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Proton transport and M-type intermediate formation by 13-*cis*-bacteriorhodopsin

Andrey D. Kaulen, Lel A. Drachev and Valeria V. Zorina

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow (U.S.S.R.)

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pH-titration of the dark-adapted 13-*cis*-retinal-containing bacteriorhodopsin (13-*cis*-bR) photocycle was performed in native and regenerated purple membranes, Triton X-100 solubilized and liposomal monomeric preparations. Under alkaline pH conditions the 13-*cis*-pigment component of these bR preparations was shown to enter into photochemical conversions, including the M-type intermediate, and was coupled to the transmembrane proton transfer. We observed that in the case of the monomeric detergent-treated bR the process of M formation in the 13-*cis*-bR photocycle is accompanied by a release of protons, subsequently uptaken simultaneously with the decay of the longwave O-like intermediate. In the 'collodion film-liposomal bR' system an electric response directly indicated a proton translocating activity in the 13-*cis*-bR at high pH.

Introduction

Bacteriorhodopsin (the light-driven proton pump from halobacterial purple membranes), when placed in the dark or exposed to light, was shown to exist in two states differing in the content of the all-*trans* and 13-*cis* isomers of the retinal chromophore group. The dark-adapted state contains an equimolar mixture of the two isomers (66% 13-*cis* according to Ref. 1), whereas the light-adapted bR has the all-*trans* pigment only [2–9]. In the purple membranes the half-time of dark adaptation is 20 min at 35 °C [5].

According to ^{13}C-NMR and Raman spectroscopy [10,11], the dark-adaptation is accompanied by isomerization around C13 = C14 and C = N double bonds, namely, all-*trans*, 15-*anti* configuration changes for 13-*cis*, 15-*syn* configuration. It should be mentioned that the light-adapted pigment photocycle includes reversible all-*trans* ⇌ 13-*cis* isomerization without any isomerization around the C = N bond.

The 13-*cis* component of the dark-adapted bR, after light absorption, undergoes cyclic photoconversion with the longwave intermediate(s) involved. At room temperature, the bathointermediate was identified according to a differential maximum at 610 nm and 37 ms lifetime [5]. The thermal bathointermediate decay was found to require $t > -140$ °C [12,13]. The quantum yield of the bathointermediate formation is about 0.3 [13]. No deprotonated M-type intermediate was described in the 13-*cis* photocycle [5,14]. In any case, 13-*cis* photocycle is usually regarded as non-coupled to H^+ translocation [14–19]. Under illumination the 13-*cis*bR with $\varphi =$ 0.03–0.08 is transferred to the all-*trans* pigment [12,20,21]. This process is carried out at relatively low excitation intensities involving the thermal conversion of bathointermediates of the 13-*cis*-bR photocycle.

At higher excitation energies, another light adaptation process contributes to the 13-*cis* → all-*trans* isomeric pigment conversion. According to Kalisky et al. [21], a nanosecond laser flash of high intensity causes direct photochemical conversion of the 13-*cis*-bR or its bathointermediate to the all-*trans*-bR or its bathointermediate. In addition, this process leads to M formation attributed to the all-*trans*-bR photocycle. Sperling et al. [22] observed that photoinduced interconversion of the isomeric forms in the dark- and light-adapted purple membranes resulted in a final mixture consisting of 80% all-*trans*-bR and 20% 13-*cis*-bR. The authors suggest an explanation based on the existence of light-induced pathways connecting the photocycles of the all-*trans*- and 13-*cis*-bR. Balashov and Imasheva [23]

Abbreviations: all-*trans*-bR, all-*trans*-retinal-containing bacteriorhodopsin; 13-*cis*-bR, 13-*cis*-retinal-containing bacteriorhodopsin.

Correspondence: A.D. Kaulen, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, U.S.S.R.

detected a low-temperature photoreaction originating from the 13-*cis* photocycle and leading to pigment P582; φ is 0.003. This pigment appeared to be competent in the photoinduced formation of K, L and M intermediates. At $t > -60\,^{\circ}C$ it converts to all-*trans*-bR.

Besides the purple membranes, the processes of dark- and light-adaptation were described in the reconstituted systems, i.e., bR proteoliposomes [24] and detergent micelles [25]. However, here the isomeric composition of dark and light-adapted states was shown to differ from that in purple membranes. For example, in the light-adapted state, both the liposomal and monomeric detergent-treated bR preserve a certain amount of 13-*cis* isomer.

The dark adaptation rate strongly depends on pH [14] and protein–protein and protein–lipid [24,25] interactions.

In the present paper we examine the 13-*cis*-bR photocycle of the dark-adapted bacteriorhodopsin in liposomal and Triton X-100-treated monomeric preparations, as well as in purple membranes and white membranes, regenerated with artificial 13-*cis*-retinal. It was found that under certain pH conditions, the 13-*cis*-bR forms an M-like intermediate in a single photon process. The 13-*cis*-bR photocycle is accompanied by electrogenesis closely resembling that of the photocycle of the light-adapted all-*trans*-bR. Some of these results were reported in a preliminary form elsewhere [26].

Materials and Methods

Purple and white membranes were isolated according to the standard procedure [27] from *Halobacterium halobium* ET1001 and JW5 strains, respectively.

To prepare bacteriorhodopsin liposomes a suspension of purple membranes (1 mg protein $\cdot$ ml^{-1}) was mixed with asolectin (15 mg $\cdot$ ml^{-1}) in a medium containing 100 mM NaCl and 50 mM Mes (pH 6.0) and sonicated for 6 min. Then the liposome suspension was centrifuged at 100 000 $\times$ *g* for 20 min. According to CD measurements, this proteoliposomal preparation contains only monomeric bR.

To prepare dark-adapted Triton X-100, solubilized bR purple membranes were incubated in a 2% (w/v) Triton X-100 solution for 24 h in a medium containing 2 mM potassium phosphate buffer at pH 7 in the dark.

Purple membranes and bR liposomes were dark-adapted for 24 h in bidistilled water or in 100 mM NaCl, respectively, slightly buffered by Mes at pH 6.0 and room temperature in the dark. The fully light-adapted state of purple membranes and liposomes is attained under constant illumination from a 70 W halogen lamp. The light was passed through a 2 cm thick heat filter, containing 5% $CuSO_4$ solution. The solubilized bR preparation was light-adapted by means of four white photoflashes (30 mJ per flash, $t_{1/2} = 400$ μs). In the case of optical measurements, $1 \cdot 10^{-5}$ M gramicidin A was added to the samples with the bR proteoliposomes to prevent the formation of the H^+ gradients.

