TIME RESOLUTION OF THE INTERMEDIATE STEPS IN THE BACTERIORHODOPSIN-LINKED ELECTROGENESIS

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1. Introduction

Bacteriorhodopsin, the retinal-containing protein from *Halobacterium halobium*, was discovered by Oesterhelt and Stoeckenius in 1971 [1]. The study of this compound revealed that it functions as an electrogenic light-dependent proton pump [2–4]. Electric potential and current generation mediated by bacteriorhodopsin was directly measured in this group [5–9]. Another line of investigation demonstrated several intermediate spectral forms of bacteriorhodopsin, participating in light-induced H^+ translocation [10–13].

The objective of this work was to obtain the time resolution of the early events in bacteriorhodopsin-mediated electrogenesis.

Electric potential generation accompanying a single turnover of bacteriorhodopsin was estimated by electrodes immersed into solutions on both sides of a phospholipid-impregnated collodion film with bacteriorhodopsin proteoliposomes or membrane fragments attached to one of its surfaces. Potential generation (plus on the bacteriorhodopsin-free side of the film) was found to be composed of two phases, one correlating with the 570 nm → 412 nm spectral transition (τ = 25–30 μs) and the other with the reversal to the 570 nm state (τ = 6–12 ms). The latter phase was specifically inhibited by La^{3+}. An early photopotential of the opposite direction was also revealed ($\tau < 0.3$ μs). Both direct phases were decelerated by D_2O and abolished at pH < 2, while the amplitude of the opposite phase increased. The amplitudes of the direct phases decreased when bacteriorhodopsin was kept in the dark (τ = 10–20 min). Illumination for 30–60 s abolished the dark adaptation influence.

2. Materials and methods

Purple sheets (bacteriorhodopsin-containing membrane fragments) were isolated from *Halobacterium halobium*, strain R_1, as in [14]. The mixture for proteoliposome reconstitution contained bacteriorhodopsin sheets (0.6 mg protein/ml), azolectin (30 mg/ml), 2% sodium cholate and 50 mM potassium phosphate, pH 7.0. This mixture was sonicated in a USDN-1U4.2 disintegrator for 4 min and dialyzed for 20 h against 50 mM potassium phosphate, pH 7.0.

The proteoliposomes were separated by centrifugation at 200 000 × *g* for 30 min and suspended in 50 mM Tris–HCl, pH 7.0. To measure electric responses of bacteriorhodopsin, the method of proteoliposome association with phospholipid-impregnated membrane filter [15–17] has been applied. However, collodion film was used instead of Millipore or other membrane filters, since only the former proved suitable for measuring fast electrogenic events in the bacteriorhodopsin cycle. The advantage of the collodion film is apparently due to lesser thickness of the phospholipid layers in the pores and, hence, higher electric capacity. The collodion film was formed on the water surface by addition of 1% amylacetate solution of nitrocellulose. The film was transferred to the air with a ring, dried, for 1 h, impregnated with a decane solution of azolectin or lecithin (100 mg/ml), and fixed in the partition of a dismountable Teflon chamber separating two com-

partments with electrolyte solutions. The electric potential difference between the two compartments was measured with Ag/AgCl electrodes immersed into the electrolyte solutions and connected with an operational amplifier (Analog Devices 48 K). The potential was monitored with an S8-13 storage oscillograph. The electrodes were screened with black polyethylene to prevent light-induced artifacts. The resistance of the film was $0.5-1.0 \times 10^8\ \Omega \times cm^2$. A 0.3 μs electric impulse was transmitted through the experimental system without distortions.

To incorporate bacteriorhodopsin into the collodion film, 2 procedures were used:

(1) Bacteriorhodopsin proteoliposomes were added to one of the two compartments of the Teflon chamber separated by azolectin-impregnated collodion film (final protein conc. 30–50 μg/ml); then the solutions in both compartments (50 mM Tris–HCl, pH 7.0) were supplemented with 15–30 mM $CaCl_2$.

