Photovoltage evidence that Glu-204 is the intermediate proton donor rather than the terminal proton release group in bacteriorhodopsin

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Abstract Electrogenic events in the E204Q bacteriorhodopsin mutant have been studied. A two-fold decrease in the magnitude of microsecond photovoltage generation coupled to M intermediate formation in the E204Q mutant is shown. This means that deprotonation of E204 is an electrogenic process and its electrogenicity is comparable to that of the proton transfer from the Schiff base to D85. pH dependence of the electrogenicity of M intermediate formation in the wild-type bacteriorhodopsin reveals only one component corresponding to the protonation of D85 in the bacteriorhodopsin ground state and transition of the purple neutral form into the blue acid form. Thus, the p*K* of E204 in the M state is close to the p*K* of D85 in the bacteriorhodopsin ground state (<3) and far below the p*K* of the terminal proton release group (~ 6). It is concluded that E204 functions as the intermediate proton donor rather than the terminal proton release group in the bacteriorhodopsin proton pump.

Key words: Bacteriorhodopsin; Photocycle; Photovoltage response; Proton transport; *Halobacterium salinarium*; E204Q mutant

1. Introduction

Bacteriorhodopsin (bR) from *Halobacterium salinarium* is a proton pump that converts light energy into a proton electrochemical gradient across the cell membrane (for reviews, see [1–6]). The chromophore group of bR is a retinal covalently bound to the ε-amino group of the K216 via protonated Schiff base. Light absorption induces an all-*trans* to 13-*cis* isomerization of the chromophore and a complex photochemical cycle including intermediates K, L, M, N and O. The chromophore isomerization results in the proton transfer from the Schiff base to the internal proton acceptor D85 during L→M transition. D85 stays protonated until the end of the photocycle [7,8]. The L→M transition is accompanied by a proton release from the protein at pH > 6. At lower pH, the proton release is delayed and parallels the O→bR transition [9]. Such pH dependence of the proton release was explained in a suggestion of the existence of a proton release group X protonated in the bR ground state. It was assumed that the p*K* of X decreases up to ~5.8 during L→M transition [9]. R82, E204 and E194, besides D85, were identified as the residues taking part in the proton transfer through the outward proton pathway [10–18]. Complicated pH dependences of the rate of dark adaptation and titration of D85 were explained in terms of coupling of p*K* of two residues, namely D85 and the terminal proton release group. This coupling probably plays the crucial role in the proton transfer reactions during L→M transition [11–18]. E204 was speculated to operate as the terminal proton release group [13,14].

In this paper we report that deprotonation of E204 is accompanied by the electric potential generation. The magnitude of this potential is comparable to that of proton transfer from the Schiff base to D85. The pH dependence of the electrogenic reactions in question indicates that the p*K* of E204 in the M state is <3 like that of D85 in the bR ground state. Thus, E204 functions as the intermediate proton donor rather than the terminal proton release group. These data were presented on the 8th International Conference on Retinal Proteins [19]. After these data had been obtained, Dioumaev et al. published a paper where a similar conclusion was drawn on the basis of a quite different approach: time-resolved protonation changes of D194 in the E194D mutant were detected revealing that E194 rather than E204 is the terminal proton release group [20].

2. Materials and methods

A phospholipid-impregnated collodion film was used to separate two compartments of a Teflon cuvette filled with the reaction mixture. The PM sheets were adsorbed onto the positively charged film impregnated with 10% (w/v) L-α-phosphatidylcholine (type V-E, from frozen egg yolk, Sigma) and 0.1% octadecylamine solution in *n*-decane. The bR-containing ultrasonic proteoliposomes [protein/lipid (w/w), 1:100] were adsorbed onto the collodion film impregnated with 10% L-α-phosphatidylcholine (type II-S, from soybean, Sigma) solution in *n*-decane in the presence of 30 mM $MgSO_4$. Subsequently, both compartments were washed with a 20-fold volume of the assay buffer to remove excess bR. For measuring photoelectric responses and processing of kinetic data, see [21–25]. The instrument constant was 0.1 μs.

The bR photocycle was monitored with a single beam spectrophotometer [21–25]. Photoexcitation of bR was carried out with a YG-481 Qantel 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.

For calculation of the sum of amplitudes of the microsecond electrogenic phases shown in Figs. 3 and 4, the amplitudes of the exponential components whose rates are comparable to those of the M intermediate formation were used. The fit of the experimental curves to the sum of exponentials was obtained with the DISCRETE program [26].

Freshly prepared PM for the *H. salinarium* wild-type ET1001 and E204Q mutant strains were used. The E204Q strain was kindly provided by Prof. J. Lanyi and Prof. R. Needleman.