Highly purified 13-*cis*-retinal ($\ll 1\%$ other isomers) was kindly supplied by Dr. B.I. Mitsner from M.V. Lomonosov Institute of Fine Chemical Technology, Moscow. The white membranes were regenerated with 13-*cis*-retinal at pH 6 in a spectrophotometric cuvette. Regeneration was completed within 8 min in an incubation volume of 800 μl. The medium contained 5 mM Mes and 100 mM NaCl (pH 6.1). Immediately before measurement the sample was diluted to 1.6 ml by a room-temperature complex buffer, containing 100 mM K^+-citrate-phosphate-borate solution at the required pH. The resultant temperature of the sample (also, during measurements) was 10 °C. The sample was studied spectrophotometrically. Light-adaptation was performed under constant illumination for 5 min at room temperature. To obtain a dark-adapted sample, both light-adapted and 13-*cis*-bR samples were incubated for 3 h at room temperature in the dark at pH 6.1.

A conventional technique using the pH-indicator pyranine was employed for measuring the transient pH changes in the sample [28,29]. Photochemical conversions in the sample were carried out by the flash photolysis technique, described previously [30,31]. To improve the signal-to-noise ratio, 4–5 optical curves were summed (20–25 in the case of pH measurement experiments). The absolute and differential spectra were recorded in the sample with a Hitachi U-3400 spectrophotometer, the cuvette positioned near the photomultiplier.

Electric measurements were made in a system containing bR proteoliposomes attached to the asolectine-impregnated collodion membrane, as described previously [30,31]. For the purpose of dark-adaptation, the sample was incubated overnight within the experimental system at room temperature in the dark. The incubation medium contained 100 mM NaCl, 15 mM $CaCl_2$ and 5 mM Mes (pH 6.0). Prior to electric measurements, the incubation medium was changed for a 100 mM K-citrate-phosphate-borate buffer at the required pH. For light adaptation, proteoliposomes attached to the collodion film were exposed to 5 min illumination. Assuming that the dark-adapted sample is composed of an equimolar ratio of all-*trans* and 13-*cis* pigments [2–6], we calculated the electric response of the 13-*cis*-bacteriorhodopsin as a difference between the response of the dark-adapted sample and half of the response of the light-adapted one.

All the data shown in the figures were obtained at saturating laser energy (5–15 mJ/cm^2 per flash). In Fig. 7A the energy was 0.3 mJ/cm^2.

Results and Discussion

The light-adapted state of bacteriorhodopsin in purple membranes and proteoliposomes observed as the red shift of the spectrum can be easily attained either by the constant light illumination for 5 min or by the impulsed light excitation. White photoflashes or green laser flashes are equally efficient. All the three techniques yielded similar results. In the case of monomeric Triton X-100 treated bacteriorhodopsin we used the white photoflash, otherwise the pigment denatured under constant light at pH > 7. The effect of green laser excitation was insignificant (40–50 flashes caused only 10–15% of 13-*cis* → all-*trans* converstion in the dark-adapted sample, whereas in the purple membranes the light adaptation process was completed under the same conditions). Four white flashes were shown to induce almost complete light adaptation without any decrease in the functional activity of the pigment. The same result was obtained when the consecutive green and blue flashes were applied to the sample, the time interval between the flashes being several milliseconds. Combination of green laser with any longwave flash was ineffective. A similar phenomenon was previously found by Shkrob and Rodionov [32]. This observation led us to propose the existence of a shortwave intermediate in the 13-*cis*-bR photocycle, capable of effective photoconversion to the all-*trans*-bR. Further experiments on the light–dark adaptation expanded our knowledge of the subject.

In the process of light adaptation the purple membranes spectrum is 10 nm red-shifted with a 10% increase in extinction at a maximum (Fig. 1). A similar pattern of changes appears in the monomeric detergent-treated bR, although the maximum shift is smaller (4–7 nm), and the change of the extinction coefficient is negligible. The differential spectra of both the purple membrane and monomeric detergent-treated bacteriorhodopsin display the same general characteristics, even though they differ in the extent of changes caused by light adaptation.

In the same experiment we examined the photoinduced transient changes in the shortwave region (recorded at 400 nm). Assuming that (i) the properties of the all-*trans*-bR are the same in the dark- and light-adapted samples and (ii) the 13-*cis* component does not contribute to the transient changes at 400 nm, after a laser flash, one should expect the response amplitude in the light-adapted state to be twice as high as compared with the dark-adapted one. That was the case for the purple membranes but not for detergent-treated monomeric bR. At pH > 7 the amplitudes at 400 nm of solubilized bR proved to be in fact the same in the light- and in the dark-adapted samples (Fig. 1c, f), approaching that of the light-adapted purple sheets. It was also shown that the 400 nm decay in solubilized bR