(2) Suspension of the bacteriorhodopsin sheets, instead of proteoliposomes, was added to one of the compartments. A mixture of 20 mM potassium salts of phosphate, citrate and borate and 10 mM ammonium acetate (pH 6.0) was used as the incubation medium. The collodion film was impregnated with lecithin.

In both cases, the mixtures were stirred overnight.

As a source of continuous light, a 150 W halogen lamp was used. A 5 cm water filter was placed between the lamp and the chamber to prevent heating. The maximal light intensity of the lamp on the membrane was 3 mW/mm^2. In the flash study, 2 light sources were used:

(i) An ISSH-100 flash lamp with a light impulse energy on the membrane of 4 MJ and 5–6 μs, impulse duration.

(ii) A neodymium Q-switched laser of doubled light frequency (λ = 530 nm, flash duration 15 ns, light impulse energy 8 MJ).

3. Results and discussion

Figure 1A demonstrates an effect of continuous light on a collodion film treated with bacteriorhodopsin proteoliposomes. It is seen that switching on the light results in a 300 mV electric potential difference across the film being generated (plus on the bacteriorhodopsin-free side of the film). Figures 1B–D illustrate the effects of a 5 μs flash which is 3 orders shorter than the time of a single bacteriorhodopsin turn-over. It is clear that the electric potential difference produced by a single photochemical cycle of bacteriorhodopsin can be easily measured by the technique used.

Measurements of single-flash response with different time resolutions revealed the existence of at least 4 phases, the first 3 being associated with bacteriorhodopsin.

1. The first phase is formation of a small but measurable potential difference of the direction opposite to that induced by continuous light (minus on the bacteriorhodopsin-free side of the film) (fig.1E). Its τ is < 0.3 μs and cannot be estimated by the measuring system employed in these experiments. In any case, this time is much shorter than that of the 570 nm → 412 nm bacteriorhodopsin transition and hence must be attributed to the earliest light-induced events of the bacteriorhodopsin cycle, i.e., a 590 nm intermediate formation.

2. The τ of the second phase can be measured by our techniques (fig.1D). It was found to be equal to 25–50 μs. The direction of the electric vector proves the same as in continuous light. Judging from the τ measurement, this phase is most probably related to the 570 nm → 412 nm transition (τ = 55–70 μs [10,13]). Such spectral shift is accompanied by a light-induced H^+ transfer from the bacteriorhodopsin Schiff base localized in the hydrophobic part of the membrane, to the extramembrane space which charges positively under illumination [18]. Apparently, it is this electrogenic process that reveals itself as the second phase of the single flash-initiated photoeffect.

3. The third phase is directed as the second (fig.1C). Its τ is 6–12 ms which roughly corresponds to the τ value of reversal of bacteriorhodopsin from the 412 nm form back to the 570 nm form (τ = 7–14

