Flash-induced voltage changes in halorhodopsin from *Natronobacterium pharaonis*

Inna V. Kalaidzidis, Yannis L. Kalaidzidis, Andrey D. Kaulen*

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

Received 20 March 1998

Abstract The flash-induced voltage response of halorhodopsin at high NaCl concentration comprises two main kinetic components. The first component with $\tau \approx 1$ μs does not exceed 4% of the overall response amplitude and is probably associated with the formation of the L (hR520) intermediate. The second main component with $\tau \approx 1$–2.5 ms which is independent of Cl^- concentration can be ascribed to the transmembrane Cl^- translocation during the L intermediate decay. The photoelectric response in the absence of Cl^- has the opposite polarity and does not exceed 6% of the overall response amplitude at high NaCl concentration. A pH decrease results in substitution of the Cl^--dependent components by the photoresponse which is similar to that in the absence of Cl^-. Thus, the difference between photoresponses of chloride-binding and chloride-free halorhodopsin forms resembles that of bacteriorhodopsin purple neutral and blue acid forms, respectively. The photovoltage data obtained can hardly be explained within the framework of the photocycle scheme suggested by Varo et al. [Biochemistry 34 (1995), 14490–14499]. We suppose that the O-type intermediate belongs to some form of halorhodopsin incapable of Cl^- transport.

Key words: Halorhodopsin; Bacteriorhodopsin; Photocycle; Photovoltage response; Cl^- transport; *Halobacterium salinarium*; *Natronobacterium pharaonis*

1. Introduction

Halorhodopsin (hR) is a light-driven chloride ion pump in the cytoplasmic membranes of halobacteria (for review, see [1–4]). The amino acid sequence of different hRs from various halobacterial species and the overall three-dimensional structure of hR bear great similarity to the proton pump, bacteriorhodopsin (bR). Like bR, photoisomerization of all-*trans* to 13-*cis*-retinal initiates the photoconversion cycle, but instead of a proton, a chloride ion is translocated in the reverse direction (to the cytoplasm). Nevertheless, under some conditions, bR displays the properties of the chloride ion pump (acid purple bR and bR mutants D85S and D85T [5–9]). On the other hand, hR is probably capable of proton transport especially in the presence of azide [10,11].

A planar lipid membrane with attached hR liposomes was used for the study of the electrogenicity of the hR transport under continuous illumination [10,12,13]. The flash-induced electrical signal in the gel containing oriented membrane hR fragments was described by Dèr et al. [14]. A signal with a fast unresolved rise and a biexponential decay with time constants of 0.4 and 2 μs was observed. Both components did not depend on Cl^- concentration. All the above-mentioned photoelectric measurements were performed with hR from *Halobacterium salinarium*. We have studied kinetics of the flash-induced voltage changes of hR from *Natronobacterium pharaonis*. This hR has several advantages in comparison with *H. salinarium* hR: lack of the light-dark adaptation, low K_D for Cl^- ($\sim$1 mM) and pronounced dependence of the photocycle kinetics on the Cl^- concentration [15–17]. Varo et al. [16,17] suggested the following photocycle scheme for the Cl^--binding hR form from *N. pharaonis*: $hR \Rightarrow K \Leftrightarrow N \Leftrightarrow N \Leftrightarrow O \Leftrightarrow hR' \Rightarrow hR$. Cl^- release and uptake is supposed to take place during $N \Rightarrow O$ and $O \Rightarrow hR'$, respectively. Flash excitation of the Cl^--free form leads to the formation only of the red-shifted intermediate.

In this paper, we have examined the photovoltage changes of the Cl^--binding and Cl^--free forms. It is concluded that kinetic data on the photovoltage responses of the Cl^--binding form can hardly be explained within the framework of the photocycle scheme suggested by Varo et al. [16,17]. We suggested that the O-type intermediate belongs to some form of hR incapable of Cl^- transport.

2. Materials and methods

H. salinarium strain L33 transformed with vector containing the *N. pharaonis hop* structural gene was kindly provided by Prof. J. Lanyi and Prof. R. Needleman. Growth of the bacteria and preparation of cell membrane fragments containing cloned hR were performed as described by Varo et al. [16]. The membrane sheets were collected from the lysed cell by centrifugation at $40\,000 \times g$ for 1 h and washed with 0.2 M NaCl. The membrane sheets were stored in the presence of 0.2 M NaCl at 4°C.

A phospholipid-impregnated collodion film was used to separate two compartments of a Teflon cuvette filled with the reaction mixture. The hR membrane sheets were adsorbed onto the positively charged film impregnated with 10% (w/v) egg lecithin and 0.1% octadecylamine solution in *n*-decane. The hR-containing ultrasonic proteoliposomes [protein/lipid (w/w), 1:100] were adsorbed onto the collodion film impregnated with 10% asolectin 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 hR. For measuring photoelectric responses and processing of kinetic data, see [9,18–20]. The instrument constant was 0.1 μs.

