

Cl[−]-dependent photovoltage responses of bacteriorhodopsin: comparison of the D85T and D85S mutants and wild-type acid purple form

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Abstract Laser flash-induced photovoltage responses of the D85S and D85T mutants as well as of the wild-type acid blue form are similar and reflect intraprotein charge redistribution caused by retinal isomerization. The Cl[−]-induced transition of all of these blue forms into purple ones is accompanied by the appearance of electrogenic stages, which is probably associated with Cl[−] translocation in the cytoplasmic direction. Cl[−] translocation efficiency of these purple forms is much lower than that of the proton transport by the wild-type bacteriorhodopsin. The values of the efficiency do not exceed 15, 8 and 3% for the D85T, D85S and wild-type acid purple form, respectively. Cl[−] induces an additional electrogenic phase in the photovoltage responses of the D85S mutant and the wild-type acid purple form. This phase is supposed to be associated with the reversible Cl[−] movement in the extracellular direction. It is interesting that this component is absent in the photovoltage response of the D85T mutant which has, like halorhodopsin, a threonine residue at position 85.

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Key words: Bacteriorhodopsin; Photocycle; Photovoltage response; Cl[−] transport; D85S and D85T mutants; *Halobacterium salinarum*

1. Introduction

Light-driven proton pump bacteriorhodopsin (bR) (for reviews, see [1,2]) and chloride ion pump halorhodopsin (hR) (for review, see [3]) contain the anionic aspartate (D85) and threonine, respectively, in the same position, i.e. in the immediate vicinity of the protonated Schiff base. Protonation of the D85 converts bR into an acid blue form incapable of proton transport. Further Cl[−] binding gives an acid purple form [4–14]. bR mutants D85T and D85S in the absence of Cl[−] are also blue pigments. After Cl[−] binding, these pigments are transformed into purple forms which are capable, like hR, of transporting Cl[−] in the cytoplasmic direction [15–17]. According to Dér et al. [18,19], the acid purple form of the wild-type bR can translocate Cl[−] in the cytoplasmic direction. However, Moltke and Heyn [20] did not confirm this conclusion. The photocycle of the acid purple form as well as D85T and D85S mutants is characterized by the presence of batho-intermediates and absence of the blue-shifted M intermediate [5,9,15,21].

In the present work, we have compared the photovoltage responses of the acid purple form of bR as well as of the

D85T and D85S mutants. Electrogenic stages which are probably due to the transmembrane Cl[−] transport in the cytoplasmic direction were revealed in all cases. At the same time, an electrogenicity likely associated with Cl[−] movement in the extracellular direction has been described for the acid purple form and the D85S mutant. A similar component in the D85T mutant is observed only at low pH.

2. Materials and methods

A phospholipid-impregnated collodion film was used as a partition between the two compartments of a Teflon cuvette filled with the reaction mixture. Freshly prepared purple membranes (PM) from the *Halobacterium salinarum* wild-type strain ET1001, or its D85S and D85T mutants, were adsorbed to the positively charged film impregnated with 10% (w/v) egg lecithin and 0.1% octadecylamine solution in decane. PM incorporated into liposomes were associated with the collodion film impregnated with 10% asolectin solution in decane in the presence of 30 mM MgSO₄. Subsequently the assay medium was changed to remove excess bR. For measuring photoelectric responses and processing of kinetic data, see [6,8,9]. The instrument time constant was > 0.2 μs.

The mutant strains were kindly donated by Prof. J. Lanyi and Prof. R. Needleman.

The bR photocycle was monitored with a single beam spectrophotometer [6,9]. Photoexcitation of bR was carried out with a YG-481 Quantel Nd laser operated in doubled frequency mode (wavelength, 532 nm; pulse half-width, 15 ns; output, 10 mJ).

The measurements were performed at room temperature.