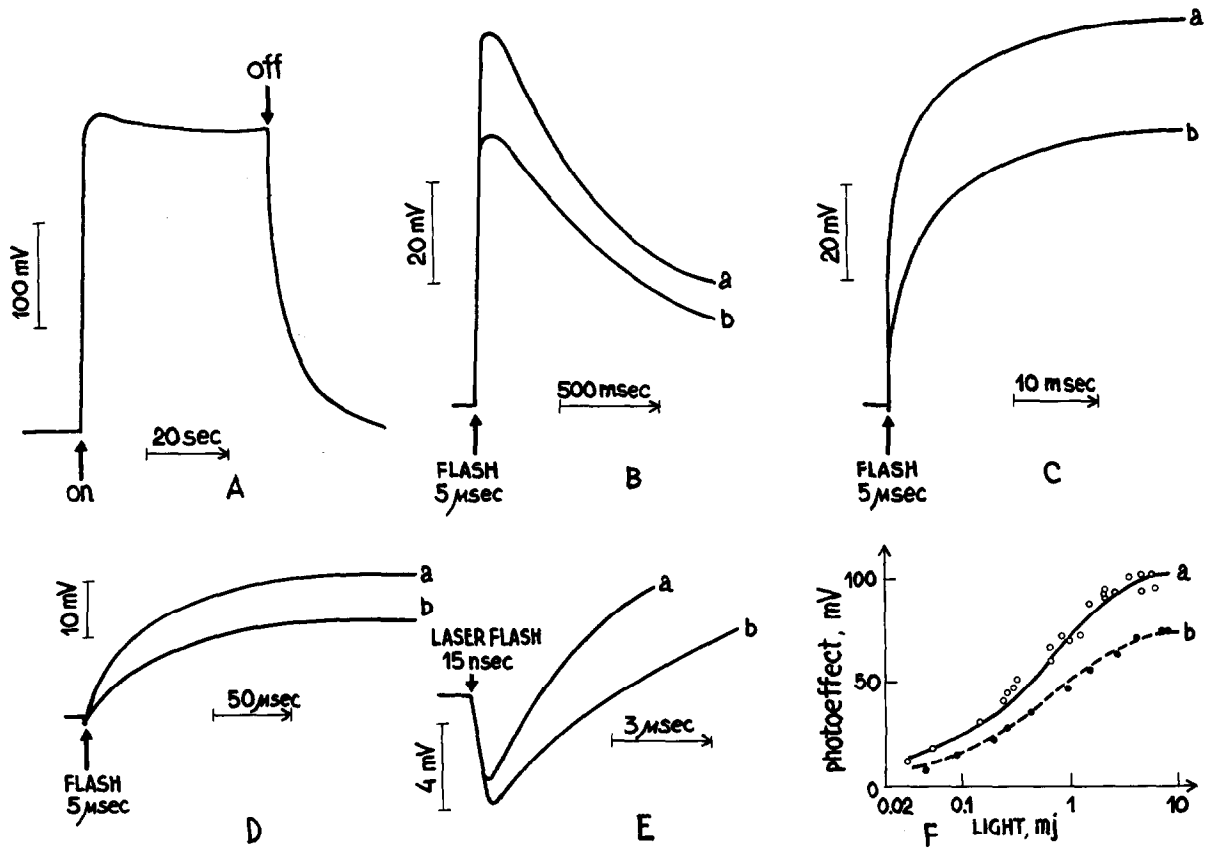

Fig.1. Generation of electric potential difference across an azolectin-impregnated collodion film covered on one side with bacteriorhodopsin proteoliposomes. Positive charging on the bacteriorhodopsin-free side of the film is indicated as upward direction of the curve. (A) Continuous light; (B–D) lamp flash; (E,F) neodymium laser flash. In fig.1 bacteriorhodopsin photoeffect is plotted against laser flash energy. Curves (a) without dark adaptation; curves (b) after 1.5 h storage of bacteriorhodopsin-treated film in the dark.

ms) [10,12,13]). One may suppose that this phase is due to the H^+ transfer to the Schiff base from the extramembrane space which charges negatively.

4. The last phase (τ = 1 s) is discharge of the flash-induced electric potential (fig.1B).

The use of a neodymium laser, instead of an ISSH-100 lamp, for producing light flash showed that all phases but the first have characteristics similar to that described above. As to the first phase, it proved more demonstrative (see fig.1E), apparently due to much shorter illumination in the laser experiment (15 ns with the laser against 5 μs with the lamp).

The action spectrum of the first, second and third phases was found to be the same as the bacteriorhodopsin absorption spectrum; it has a broad $A_{500-600}$ max.

In fig.1 an effect of dark adaptation of bacteriorhodopsin is also shown. It was found that the amplitude of the second and third phases of the flash-induced response decreases after dark storage of the system. τ of the dark adaptation was about 10–20 min coinciding with τ of the 570 → 560 nm spectral transition. Illumination for 30–60 s reversed the effect of the dark. The τ values (as well as ratio of amplitudes) of the second and third phases are not affected by dark adaptation. The amplitude of the

first phase is slightly higher in the dark-adapted samples.