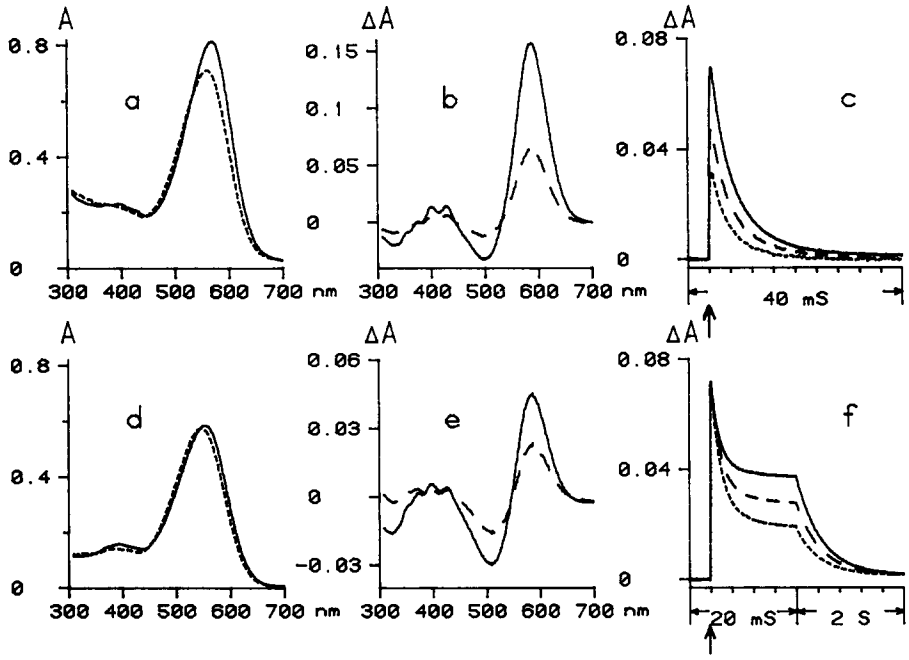

Fig. 1. Absolute and differential spectra, kinetics comparison of photoresponses at 400 nm of the light- (———) and dark-adapted (- - - - -) purple membranes (a, b, c, respectively) and 2% Triton X-100-solubilized bR (d, e, f, respectively). The widely dashed (— — —) curves signify the intermediate state of light adaptation in both purple membrane (b, c) and Triton X-100 treated bR (e, f). The incomplete light adaptation in the purple membrane was attained after 1 min illumination of the sample. The respective state in the solubilized bR was obtained after a single white flash applied to the sample. The probe contained: 12,5 μM bR, 100 mM K-citrate-phosphate-borate buffer (pH 7), 23° C. 2% (w/v) Triton X-100 was added in the case of d, e, f. Here and in the rest of the article, vertical arrows indicate the laser flash.

is clearly biphasic: a portion of 400 nm decomposes in the millisecond, the rest – in the seconds time-scale. The amplitude contribution of long-lived component increased after light adaptation. The continuous dark adaptation of the light-adapted monomeric samples resulted in the increased contribution of the short-lived phase at the expense of the long-lived one. The process was accelerated at lower pH. In purple sheets the long-lived M intermediate is absent and the decay is completed within 20 ms.

The data in Fig. 1 provide a basis for the assumption that in the solubilized bR preparation not only all-*trans*- but also 13-*cis*-bR is competent in the formation of M.

The simplest explanation of the 400 nm biphasic decay is that the M intermediate originating from all-*trans*-bR is long-lived, whereas that from 13-*cis*-bR is short-lived.

Further experiments showed, however, that the situation is, in fact, more complex. An indication was obtained that the M intermediate formed from 13-*cis*-bR contributes to some extent to slow 400 nm decay, while the all-*trans* component is present in the fast process.

The long-lived M displayed a strongly pH-dependent character. At higher pH its decay was found to decelerate with concomitant amplitude increase, while the fast phase to be insensitive to pH conditions within a pH range of 5–9 (Fig. 2A).

At pH > 8 an intermediate process was separated in the 400 nm decomposition of the dark-adapted sample (indicated in Fig. 2A by an asterisk). The time constant closely resembles that of the decay of the longwave intermediate, isolated spectroscopically around 610 nm. The latter is slowed down with pH increase (Fig. 2B, $t_{1/2}$ = 1.5–3, 15, 80 ms at pH 6.5, 7.6, 8.6, respectively), whereas the rise time of the optical changes at 610 nm is indifferent to pH conditions and repeats the character of the fast M decomposition. The absorption changes at both 400 and 610 nm, responsible for the longwave intermediate, are considerably suppressed under light (Fig. 2B), suggesting its 13-*cis* origin.

In our opinion, the coincidence of kinetics between the process of longwave intermediate formation and the fast M decay indicates a fast equilibration process occurring between the two forms in photochemical conversions. The photoresponse kinetics are reproduced under green laser flashes, thus suggesting the photocyclic character of the whole process.

Fig. 3 represents the light-induced pH changes in a solution of bR in 2% Triton X-100. To follow these changes we applied the conventional technique with the pH indicator dye [28,29]. The pH response, recorded after a laser flash, displayed a biphasic relaxation kinetics. In the dark-adapted bR, the contribution of the fast phase was higher than in the light-adapted one. Comparing these data with the kinetics of M decay and the longwave intermediate formation – decomposition – one may conclude that proton uptake takes place together with the decay of the O-like intermediate of 13-*cis*-bR (the faster phase) and the slow decomposition of M in the all-*trans*-bR photocycle (the slower phase). It is remarkable that the amplitude of pH responses in both dark- and light-adapted solutions do not differ significantly. Thus, the 13-*cis*-bR photocycle is accompanied by reversible protonation-deprotonation of bR.

As shown in Fig. 4, the major features of the light-induced difference spectra associated with the fast and slow components of relaxation at 400 nm in the dark-adapted solubilized sample include negative and positive bands with a crossover around 450 nm. Both shortwave bands are near 410 nm and the longwave

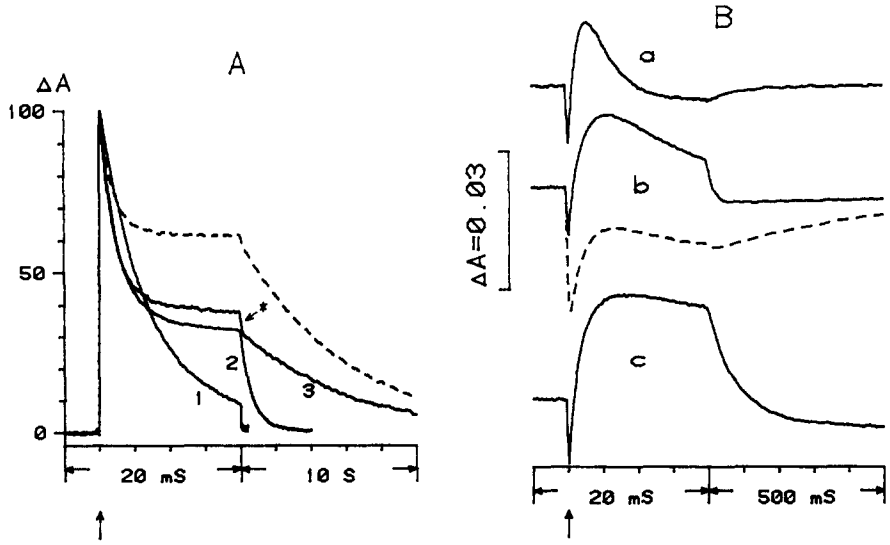

Fig. 2. Kinetics comparison of photoresponses at 400 nm (A) and kinetic changes at 620 nm (B) in the light- (- - - - -) and dark-adapted (———) samples of solubilized bR. The probe composition, as in Fig. 1, d–f. pH conditions in A: pH 4, 7.3 and 8.3 correspond to 1, 2, 3 curves, respectively; in B: pH 6.5, 7.6 and 8.6 correspond to 1, b and c. The light-adapted state curve at 400 nm was recorded at pH 8.3, 23° C.

