Photoelectric response of the N intermediate of bacteriorhodopsin and its mutant T46V

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Abstract Double flash experiments were performed in order to gain information about the characteristics of the N intermediates of the photocycle of bacteriorhodopsin. The N intermediates of wild-type bacteriorhodopsin and mutant T46V were excited at different delay times after the first laser flash which induced the photocycle and the electric responses were registered. These electric signals revealed that charge motions occurred in both cases, though charge translocation, i.e. H^+ pumping, could not be observed. The delay time dependence of the electric signals is characterized by two distinct processes corresponding to two substates of the N intermediates.

Key words: Retinal protein; Bacteriorhodopsin mutant; Double flash excitation; Oriented purple membrane; Proton pumping

1. Introduction

After absorption of a photon the bacteriorhodopsin (bR) molecule from *Halobacterium salinarum* is excited and relaxes through intermediates, named J, K, L, M, N and O, to its ground state. These intermediates, members of the photocycle of bR, are characterized by the maximal wavelength of their absorption and their life time (recent reviews [1–3]). The intermediates may also absorb photons and have their own photocycle ending in the ground state of bR. The study of the photocycle of the intermediates is important because it offers information on the photocycle of bR itself and because the behavior of bR under physiological conditions, i.e. under the continuous spectrum of the sunlight which excites the intermediates too, may be better understood [4]. Probably these are the reasons for the numerous reports dealing with the photoexcitation of the intermediates (see the review by Balashov [5]).

In this paper we report measurements of the photoelectric responses of the N intermediates of wild-type bR (WTbR) and mutant T46V, known to have a long-living N intermediate [6,7]. Photoexcitation of the N intermediate of these species has already been studied with light absorption measurements [6,8,9]. The study that previously reported on photoelectric responses of the N intermediate left the question of charge translocation during the N photocycle open [10]. Here we show that charge translocation does not occur in the case of the N intermediate of the wild-type bR or the mutant T46V.

2. Materials and methods

Purple membrane (pm) containing WTbR was separated from *H. salinarum* strain R_1M_1. The pm with T46V mutant expressed in strain L-33 was supplied by J.K. Lanyi. The membrane fragments were oriented and immobilized in gel as described in [11]. Slabs measuring $1.6\times1.6\times0.18$ cm were cut and soaked overnight at least in a solution (≈100 cm^3) containing 50 μM $CaCl_2$ at pH 9.5 for WTbR and pH 7.5 for the mutant and placed in the same solution into cuvettes.

The samples were illuminated with two lasers: a frequency-doubled Nd YAG laser (Surelite I-10, Continuum), and an excimer laser-driven dye laser (Lambda Physik, EMG 101 MSC). The wavelengths were 530 nm and 580 nm (using Rhodamine 6G dye), respectively. The energy varied between 1 and 2 mJ. Platinized Pt electrodes immersed into the solution picked up the electric signals, which were amplified by a home-made current amplifier based on a Burr-Brown 3554 operational amplifier with band width set at 100 kHz for the short and at 1 kHz for the long time recordings, and digitized by computer-controlled transient recorders (Thurlby DSA-524 with 1000 channels or LeCroy 9310 L with 10 000 channels). A home-made generator controlled the time delay between the two flashes. Data recording could be triggered either from the first or the second flash. The NdYAG laser was always used as first flash.

Absorption changes were monitored at 632 nm from a He-Ne laser in the case of WTbR and at 575 nm selected with two interference filters from the light of a 250 W tungsten lamp in the case of mutant T46V. Application of the He-Ne laser in such complicated systems is technically easier but the light absorption changes in the case of mutant T46V were so small that we had to apply monitoring light of 575 nm. The light intensity was measured with a photomultiplier and digitized with transient recorders. Light beams from the three different sources (first laser flash, second laser flash and the monitoring light) were focused onto the same area of the sample as accurately as possible.

The measurements were performed at room temperature (22–24°C).