Figure 1F demonstrates photoelectric effect as a function of laser flash intensity. The dark adaptation was found to entail a 1.4–1.5-fold decrease in the amplitude of the second and third phases at any flash energy.

Dark adaptation is usually explained by partial transition of all-*trans*–13-*cis* retinal [19,20]. According to [21], only all-*trans* retinal-containing bacteriorhodopsin can give the 412 nm intermediate in the light, while 13-*cis* isomer is transformed via some long-wave derivatives to all-*trans* retinal bacteriorhodopsin.

The above effect of the dark adaptation on the amplitude of the second and third phases can be easily explained if we assume that *cis*-retinal bacteriorhodopsin fails not only to form the 412 nm intermediate but also to transport vectorially H^+ ions. This assumption seems quite reasonable if we take into account the central position of the 412 nm intermediate in the H^+-translocating bacteriorhodopsin cycle. Measurements of the 570 nm → 412 nm spectral shift in the dark-adapted proteoliposomes showed that formation of the 412 nm intermediate was 1.4–1.6-fold lower than in light-adapted proteoliposomes.

The fact that the first phase is not inhibited by dark adaptation indicates that both all-*trans* and 13-*cis* isomers are competent in producing this photoelectric effect.

This may be compared with the data [21] that both all-*trans* and 13-*cis* isomers are competent in the light-dependent formation of a bathointermediate.

In fig. 2A–C effects of D_2O on te process studied are shown. The curves marked as 'H_2O' demonstrate light flash responses in water solution of 50 mM Tris–HCl, pH 7.0. The curves marked as 'D_2O' show

