Combined Optical and Photoelectric Study of the Photocycle of 13-*cis* Bacteriorhodopsin

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ABSTRACT The photocycle of the 13-*cis* retinal containing bacteriorhodopsin was studied by three different techniques. The optical multichannel analyzer monitored the spectral changes during the photocycle and gave information about the number and the spectrum of the intermediates. The absorption kinetic measurements provided the possibility of following the absorbance changes at several characteristic wavelengths. The electric signal provided information about the charge motions during the photocycle. The results reveal the existence of two intermediates in the 13-*cis* photocycle, one with a short lifetime having an average of 1.7 μs and an absorption maximum at 620 nm. The other, a long-living intermediate, has a lifetime of about 50 ms and an absorption maximum around 585 nm. The data analysis suggests that these intermediates are in two parallel branches of the photocycle, and branching from the intermediate with the shorter lifetime might be responsible for the light-adaptation process.

INTRODUCTION

Bacteriorhodopsin (BR), the light-driven proton pump from *Halobacterium halobium*, exists in two forms: dark- and light-adapted BR, depending on the conditions of illumination. In the dark-adapted BR, the retinal contains two conformations: 13-*cis* and all-*trans*, with an isomeric ratio of 13-*cis*/all-*trans* about 2:1 (Scherrer et al., 1987). The configuration of the so-called 13-*cis* retinal in the dark-adapted BR is 13-*cis*-15-*syn* (Harbison et al., 1984). In the K, L, M, and N intermediates of the all-*trans* photocycle, the retinal configuration is also 13-*cis*, but it is 13-*cis*-15-*anti* (Braiman and Mathies, 1982). Upon prolonged exposure to light, the 13-*cis* retinal isomerizes to the all-*trans* form, giving the light-adapted BR. At low light intensity, the light-adaptation takes place with an apparent quantum efficiency of 0.035 (Kalisky et al., 1977), which increases with light intensity (Bryl et al., 1992). Although this process is light-driven, the dark adaptation occurs through a thermal relaxation with a lifetime of about a half-hour at pH 7 and room temperature (Stoeckenius et al., 1979; Roepe et al., 1988; Váró and Bryl, 1988; Brown and Chamorovsky, 1993). The effect of pressure on the dark adaptation was also studied (Kovács et al., 1993). The state of the protein surrounding the retinal plays an important role in the isomerization process, which is suggested by the fact that the mutation of Arg-82 to Ala has a drastic effect on the dark adaptation rate constant (Balashov et al., 1993). Various pathways have been proposed for the light adaptation, with all steps of the 13-*cis* photocycle as possible branching points (Kalisky et al., 1977; Sperling et al., 1977; Váró and Bryl, 1988; Bryl et al., 1992). Some experimental data point toward the possibility that the branching occurs early in the excited state (Korenstein and Hess, 1977; Váró and Bryl, 1988). The same conclusion was drawn from molecular dynamic calculations (Logunov et al., 1994).

Although the photocycle of the BR containing all-*trans* retinal has been extensively studied, only a few papers have appeared about the photocycle of the 13-*cis* retinal containing BR (Sperling et al., 1977; Kalisky et al., 1977; Korenstein and Hess, 1997). The proposed photocycle has a K-like intermediate with a very fast decay. At −90°C, this intermediate decays in less then 0.5 μs to a long-living O-like intermediate, which has a decay time of 40 ms at 20°C and absorption maximum at 610 nm (for review, see Stoeckenius et al., 1979). The most complete study of the 13-*cis* photocycle (Hofrichter et al., 1989) contains the SVD analysis (singular value decomposition) of the time-resolved difference spectra measured on light- and dark-adapted BR, revealing the existence of at least three components during the 13-*cis* photocycle.

Usually, the analysis of the measurements uses the assumption that in the 13-*cis* photocycle there is no M intermediate and no proton transport. Today, it is generally believed that this assumption is true for BR only at neutral or lower pH, because some observations show that at above pH 7 the 13-*cis* photocycle not only produces M form but, related to it, also transports protons (Drachev et al., 1988; Kaulen et al., 1990; Drachev et al., 1993). An appropriate method to study the process of light- and dark adaptation is the measurement of the electric signals that belong to the M form (Trissl and Gärtner, 1987; Váró and Bryl, 1988) because this type of experiment does not need monitoring light. The existence of charge motions during the 13-*cis* photocycle was also observed (Drachev et al., 1978; Váró, 1987).

In this paper, new results are presented concerning the photocycle of the 13-*cis* retinal, studied by optical spectroscopic multichannel analyzer technique and optical kinetic measurements performed at two wavelengths (410 and 610

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nm). The spectrum and the time course of a fast and a slow decaying intermediate are presented. In addition, by electric measurements the charge motions during the 13-*cis* photocycle were investigated, and the same components were found. From the analysis of all the collected data, the intermediates of the 13-*cis* photocycle were resolved, and some possible conclusions emerge in connection with a branching in an early step of the photocycle. It was concluded that one of the branches might be responsible for the light adaptation process.

MATERIALS AND METHODS

Purple membrane suspension was obtained by the standard procedure from *Halobacterium halobium* strain S9 (Oesterhelt and Stoeckenius, 1974). During the optical measurements, the purple membrane suspension contained 30 μM BR, 100 mM NaCl, and 50 mM phosphate buffer. In the case of the electric signal measurements, no salt or buffer was added to the suspension. The experiments were carried out at room temperature (20°C).