3. Results

The difficulty in measuring either the light absorption changes or the photoelectric signals from the intermediates is due to the unavoidable excitation of the ground state and other intermediates absorbing at the exciting wavelength. In order to avoid this problem, we applied the following protocol:

(1) The absorption change is measured at 632 or 575 nm. The registered curve is the sum of the positive absorption change due to the N intermediate and the negative one due to the bleached ground state of bR because the absorption maxima of the two forms are near to each other. This involves the assumption that the contribution of other intermediates is negligible. The longest time constant and the maximum absorption (τ_1, A_{max}) of the appearing negative absorption curve are determined, an exponential is constructed with these parameters and subtracted from the measured curve. The resulting absorption change $A_N(t)$ is due to the N intermediate. This process is illustrated in Fig. 1 for the WTbR (line a is

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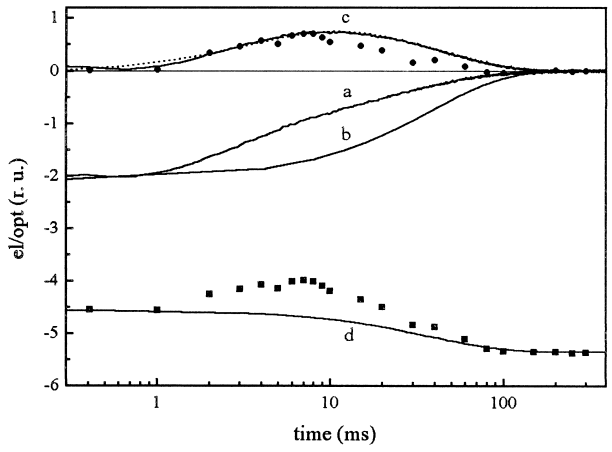

Fig. 1. Processing of the electric and optical data in the case of wild-type bacteriorhodopsin. Line a: light absorption change at 632 nm; line b: calculated function for the bleaching of bR ($-1.96\times\exp(-(t-0.8)/38)$ where the time values are given in ms); line c: difference between lines a and b, representing time dependence of the N intermediate, dotted line is the fit: $\tau_{rise} = 4.0 \pm 0.3$ ms, $\tau_{decay} = 38.1 \pm 0.9$ ms. Squares: integrated values of the first fast electric signal measured at different delay times after the first flash; line d: the calculated contribution from the excitation of the ground state ($-0.8\times(1-\exp(-(t-0.8)/38))-4.6$), circles: differences between the squares and line d. Data are scaled in order to show the time behavior of the processes. pH 9.5, temperature 24°C.

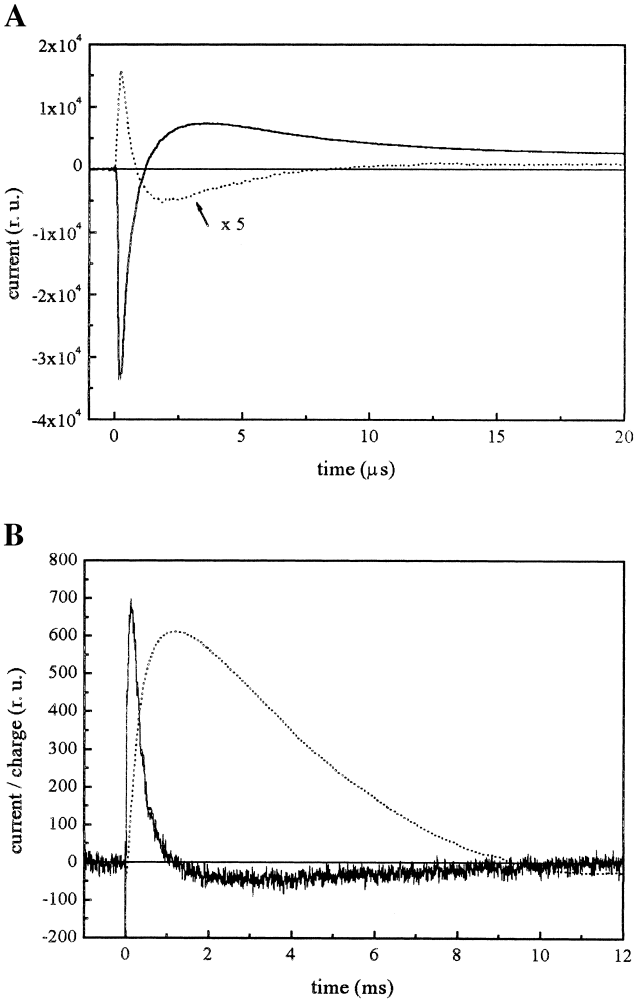

Fig. 2. Electric signals attributed to the photoresponse of the N intermediate in the case of wild-type bR. A: μs time domain, solid line signal from the excitation of the ground state, dotted line from N intermediate. B: ms time domain, solid line the measured electric current, dotted line its time integral representing the charge transport. Time delay between the two flashes was 7 ms. Data are scaled. Solution as in Fig. 1.

the measured absorption change, b is the constructed exponential and c is $A_N(t)$.