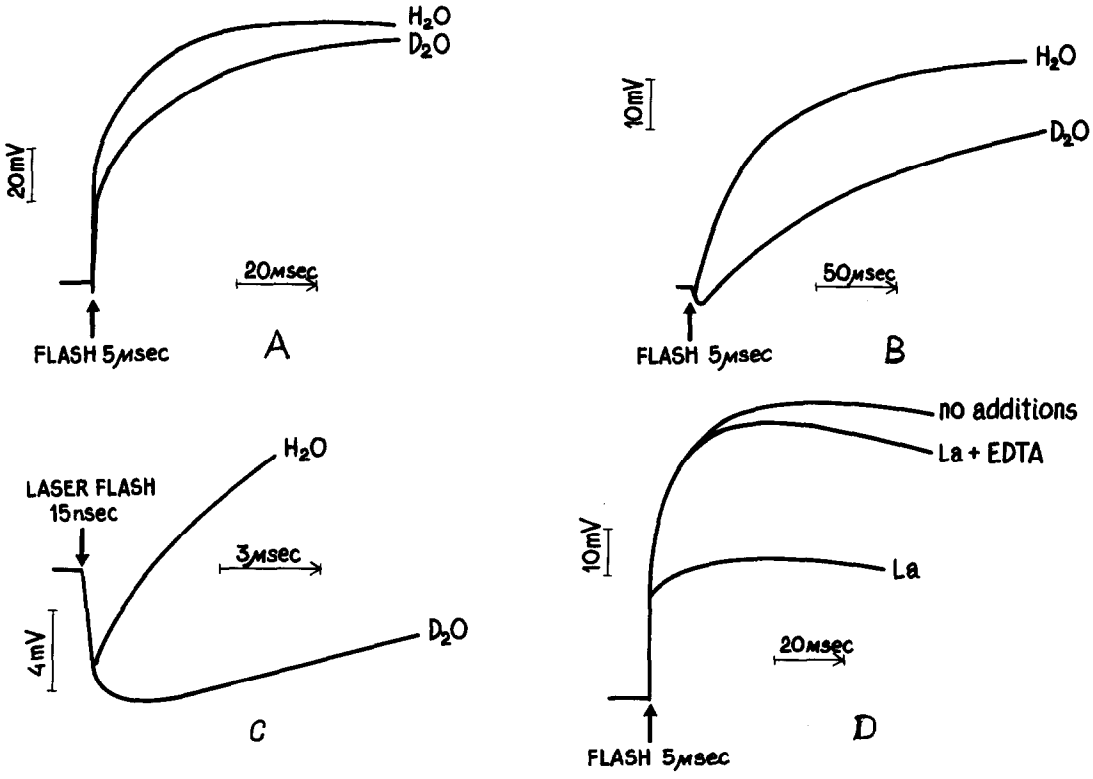

Fig.2. Actions of D_2O (A–C) and La^{3+} (D) on bacteriorhodopsin photoeffect. In fig.3D the incubation mixture was supplemented with 10 mM $La(NO_3)_3$ (curve 'La') and 10 mM $La(NO_3)_3$ plus 15 mM EDTA (curve 'La + EDTA').

responses of the same bacteriorhodopsin-containing film after H_2O solution was replaced by a D_2O solution of the same composition. It is seen that D_2O brings about a pronounced increase in the amplitude of the first phase. On the other hand, the second and third phases are decelerated by factors 4.5–5 and 2, respectively. When H_2O was again used, the original photoeffect was obtained.

The D_2O action shown in fig.2 gives an independent piece of evidence of H^+ being involved in the second and third phases of electrogenesis. It can be noted in this connection that, according to [13], D_2O decelerates the 570 nm → 412 nm transition 3.5 times and the 412 nm → 570 nm transition 2.5 times, which corresponds to the above inhibition of the second and third phases, respectively.

In fig.2D the effect of La^{3+} on the flash-induced photoelectric responses is demonstrated. This cation was found to inhibit the photoeffect supported by continuous light. In the experiments [9], La^{3+} addition even changed the sign of the photoeffect of proteoliposomes associated with the planar phospholipid membrane. The absolute values of the light-dependent potential differences in these experiments were lower than in this study (60–80 mV previously, against 200–300 mV now). This and some other observations indicate that the slightly different procedure of the proteoliposome reconstitution, applied in this study, gives vesicles with much lesser contamination by oppositely-oriented bacteriorhodopsin ejecting H^+ ions from proteoliposomes. These preparations were shown to respond to La^{3+} addition by strong inhibition of the third phase with no effect on the first and second phases. Addition of EDTA completely reversed the inhibition. The sign of the photoeffect did not change (fig.2D).

These data can be accounted for by the assumption that La^{3+} ions, adsorbing on the surface of proteoliposomes associated with the collodion film, bring about positive charging of the membrane/water interface, the effect hindering transport of H^+ ions from the extraproteoliposomal water to the Schiff base in the membrane, preventing thereby, according to the above concept, the third phase of electrogenesis. La^{3+} ions that are non-penetrants to proteoliposomal membrane, cannot diffuse to the intraproteoliposomal water and fail to inhibit those bacteriorhodopsins which transfer H^+ ions from the proteoliposome interior to the extraproteoliposomal space. If the amount of these bacteriorhodopsins is sufficiently large, the reversal of the direction of the photoinduced electric potential can occur.

In the last series of the experiments, another procedure for bacteriorhodopsin incorporation into collodion film was used. The film was impregnated with lecithin (instead of azolectin in the above experiments), and bacteriorhodopsin sheets were added on one side of the film. It was found that at pH 6 spontaneous incorporation of sheets into the film took place, the process being over within several hours. The obtained system was found to generate a 300 mV potential difference when illuminated by continuous light.

In fig.3A a laser flash-induced photoresponse of this system is shown. Again, three electrogenic stages can be revealed. The amplitude of the second phase to that of the third was 1:4. Assuming that the low dielectric part of the membrane profile of the bacteriorhodopsin sheet is homogeneous, we can roughly estimate the distance between the protonated Schiff base of retinal and the membrane surface to which H^+ ion is transported after absorption of a photon. This distance should be equal to one-fourth of the membrane thickness, e.g., about 12 Å.

In the same system, the effect of low pH on the photoresponse has been studied. It was found that at pH below 2 one can observe the opposite phase only, whereas both direct phases completely disappear (fig.3B,C). The inhibition of the second and the third phases proved reversible.