The setup for the optical spectroscopic multichannel analyzer (OSMA) measurements was similar to that described elsewhere (Zimányi et al., 1989), with the fastest spectrum acquisition of 400 ns after excitation. The only modification was the use of a home-made stopped flow cuvette, synchronized to the trigger signal. The cuvette had a volume of about 0.4 ml and was connected to a thermostated reservoir of 1 l. By changing the suspension in the cuvette after every excitation, it was assured that the sample was in the same condition as in the reservoir (dark- or light-adapted) during the measurements.

The same stopped flow cuvette was used for optical kinetic measurements performed at two wavelengths (410 and 610 nm), with the setup described elsewhere (Váró and Keszthelyi, 1983).

The electric signal measurements were carried out with the method described by Keszthelyi and Ormos (1980, 1983) and modified as follows. The 2 × 15 × 10 mm cuvette contained three parallel, platinized platinum electrodes. An orienting field of 15–20 V/cm was connected for 0.5 s, the middle electrode at the positive pole and the two lateral equidistant electrodes at the negative pole. By this, an opposite orientation of the two half cuvettes was achieved. The lack of salts from the suspension assured the orientation of the purple membranes by a relatively weak electric field, and no electrolysis appeared at the surface of the electrodes. The electric signal was measured between the two lateral electrodes 5 ms after switching off the orienting field, when the orientation of the purple membranes still persisted. Only one of the two half cuvettes was excited by the laser. In the two opposite oriented parts of the cuvette, all signals, because of orientation and other artefacts, were annihilated. From the electrical point of view, the measuring system was similar to that described by Liu and Ebrey (1988). The detected electric signal corresponded to the charge motions during the photocycle, produced in the excited half cuvette. The cuvette was connected to the stopped flow instrument described above, which changed the suspension after every measurement. The electric signal showed a good stability up to the millisecond time range, but at longer times the slow baseline changes made the signal unreliable. To double-check the reproducibility of the electric signals, these experiments were repeated several times. Each data collection consisted of 100 signals averaged. The data collection for absorption kinetic and electric signals was performed as described by Gergely et al. (1993).

For the OSMA measurement, an excimer laser-pumped dye laser was used. In all of the other cases, a nitrogen laser-pumped dye laser gave the excitation. In both cases, the dye was Coumarin 153, with an emission wavelength of 540 nm.

Every experiment contained two sets of data, one taken on dark- and one on light-adapted BR. The signals corresponding to the dark-adapted BR were collected after keeping the suspension overnight in dark. During the measurement the reservoir containing the purple membrane suspension was also in dark. Before measurements on light-adapted BR, the reservoir was illuminated with a 200 W mercury lamp, using heat and yellow light filters, for about 15 min, and this light was on for the entire measuring period. All of the measurements were performed between pH 4 and 7, where there is no M intermediate corresponding to the 13-*cis* photocycle (Drachev et al., 1993) and the light-adaptation does not produce other type of isomers (Koyama et al., 1993). From the dark-adapted BR signal was subtracted the light-adapted one multiplied with a factor. This factor was determined in such a way that the resulting signal around 410 nm, corresponding to the M intermediate, disappeared. In the calculations, it was supposed that the light-adapted BR contains only all-*trans* retinal, which is generally accepted (Scherrer et al., 1987). Even if this last assumption is not valid in all conditions, the subtraction does not introduce systematic error in calculations; only the size of the difference is diminished as a small part of the 13-*cis* signal is also subtracted. After the subtraction, the remaining part is that belonging to the 13-*cis* photocycle.

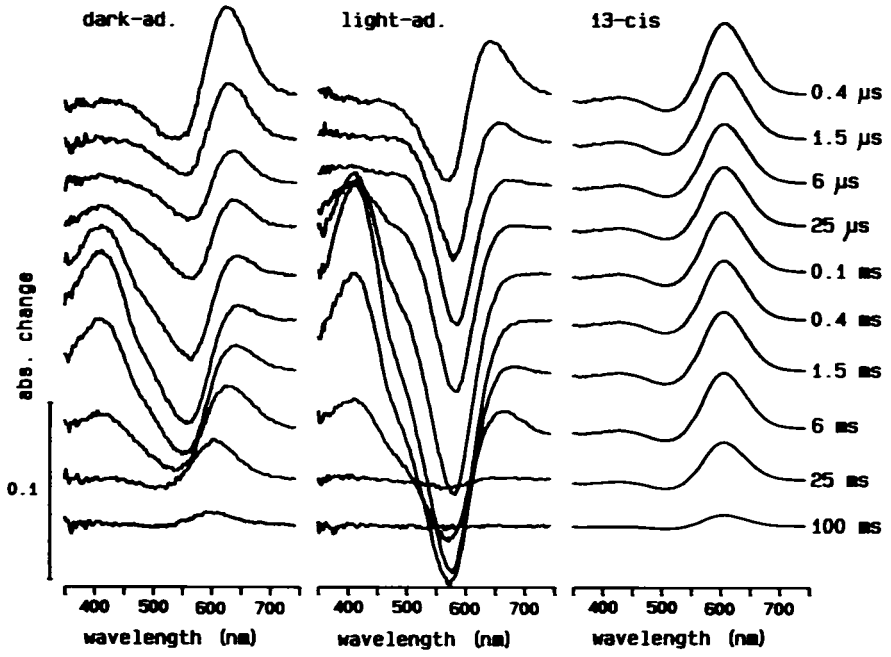

FIGURE 1 The OSMA difference spectra measured at different time delays after the excitation for the dark-adapted (*first column*) and light-adapted BR (*second column*). The suspension contained 100 mM NaCl, 50 mM phosphate buffer at pH 7, 20°C. The third column contains the SVD-filtered difference spectra corresponding to the 13-*cis* photocycle (see text).

