Membrane potential stabilizes the O intermediate in liposomes containing bacteriorhodopsin

Inna V. Kalaidzidis, Ilya N. Belevich, Yannis L. Kalaidzidis, Andrey D. Kaulen*

Department of Photobiochemistry, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia

Received 20 August 1999

Abstract In the bacteriorhodopsin-containing proteoliposomes, a laser flash is found to induce formation of a bathointermediate decaying in several seconds, the difference spectrum being similar to the purple–blue transition. Different pH buffers do not affect the intermediate, whereas an uncoupler, gramicidin A, and lipophilic ions accelerate decay of the intermediate or inhibit its formation. In the liposomes containing E204Q bacteriorhodopsin mutant, formation of the intermediate is suppressed. In the wild-type bacteriorhodopsin liposomes, the bathointermediate formation is pH-independent within the pH 5–7 range. The efficiency of the long-lived O intermediate formation increases at a low pH. In the wild-type as well as in the E204Q mutant purple membrane, the O intermediate decay is slowed down at slightly higher pH values than that of the purple–blue transition. It is suggested that the membrane potential affects the equilibrium between the bacteriorhodopsin ground state (Glu-204 is protonated and Asp-85 is deprotonated) and the O intermediate (Asp-85 is protonated and Glu-204 is deprotonated), stabilizing the latter by changing the relative affinity of Asp-85 and Glu-204 to H^+. At a low pH, protonation of a proton-releasing group (possibly Glu-194) in the bacteriorhodopsin ground state seems to prevent deprotonation of the Glu-204 during the photocycle. Thus, all protonatable residues of the outward proton pathway should be protonated in the O intermediate. Under such conditions, membrane potential stabilization of the O intermediate in the liposomes can be attributed to the direct effect of the potential on the pK value of Asp-85.

Key words: Bacteriorhodopsin; Photocycle; Proton pumping; Purple membrane; E204Q mutant; *Halobacterium salinarium*

1. Introduction

Bacteriorhodopsin (bR) from *Halobacterium salinarium* is a proton pump that converts light energy into a proton electrochemical gradient across the bacterial membrane (for reviews, see [1–4]). The chromophore group of bR is a retinal covalently bound to the ε-amino group of the Lys-216 via protonated Schiff base. Light absorption induces an all-*trans* to 13-*cis* isomerization of the chromophore and a complex photochemical cycle including J, K, L, M, N and O intermediates. During the L→M transition, the chromophore isomerization initiates the proton transfer directed to the outer membrane surface, occurring from the Schiff base to the proton acceptor group of Asp-85. The following M→N transition is coupled with the Schiff base reprotonation by the proton donor group of Asp-96 localized between the chromophore and the cytoplasmic surface. The N decay and bR initial state restoration is accompanied by reprotonation of the Asp-96 and the chromophore 13-*cis*-all-*trans* re-isomerization.

The O→bR transition seems to be the reaction which completes the bR photocycle. The O intermediate contains all-*trans*-retinal [5], while in the L93A mutant, long-lived O intermediate containing 13-*cis*-retinal was found [6]. Investigation of pH-dependence of the O intermediate in the wild-type bR and comparison between kinetics of the O intermediate and proton release and uptake, as well as studies of the mutant with substituted Glu-194 and Glu-204, led to the conclusion that the O→bR transition is accompanied by intraprotein proton transfer from Asp-85 to Glu-204 [4,8–11].

Nasuda-Koyama et al. [12] described formation of a blue form in bR liposomes under continuous illumination. The authors suggested that this effect is due to the formation of the blue acid bR form induced by a large decrease in the intravesicular pH. Hellingwerf et al. [13] found that the decay kinetics of the flash-induced O intermediate are slightly slower under steady-state illumination than in the dark, but can be accelerated by protonophore uncoupler or nigericin+valinomycin. Quintanilha [14] ascribed this phenomenon mainly to the inhibition of the decay of the preceding M intermediate by an electric field. The effect of the membrane potential on the M decay was also investigated in the whole cells [15,16]. Hellingwerf et al. [13] noted the existence of a slow component in the bR photocycle measured at a long wavelength, which was attributed to light scattering processes.

