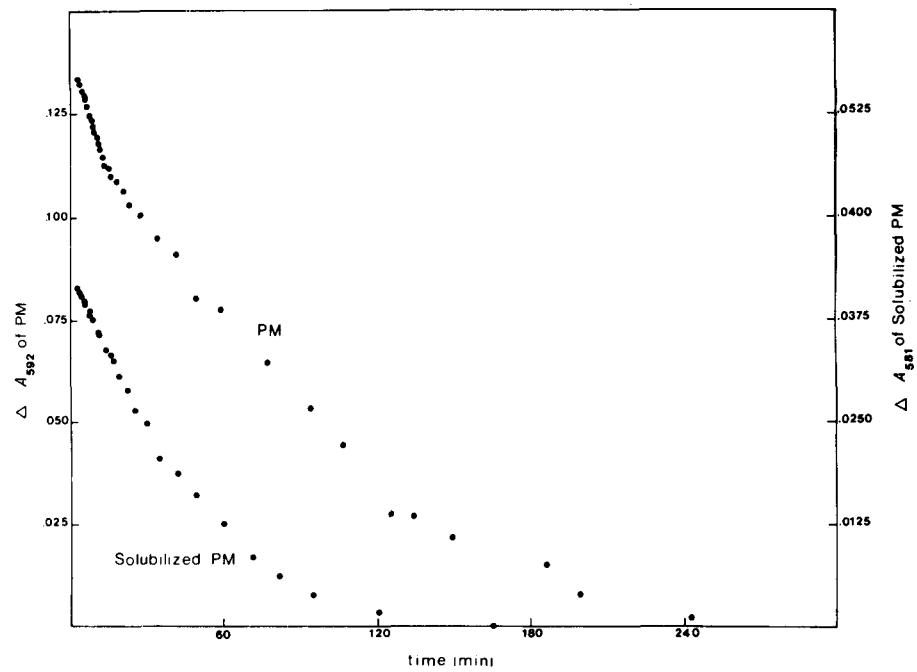

Fig. 3. Kinetics of dark adaptation for intact and solubilized purple membrane (PM) at 26°C. The decrease in the maximum of the light-adapted minus dark-adapted difference spectra is plotted as a function of time. Both preparations were at pH 5.0; spectra were taken with the integrating sphere to minimize the effect of aggregation occurring in the intact purple membrane suspension.

sive increase in 380 nm absorbance, most likely indicating a partial unfolding of the protein and possibly release of retinal. An example for a moderate amount of bleaching is shown in Fig. 4. Note that the increase in absorbance of the light-adapted form in comparison to that of the dark-adapted one after illumination is 4%, the same as for shorter illuminations where no measurable bleaching occurs. Following the dark adaptation of a bleached sample for a very long time, it is possible to detect some regeneration of color (1% regeneration occurs in 24 h for a preparation with 4% bleaching). Therefore, the dark-adaptation kinetics we measure should not be complicated by any effect due to spontaneous reconstitution of bleached material. Another very slow effect, which has not been observed consistently but nevertheless appears to be real, is a bleaching in the dark of a light-adapted sample. This clearly requires further study, but also appears to be too slow to affect our light-dark adaptation results. The earliest time at which we have observed the beginning of this effect is 7 h after illumination when dark adaptation is essentially complete.

We have extracted the retinal both from intact and solubilized light and dark-adapted purple membrane (Table I). At pH 5 extractions of retinal from intact dark-adapted purple membrane yields 45% 13-*cis* and 55% all-*trans* isomers. Upon light adaptation, 18% 13-*cis*-retinal is still present. This finding is different from results obtained at higher pH values where only negligible amounts of 13-*cis* are found but is in agreement with observations showing