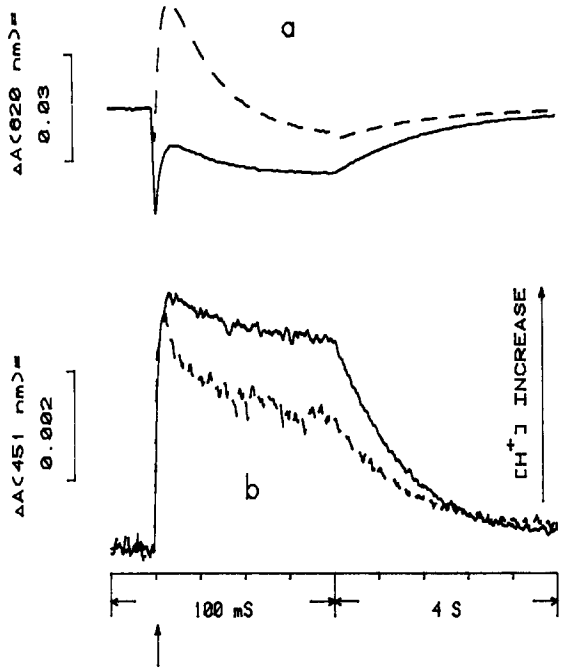

Fig. 3. Kinetics comparison between the optical response at 620 nm (a) and the light-induced pH changes (b) in the Triton X-100 solubilized dark- (- - - - - -) and light-adapted (———) samples. The probe composition: 12.5 μM bR, 200 mM NaCl, 40 μM K-citrate-phosphate-borate buffer, pH 7.5, 23° C (in b, +100 μM pyranine).

band of the slow phase differential spectrum is 10–20 nm red-shifted against that of the fast phase. The intensity of the slow component longwave band is 1.7-times that of the 410 nm band, as compared with the 1.2 ratio characteristic of the fast component spectrum. It is noteworthy that the 1.7 ratio is inherent in the all-*trans*-bR photocycle of purple membranes. Light adaptation of the monomeric sample resulted in a red shift of the fast longwave band with a consequent increase in the minimum/maximum ratio. The differential spectrum of the slow component remained unaltered.

These changes may suggest that some part of the all-*trans* pigment molecules has a photocycle with the short-lived M-intermediate and their contribution decreases at higher pH. In addition, the short-lived M may interfere with the differential spectra of both short- and long-lived M components indirectly, for according to our data [33–36] it gives rise to the slow P intermediate in the all-*trans*-bR photocycle. In solubilized bR, the recovery of the ground state from P may occur in the seconds time-scale at pH > 7.

13-*cis*-bR gradually loses the ability to form M at pH < 7, as the difference between the amplitudes of transient responses at 400 nm of the light and dark preparations becomes greater. At pH < 5 it reaches 1.3–1.4 (Fig. 5).

Fig. 6a, b represents the pH dependence of the 400 nm maximal amplitude in both light- and dark-adapted proteoliposomal monomeric preparations and the light-to-dark amplitude ratio. At pH 6.0 the response of the light-adapted preparation at 400 nm was shown to have an amplitude twice as high compared with the dark-adapted one, while at pH 9.2 both amplitudes appeared nearly equal. In the purple sheets the respective difference at pH 5–6 is 2–2.2-fold. At pH > 9.5 the difference is confined to 1.4–1.5, the curve reaching a relative plateau (Fig. 6c, d). The process is developed with apparent pK 8.5; in the low ionic conditions the pK value is one pH unit shifted to the higher pH (not shown). Thus, the 13-*cis* photocycle of the purple sheets is less efficient in M formation as compared with the monomeric bR samples.

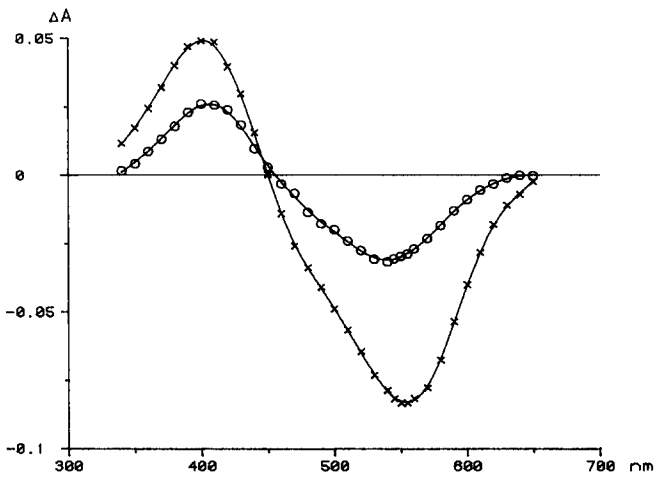

Fig. 4. Light-induced difference spectra associated with the fast (×) and slow (○) processes in the transient relaxation at 400 nm of the dark-adapted solubilized sample. The sample composition, as in Fig. 1, –f, pH 8.5, 23° C. The differential spectrum of the slow phase was obtained 550 ms after the laser flash. The differential spectrum of the fast phase was obtained as the difference between the optical changes at 140 μs and 550 ms after laser flash., i.e., between the maximal M accumulation and the completed O decay.

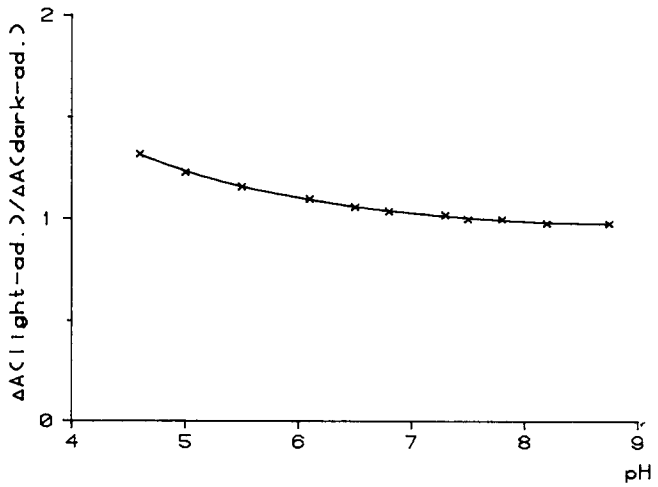

Fig. 5. pH dependence of the light-to-dark photoresponse amplitude ratio, recorded at 400 nm in the Triton X-100-solubilized bR. Sample composition, as in Fig. 1, d–f, 23° C.