In our experiments on bR liposomes with a high degree of protein orientation asymmetry, we have found that this slow process is due to the slow relaxation of the flash-induced O-like intermediate. We have concluded that this phenomenon is a consequence of stabilization of the O intermediate by the membrane potential, affecting the equilibrium between the O intermediate and the bR ground state.

2. Materials and methods

Freshly prepared purple membrane sheets from the halobacterial wild-type ET1001 and E204Q mutant strain were used. The E204Q strain was kindly provided by Prof. J. Lanyi and Prof. R. Needleman.

To prepare bR liposomes, a suspension of L-α-phosphatidylcholine (type II-S from soybean, Sigma) (50 mg/ml) in a medium containing 30 mM Na_2SO_4, 1 mM MES, pH 6.0, was sonicated (UZDN-4 disintegrator, 22 kHz) for ≈4 min until it became transparent. After addition of the purple membranes (0.5 mg protein/ml), the suspension was sonicated once more for 4 min. Then, the proteoliposome suspension was centrifuged at 160 000×*g* for 40 min.

The bR photocycle transient absorbance changes were measured using a home-made single-beam spectrophotometer as previously described [17,18]. To measure flash-induced pH changes, the difference

*Corresponding author. Fax: (7) (95) 939 3181.
E-mail: kaulen@phtbio.genebee.msu.su

PII: S0014-5793(99)01193-X

between spectral responses at 453 nm in the absence and in the presence of pyranine was detected [19,20].

Light flashes were provided by a frequency-doubled Quantel Nd-YAG-481 laser (wavelength 532 nm, pulse half-width 15 ns, energy 10 mJ).

Measurements were performed on the light-adapted proteoliposome suspension (5–10 μM bR) at room temperature.

3. Results and discussion

In the open purple membrane sheets, the half-time of the O intermediate decay is known to be several milliseconds [2,4]. On the other hand, in the bR proteoliposome suspension, the relaxation of the flash-induced optical changes at 640 nm proved to be in the time range of several seconds (Fig. 1A). The difference spectrum of this slow relaxation is similar to that of the bR transition into the blue acid form (Fig. 1B). This may mean that in the long-lived intermediate, the Asp-85 residue is protonated. Note that the slow components are absent in the kinetics of relaxation of the optical changes at 400 and 340 nm (Fig. 1A). Taking into account that optical changes at 340 nm are sensitive to the isomeric retinal state and relaxation of the N intermediate [21], it can be concluded that both the M and N intermediates are not in equilibrium with the long-lived intermediate. Thus, the latter probably contains the *trans*-retinal and can be considered either as the blue acid form or a long-lived O-type intermediate other than the O-like intermediate with 13-*cis*-retinal found in the L93A and some related mutants [6,22].

The main difference between the purple membranes and the proteoliposomes is that the proteoliposomes represent closed membrane vesicles capable of maintaining a protonmotive force. Thus, the reason for the long-lived intermediate formation may be formation of the light-dependent protonmotive force on the proteoliposome membrane. In line with such an assumption, an increase in the membrane permeability by the protonophorous uncoupler FCCP is found to result in acceleration of the O-type intermediate decay (Fig. 2A). However, gramicidin A decreases only the amplitude of this intermediate (Fig. 2B). The difference in behavior of two uncouplers is not surprising. Most probably, incorporation of a single gramicidin A molecule in a proteoliposome induces a so high membrane permeability increase (a microsecond membrane potential discharge rate [17]) that the residual responses in the presence of gramicidin are due to the proteoliposomes containing no gramicidin molecules.

Inasmuch as FCCP and gramicidin A induce the dissipation of the electric potential difference as well as the proton gradient, the above data do not allow us to discriminate between two factors that can be responsible for the long-lived intermediate formation, namely, the membrane potential and a decrease in the intravesicular pH.