3. Results and discussion

Laser flash excitation of the planar lipid membrane with adsorbed mutant D85S or D85T proteins induces generation of an electric potential difference with an amplitude of 1–2 mV and a half-risetime of < 0.2 μs in the absence of Cl[−] (Fig. 1A, curve a, and Fig. 3A, curve a). The photoelectric response decays on a millisecond time scale. Similar types of voltage responses are observed in mutant PM associated with the positively charged planar membrane and in proteoliposomes with bR attached to the negatively charged lipid membrane in the presence of divalent cations. The polarities of the responses are identical and their characteristics are similar to those of the photoelectric response of the wild-type blue acid form. This form is incapable of proton transport, and its response possibly reflects charge redistribution within the chromophore and/or its nearest environment [6,8,9]. The polarity of the response (the so-called negative phase) is opposite to the direction of the proton transport by the neutral purple bR. Addition of sulfate anions up to 0.8 M neither affects the photoelectric responses nor changes the blue spectra of the mutant proteins.

Addition of Cl[−] results in an increase in the photoelectric response amplitude of the D85S mutant (Fig. 1A); electro-

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Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; PM, purple membrane

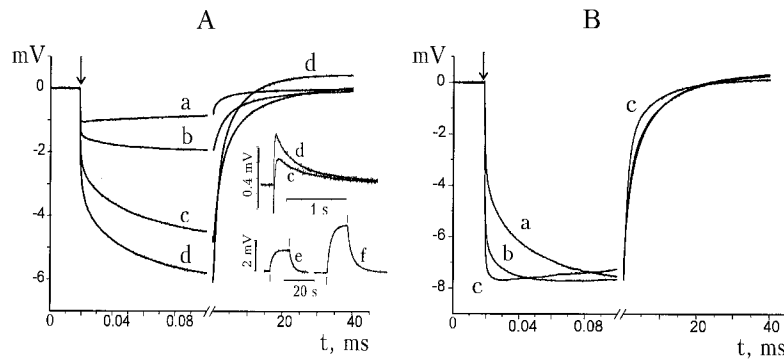


Fig. 1. Photovoltage responses of the dark-adapted D85S mutant (PM absorbed on the collodion film). A: The Cl^- dependence of the photovoltage responses induced by laser flash (a–d) and by continuous illumination (e, f). The assay medium: 30 mM Na_2SO_4 , 10 mM MES, pH 5 (a–d); 30 mM Na_2SO_4 , 10 mM HEPES, pH 7 (e, f). a, no addition; b, 30 mM NaCl; c, 300 mM NaCl; d, 2 M NaCl; e, 1 M NaCl; f, 2 M NaCl. B: pH dependence of the laser flash-induced response of the D96S mutant. The assay medium: 2 M NaCl, 30 mM Na_2SO_4 , 10 mM MES. a, pH 5; b, pH 1.6; c, pH 0.4.

genic components with τ about 15 and 150 μs (at pH 5) appeared. pH decrease at saturated Cl^- concentration induces a decrease in the half-rise time to $< 1 \mu\text{s}$ (Fig. 1B). The relaxation rate slightly depends on the concentration of Cl^- or H^+ . The major feature of the photoelectric response in the presence of Cl^- is the distinct 'positive overshoot' with amplitude depending on the Cl^- concentration (Fig. 1A, curves c and d). Relaxation of the overshoot to the initial level is complete within seconds with the time constant equal to that of the passive discharge. Cl^- addition also leads to the appearance of the electrical response induced by continuous illumination ($\lambda > 540 \text{ nm}$) (Fig. 1A, curves e and f). Polarity of the overshoot and the photoelectric response on the continuous illumination corresponds to the H^+ transport by bR in the extracellular direction or the Cl^- transport in the cytoplasmic direction. This is in line with the data of Lanyi's laboratory [15,16], demonstrating that the mutant proteins are competent in Cl^- transport in the cytoplasmic direction.

Fig. 2A shows the dependence of the amplitude of the 'negative' phase on the Cl^- concentration. The observed effect correlates fairly well with transition of the mutant bR into the purple form (data not shown). Both of them are pH-dependent. According to Brown et al. [16], the effect of pH on the blue-to-purple transition is due to the contribution of the protonation state of Glu-204.