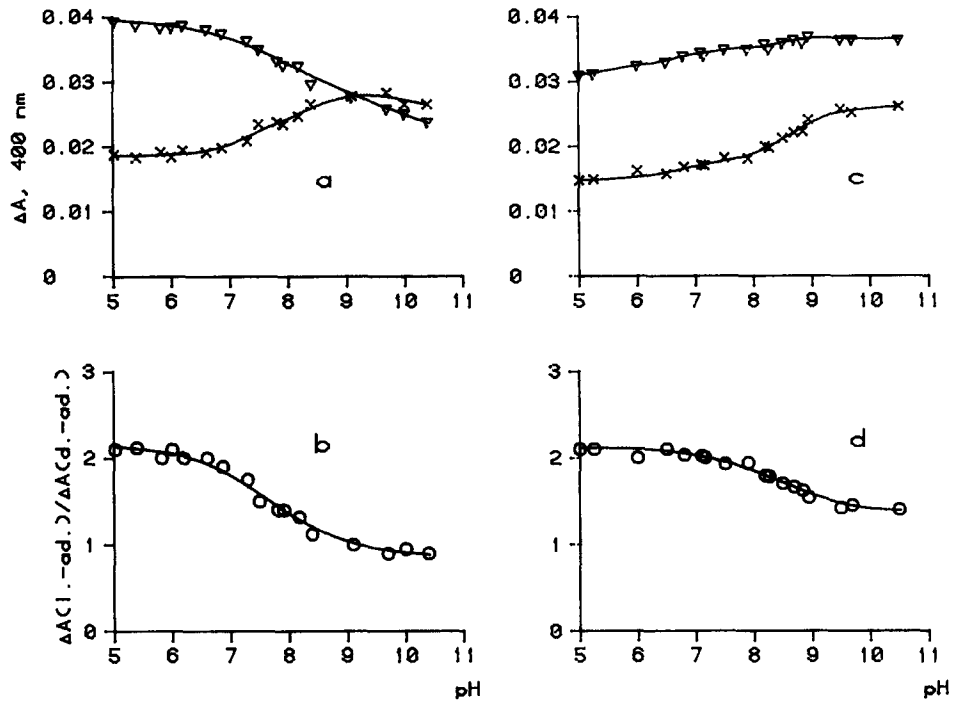

Fig. 6. pH-dependence of the photoresponse amplitudes recorded at 400 nm in the light- (▽) and dark-adapted (×) samples and light-to-dark amplitude ratio (○) at 400 nm in bR proteoliposomes (a, b) and purple membranes (c, d). The probe contained: 12 μM bR, 100 mM K-citrate-phosphate-borate buffer. In a and b $1 \cdot 10^{-5}$ M gramicidin A was added to the sample.

To anticipate the possible objections here, we want to especially emphasize that the envisaged pH dependence of bR optical responses is considered similar to pH changes of functional activity of an enzyme. Our observations are not meant to characterize the isomeric state of pigment appearing in the dark and after illumination at higher pH (for example) in stationary conditions, but implies that the isomeric composition does not change with pH relative to that existing at pH 7. In all cases both dark- and light-adapted samples were prepared in the same conditions of neutral pH, then pH was rapidly changed to the required point and the sample was immediately subjected to the optical measurements. The rapid change from neutral to alkaline pH and back again restored the initial amplitude and kinetics of the optical responses, indicating that no significant changes in the isomeric composition occur in the sample during this procedure.

Fig. 7 shows the relaxation kinetics of both light- and dark-adapted purple membranes recorded at 400 nm, pH 10.5. A comparatively low laser intensity was applied to minimize cooperative effects in trimers [37, 38]

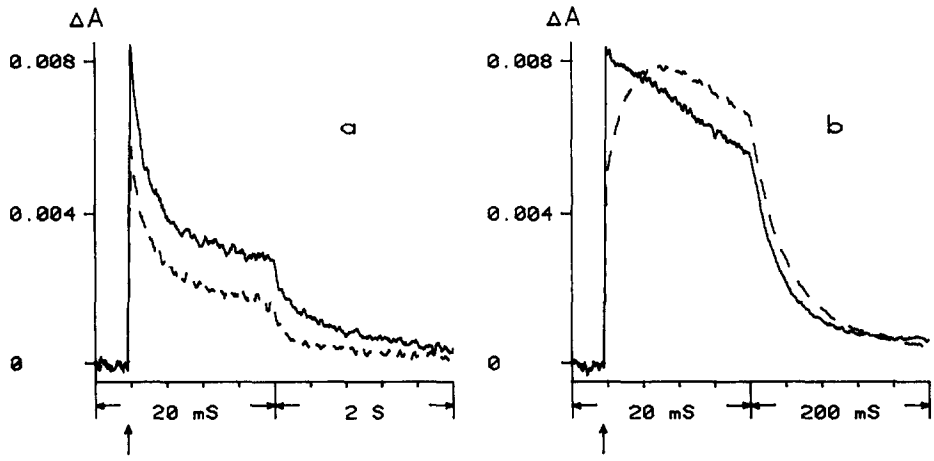

Fig. 7. Optical responses recorded at 400 nm (a) in the light (———) and dark-adapted (- - - - - -) purple membranes. The laser intensity was reduced to 0.3 mJ. The calculated (see Materials and Methods) 13-*cis*-bR optical response at 610 nm (b), recorded at pH 7 (———) and 10.5 (- - - - - -).

produced on the transient kinetics. One can see that after light adaptation, a 1,4-fold change of the whole response is accompanied by a 2-fold change of the slow component amplitude. In agreement with our previous observations in solubilized bR we conclude that the decay of M intermediate in the 13-*cis*-bR photocycle contributes to the fast component of relaxation kinetics at 400 nm, while the slow component belongs to M of all-*trans*-bR.