It was also found that the photoeffect in the sheet-treated film is much less sensitive to La^{3+} than in the proteoliposome-treated one (not shown). This may be due to differences in the ability of the incorporated proteoliposomes and sheets to bind La^{3+}.

In conclusion, the obtained data demonstrate the existence of 2 main unidirectional phases of bacteriorhodopsin-mediated electrogenesis: the fast, corresponding to the H^+ transfer from the bacteriorhodopsin Schiff base to water (the 570 nm → 412 nm spectral transition); and the slow, related to the H^+ transfer to the Schiff base from the water on the other side of the membrane (the 412 nm → 570 nm transition). Besides, a very fast phase of the opposite direction ($\tau < 0.3$ μs) and of a small amplitude was revealed. Its rate is below the time resolution of the measuring

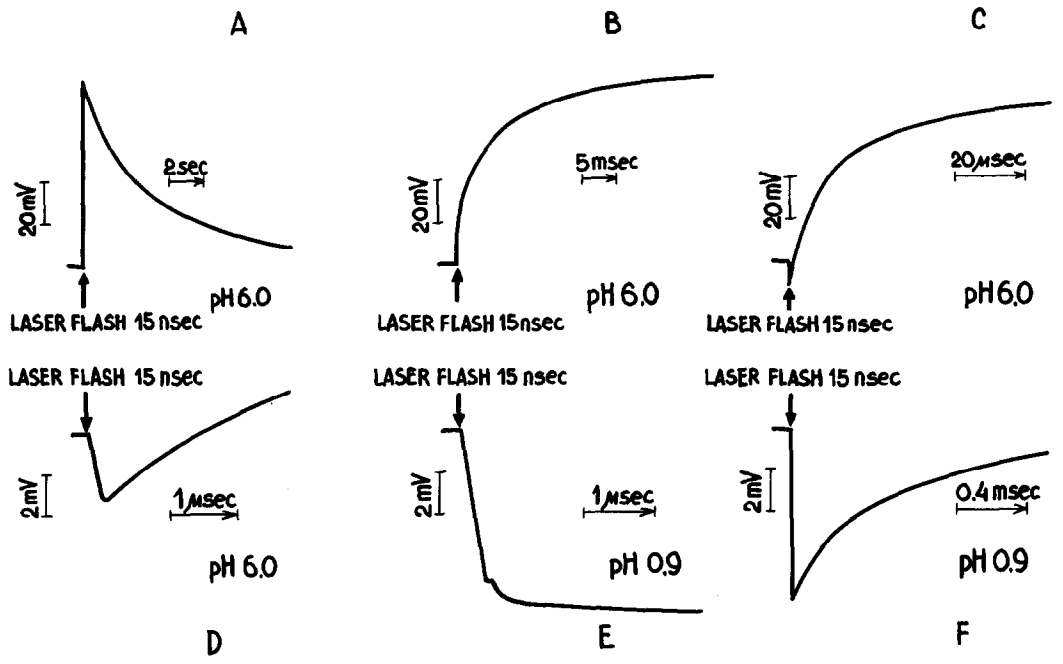

Fig.3. Generation of electric potential difference across a lecithin-impregnated collodion film covered on one side with bacteriorhodopsin sheets.

technique employed. On the other hand, the τ values of the main 2 phases are

(i) Clearly above the τ of the measuring technique.

(ii) Independent of light intensity and amount of bacteriorhodopsin.

These facts support the conclusion that the measured τ values of 2 main phases are true time characteristics of the bacteriorhodopsin generator. In proteoliposomes, the slow main phase is specifically inhibited by La^{3+}, presumably due to the positive charging of the membrane surface, preventing reprotonation of the 412 nm intermediate by H^+ ions of the medium. Both main phases, but not the opposite one, are abolished at pH < 2, due to protonation of the group(s) responsible for light-induced proton ejection from the membrane or, alternatively, as a consequence of a non-specific reversible damage to the bacteriorhodopsin generator.

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