The data analysis of the optical kinetic and electric signals of the 13-*cis* photocycle was performed as described earlier (Gergely et al., 1993). The singular value decomposition (SVD analyses) (Cao et al., 1993) of the OSMA spectra corresponding to the 13-*cis* photocycle and the global fitting of the kinetics were performed with the program SPSERV described by Bagyinka et al. (1993).

RESULTS

The absolute spectra of the unphotolyzed dark- and light-adapted BR, measured with OSMA as reference spectra, were similar to that measured with a Shimadzu UV-Vis 160 spectrophotometer (data not shown), and during the whole data collecting period they remained unchanged. This gave a control over the stability of the sample.

The 28 OSMA difference spectra of the dark- and light-adapted BR measured at pH 7 show complex changes within the measured time period (Fig. 1, *first two columns*). To subtract the light-adapted spectra in such a way that the peak corresponding to the M intermediate around 410 nm disappears, they had to be normalized with a factor of 0.52, giving a 13-*cis*/all-*trans* isomeric ratio in the dark-adapted form of 1 : 1. For a long time, the isomeric ratio was accepted to be 1 : 1 (Stoeckenius et al., 1979), but later it was modified to 2 : 1

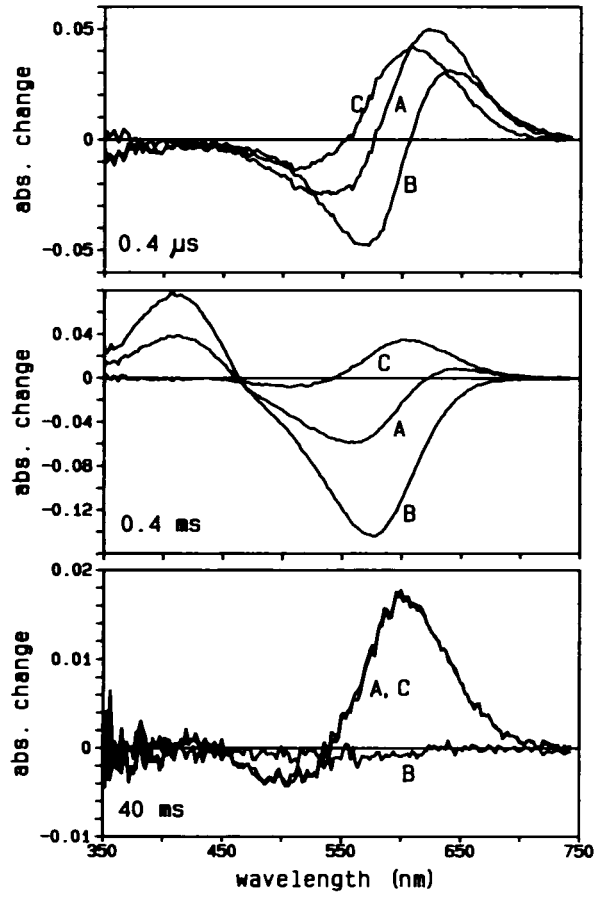

FIGURE 2 The dark-adapted (*A* spectra), light-adapted (*B* spectra) difference spectra measured at three different time delays and their difference after the light-adapted spectrum was multiplied with a normalization factor of 0.52 (*C* spectra). Difference spectra *C* correspond to the 13-*cis* photocycle.