3. Results and discussion

Fig. 1 shows laser flash-induced voltage responses of the wild-type bR and the E204Q mutant. The proteins were pre-

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Abbreviations: bR, bacteriorhodopsin; PM, purple membrane

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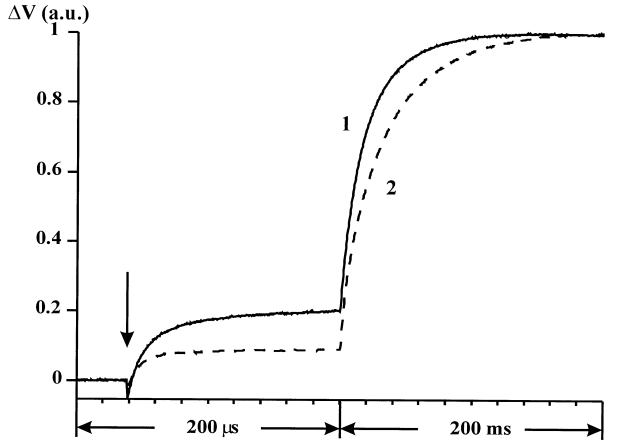

Fig. 1. Comparison of the flash-induced voltage responses of the wild-type bR (1) and E204Q mutant (2). bR-containing proteoliposomes were attached to the lipid-impregnated collodion film. Assay medium: 100 mM NaCl, 5 mM HEPES, pH 7.

viously incorporated into liposomes. The proteoliposomes obtained were adsorbed onto the lipid-impregnated collodion film. It is well known [21–24] that the photovoltage response of the wild-type bR comprises two main proton-translocating 'positive' phases: the fast microsecond phase associated with M intermediate formation and the slow millisecond phase associated with M decay and regeneration of the bR ground state. In the E204Q mutant, (i) the magnitude of the microsecond phase proved to be two-fold lower and (ii) the millisecond phase was slower than in the wild-type protein. The latter effect was most probably due to the slower rate of bR ground state regeneration resulting from the appearance of a long-living O intermediate in the E204Q mutant photocycle [13,18]. In liposomes qualitatively the same photocycle changes are revealed (not shown).

The ratio of the amplitudes of the two main electrogenic phases was shown to be constant when the photovoltage was measured on proteoliposomes. In attached PM, however, this parameter proved to be variable, possibly due to the leaky contact between the planar membrane and at least some of the adsorbed PM. So, a different approach was used to analyze the photoresponse in the PM. Acidification of the bR suspension is known to induce protonation of D85 and formation of the blue acid form incapable of proton transport. The photovoltage response of this form has the opposite direction to the main proton translocating phases in the purple neutral form photoresponse. Its amplitude does not exceed 10% of the overall photoresponse of the purple neutral form [21–23,25]. Probably, this photoresponse is a result of the charge redistribution in bR, induced by retinal isomerization. Due to a lack of proton transport, one may hope that the photoresponse of the blue acid form will be insensitive to the E204Q substitution. If it were the case, the photoresponse of the blue acid form would be used as an internal standard to normalize the photovoltage responses of the neutral form. Such an approach (Fig. 2) confirmed that the microsecond phase of the mutant neutral form is approximately two times smaller than that of the wild type. The rate of the neutral form microsecond phase was faster than in the wild type due to the faster rate of M intermediate formation. The flash-induced maximal amplitude of the M intermediate proved to be the same in mutant and wild-type bR in PM (see below, Fig. 4A,B) as well as in proteoliposomes (not shown). Thus, the above effect of mutation on the microsecond electrogenic phases cannot be ascribed to a shift of equilibrium between the M and L intermediates.

The simplest explanation of the obtained data is presented in Fig. 3A. We suppose that M intermediate formation in the wild-type bR is accompanied by two proton translocating reactions with comparable electrogenicities, namely (1) proton transfer from the Schiff base to D85 and (2) from E204 to the water medium or to some acceptor in the interphase. In the case of the E204Q mutant, only the former process takes place. Our scheme does not imply any protein conformational changes during the proton release phase. But if such changes exist, they are probably not electrogenic.

It is noteworthy that Govindjee et al. [16] observed at pH 9.0 two photocurrent components with equal electrogenicity in the oriented PM containing mutant R82Q. These components were attributed to the proton transfer from the Schiff base to D85 and to the deprotonation of the proposed release group. The data mentioned are in line with our interpretation of the electrogenic events coupled with M intermediate formation.