(2) The first component of the electric signal, assumed to be proportional to the number of the excited photocycle [12], is measured depending on the time delay between the two flashes. In this case the recording is triggered with the second flash. The value of the time integral of the signal (I_t) is plotted versus the delay time, I_∞ being the value without preflash. These signals represent charge motion in a 'negative' direction, i.e. opposite to the direction of the proton translocation. An important assumption is that at the shortest time delay the signal (I_s) originates primarily from the excitation of the ground state only because the N intermediate is not yet built up and no other intermediates absorb at 580 nm. With this assumption the signals due to the N intermediate are calculated for every delay time t as:

$$I_N(t) = I_t - \{I_S + (I_\infty - I_S)(1-e^{-t/\tau_1})\} \quad (1)$$

The function $I_S + (I_\infty - I_S)(1-e^{-t/\tau_1})$ is line d in Fig. 1. If the assumption is correct then the normalized values of $I_N(t)$ and $A_N(t)$ coincide. Fig. 1 contains these data for WTbR (squares correspond to I_t, circles to $I_N(t)$. It will be shown that this assumption does not hold in the case of mutant T46V.

(3) Detailed measurements of the electric signals in the photocycle of N intermediates were performed at those delay times when $I_N(t)-s$ were at a maximum.

3.1. Wild-type bR

In these measurements, the pH of the bathing solution was set at 9.5 in order to obtain long living N intermediates as in [9]. Fig. 1 shows that the differences calculated for the excitation with the second, 580 nm flash (circles) are near to the derived time dependence of the N intermediate ($A_N(t)$, line c). $I_N(t)$ deviates from $A_N(t)$ at longer delay times.

Electric signals were measured at a delay time of 7 ms and

Table 1
Parameters of the exponentials fitted to the calculated absorption curve $A_N(t)$, to the difference curve $I_N(t)$ representing the excitation of the N intermediate of the mutant T46V and to the electric response of the ground state excitation *El(GS)*

Function	τ_1	A_1	τ_2	A_2	τ_3	A_3
$A_N(t)$	0.68 ± 0.02	−0.57 ± 0.01	16.2 ± 2.4	−0.15 ± 0.01	131 ± 3	0.77 ± 0.01
$I_N(t)$	n.d.	n.d.	20 ± 1	0.21 ± 0.05	n.d.	n.d.
El(GS)	1.1 ± 0.1	0.81 ± 0.01	5.7 ± 0.1	0.187 ± 0.004	170 ± 90	0.0020 ± 0.0006

Life time values τ_1, τ_2, τ_3 are in ms, the amplitudes A_1, A_2, A_3 in relative units.

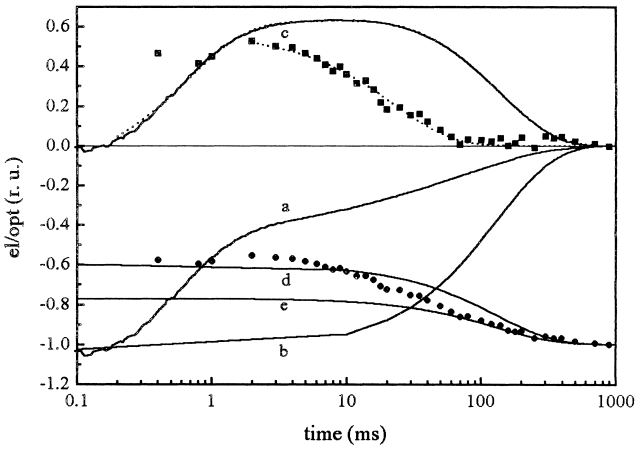

Fig. 3. Processing of the electric and optical data in the case of the mutant T46V. Line a: light absorption change at 575 nm; line b: calculated function for the bleaching of bR ($-1.025\times\exp(-(t-0.2)/131)$ where the time values are given in ms); line c: difference between lines a and b, representing the time dependence of the N intermediate, dotted line is the fit (values are given in Table 1). Circles: integrated values of the first fast electric signal measured at different delay times after the first flash; line d: $-0.4\times(1-\exp(-(t-0.2)/131))-0.6$; line e: $-0.23\times(1-\exp(-(t-0.2)/131))$ are the calculated contributions from the excitation of the ground state. Squares: differences between the circles and line e, dotted line is the fit (data in Table 1). Data are scaled in order to show the time behavior of the processes. pH 7.5, temperature 24°C.

the differences calculated. The signals are plotted in Fig. 2A,B for the μs and ms time domain, respectively. The first component of the electric signal for the N intermediate is positive followed by a negative component (Fig. 2A). In the ms time range positive and negative components appear (Fig. 2B). The time integral of the electric signal goes to zero indicating that charge translocation does not occur in this process.