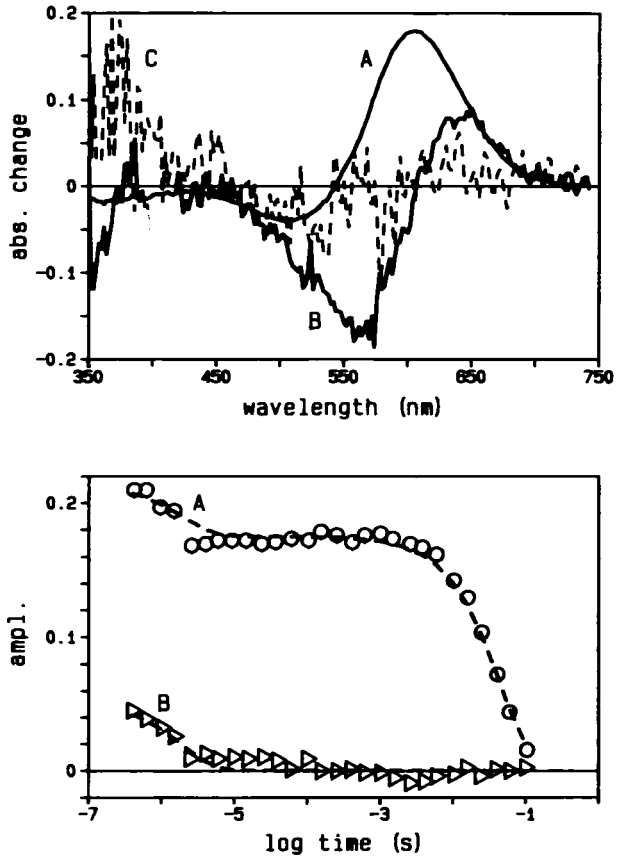

FIGURE 3 The first (*A*), second (*B*), and third (*C*) spectral component of the SVD analysis (*top* figure) and the weighted amplitude of the first two components (symbols of the *bottom* figure). The dotted lines are the fits of the amplitudes with two exponentials.

(Scherrer et al., 1987), and its temperature dependence was measured. The isomeric ratio could depend not only on temperature, but also on the pH, ionic strength, and other experimental conditions, so the 1 : 1 ratio found by us is reasonable.

The dark- and light-adapted spectra and their normalized difference, which is characteristic for the 13-*cis* photocycle, measured at three different time intervals after the excitation, are shown in Fig. 2. It can be seen that the spectra belonging to the 13-*cis* photocycle were moderately noisy. The SVD analysis of these difference spectra showed a dominant component spectrum with weight factor of 0.97 and a second spectrum with weight factor of 0.07. All other spectra contained only noise and had a weight factor less than 0.03. Although the time-dependent amplitudes corresponding to the first two components had a well determined feature, those corresponding to the other spectra had no structure, containing only noise. The difference spectra of the 13-*cis* photocycle were reconstructed with a very effective filtration of the noise (Fig. 1, *third column*) by using the first two component spectra (Fig. 3, *top*), their weight factor and the corresponding time-dependent amplitudes as described in Cao et al. (1993). These spectra show a constant feature with small amplitude change in the microsecond time range that disappears after 10 ms. The amplitudes of the first two

components, multiplied by their weight factor (Fig. 3, *symbols*), were fitted simultaneously with two exponentials (Fig. 3, *dotted line*). The two lifetimes were found to be 1.9 ± 0.5 μs and 46 ± 4 ms. Although a 2-μs lifetime was observed in the 13-*cis* photocycle only by Hofrichter et al. (1988), the long-living component was reported much earlier (Dencher et al., 1976; Kalisky et al., 1977).

From the absorption kinetic measurements performed at 410 and 610 nm (Fig. 4), the kinetics of the 13-*cis* photocycle at 610 nm were calculated. After the subtraction of the measured signals by using a normalization factor of 0.5 ± 0.03, the absorbance change at 410 nm is at the noise level (Fig 4, 410 nm, curve *C*). This shows that in both light- and dark-adapted cases the kinetics of the M is the same, supporting that in our measuring conditions only the all-*trans* photocycle produces this intermediate. On the kinetic curve of the 13-*cis* photocycle at 610 nm (Fig. 4, 610 nm, *solid line C*), the time dependence of the 610 nm amplitude from the OSMA spectra is drawn in (Fig 4, *symbols*), normalized to one point, showing that in both cases the kinetics were the same. The exponential fit to the measured curves revealed two lifetimes: 1.5 ± 0.4 μs and 52 ± 4 ms.

Absorption kinetic measurements were performed at seven pH values between 4 and 7, but no kinetic changes

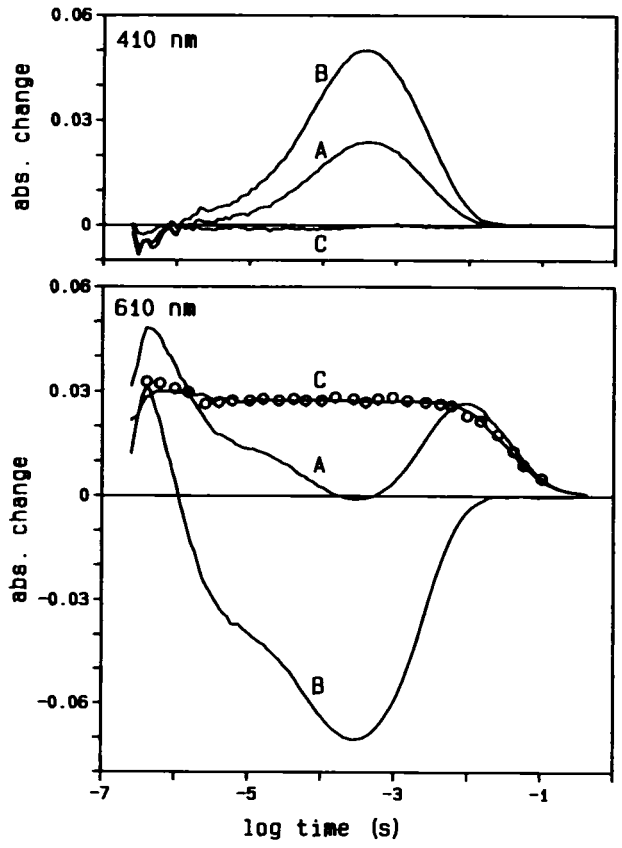

FIGURE 4 The optical kinetic measurements of the dark-adapted (curve *A*), light-adapted BR (curve *B*) measured at two wavelengths and the normalized difference of them (curve *C*). The measuring conditions are the same as for Fig. 1. The symbols are the readings of the maxima of the difference spectra from the OSMA measurements.