If the electrogenicity of deprotonation of E204 were equal to that of the Schiff base and E204 were the terminal proton release group, the pH dependence of the amplitude of the microsecond electrogenic phase in the wild-type bR would

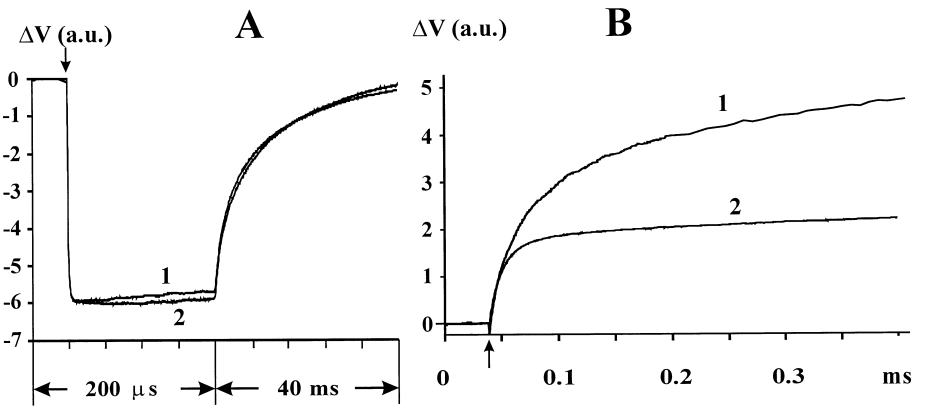

Fig. 2. Comparison of the flash-induced voltage responses of the wild-type bR (1) and E204Q mutant (2). PM were attached to the lipid-impregnated collodion film. A: pH 1. B: pH 7. The electrical signals measured at pH 1 were used for normalization of the responses measured at pH 7. The assay medium: 30 mM Na_2SO_4, 5 mM HEPES. A.u., arbitrary units.

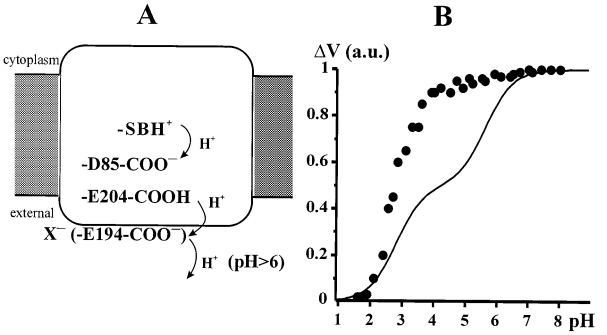

Fig. 3. A: A proposed scheme of the proton transfer reactions coupled with M intermediate formation. B: pH dependence of the sum of the microsecond electrogenic phase amplitudes for the wild-type bR (PM). The solid curve represents the expected pH dependence with pK 5.8 and 2.3 and equal contributions (see text for details). Assay medium: 30 mM Na_2SO_4, 2 mM Na-citrate, 2 mM MES, 2 mM HEPES.

comprise two components of similar amplitudes and different pK. One pK would be equal to the pK of the terminal proton release group in the M state ($\sim$5.8) and another to the pK of D85 in the bR ground state. However, experimental data (Fig. 3B) reveal the existence of a single transition with low pK corresponding to the purple neutral $\rightarrow$ blue acid form transition. Note the small ($\leq$10%) decrease in amplitude induced by a pH decrease from 7 to 4. This decrease is accompanied by the similar lowering of the M level due to most probably a pH dependence of the M $\Leftrightarrow$ L equilibrium. An equilibrium sensitive to the pK of the proton release group in the M state is quite obvious in the D115N and D96N mutants [9] but is also revealed in the wild-type bR [27].

Thus, one may conclude that there are no electrogenic processes involved in M intermediate formation with p$K\sim$5.8. In fact, these data support our previous observation on pH dependence of the microsecond phase amplitude in PM and the bR proteoliposomes [22,23]. Moreover, Ormos et al. [28] did not find any changes in the microsecond components of the photocurrent measured in the oriented purple membrane at $4.5 < pH < 10.5$. Liu [29], using the same photocurrent method, described a pH dependence of the microsecond component quite similar to ours. In our opinion, the data obtained indicate that E204 is deprotonated even at low pH, and its pK value in the M state is comparable to that of D85 in the bR ground state. Thus, E204 is not the terminal proton release group. This means that another terminal group (X) exists. It is obvious, that if X is the only proton release group between E204 and the bulk water phase, it should be deprotonated in the bR ground state, and its pK should increase during M formation and deprotonation of E204. When this paper was in preparation, the article by Dioumaev et al. [20] was published in which such behavior was found for D194 (the time-resolved protonation of D194 in the E194D mutant was measured). The authors concluded that E194 is the terminal proton releasing group accepting a proton from E204. Our photovoltage data are in line with such a model.