Light adaptation results in some decrease in the amplitude

of the 'negative' phase and a 2–3-fold increase in the overshoot (Fig. 2B). In addition, light adaptation leads to an increase in the amplitude of the fast decaying bathointermediate ($\tau = 5 \text{ ms}$) and a decrease in the amplitude of the slowly decaying one ($\tau = 50 \text{ ms}$) (not shown). Both the electrical and optical measurements reveal the fast rate of the dark adaptation ($t_{1/2} < 30 \text{ s}$ at pH 5). One may assume that that 'negative' phase is due to the photocycle including both 13-*cis*- and all-*trans*-bRs, whereas the overshoot is related to the photocycle of the all-*trans*-bR with no 13-*cis*-bR involved.

The photoelectric response of the D85T mutant in the presence of Cl^- at pH > 4 differs from that of the D85S mutant. The Cl^- addition does not affect the 'negative' phase but induces the overshoot which decays in seconds (Fig. 3A). As in the D85S mutant, Cl^- induces the photoelectric response upon continuous illumination (data not shown). pH decrease in the presence of Cl^- leads to a distinct increase in the 'negative' phase amplitude and some decrease in the overshoot (Fig. 3B). Thus, at pH < 3 the photoelectric response of the D85T mutant is similar to that of the D85S mutant. The light-dark adaptation does not affect the photoelectric response of the D85T mutant.

The Cl^- effect on the photoelectric response is very slow. It takes several minutes in spite of vigorous mixing. Reversal of the effect after Cl^- removal is equally slow. The phenomenon is likely related to the binding of Cl^- ions which come from

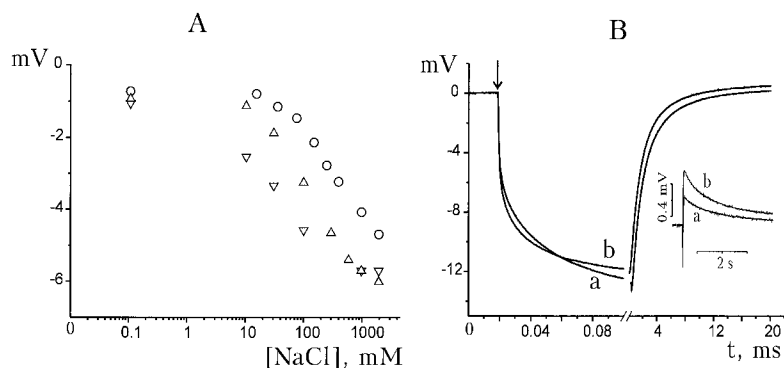


Fig. 2. A: The Cl^- dependence of the 'negative' phase amplitude of the laser flash-induced photovoltage response of the dark-adapted D85S mutant at different pH values (PM absorbed on the collodion film). The assay medium: 30 mM Na_2SO_4 , 10 mM MES. ∇ , pH 3.5; Δ , pH 5; \circ , pH 6. B: Light-dark adaptation effect on the laser flash-induced photovoltage response of the D85S mutant. The assay medium: 2 M NaCl, 30 mM Na_2SO_4 , 10 mM HEPES, pH 5. a, dark-adapted sample; b, light-adapted sample.