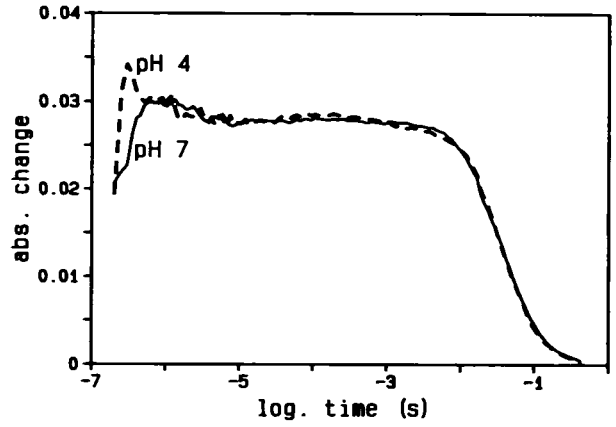

FIGURE 5 The 610 nm difference signal of the optical kinetic measurements at pH 4 and 7. The measuring conditions are the same as for Fig. 1

were found (Fig. 5), pointing out that in this pH interval the observed processes belonging to the 13-*cis* photocycle are pH-independent. It is interesting to note that in the all-*trans* photocycle, the kinetics of the M intermediate, measured at 410 nm, does have changes in this interval (not shown), an already studied effect (Váró et al., 1990). Additionally, the signal measured at 610 nm shows changes not only in the kinetics but in its form as well (not shown), because by lowering the pH the O of the all-*trans* cycle intermediate is more and more accumulated (Váró et al., 1990).

The electric signal measurements were performed at pH 7 with no salt or buffer added in the suspension. It is known that the lack of the salts affects the pH in such a way that the local pH at the surface of the membrane is lower than the pH measured in bulk (Szundi and Stoeckenius, 1989). This difference can be as high as 2 pH units, but as it was shown above, the observed processes are pH-independent. The absorption kinetic measurements at the same two wavelengths were similar to those measured in the presence of salt and buffer. The same subtraction procedure was used to determine the signal corresponding to the 13-*cis* photocycle, which provided the same normalization factor and same kinetics at 610 nm (not shown). From the current signal of the dark-adapted form was subtracted the light-adapted one by using the above determined normalization factor. The net current signal corresponding to the 13-*cis* photocycle, obtained in this way, has a positive peak after a fast negative one, similar to that observed on dried oriented samples (Váró, 1987). The positive peak is followed by a negative component (Fig. 6 *A*, *solid line*), which has no equivalent in the dried sample. Both the current signal and its integral, which is proportional to a voltage (Fig. 6 *B*, *solid line*), revealed these two components after the fast negative signal. The fitted lifetimes are 1.5 ± 0.6 and 17.5 ± 2 μs (Fig. 6, *A* and *B*, *dotted line*).

DISCUSSION

The interpretation of the SVD results of the difference spectra belonging to the 13-*cis* photocycle, suggests the existence of two intermediates in the analyzed time period, decaying

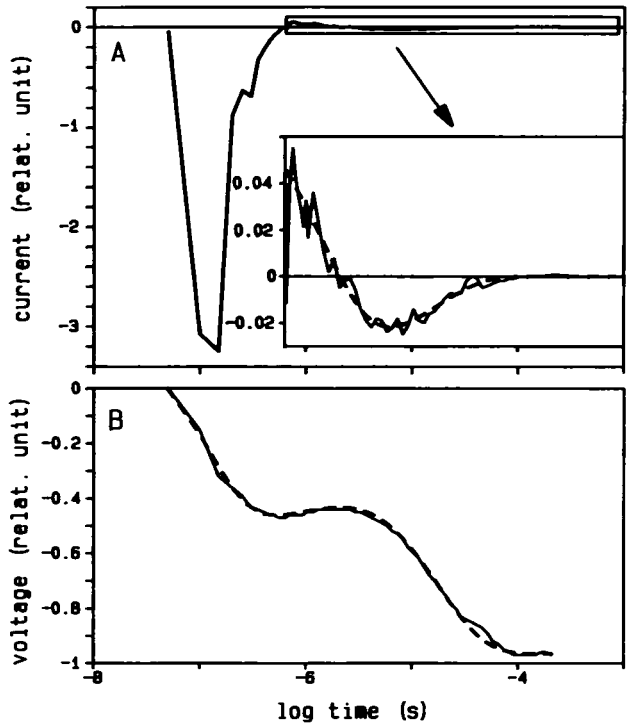

FIGURE 6 The current (*A*) and its integral (*B*) signal (——) and the fit with two exponents (- - - -). The measurements were performed at pH 7 in distilled water, no salt and buffer added.