As experiments showed, variation in the buffers used (50 mM MES, HEPES, TRIS, bis-tris-propane, acetate or citrate) did not influence the long-lived intermediate formation. The buffers were without effect being added either to medium for incubation of the liposomes or to the medium for their preparation (not shown). Thus, these data indicate that internal acidification is hardly the main factor inducing the appearance of the long-lived intermediate.

On the other hand, lipophilic ions affect the optical signal at 640 nm (Fig. 2C,D). Lipophilic anions at a low concentration distinctly increase the rate of the O decay. At high concentrations of anions, the fast O intermediate similar to that in the open purple membrane sheets is observed. Inasmuch as membrane potential induces the lipophilic ion transport (the fast phase of the O decay) and correspondingly formation of the ion gradient, the slow components of signal decay are possibly due to the relaxation of the membrane potential when the electrochemical potential difference of lipophilic ions is close to zero. The absence of the fast O intermediate at a high concentration of cations is possibly due to an addition direct effect of the cations on the photocycle kinetics.

It is noteworthy that such agents as ammonium salts, imidazole and 9-aminoacridine were shown to decrease the magnitude of the flash-induced optical changes at 640 nm and slightly accelerate its decay. All these agents are pH buffers

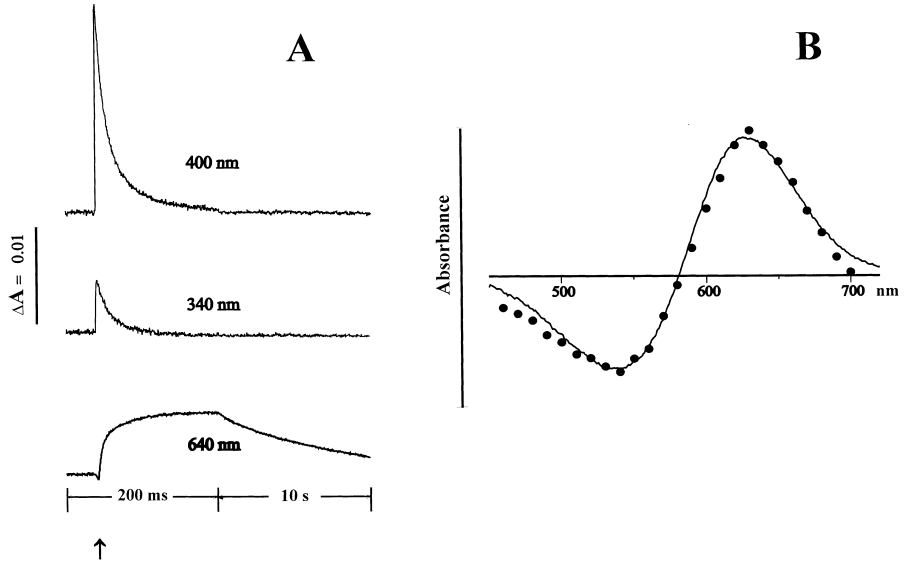

Fig. 1. Flash-induced optical changes (A) in bR proteoliposomes at pH 6.0 and comparison of the difference spectrum (B) of the long-lived intermediate (●), measured at pH 6.0 in 200 ms after the flash, with that of purple→blue transition (solid line) in bR proteoliposomes (pH 6.0–3.5). The assay medium: 30 mM Na_2SO_4, 1 mM MES. Here and below, a vertical arrow indicates the laser flash.