In the longwave range of the spectrum (Fig. 7b) we compared the formation and decay of 13-*cis*-bR intermediates appearing at pH 6.0 and 9.2. At pH 6.0 the fast rise of absorption at 610 nm indicated the formation of bathointermediate, referred to elsewhere as K610, decaying at $t_{1/2} = 34$ ms. At pH 9.2 the amplitude of the unresolved rise is reduced by 30–40%. The slower positive phase pursues the fast M decay kinetics and seems to be responsible for the formation of an O-like analog in the 13-*cis*-bR photocycle.

Fig. 8 shows the absorption changes at 400 nm, recorded in a suspension of white membranes, regenerated with 13-*cis*-retinal. At pH 6.1 no M formation is actually observed. The traces of M are most likely connected with thermal *cis* ⇌ *trans* equilibration, accelerated in the regenerated pigment in comparison with the native purple membranes (our unpublished observations). Light-adaptation significantly increased the amplitude. The subsequent dark-adaptation decreased it 2-fold. At pH 9.8 the 13-*cis*-bR response amplitude is equal to that of the light-adapted sample and slightly reduced in comparison with the light-adapted sample at pH 6.1 owing to 460 nm pigment formation (see below). At higher pH the response ampiltude of regenerated 13-*cis*-bR sample is even higher than that after light-adaptation (not shown). The two pigments exhibit a pronounce difference in the relaxation kinetics at 400 nm. The subsequent dark-adaptation of both pigments produced intermediate effect.

The differential spectrum of the M intermediate of the regenerated 13-*cis*-bR corresponded roughly to that of the light-adapted sample (not shown). Therefore, in these conditions direct measurement of the 13-*cis*-bR optical response confirms that the M intermediate is formed in the 13-*cis*-bR photocycle.

Similar results were obtained with the pigment regenerated from apomembranes (not shown). However, in this case the formation of the 460 form is less efficient at the respective high pH values and M intermediate in the photocycle of 13-*cis*-bR is produced to a lesser extent than that in the all-*trans*-bR photocycle. This is closely correlated with the results obtained with the native purple membranes (see above).

At high pH we examined the electrogenic activity of 13-*cis*-bR in comparison with the well-established pattern for the all-*trans* pigment in the neutral medium. The interpretation of the kinetics data obtained in a system designed in our laboratory, as described in [15,16,28,30,31], includes several points. It is shown that the electric potential generation of the all-*trans* pigment at neutral pH comprises three phases: one negative and two positive – the micro- and the millisecond ones. The low amplitude negative phase may reflect charge displacement within the chromophore during bathointermediate formation. The positive micro- and millisecond phases follow proton movements within the membrane. The microsecond phase correlated with the formation of

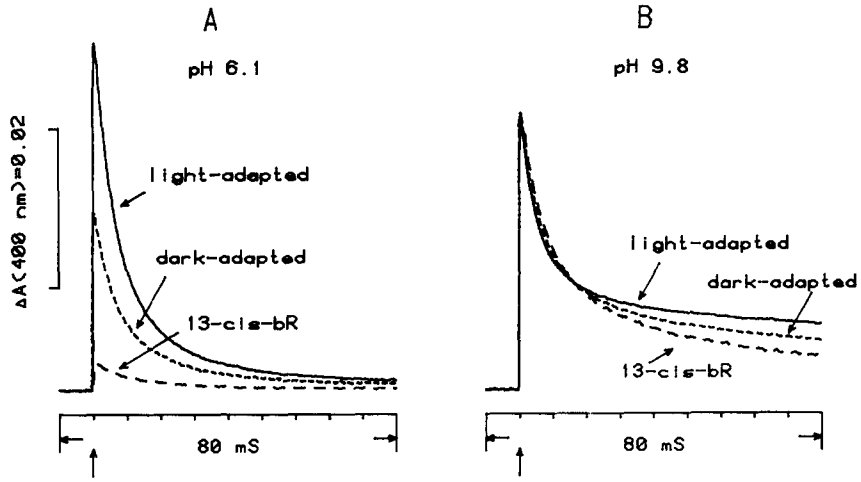

Fig. 8. Absorption changes at 400 nm, recorded at pH 6.1 (A) and 9.8 (B) in regenerated purple membranes suspension. The white membranes were regenerated at pH 6 in a spectrophotometric cuvette. Regeneration was completed within 8 min. The medium contained 5 mM Mes/100 mM NaCl, 0 °C. The incubation volume was 800 μl. For measurement, the sample was diluted with the complex buffer at room temperature. The final probe contained: 12 μM bR, 100 mM K-citrate-phosphate-borate buffer of required pH, 10 °C. The curves indicated by solid, dashed and rarely dashed lines are related to the shortwave absorption recovery of light- and dark-adapted samples and 13-*cis*-bR. For dark-adaptation the light-adapted sample was kept in darkness for 3 h at 23 °C.

intermediate M and proton release in the medium. The millisecond phase is associated with the decay of M intermediate and proton uptake [28,31].

Fig. 9 shows the electric responses of light- and dark-adapted protoliposomes, recorded at pH 6.0 (A) and 9.2 (B). The 13-*cis*-bR response was obtained by the subtraction method assuming that the light-adapted state at both pH contained only all-*trans* pigment and the dark-adapted state included an equimolar ratio of both isomeric forms. At pH 6.0 the 13-*cis*-bR electric response is represented by a relatively small signal consisting of two phases: the fast negative and the millisecond positive ones. The positive microsecond phase is not detected.

The negative phase in the dark-adapted purple membranes and proteoliposomes was previously observed in this group [15,16] and lately confirmed in Refs. 18 and 19. Most likely it can be attributed to the existence of the longwave intermediate, found by many authors [5,12,13] in the 13-*cis*-bR photocycle. At neutral pH the longwave intermediate largely returns to the initial state and is partly transferred to the all-*trans*-bR. The latter process produced the positive millisecond phase. It is accompanied by charge displacements within the molecule. According to our observations one positive charge is shifted to not less than 0.5 of the membrane thickness. A similar result was obtained in the same system [19,40], when the 13-*cis*-bR electric signal was recorded immediately after pigment regeneration with the synthetic 13-*cis*-retinal.