At high pH (Fig. 4A,B), the rate of M formation is greatly accelerated in the wild-type bR but not in the E204Q mutant [14]. The acceleration of M intermediate formation in the wild-type bR is possibly due to E204 deprotonation in the bR ground state [12,14,30]. In the wild-type bR, the acceleration of M intermediate formation is accompanied by a two-fold decrease in amplitude of the microsecond electrogenic phase (Fig. 4C). This decrease is also coupled to the acceleration of the microsecond electrogenic phase, coinciding with the acceleration of M intermediate formation. A similar in-

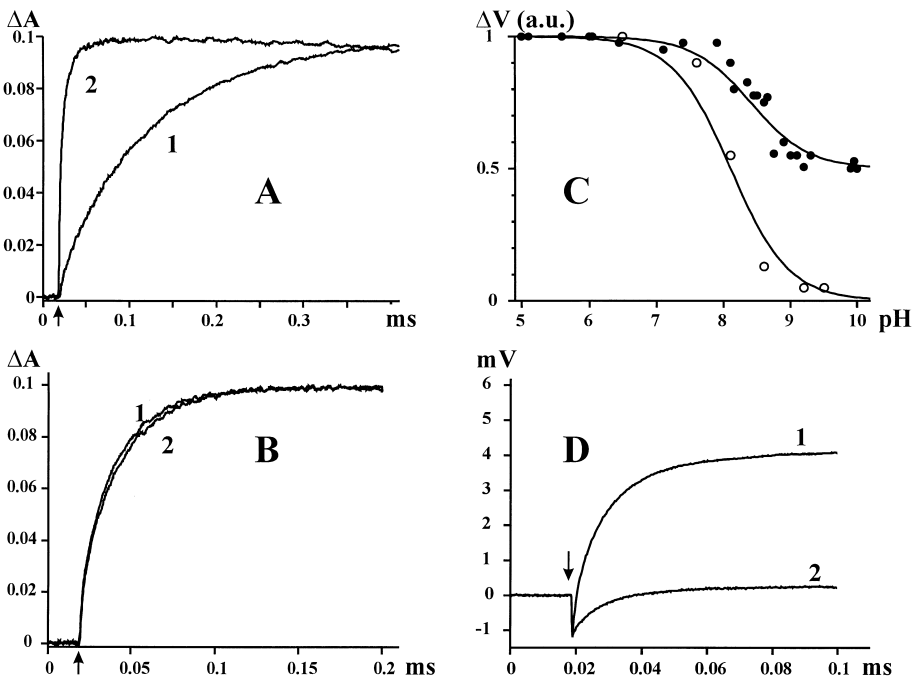

Fig. 4. A, B: Flash-induced optical changes at 400 nm at pH 6 (1) and 10 (2) in the wild-type bR and E204Q mutant, respectively (PM). C: pH dependence of the sum of the microsecond electrogenic phase amplitudes for the wild-type bR (●) and E204Q mutant (○) (PM). Solid curves represent the titration of the protonatable group with pK 8.4 (upper curve) and 8.1 (lower curve). D: Comparison of the microsecond electrogenic phases in the E204Q mutant at pH 6 (1) and pH 9 (2). Assay medium: 0.5 M Na_2SO_4, 5 mM HEPES, 5 mM Tris, 5 mM CHES, 5 mM CHAPS.

crease in rate of the microsecond phase and decrease in the sum of their amplitudes were detected in photocurrent measurements in oriented PM suspension by Kono et al. [30] and Liu [29]. All these observation are in line with the conclusion on the electrogenic nature of the E204 deprotonation. The deprotonation of E204 in the bR ground state at high pH should lead to a decrease in electrogenicity of the M formation.

However, some unusual results were obtained on the E204Q mutant. Taking into account the absence of E204 and the independence of the M amplitude of pH (Fig. 4A,B), it would be expected at high pH that the microsecond electrogenic component in the mutant would be pH-independent. Nevertheless, a pH increase results in disappearance of this electrogenic component with a p*K* slightly lower than that of transition of the wild-type bR into the alkaline form (Fig. 4C). Note the pH independence of the 'negative' phase associated with K and L intermediate formation (Fig. 4D). We suppose that deprotonation of the unknown group Y leads to some kind of distortion of the outward proton pathway. For example, an increase in dielectric permeability near D85 due to the appearance of some water molecules should lead to a decrease in the measuring amplitude of electric dipole generation as a result of proton transfer from the Schiff base to D85. However, some other possibilities (such as structural changes and so on) cannot be excluded. In spite of this, E204 in deprotonated state seems to stabilize the outward proton pathway at high pH. Moreover, it is possible that the p*K* of E204 in the bR ground state may be determined by the p*K* of Y.

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