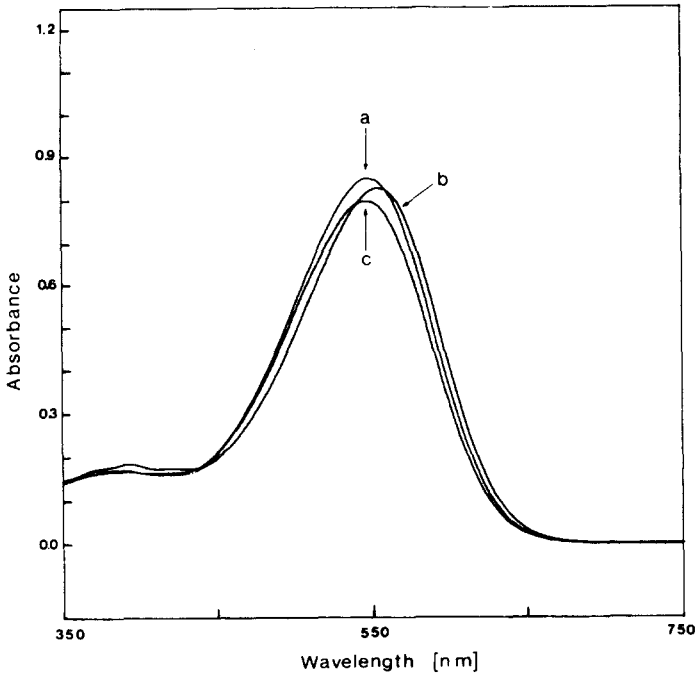

Fig. 4. Bleaching of solubilized purple membrane at high light intensity. Curve a shows the absorption spectrum of the dark-adapted sample before illumination, λ_{max} = 549 nm; curve b after illumination with $7 \cdot 10^5$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1} for 2.5 min, λ_{max} = 553 nm, curve c same preparation after 6 h at 24°C in the dark, λ_{max} = 549 nm.

TABLE I

EXTRACTION OF RETINAL (% ISOMER COMPOSTION)

	Purple membrane		Solubilized	
	13-*cis*	All-*trans*	13-*cis*	All-*trans*
Dark-adapted	45 ± 1	55 ± 1	58 ± 3	42 ± 2
Light-adapted	18 ± 1	82 ± 4	38 ± 2	62 ± 2

The yield of retinal was 5—15%. As controls all-*trans*- and 13-*cis*-retinal were solubilized with 1.5% Triton X-100 in 0.1 M acetate buffer at pH 5.0; of the retinal recovered in the extract 99 ± 1% was in the isomeric form added.

increasing amounts of 13-*cis* isomer in light-adapted purple membrane as the pH is decreased [9]. After solubilization in Triton in the dark, we find 58% 13-*cis* and 42% all-*trans*-retinal, i.e. in the monomer the equilibrium of the isomers is shifted towards the 13-*cis* form. After light adaptation under conditions of negligible bleaching, we obtained 38% 13-*cis* and 62% all-*trans* isomer, i.e. only 35% of the 13-*cis*-retinal is converted to all-*trans* upon full light adaptation.

Discussion

The addition of Triton X-100 to a purple membrane suspension causes a fast 10 nm blue shift and a slow decrease in absorbance. The blue shift cannot be correlated with dissociation of the lattice because it is essentially complete after 10 min when the membrane according to CD and centrifugation data is still intact. Also, glutaraldehyde-cross-linked membrane in Triton shows the blue shift but the lattice remains intact even after exhaustive Triton treatment (our unpublished observations). The blue shift is probably at least in part due to a direct interaction of Triton X-100 with the chromophore, because Triton X-100 is not very effective in removing lipids from purple membranes and an almost complete removal of lipids from purple membrane by deoxycholate results in a much smaller blue shift [13].

Light-dark adaptation during solubilization is difficult to measure because the absorbance decrease due to solubilization cannot be clearly separated from that due to dark adaptation and without a postillumination dark-adapted spectrum, saturation of the light adaptation and bleaching cannot be assessed. Nevertheless, we note here, that the red shift occurring upon light adaptation of purple membrane after 10 min Triton X-100 treatment is almost as large as in intact purple membrane. Furthermore, glutaraldehyde-cross-linked purple membrane even after 48 h in Triton shows a red shift and absorbance increase very similar to that of intact membrane (our unpublished observations). The aggregation state of bacteriorhodopsin in the membrane, therefore, seems to influence the light-dark adaptation reaction. However, the lipid environment in these modified membranes must be assessed before any firm conclusions can be drawn.