3.2. Mutant T46V

Fig. 3 contains the optical and electric data. The optical data were processed as in Fig. 1: line a shows the absorption change at 575 nm, b is the recovery of the ground state of bR, c is the calculated time dependence of the N intermediate $A_N(t)$ which is fitted with three exponentials (the parameters are in Table 1). This process involves the assumption, also taken in [6,7], that after the decay of the M intermediate no other intermediates absorbing at 580 nm (for example L intermediate) prevail. The procedure dealing with the electric signals, as in Eq. 1, leads to curve d in Fig. 3. Subtracting it from the measured values (not shown), positive and negative lobes would appear. The time course of the electric signals determined at the maximum of the negative lobe at delay time 70 ms does not differ from those without preflash, consequently, they may not be considered as originating from the N intermediate. Accordingly, curve e was constructed and subtracted from the data yielding the difference $I_N(t)$ (circles). It is clear that $I_N(t)$ does not follow $A_N(t)$. The deviating point at 0.4 ms delay may be attributed to the possible excitation of the L intermediate, and, more remarkably, the main component of $I_N(t)$ decays with a life time of 20 ± 1 ms (Table 1). The life time of the following part cannot be determined.

A delay time of 3 ms was selected for measuring the electric signals due to the excitation of the N intermediate. Records at 3 ms delay are shown in Fig. 4A,B. The first fast signal is

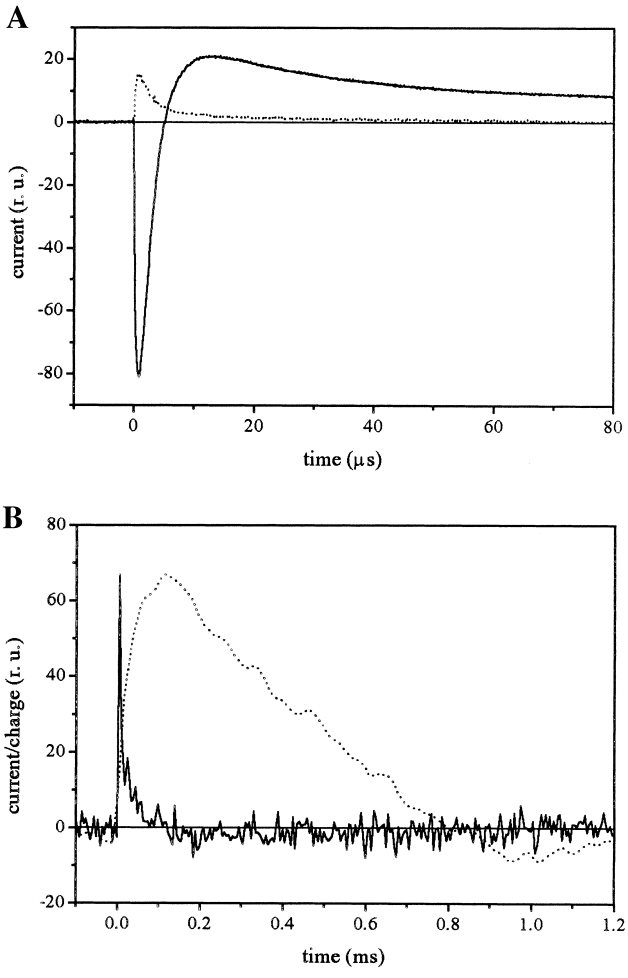

Fig. 4. Electric signals attributed to the photoresponse of the N intermediate in the case of mutant T46V. A: μs time domain, solid line signal from the excitation of the ground state, dotted line from N intermediate. B: ms time domain, solid line the measured electric current, dotted line its time integral. Time delay between the two flashes was 3 ms. Data are scaled. Solution as in Fig. 3.