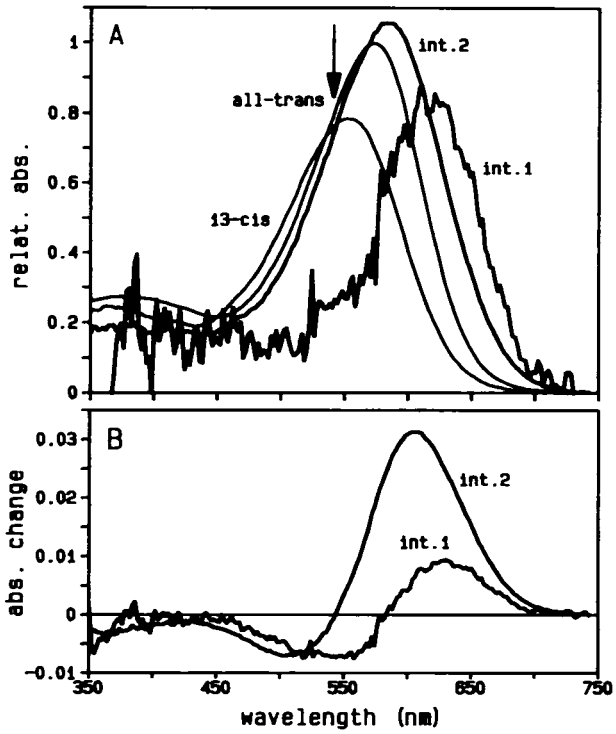

FIGURE 7 The difference (*B*) and absolute spectra (*A*) of the intermediates, when two parallel branches were considered (see text). As a reference, on Fig. *A* the spectra of the 13-*cis* and all-*trans* retinal containing BR were also plotted. The arrow shows the excitation wavelength of the dye laser.

with time constants 1.9 μs and 46 ms, respectively. Two models could be considered for this type of kinetics: a) a parallel model that could originate from a very early branching, or b) a sequential model. By using the two component spectra and the fitted amplitudes, it was possible to reconstruct the model-dependent difference spectrum of the intermediates. Adding back the absolute spectrum of the 13-*cis* BR, calculated earlier from the OSMA reference measurements (see Fig. 7 *A*), the absolute spectrum of the intermediates could be estimated. This calculation gives in the same time the relative value of the excitation produced by laser flash. The procedure of calculation was described elsewhere (Váró and Lanyi, 1991). From the light-adapted difference spectra, it was estimated that 19 ± 1% of BR was excited.

In the case of the parallel model,

$$\mathrm{BR} \rightarrow \cdots \rightarrow \begin{cases} \cdots \rightarrow \mathrm{Int1} \rightarrow \mathrm{BR} \\ \cdots \rightarrow \mathrm{Int2} \rightarrow \mathrm{BR} \end{cases}$$

the reconstructed difference and absolute spectra of the intermediates are shown on Fig. 7. Although the difference spectrum of the first intermediate looks similar to that of the K intermediate in the all-*trans* photocycle, it can be ruled out that just an erroneous subtraction let a small quantity of K in the analyzed signals, because its lifetime is 3 times slower than that corresponding to the decay of the K. The decay time of the K was found to be 0.6 μs, compared with the first intermediate in 13-*cis* photocycle, which decays with 1.7 μs. In the all-*trans* photocycle, there was no lifetime close to that in question. The calculations revealed that the laser excited roughly 9 ± 0.5% 13-*cis* BR if the normalization factor of 0.5 is considered. This 9% branched in such a way that 1.5% was in the fast decaying intermediate and about 7.5% was in the slow decaying intermediate. Hofrichter et al. (1989) in their analysis observed a lifetime of 2 μs, and they proposed that it could have been related "to the formation of an all-*trans* chromophore." The relative quantum efficiency of the fast decaying component, defined as the part of the absorbed quanta producing the desired effect, is roughly 0.05 in our measuring conditions of the OSMA spectra. As it was shown in the introduction, the relative quantum efficiency of the light-adaptation at low light intensity is 0.035 (Kalisky et al., 1977) and increases with the excitation intensity (Bryl et al., 1992). At a relative high excitation, it could reach the value of 0.05. All these suggest that the fast branch of the 13-*cis* photocycle is related to the light adaptation process of the BR. The branching occurs in the early part of the photocycle, in good agreement with the observation (Váró and Bryl, 1988) and theoretical calculation (Logunov et al., 1994) that it happens in the excited state. Measurements performed on dried BR samples show that the removal of the water blocks the main path of the 13-*cis* photocycle, but the process of light adaptation still exists, pointing also to an early branching after excitation (Korenstein and Hess, 1977).

The same calculations made for the sequential model

$$\mathrm{BR} \rightarrow \cdots \rightarrow \mathrm{Int1} \leftrightarrow \mathrm{Int2} \rightarrow \mathrm{BR}$$

raised a contradiction. The 13-*cis* photocycle gave good in-

termediate spectra (not shown) for about 7.5 ± 0.5% excitation, which corresponds to a total excitation of only 15–16%, instead of the 20% calculated earlier, showing that the parallel model seems to be the correct one, which describes properly the 13-*cis* photocycle of the BR. Raising the excitation to over 8% yielded unacceptable spectra for the intermediates.

The absorption kinetic measurements gave almost the same two lifetimes (1.5 μs and 52 ms). The pH independence of the kinetic signals in the range between pH 4 and 7 (Fig. 5) shows that the observed processes are independent from the proton concentration and from the changes connected to the eventually titrated amino-acids, suggesting that these events occur deep inside the protein, close to the retinal.