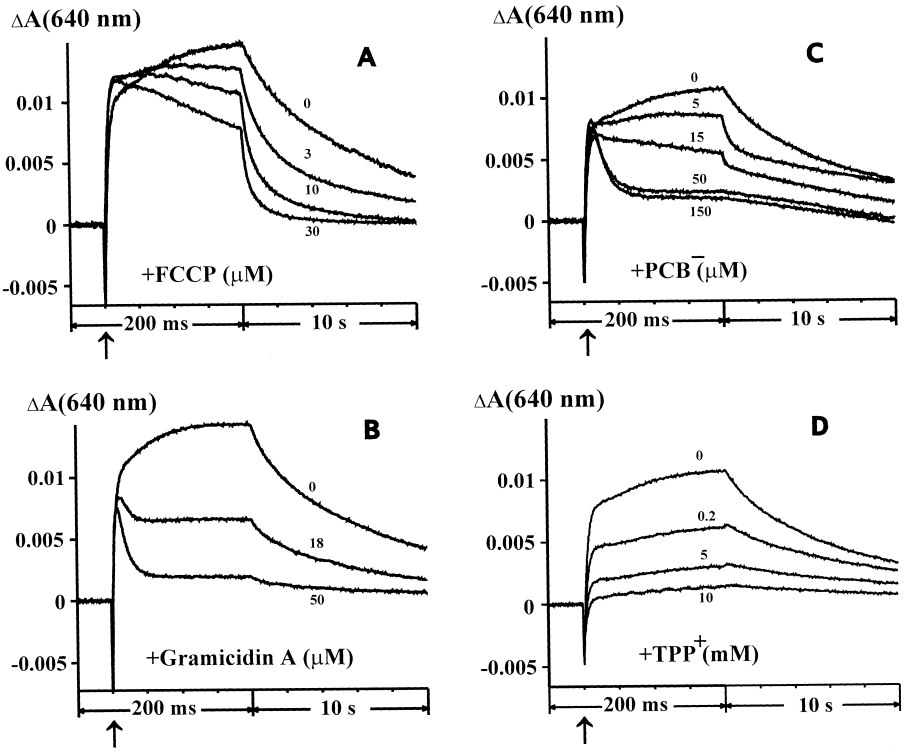

Fig. 2. Effects of protonophorous uncoupler FCCP (A), gramicidin A (B), phenyldicarbaundecaborane anion PCB^- (C) and tetraphenyl phosphonium cation TPP^+ (D) on the flash-induced optical changes at 640 nm in proteoliposomes. The assay medium: 30 mM Na_2SO_4, 1 mM MES, pH 6.0.

and are able to penetrate through the membrane in their neutral forms. Hence, these data might indicate that the acidification of the intravesicular volume is the main reason for the long-lived intermediate formation. However, ethanol (5–8%) and DMSO (20–30%) produce the same effect. Since ethanol and DMSO have no proton acceptor groups but can penetrate into the membrane, we suppose that all these effects are due to the unknown direct action on the membrane properties that are not related to the intravesicular pH changes.

Thus, the above data indicate that the membrane potential is the main cause for the flash-induced formation of the long-lived intermediate. However, they cannot exclude that its formation under continuous illumination may also be caused by acidification of the intravesicular volume as was suggested by Nasuda-Koyuama et al. [12]. It is noteworthy that light adaptation increases the magnitude of the O intermediate concurrently with the increase in the M intermediate magnitude (data not shown). Apparently, the long-lived O intermediate may be formed both from the *trans*-bR and 13-*cis*-bR molecules. This contradicts the conclusion of Nasuda-Koyuama et al. [12] that only 13-*cis*-bR is able to give the bathoform in response to low pH in the intravesicular volume.

There are two additional pieces of evidence confirming our conclusion. The proteoliposome suspension could be roughly separated into two fractions by centrifugation at $160\,000 \times g$ by 40 min. The ratios of the maximal amplitudes of the responses at 640 and 400 nm are 20–30% and 50–60% in the pellet and supernatants, respectively. The M intermediate decay is slower in the supernatant (Fig. 3A). Addition of gramicidin A accelerates the M decay in both fractions to the same extent. It is known that the M decay is retarded by the membrane potential [14,16]. Thus, correlation between the amplitude of the signal at 640 nm and the rate of the M decay confirms the main role of the membrane potential in the long-lived O-type intermediate formation. However, the reason for the differences between the liposome fractions is not clear yet. One of the possibilities is the different degree of the bR asymmetry in the proteoliposome membrane.