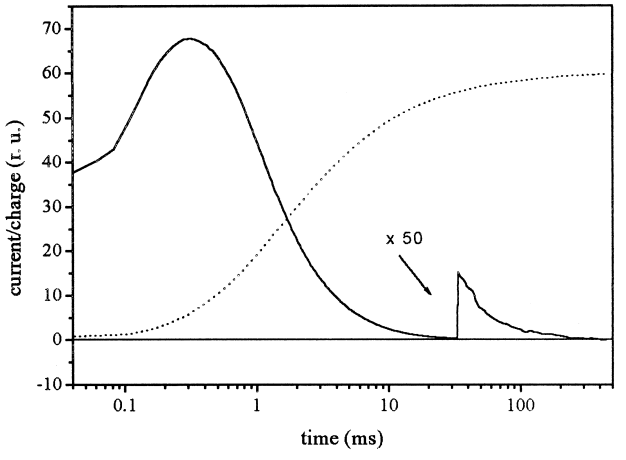

Fig. 5. Electric response of the excitation of the ground state of mutant T46V. Solid line: the measured electric current, dotted line its time integral. Data are scaled. Solution as in Fig. 3.

positive (Fig. 4A) as in the case of WTbR, the signal in the ms range is very small, its time integral tends to zero (Fig. 4B).

During these studies, the electric responses of the ground state excitation were also registered. We show the electric signal and its time integral for the mutant T46V in Fig. 5 and the parameters of the three exponential fits of its decay in Table 1. It is noticeable that a large fraction ($\approx 88\%$) of the proton transport occurs with short life times of 1.1 and 5.7 ms. The proton transport in the 130 ms component (its life time is determined with large error) is small but surely existing. Experiments performed with the proton sensitive dye, pyranine, clearly demonstrate that the protons are in the bulk for this long time (not shown). Experiments with pyranine support that protons do not exchange with the bulk during the excitation of the N intermediate.

4. Discussion

The main results of the study of the electric signals of the N intermediate of WTbR and the mutant T46V are the following:

1. two distinct processes characterize the electric signals (deviation of $A_N(t)$ and $I_N(t)$;
2. charge motion occurs in both cases;
3. charge translocation does not occur.

Previous studies pointed out that residues D96, T46 and R227 and some water molecules form a cluster at the cytoplasmic side of bR which is mainly responsible for the reprotonation of the Schiff base and proton uptake from the bulk [6–9]. The reaction pathway contains the following transitions in WTbR: $M_2^{(-1)} \leftrightarrow N^{(-1)} \leftrightarrow N^{(0)} \rightarrow bR$, where $M_2^{(-1)}$ is the second M intermediate with deprotonated Schiff base. In intermediate $N^{(-1)}$ the Schiff base is reprotonated from residue D96 which gets a proton from the bulk via the other components of the cluster and so $N^{(0)}$ is formed. The introduction of the two N intermediates is substantiated by the observation that the proton uptake is faster than the restoration of bR [8]. The configuration of retinal changes from 13-*cis* to all-*trans* in the transition $N^{(0)} \rightarrow bR$.

This pathway has been confirmed in the recent optical and electrical measurements of Ludmann et al. [13,14] in the case of WTbR, though they did not assign two substates to the N intermediate. The distinction of the two N substates postulated in [8] also appears in the present electrical measurement as seen in Fig. 1. The circles representing $I_N(t)$ follow $A_N(t)$ until 10–15 ms, but then deviate from it. The time integral of the longer living components of the electric signal measured at a delay of 7 ms indicates that charge translocation does not occur though internal charge motions take place (Fig. 2). Optical studies indicate that the excitation of the N intermediate leads to its thermal equilibration with the M intermediate, i.e. the N cycle returns to the original cycle [6,8]. Consequently, additional charge translocation is not expected. The zero charge translocation found in our experiment supports this reaction path. The difference signals in the second time period when the deviations appear are too small to determine whether charge translocation occurs or not.

In the photocycle of mutant T46V, the M intermediate decays within 1 ms and long living N intermediate arises. The N intermediate does not equilibrate with the M intermediate. The N intermediate, as shown in our measurements, exhibits two long living components in $A_N(t)$ and one in $I_N(t)$, represented by circles, decaying with a life time of ~ 20 ms (Fig. 3 and Table 1). The electric signals of 20 ms life time are assigned to the first long living component of $A_N(t)$. In the following, second long living time domain electric responses from the N intermediate cannot be detected.

In conclusion, based on the observation in this work and also on the results in [6], it seems to be evident that the N intermediates of WTbR and the mutant T46V are similar. The similarity appears in their absorption spectra, their place in the photocycle and in their electric responses for excitation. The N intermediates have two substates clearly demonstrated by the time behavior of the electric responses. Therefore, the pathway, shown above for WTbR, also holds for T46V.

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