Another situation appared at pH 9.3, where the 13-*cis*-bR is capable of M intermediate formation. In contrast to the 13-*cis*-bR response at pH 6.0, the electric response at pH 9.2 comprises three phases instead of two, the kinetic pursues the general patatern of electric response inherent in the proton translocating all-*trans*-bR at pH 6.0. The amplitude of the dark preparation appeared even higher than that of the light-adapted state (Fig. 9B). The relaxation kinetics of both light- and dark-adapted signal are identical and determined by the RC-time constant for the lipid-impregnated collodion film (not shown). Thus taking into account the ability of detergent-treated 13-*cis*-bR to release and uptake H^+, respectively, in the course of M intermediate formation and relaxation of the initial state, we conclude analogously to the *trans*-bR [28,31] that the 13-*cis*-bR electric response at pH 9.2 is due to transmembrane H^+ transfer, while its micro- and millesecond phases are associated with the H^+ release and uptake.

The change of 13-*cis*-bR photoconversion mode is not the only process which develops at higher pH. For illustration we return to Fig. 6, showing the pH-dependence of proteoliposomes and purple membranes transient responses at 400 nm. In the purple membranes suspension, the amplitude of transient changes remains practically unaltered within the pH range 5–10.5 (it increases slightly at higher pH). The amplitude curve of light-adapted proteoliposomes, however, declines significantly at pH > 6. At pH > 9 the transient response in the dark-adapted sample has an even higher amplitude than in the light-adapted one. Assuming that the general patterns of M formation in the all-*trans* photocycle are similar for purple membranes and proteoliposomes, the observed effect was attributed to another process emerging in the all-*trans*-bR at higher pH. Further investigations showed that the pK_a of the observed changes coincided with the formation of the shortwave pigment having the differential maximum at 460 nm (Fig. 10). Within the range between pH 7 and 11, the form of the curve with positive and negative bands at 460 and 575 nm, respectively, remains unchanged, while the intensity of both spectral bands in the light-adapted state is 1.6–1.8-fold that in the dark-adapted sample. (Fig. 10). This ratio, probably, confirms the all-*trans*-bR origin of the alkaline 460 nm pigment. A similar form

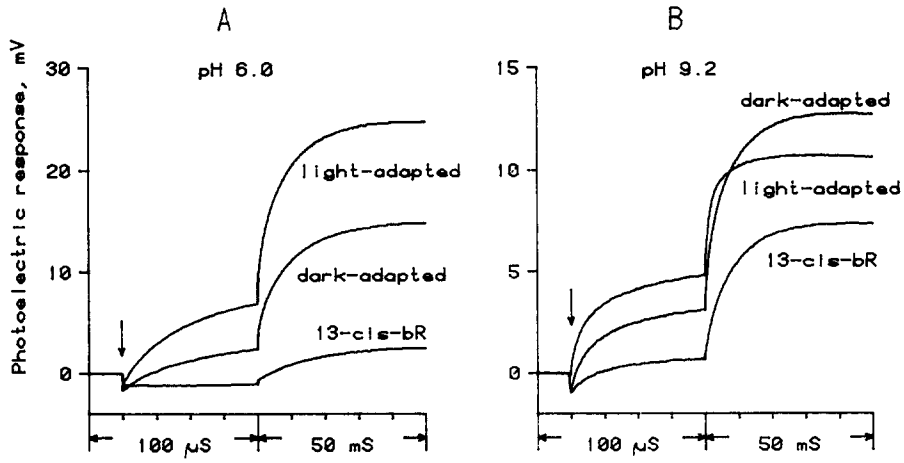

Fig. 9. Electrogenesis in the system of bR proteoliposomes, attached to the asolectin-impregated collodion film at pH 6.0 (A) and 9.2 (B) in 100 mM K-citrate-phosphate-borate buffer. 13-*cis*-bR response is a contribution of 12-*cis*-bR component to the electric signal of dark-adapted bR (see Materials and Methods).

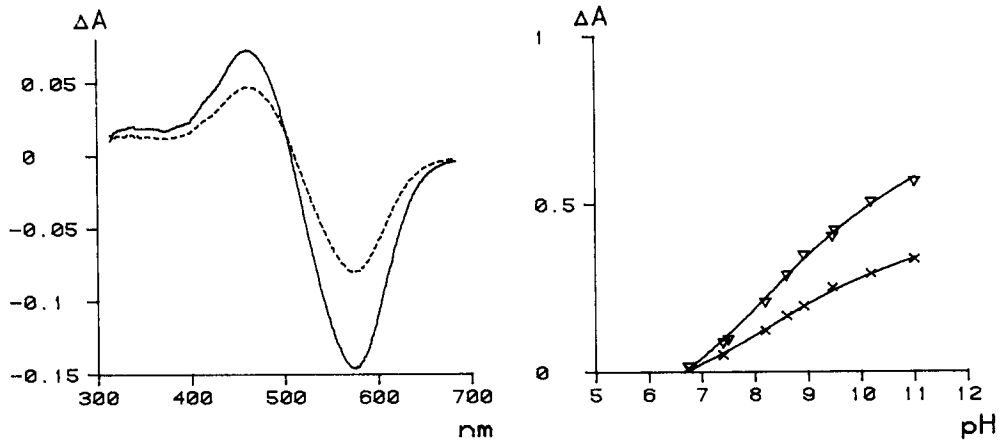

Fig. 10. Differential spectra of the light (———) and dark-adapted (- - - - -) bR proteoliposomes, recorded at pH 8.2 against pH 6. pH-dependence of the 460-to-575 nm difference amplitude in the light- (▽) and dark- (×) adapted samples. The sample contained: 12 μM bR, 100 mM K-citrate-phosphate-borate buffer, $1 \cdot 10^{-5}$ M gramicidin, 23°C.

was found in purple membranes at pH > 11. The alkaline pigment appeared photoactive and, in its turn, produced a slowly decaying shortwave M-like intermediate. One can see in the amplitude differential spectra recorded 0.6 ms after the flash (Fig. 11) a spectral shoulder around 460 nm, which is more pronounced at pH 9.5 as compared with that at pH 6.0. In the differential spectrum recorded 1 s after flash at pH 9.5 a shortwave-shifted band of absorption decrease appears together with a positive band around 390 nm. Therefore, this form is the source of the most long-lived shortwave intermediate in proteoliposomes.