In Fig. 3B, the O intermediate decay and the passive proton release are compared. The faster rate of the O decay can be explained by the fact that the membrane potential discharge may be faster than the passive transmembrane proton flow due to participation of ions other than H^+ in the discharge. An increase in the flash energy induces the proportional rise of the amplitudes of the M and O intermediates (not shown), indicating that the O formation depends on the number of protons translocated by bR. A decrease in the flash intensity results in a slight decrease in the O intermediate decay rate (Fig. 3C). The most probable explanation for this consists in existence of the potential-dependent equilibrium between the O intermediate and the bR ground state. In this case, some acceleration of the O decay at high flash intensity may reflect some non-linearity of the membrane resistance at a high voltage. On the contrary, if the membrane potential simply slows down the O decay, one should expect the O decay acceleration at a low flash intensity and, hence, at a low membrane potential. The data obtained contradict the latter possibility.

Fig. 4A shows the pH-dependence of the ratio between the maximal O and M amplitudes. The ratio is pH-independent in the pH 5–7 range, pointing to that external H^+ ions are not involved in the $O \leftrightarrow M$ equilibrium. Apparently, a proton transfer between Asp-85 and some other intraprotein group takes part in this process. The most plausible candidate is

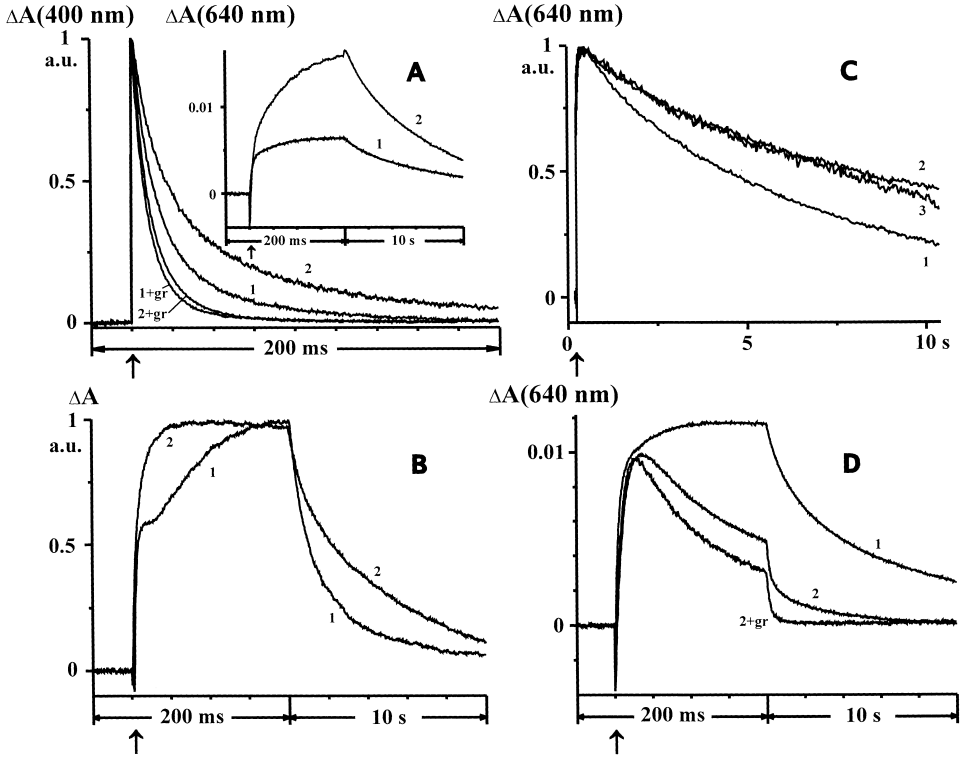

Fig. 3. Some properties of the long-lived O intermediate. A: Comparison of the M and O kinetics in the different proteoliposome fractions. 1, Proteoliposomes from the pellet fraction (160 000×*g*, 40 min). 2, Proteoliposomes from the supernatant. gr, 60 μM gramicidin A is added. The assay mixture: 30 mM Na_2SO_4, 1 mM MES, pH 6.0. B: Comparison of the O intermediate kinetics (1) and flash-induced pH changes in proteoliposome suspension (2). The assay medium: 50 mM NaCl, 100 μM pyranine, pH 7.0. C: Comparison of the O intermediate decay at different flash intensities. The ratio between signal amplitudes 1:2:3 is equal to 1:0.4:0.1. The assay mixture: 30 mM Na_2SO_4, 1 mM MES, pH 6.0. D: Comparison of the O intermediate kinetics in the proteoliposomes with the wild-type bR (1) and with the E204Q mutant bR (2). gr, 60 μM gramicidin A is added.