Since we are comparing solubilized BR to intact membrane, we must show

that we have reached the photosteady state in the solubilized membrane. This is complicated by the bleaching phenomenon which causes a decrease in absorbance superimposed on the increase due to light adaptation. For this reason we followed in all cases the dark readaptation of the solubilized material to measure the degree of bleaching and found that it is possible in fully solubilized membrane to obtain complete light adaptation without significant bleaching. This is important, because the extent of bleaching can always be measured by waiting for dark readaptation, but the extraction results of light-adapted preparations might be falsified if significant bleaching had occurred, because we do not know which isomeric conformation the bleached chromophore assumes. We find that only 35% of the 13-*cis*-retinal in solubilized membrane is converted to the all-*trans* isomer after complete light adaptation. This is consistent with the smaller red shift and absorbance increase compared to intact purple membrane. However, it is not consistent with the suggested mechanism for light adaptation in intact membrane unless only a fraction of the whole population of solubilized purple membrane light-adapts. This would imply that our solubilized preparations are inhomogeneous. Analytical ultracentrifugation and gel filtration chromatography rule out any significant concentration of BR oligomers. This leaves differences in lipid content of the BR-Triton micelle as the only likely source of inhomogeneity. We have therefore first extracted the lipids from intact purple membrane with deoxycholate [13] and then solubilized with Triton. The light adaptation of this preparation was the same as that of solubilized membrane, which effectively rules out this explanation. A difference in the mechanism of light adaptation between intact and solubilized membranes, therefore, has to be considered. Sperling et al. [14] have partially characterized the 13-*cis* photoreaction cycle in dark-adapted purple membrane and shown, that light adaptation occurs through a partial conversion in every cycle of one of the 13-*cis* cycle intermediates into BR_{570}^{LA}. The light-adapted cycle has been known for some time [15]. We assume that in solubilized material the same two photocycles exist and that their kinetics are similar to those of intact purple membrane. We also have preliminary evidence from flash spectroscopy that this is indeed the case (unpublished results with R. Lozier). If we do not accumulate 100% all-*trans*-retinal after saturating illumination, a fast backreaction to the 13-*cis* cycle must exist. Obviously the dark adaptation is too slow a process to account for the large amounts of 13-*cis*-retinal extracted from light-adapted solubilized BR; only a significant backreaction from an intermediate of the all-*trans* photoreaction cycle to 13-*cis*-BR or one of its photocycle intermediates can satisfactorily explain the results (Fig. 5). This explanation implies that the degree of light adaptation in solubilized BR may be wavelength dependent. By preferentially illuminating one isomer we should be able to change the concentration of intermediates in the two reaction cycles and therefore, shift the isomer ratio attained in the photosteady state.

It is possible to calculate the spectra of 13-*cis*- and all-*trans*-retinal-containing solubilized BR, because the ratio of the two isomers is known from the extraction data for both light and dark-adapted BR; they are shown in Fig. 6A. The difference spectrum (Fig. 6B) shows that the all-*trans* isomer absorbs more at 570—590 nm and the 13-*cis* isomer, more at 490—510 nm. If our proposed

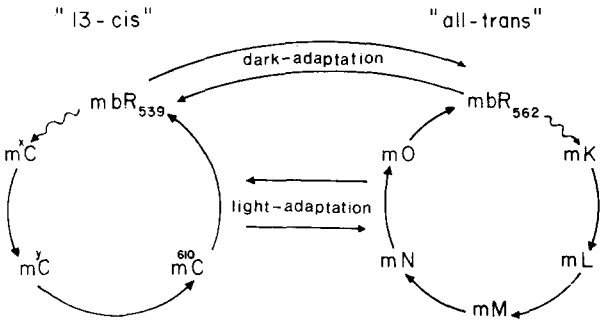

Fig. 5. Model for the light-dark adaptation pathways in solubilized purple membrane. The 13-*cis* cycle with its intermediates is taken from Sperling et al. [14], the all-*trans* cycle from Lozier and Stoeckenius [15]. The prescript m denotes that BR is in its monomeric form; the subscripts indicate the absorption maxima for 13-*cis*- and all-*trans*-retinal-containing BR. (Note that except for M we have only very preliminary evidence that intermediates corresponding to those in intact purple membrane also occur in solubilized purple membrane.) The upper pair of arrows shows the slow equilibration between 13-*cis*- and all-*trans*-retinal-containing BR in the dark; the lower pair of arrows indicates equilibration between intermediates of the reaction cycles; which intermediates are involved has not been established except for the observation by Sperling et al. [14] that in intact purple membrane branching occurs at the ^{610}C intermediate.