At higher pH we observed a gradual decrease in the amplitude of optical signal at 400 nm connected with the alkaline pigment formation. We explain this effect by the lesser efficiency of M formation in the photocycle of the alkaline pigment as compared with the same process in the all-*trans*-bR photocycle.

The pH dependence data of M formation shown in Figs. 5 and 6 were obtained with the saturating laser flash. All the three preparations, however, displayed a common pattern of behaviour when the exciting light energy was varied from 0.2 to 20 mJ per flash (1–20% bR molecules enter the photocycle). The range within 0.2–0.8 mJ per flash is characterized by a linear dependence between the optical response amplitude and the light intensity. Thus, any multi-photon processes in the 13-*cis*-bR photocycle. such as discussed in Refs. 21–23 can be excluded.

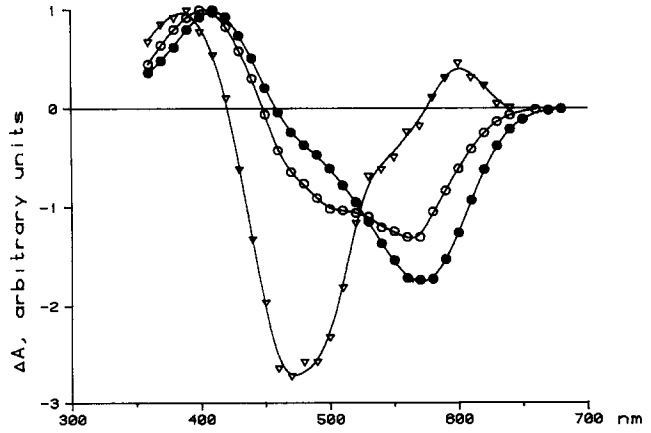

Fig. 11. Light-induced differential spectra of bR proteoliposomes, recorded 0.6 ms (○ and ●) and 1 s (▽) after laser flash. ○ and ▽, pH 9.5; ●, pH 6.5. Probe composition as in Fig. 10.

According to the data discussed above, the 13-*cis*-bR is competent in the formation of deprotonated M intermediate and transmembranous H^+ transfer at higher pH. This intermediate M was identified in solubilized bR, when excited by a blue flash. The process was shown to produce light-adaptation effect, observed as the longwave shift of the spectrum. Besides, the formation of M during the 13-*cis*-bR photoconversions of all the three preparations was accompanied by the decrease of the extent of light-adaptation under the exciting green laser flashes, thus, indicating the cyclic character of photoconversions. These observations suggest the general similarity of 13-*cis*-bR and all-*trans*-bR functional activity. It is illustrated by the scheme in Fig. 12. The groups marked as Y and Y_1 were introduced to explain the pH-dependent switch of the photocycles, electrogenesis and proton-translocating activity of both pigments.

We tentatively suppose that the formation of M intermediate coupled to H^+ release by bacteriorhodopsin is regulated by the protonation-deprotonation of an acceptor group, which receives proton directly from the Schiff base. The decrease of pH < 4 resulted in the appearance of the blue acidic pigment incapable of proton transfer and M formation [8,31,41]. The inhibition of the bacteriorhodopsin proton translocating activity as low pH can be accounted for by the protonation of the acceptor group (from the solution). Consequently, this group cannot accept another proton

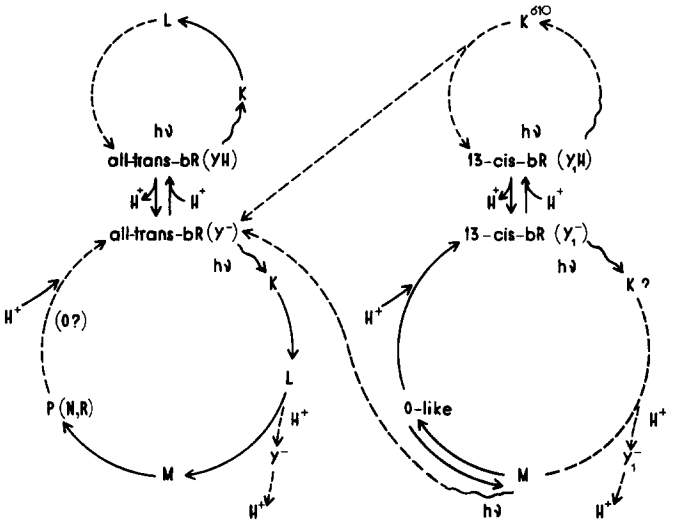

Fig. 12. A tentative scheme of the all-*trans*- and 13-*cis*-bR photochemical conversions.

from the Schiff base. But before excitation the chromophore is possibly unaware of the protonation of the group and has to pass through K and L intermediates to probe it. Thus, photocycle appears reduced to the longwave intermediates, formed before M. Consistent with this assumption is the observation of the fast negative deflection in the electric signals of both pigments.

Combined with our present results, these data suggest that the pH-dependent switch of functional activity in both isomeric forms of bacteriorhodopsin is controlled by the group(s) with different pK_a. These can be different groups or the same group with pK_a regulated by the isomerization state of chromophore. The fast negative phase of bacteriorhodopsin electrogenesis detected in the absence of H^+ transfer may be considered as an indication of its capability to translocate H^+, which is realized when the acceptor group is deprotonated.

Thus, the primary chromophore isomerization and(or) charge displacement is a necessary but insufficient condition of proton transfer initiation. To facilitate transmembranous proton transfer both pigments are likely to acquire the 'active' state, characterized by the deprotonation of proton-acceptor group(s), located at the entrance of the outward proton conducting pathway.

Addendum

After the completion of the article by us in collaboration with Dr. B.I. Mitsner and Dr. S.V. Eremin from M.V. Lomonosov Institute of Fine Chemical Technology, extraction and determination of retinal isomer by HPLC was done according to the method as described in Ref. 42. The results obtained fully substantiate the proposal used in the article. Firstly, we have not observed any changes of the retinal 13-*cis*/all-*trans* ratio after short-term incubation of dark-adapted purple membranes at high pH. Secondly, the all-*trans*-retinal is not observed in apomembranes regenerated with the 13-*cis*-retinal and is capable of forming M intermediate under high pH. Finally, under light-adaptation of monomeric Triton X-100-treated bacteriorhodopsin by white flashes, the all-*trans*-retinal content increases about twofold, while a portion of 13-*cis*-retinal decreases respectively (the dark-adapted preparation contains 40–45% all-*trans*-retinal).

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