Glu-204 because the reprotonation of Glu-204 by Asp-85 probably is the final stage in the bR photocycle [8–11]. The three-dimensional bR structure [23,24] predicts that this transfer should be an electrogenic process. Note that the O intermediate decay is an electrogenic process too [25]. Formation of the O intermediate accompanied by the slow relaxation is strongly inhibited in the E204Q mutant (Fig. 3D). Nevertheless, in this mutant, gramicidin A accelerates the O intermediate decay. We suppose that in this case, the membrane potential slows down the Asp-85 reprotonation, whereas in the wild-type protein, the membrane potential affects the proton affinities of the Asp-85 and Glu-204, thus stabilizing the O intermediate containing protonated Asp-85 and deprotonated Glu-204.

The O/M ratio decreases at a pH higher than 7 (Fig. 4A). The reason for this effect is not clear. The apparent pK varied in different proteoliposome fractions. In liposomes with a low O/M ratio, the p*K* is within the 7.8–8.2 range. It is interesting that the p*K* of Glu-204 in all the proteoliposome preparations is 8–8.5 (the pK was determined from the pH-dependent decrease in the amplitude of the electrogenic microsecond phase, according to Kalaidzidis et al. [11]). Thus, one may assume that deprotonation of the Glu-204 in the bR ground state is the process which shifts the equilibrium between the O intermediate and bR towards the bR ground state. However, in proteoliposomes with the high O/M ratio, the p*K* is lower (in the 7.3–7.7 range), while the p*K* of the Glu-204 is higher than 8. It is possible that this effect is due to a decrease in the proton pumping activity of bR at a high pH and a high level of the membrane potential. This suggestion is currently under investigation.

The O/M ratio increases at low pH (Fig. 4A). This effect occurs within the pH range which is close to that of the bR transition into the blue acid form, measured by a decrease in the flash-induced M amplitude (Fig. 4A). It is interesting that in the wild-type purple membrane, the O intermediate decay slows down at low pH (Fig. 4B). Moreover, in the mutant E204Q purple membranes, the O intermediate formation is also retarded. A possible explanation of this phenomenon consists of protonation of Glu-194 in the bR ground state (from Fig. 4B, it may be concluded that in the ground state, the apparent p*K* of the Glu-194 in the wild-type bR is ∼3.8–4, whereas the p*K* of Asp-85 is ∼2.7–2.9), which may prevent deprotonation of Glu-204 during the photocycle and thus retard the deprotonation of Asp-85 associated with the O intermediate decay. If this assumption is correct, it may be expected that all the proton acceptor groups in the inward proton pathway are protonated during the photocycle at low pH. In this case, the O intermediate stabilization may be regarded as a consequence of a direct membrane potential-dependent increase in the p*K* of Asp-85.