scheme is correct, illumination in the long-wavelength region should result in less light adaptation, because it would preferentially drive the back reaction from the all-*trans* to the 13-*cis* cycle and vice versa. We find that illumination through a narrow band 592 nm interference filter in the photosteady state gives a 4 nm red shift and a 4% increase in absorbance, whereas a 500 nm interference filter gives a 6% increase in absorbance and a 7 nm red shift, confirming the predictions from the model, which assumes a significant backreaction from an intermediate of the all-*trans* photoreaction cycle to the 13-*cis*-containing

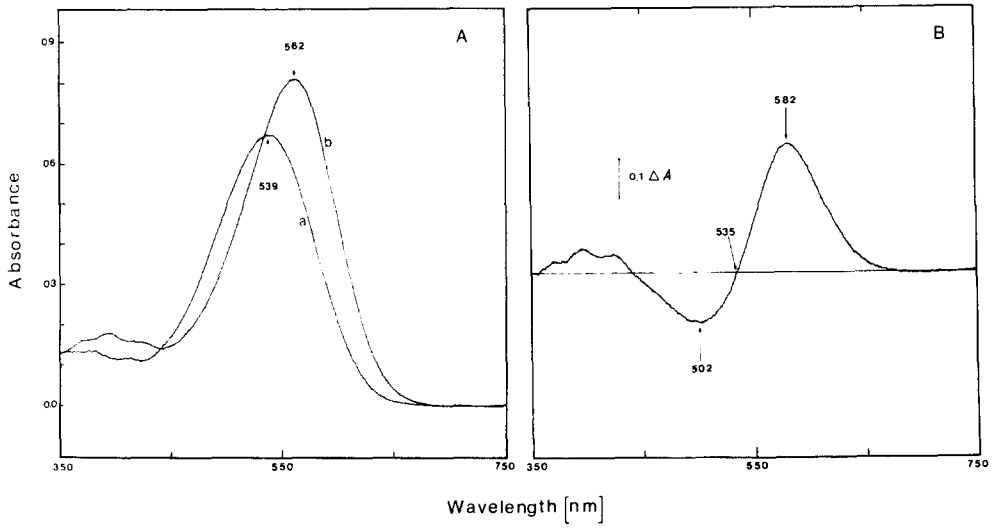

Fig. 6. A. Calculated absorption spectra for solubilized 13-*cis* (a) and (b) all-*trans*-retinal-containing BR. B. Difference spectrum b — a.

BR. Sperling et al. [14] have recently shown that light absorption by an intermediate of the all-*trans* photoreaction cycle (presumably K) causes a back-reaction to the 13-*cis*-chromophore. This, however, occurs only at very high light intensities to any significant degree, because the kinetics of the photoreaction cycle do not allow significant concentrations of intermediates to arise at low or moderate light intensities. Our preliminary results as well as those of others [12] show that the photoreaction cycle kinetics are not significantly changed after solubilization and a back photoreaction from the all-*trans* cycle to 13-*cis*-BR is, therefore, not a plausible explanation for our results.

The reaction pathway postulated here may not occur in solubilized purple membrane only. In intact purple membrane at pH 5 it may account for the 18% 13-*cis* isomer we extract from the fully light-adapted preparation, and even at higher pH we find small amounts of 13-*cis*-retinal in light-adapted purple membrane at light intensities which should preclude any photoreactions of an all-*trans* photocycle intermediate to 13-*cis*-containing BR. The results reported here are of interest not only as a contribution to understanding the mechanism of light-dark adaptation. Since only the all-*trans* photoreaction cycle translocates protons, the monomer, which for instance exists in bacteriorhodopsin-containing lipid vesicles above the phase transition [17] should be less efficient in establishing a proton gradient. (Preliminary results bear out our expectation that the light-dark adaptation in BR-lipid vesicles shows the same decrease in the extent as the Triton-solubilized preparations.) To further investigate this aspect it will be important to establish the cross-over point from the all-*trans* photocycle to the 13-*cis* photocycle.

Acknowledgements

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