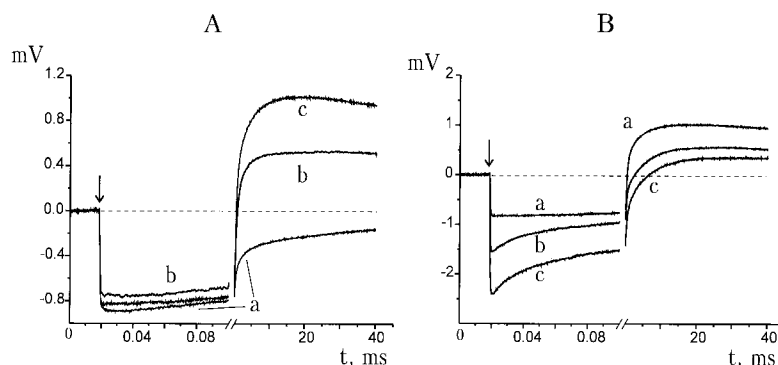


Fig. 3. Laser flash-induced photovoltage response of the D85T mutant (PM absorbed on the collodion film). A: The Cl^- dependence of the photovoltage response. The assay medium: 30 mM Na_2SO_4 , 10 mM MES, pH 5. a, no addition; b, 500 mM NaCl; c, 2 M NaCl. B: pH dependence of the photovoltage response. The assay medium: 2 M NaCl, 30 mM Na_2SO_4 , 10 mM MES. a, pH 5; b, pH 3; c, pH 2.

the external surface in the ground bR state (remember that the bR molecules attached to the planar membrane are exposed to the bulk water phase by their cytoplasmic surface).

Relaxation of the 'negative' phase and formation of the overshoot in the photovoltage response at pH 5 includes components of $\tau=0.4$ ms (65%) and 3 ms (35%) that are close to the slow component of the bathointermediate formation ($\tau=0.3$ ms) and its decay ($\tau=3$ ms), respectively. These spectral components correspond to the Cl^- release and subsequently its uptake from the opposite bR surface [16].

The blue acid form of the wild-type bR with protonated Asp-85 gives the purple acid form after Cl^- binding [4–14]. This transition is very similar to the corresponding transition in the D85T and D85S mutants. The photoelectric response of the wild-type neutral purple form consists of three main phases: small and very fast 'negative' and two 'positive' ones proceeding on the microsecond and millisecond time scales, respectively (Fig. 4). The two 'positive' phases represent the transmembrane proton transport, whereas the 'negative' one has been interpreted as the intraprotein charge redistribution during bR \rightarrow K \rightarrow L transitions [6,8,9]. The photoelectric response of the blue acid form comprises only the 'negative' phase with either HCl or H_2SO_4 used to produce the blue form. The Cl^- binding results in a significant increase in the amplitude of the 'negative' phase (Fig. 4) [6,9,20] and in formation of the acid purple form. According to Dér et al. [18,19], the acid purple form is capable of light-driven transmembrane Cl^- transport. This idea was based on the investigation of the oriented purple membrane in polyacrylamide gel. However, Moltke and Heyn [20] did not confirm this conclusion when they used PM absorbed to the planar membrane. These authors did not observe the components of the net transport (the overshoot) induced by the laser flash. We also failed to find any overshoot using the PM absorbed to the planar membrane. However, the overshoot was always observed in bR liposomes (Fig. 4, curve c). Small electric responses upon continuous illumination were also found (data not shown). The data obtained confirm the conclusion of Dér et al. [18,19] on the ability of the acid purple form to transmembrane translocation of Cl^- . The amplitude of the overshoot is 3–6% of that of the 'negative' phase, which is in line with the data of Dér et al. [19]. According to these authors the sum of the 'positive' amplitudes exceeded the sum of the 'negative' amplitudes by 4% (see Table 2 in [19] and Discussion in [20]). The absence of the overshoot in PM is

probably due to the high permeability of the contact between the PM and planar membrane at low pH and high salt concentration. This 'leaky' contact would account for the high rate of passive membrane potential discharge in spite of the high planar membrane resistance [9].