Our conclusion on the potential-dependent equilibrium between the O intermediate and the bR ground state is in line with the data obtained by Kononenko et al. [26]. The authors observed a bathochromic shift of the maximum absorbance in oriented purple membrane film induced in the dark by external electric field of a direction coinciding with that of the transmembrane field generated by the bR proton pump. Ko-

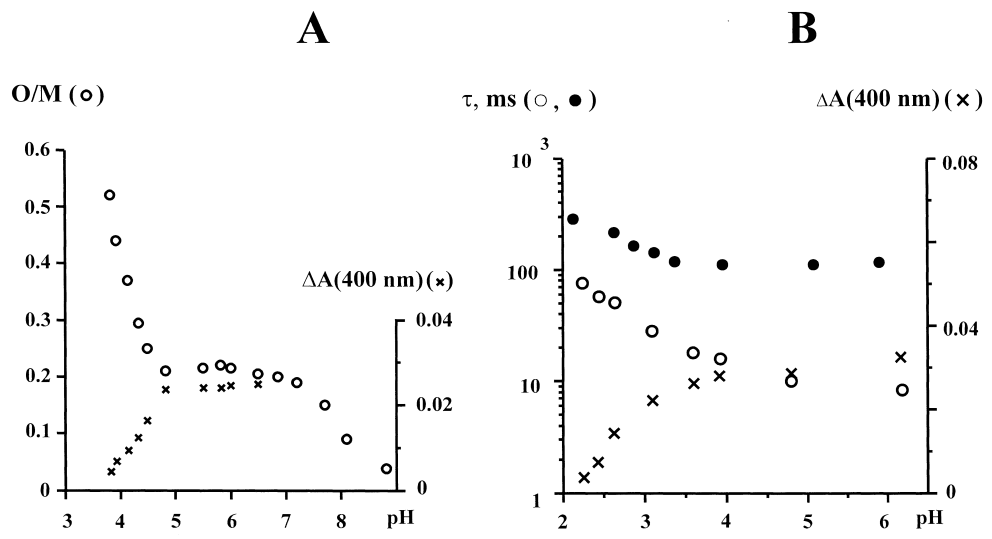

Fig. 4. pH-dependence of the O and M intermediates. A: pH-dependence of the O/M ratio (o, ratio of the amplitudes in the maximum of the flash-induced optical changes at 640 and 400 nm, respectively) and of the amplitude in the maximum of the optical changes at 400 nm (x) in proteoliposomes. The assay medium: 30 mm Na_2SO_4, 1 mM MES, 1 mM HEPES, 1 mM Tris. B: pH-dependence of the rate of the O intermediate decay in the purple membrane. (o), the wild-type bR; (●), the E204Q mutant. (x), pH-dependence of the amplitude in the maximum of the optical changes at 400 nm in the wild-type bR. The assay medium: 150 mM Na_2SO_4, 1 mM MES.

nonenko et al. [26] suggested that formation of this bathoproduct plays a regulatory role in the bR proton pumping in cells by a negative feedback mechanism. However, until now, the long-lived O intermediate was not described in the literature. If this intermediate does not form in the cells, then, this may indicate that the difference between the p*K* of the Asp-85 and Glu-204 in the purple membrane is significantly higher than in proteoliposomes and the membrane potential is not able to overcome this difference. This problem is under investigation in our group.

The pool of the O intermediate seems to be heterogeneous [7,27], probably because of several consequential molecular processes that take place during the O→bR transition. It was shown that the O intermediate is in the equilibrium with a preceding intermediate [28], whereas the O→bR transition seems to be the irreversible step in the bR photocycle [4,7]. Our data indicate that the final step in the bR photocycle, i.e. proton transfer from the Asp-85 to the Glu-204, is electrogenic, being an equilibrium process at least in some conditions (for instance, under influence of the membrane potential). Thus, one of the previous steps (isomerization of the retinal and/or accompanying protein relaxation) should be irreversible.

Acknowledgements: We are grateful to Prof. J. Lanyi and Prof. R. Needleman for providing the mutant E204Q halobacterial strain. This work was supported by the Russian Foundation for Basic Research (Grant 97-04-49749), INTAS (Grant 93-2852) and ISTC (Grant 866).