The electric signal measurement (Fig. 6) was very sensitive in the studied time interval (up to 1 ms). In the time interval presented on Fig. 6, the signal was reproducible within the noise. The fast negative signal is followed by a positive one that decays with a lifetime of about 1.5 μs, corresponding to the decay of the fast intermediate. The small amplitude and the positive sign of the signal, which is opposite to the all-*trans* to 13-*cis* isomerization signal observed in the light-adapted BR, suggests that this corresponds to the 13-*cis* to all-*trans* isomerization of the retinal during the light adaptation. The second negative signal, with a decay time of 17 μs, has no optical correspondent. It might be that this is related to the relaxation of the side chain of some charged amino acids. This is supported by the fact that in dried samples, where the conformational motions are hindered, this signal was not observed (Váró, 1987). The missing optical correspondent reflects that it could happen further from the retinal, not in its surroundings.

The measurements thus reveal the existence of two intermediates in the photocycle of the 13-*cis* BR. It was shown that the most probable model is the parallel one, in which the main branch contains a slow decaying intermediate. This was observed by others from kinetic measurements and named C^{610} (Kalisky et al., 1977; Hofrichter et al., 1989), having a difference maximum at around 610 nm. From the calculated absolute spectrum, it can be seen that this slow intermediate has the absorption maximum at around 585 nm (Fig. 7*A*) and decays with a lifetime of about 50 ms back to 13-*cis* retinal containing BR. The fast decaying intermediate has its absorption maximum at around 620 nm and, possibly, it decays to the all-*trans* retinal containing BR in about 1.7 μs, being responsible for the light adaptation of the dark-adapted BR as was also suggested by others (Hofrichter et al., 1989). Because this intermediate participates in a relative small quantity (1.5%) in the photocycle and the absorbance difference between the 13-*cis* and all-*trans* retinal containing BR are not extremely large, it is hard to show that some amount of 13-*cis* retinal was converted to all-*trans* retinal during a single photocycle. As a conclusion, a possible model of the 13-*cis* photocycle is

$$BR^{13\text{-}cis} \rightarrow \cdots \begin{cases} \rightarrow \cdots \rightarrow C^{620} \rightarrow BR^{all\text{-}trans} \\ \rightarrow \cdots \rightarrow C^{585} \rightarrow BR^{13\text{-}cis} \end{cases}$$

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REFERENCES

Bagyinka, Cs., J. P. Whitehead, and M. J. Maroney. 1993. An X-ray absorption spectroscopic study of nickel redox chemistry in hydrogenase. *J. Am. Chem. Soc.* 115:3576–3585.

Balashov, S. P., R. Govindjee, M. Kono, E. S. Imasheva, E. P. Lukashev, T. G. Ebrey, R. K. Crouch, D. R. Menick, and J. Feng. 1993. Effect of the arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle. *Biochemistry*. 32: 10331–10343.

Braiman, M. S., and R. A. Mathies. 1982. Resonance Raman spectra of bacteriorhodopsin's primary photoproduct: evidence for a distorted 13-*cis* retinal chromophore. *Proc. Natl. Acad. Sci. USA*. 79:403–407.

Brown, L. S., and S. K. Chamorovsky. 1993. Light adaptation blockage in dehydrated purple membrane does not result from retinal isomerisation inhibition. *J. Photochem. Photobiol. B Biol.* 18:123–126.

Bryl, K., M. Taiji, M. Yoshizawa, and T. Kobayashi. 1992. Light adaptation of dark-adapted bacteriorhodopsin studied by nanosecond time-resolved absorption spectroscopy. *Photochem. Photobiol.* 56:1013–1018.

Cao Y., G. Váró, A. L. Klinger, D. M. Czajkowsky, M. S. Braiman, R. Needleman, and J. K. Lanyi. 1993. Proton transfer from Asp-96 to the bacteriorhodopsin schiff base is caused by a decrease of the pK_a of Asp-96 which follows a protein backbone conformational change. *Biochemistry*. 32:1981–1990.

Dencher, N. A., Ch. N. Rafferty, and W. Sperling. 1976. 13-*cis* and *trans* bacteriorhodopsin: photochemistry and dark equilibrium. Berichte der Kernforschungsanlager Julich GmbH. 1–42.

Drachev, L. A., S. V Dracheva, and A. D. Kaulen. 1993. pH dependence of the formation of an M-type intermediate in the photocycle of 13-*cis*-bacteriorhodopsin. *FEBS Lett.* 332:67–70.

Drachev, L. A., A. D. Kaulen, and V. P. Skulachev. 1978. Time resolution of the intermediate steps in the bacteriorhodopsin-linked electrogenesis. *FEBS Lett.* 87:161–167.

Drachev, L. A., A. D. Kaulen, V. P. Skulachev, and V. V. Zorina. 1988. Electrogenic photocycle of the 13-*cis* retinal containing bacteriorhodopsin with an M intermediate involved. *FEBS Lett.* 239:1–4.

Gergely, C., C. Ganea, G. I. Groma, and G. Váró. 1993. Study of the photocycle and charge motions of the bacteriorhodopsin mutant D96N. *Biophys. J.* 65:2478–2483.