Thus, taking these results together, one can suggest that the purple acid forms of the wild-type bR as well as mutants D85T and D85S can function as chloride pumps and display similar laser flash-induced photoelectric signals. We believe that the nature of the early charge redistribution within the chromophore in all blue forms is the same. If so, the magnitude of the 'negative' phase may be used as the internal standard. The amplitude of the blue acid form 'negative' phase does not exceed 10% of that in the wild-type bR 'positive' phase reflecting transmembrane transport of a single H^+ . The overshoot amplitude in D85T is not more than 1.5 times larger than the amplitude of corresponding blue form 'negative' phase. Thus, in this case the efficiency of transmembrane Cl^- transport does not exceed 15% of the efficiency of proton transport by wild-type bR. In D85S and the acid purple form, the efficiency is 4–8% (for all-*trans*-D85S-bR) and 1–3%, respectively. The low efficiency of Cl^- transport by these bR forms may be due to the fact that the cytoplasmic half-channel is closed in the bR ground state. On the other hand, the cytoplasmic half-channel is likely open in the hR ground state. Electron microscopic data indicate some structural differences in the bR and hR ground states, resembling the difference between the closed conformation of the bR ground state and the open conformation of the M intermediate, respec-

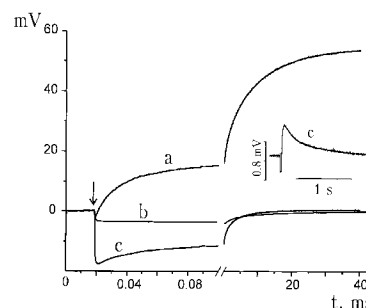


Fig. 4. Laser flash-induced photovoltage response of the wild-type bR (bR liposomes adsorbed on the collodion film). The assay medium: 30 mM Na_2SO_4 , 10 mM MES. a, light-adapted neutral purple form, pH 5; b, acid blue form, pH 0.1 (acidification by H_2SO_4); c, acid purple form, 1 M NaCl, pH 0.1 (acidification by HCl).

tively [21–23]. In addition, opposite effects of hydrostatic pressure on the photocycles of bR and hR suggest different sequences of structural changes during their photocycles [24].

The nature of the Cl[−]-dependent 'negative' phase is not clear yet. Moltke and Heyn [20] supposed that it is due to intra-protein H⁺ transport. We suggest that generation of the large 'negative' phase is the feature of the Cl[−]-containing acid purple form. Sulfate does not affect the magnitude of the blue form electric response. (Some increase in the photoresponse in the presence of sulfate took place when excess unattached PM was not completely removed.) In the neutral purple wild-type bR form, retinal isomerization results in proton transfer to Asp-85. The same isomerization should lead to the destabilization of the movable anion (Cl[−]) serving as a counterion for the protonated Schiff base in the absence of deprotonated Asp-85. This process may lead to Cl[−] transport not only to the cytoplasmic surface but also to the external surface of the PM through the open outward proton channel. The latter would induce the development of the 'negative' phase. The following Cl[−] uptake would result in the relaxation of the 'negative' phase. In those bR molecules that had released Cl[−] on the cytoplasmic surface, this uptake would contribute to the overshoot potential. Obviously, the efficiency as well as the rate of the Cl[−] transport through the proton outward channel may depend on the protonation state and on the structure of this channel depending in turn on the type of amino acid substitution in the channel. Several observations are in line with such a model. Two of them indicate the apparent competitive behavior of the 'negative' phase and the overshoot: (1) the increase in the amplitude of the D85T 'negative' phase at low pH is accompanied by the decrease in the overshoot (Fig. 3B), (2) the Cl[−] translocation efficiency of the D85T at pH > 4 is larger than the corresponding efficiencies of the D85S and acid purple form. Other indirect evidence is based on the correlation between the kinetics of the photovoltage response and of the bathointermediate that is probably similar to the bR state without bound Cl[−]. Generation of the large 'negative' phase of the acid purple form correlates with bathointermediate formation [6,9]. The rise of the 'negative' phase in the case of the D85S mutant comprises one component of the bathointermediate formation. On the other hand, relaxation of the D85T 'negative' phase and generation of the overshoot (i.e. the transmembrane Cl[−] translocation) include the components of the bathointermediate formation and subsequently its decay.

Note that the presence of a threonine but not a serine residue at position 85 prevents Cl[−] extrusion to the extracel-

lular surface at neutral pH. It may be important for the functioning of hR as an effective Cl[−] pump.

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