References

[1] Oesterhelt, D., Tittor, J. and Bamberg, E. (1992) J. Bioenerg. Biomembr. 24, 181–191.
[2] Ebry, T.G. (1993) in: Thermodynamics of Membrane Acceptors and Channels (Jackson, M., Ed.), pp. 353–387, CRC Press, Boca Raton, FL.
[3] Skulachev, V.P. (1993) Q. Rev. Biophys. 26, 177–199.
[4] Lanyi, J.K. and Varo, G. (1995) Isr. J. Chem. 35, 365–385.
[5] Smith, S.O., Pardoen, J.A., Mulder, P.P.J., Curry, B., Lugtenburg, J. and Mathies, R. (1983) Biochemistry 22, 6141–6148.
[6] Delaney, J.K., Schweiger, U. and Subramaniam, S. (1995) Proc. Natl. Acad. Sci. USA 92, 11120–11124.
[7] Cao, Y., Brown, L.S., Needleman, R. and Lanyi, J.K. (1993) Biochemistry 32, 10239–10248.
[8] Brown, L.S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995) J. Biol. Chem. 270, 27122–27126.
[9] Balashov, S.P., Imasheva, E.S., Ebrey, T.G., Chen, N., Menick, D.R. and Crouch, R.K. (1997) Biochemistry 36, 8671–8676.
[10] Dioumaev, A.D., Richter, H.-T., Brown, L.S., Tanio, M., Tuzi, S., Saito, H., Kimura, Y., Needleman, R. and Lanyi, J.K. (1998) Biochemistry 37, 2496–2506.
[11] Kalaidzidis, I.V., Belevich, I.N. and Kaulen, A.D. (1998) FEBS Lett. 434, 197–200.
[12] Nasuda-Kouyama, A., Fukuda, K., Takayoshi, I. and Kouyama, T. (1990) Biochemistry 29, 6778–6788.
[13] Hellingwerf, K.J., Schuurmans, J.J. and Westerhoff, H.V. (1978) FEBS Lett. 92, 181–186.
[14] Quintanilha, A.T. (1980) FEBS Lett. 127, 8–12.
[15] Dancshazy, Z., Helgerson, S.L. and Stoeckenius, W. (1983) Photobiochem. Photobiophys. 5, 347–357.
[16] Groma, G.I., Helgerson, S.L., Wolber, P.K., Beece, D., Dancshazy, Z., Keszthelyi, L. and Stoeckenius, W. (1984) Biophys. J. 45, 985–992.
[17] Drachev, L.A., Kaulen, A.D., Khitrina, L.V. and Skulachev, V.P. (1981) Eur. J. Biochem. 117, 461–470.
[18] Drachev, L.A., Kaulen, A.D. and Skulachev, V.P. (1984) FEBS Lett. 178, 331–335.
[19] Grzesiek, S. and Dencher, N.A. (1986) FEBS Lett. 208, 337–342.
[20] Drachev, L.A., Kaulen, A.D. and Komrakov, A.Y. (1992) FEBS Lett. 313, 248–250.
[21] Drachev, L.A., Kaulen, A.D., Skulachev, V.P. and Zorina, V.V. (1986) FEBS Lett. 209, 316–320.
[22] Kandori, H., Yamazaki, Y., Hatanaka, M., Needleman, R., Brown, L.S., Richter, H.-T., Lanyi, J.K. and Maeda, A. (1997) Biochemistry 36, 6134–6141.
[23] Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
[24] Luecke, H., Richter, H.-T. and Lanyi, J.K. (1998) Science 280, 1934–1937.
[25] Ludmann, K., Gergely, C., Der, A. and Varo, G. (1998) Biophys. J. 75, 3120–3126.
[26] Kononenko, A.A., Lukashev, E.P., Maximychev, A.V., Chamorovsky, S.K., Rubin, A.B., Timashev, S.F. and Chekulaeva, L.N. (1986) Biochim. Biophys. Acta 850, 162–169.
[27] Richter, H.-T., Needleman, R., Kandori, H., Maeda, A. and Lanyi, J.K. (1996) Biochemistry 35, 15461–15466.
[28] Chizhov, I., Engelhard, M., Chernavsky, D.S., Zubov, B. and Hess, B. (1992) Biophys. J. 61, 1001–1006.