Harbison, G. S., S. O. Smith, J. A. Pardoen, C. Winkel, J. Lugtenburg, R. A. Mathies, and R. G. Griffin. 1984. Dark-adapted bacteriorhodopsin contains 13-*cis*, 15-*syn* and all-*trans*, 15-anti retinal Schiff base. *Proc. Natl. Acad. Sci. USA*. 81:1706–1709.

Hofrichter, J., E. R. Henry, and R. H. Lozier. 1989. Photocycles of bacteriorhodopsin in light- and dark-adapted purple membrane studied by time-resolved absorption spectroscopy. *Biophys. J.* 56:693–706.

Kalisky, O., C. R. Goldschmidt, and M. Ottolenghi. 1977. On the photocycle and light adaptation of dark-adapted bacteriorhodopsin. *Biophys. J.* 19: 185–189.

Kaulen, A. D., L. A. Drachev, and V. V. Zorina. 1990. Proton transport and M-type intermediate formation by 13-*cis*-bacteriorhodopsin. *Biochim. Biophys. Acta Bioenerg.* 1018:103–113.

Keszthelyi, L., and P. Ormos. 1980. Electric signals associated with the photocycle of bacteriorhodopsin. *FEBS Lett.* 109:189–193.

Keszthelyi, L., and P. Ormos. 1983. Displacement current on purple membrane fragments oriented in a suspension. *Biophys. Chem.* 18:397–405.

Korenstein, R., and B. Hess. 1977. Hydration effects on *cis-trans* isomerization of bacteriorhodopsin. *FEBS Lett.* 82:7–11.

Kovács, L., G. U. Nienhaus, R. Philipp, and A. Xie. 1993. Pressure effects on the dark-adaptation of bacteriorhodopsin. *Biophys. J.* 64:1187–1193.

Koyama, Y., H. Nakasu, Y. Mukai, and F. Tokunaga. 1993. Isomerisation of the retinylidene chromophore of bacteriorhodopsin in light adaptation: intrinsic isomerisation of the chromophore and its control by the apoprotein. *Photochem. Photobiol.* 57:732–738.

Liu, S. Y., and T. G. Ebrey. 1988. Photocurrent measurements of the purple membrane oriented in a polyacrylamide gel. *Biophys. J.* 54:321–329.

Logunov, I., W. Humphrey, K. Schulten, and M. Sheves. 1994. Molecular dynamics study of the 13-*cis* photocycle of bacteriorhodopsin. *Biophys. J.* 66:44*a*. (Abstr.)

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of Halobacterium halobium and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–678.

Roepe, P. D., P. L. Ahl, J. Herzfeld, J. Lugtenburg, and K. J. Rothschild. 1988. Tyrosine protonation changes in bacteriorhodopsin. A Fourier transform infrared study of BR548 and its primary photoproduct. *J. Biol. Chem.* 263:5110–5117.

Scherrer, P., W. Stoeckenius, M. K. Mathew, and W. Sperling. 1987. Isomer ratio in dark-adapted bacteriorhodopsin. *In* Biophysical Studies of Retinal Proteins. T. G. Ebrey, H. Frauenfelder, B. Honig, and K. Nakanishi, editors. University of Illinois Press, Urbana-Champaign, IL. 206–211.

Sperling, W., P. Carl, C. N. Rafferty, and N. A. Dencher. 1977. Photochemistry and dark equilibrium of retinal isomers and bacteriorhodopsin isomers. *Biophys. Struct. Mech.* 3:79–94.

Stoeckenius, W., R. H. Lozier, and R. A. Bogomolni. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta.* 505:215–278.

Szundi, I., and W. Stoeckenius. 1989. Surface pH controls purple-to-blue transition of bacteriorhodopsin. A theoretical model of purple membrane surface. *Biophys. J.* 56:369–383.

Trissl, H.-W., and W. Gärtner. 1987. Rapid charge separation and bathochromic absorption shift of flash-excited bacteriorhodopsins containing 13-*cis* or all-*trans* forms of substituted retinals. *Biochemistry.* 26:751–758.

Váró, G. 1987. Photoelectric response signals of bacteriorhodopsin containing 13-*cis* retinal. *Acta Biochim. Biophys. Hung.* 22:99–105.

Váró, G., and K. Bryl. 1988. Light- and dark-adaptation of bacteriorhodopsin measured by a photoelectric method. *Biochim. Biophys. Acta.* 934:247–252.

Váró, G., A. Duschl, and J. K. Lanyi. 1990. Interconversion of the M, N and O intermediates of the bacteriorhodopsin photocycle. *Biochemistry.* 29: 3798–3804.

Váró, G., and L. Keszthelyi. 1983. Photoelectric signals from dried oriented purple membranes of Halobacterium halobium. *Biophys. J.* 43:47–51.

Váró, G., and J. K. Lanyi. 1991. Distortions in the photocycle of bacteriorhodopsin at moderate dehydration. *Biophys. J.* 59:313–322.

Zimányi, L., L. Keszthelyi, and J. K. Lanyi. 1989. Transient spectroscopy of bacterial rhodopsins with optical multichannel analyzer. 1. Comparison of the photocycles of bacteriorhodopsin and halorhodopsin. *Biochemistry.* 28:5165–5172.