The hR photocycle was monitored with a single beam spectrophotometer [18–20]. Photoexcitation of hR was carried out with YG-481 Quantel Nd laser operated in double 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

*Corresponding author. Fax: (7) (095) 9393181.
E-mail: kaulen@phtbio.genbee.msu.su

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin

PII S0014-5793(98)00394-9

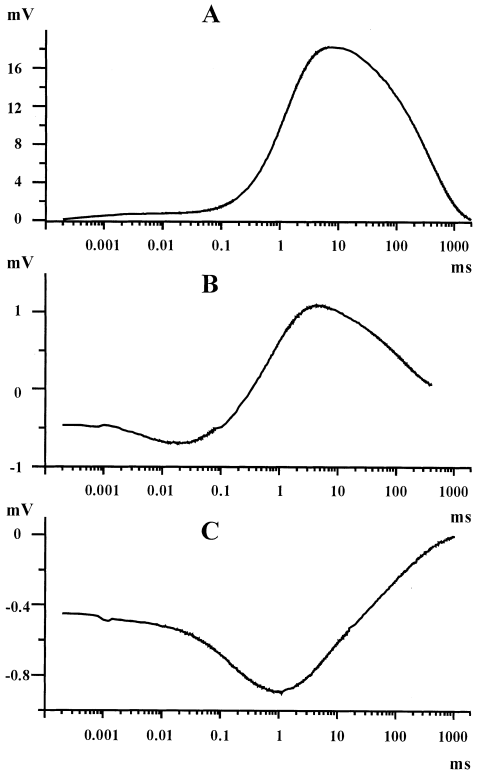

Fig. 1. Flash-induced voltage responses of hR membrane sheets adsorbed onto the collodion film. A: Cl^--binding form at 0.1 M NaCl, pH 6.8. B: Cl^--free form at 30 mM Na_2SO_4, pH 6.8. C: Cl^--free form at 0.5 M N_2SO_4, pH 6.8.

adsorbed hR membrane sheets at high NaCl concentration induces generation of electric potential difference including two distinct 'positive' phases: a small one with $\tau \approx 1$ μs whose contribution does not exceed 3–4% of the overall response amplitude, and a main phase with $\tau \approx 1$–2.5 ms (Fig. 1A). The discharge rate of the photoresponse depends on the salt concentration. Inasmuch as the photoresponse polarity is the same as in the case of bR purple membranes, one may conclude that hR membrane sheets are attached to the planar membrane by their extracellular surface. 'Positive' phases reflecting proton translocation by bR are probably associated with chloride transmembrane translocation by hR in the cytoplasmic direction. Similar types of voltage responses are observed in hR sheets adsorbed onto the positively charged planar membrane and in proteoliposomes with hR attached to the negatively charged lipid membrane in the presence of divalent cations. The absorption maximum of hR is at 577 nm in the presence of high NaCl concentration (Cl^--binding form). Surprisingly there are at least two different Cl^--free form of hR. Removal of Cl^- by washing with 0.5–1 M Na_2SO_4 shifts the maximum toward the red (to 589 nm). On the other hand, washing with 0.05 M Na_2SO_4 shifts the maximum toward the blue (to 560 nm). Photovoltage responses of these hR forms are shown in Fig. 1B,C, respectively. The polarity of the responses is opposite ('negative' phase), and their amplitudes do not exceed 6% of the overall response in the presence of NaCl. The two components are observed in 'negative' photoresponse of both chloride-free forms. The first one is limited by the time resolution of our set-up ($\tau \approx 0.1$ μs). The second component is much slower: $\tau \approx 250$ μs and 10 μs for the blue-shifted and red-shifted hR forms, respectively. During the photocycle both these chloride-free forms generate bathointermediates which decay with two time constants (350 μs and 2.5 ms for the blue-shifted form and 30 and 800 μs for the red-shifted form). Note the existence of the small (10% of the amplitude of the corresponding Cl^--binding form) 'positive' component in the response of the red-shifted form with $\tau \approx 1$–2 ms. This component may be due either to chloride contamination in concentrated sodium sulfate solution or to the transport of sulfate anion by the hR. Varo et al. [16] also observed about 14% of the anion-binding form in the concentrated sulfate solution. Chloride binding to the Cl^--free forms leads to the restoration of the hR with a maximum at 577 nm. The main difference between these forms is the rate of Cl^- binding: the red-shifted form binds Cl^- immediately whereas the completion of binding Cl^- to the blue-shifted form requires 30 min at low Cl^- concentration. The blue-shifted form has a significantly higher apparent binding constant for Cl^- (10–20 mM) than the red-shifted form (1–2 mM). Cl^- addition induces a gradual replacement of the photovoltage response of the chloride-free form by the response typical for the chloride-binding form (Fig. 2A). Fig. 2B shows the dependence of the main Cl^--dependent electrogenic component on the Cl^- concentration. Note that the Cl^--dependent increase in the amplitude (the half-maximal chloride concentration is about 6 mM) is not accompanied by any measurable changes in its time constant. An unusual difference of two chloride-free

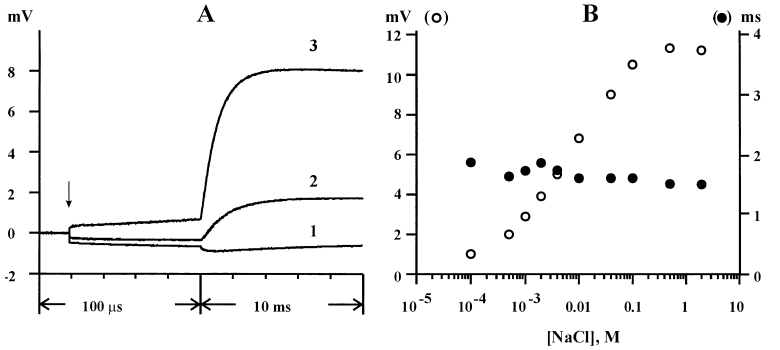

Fig. 2. Cl^- dependence of the hR photovoltage response. A: Cl^--free hR sheets incorporated at 30 mM Na_2SO_4, pH 6.8. 1, no addition; 2, 1 h after addition of 5 mM NaCl; 3, 1 h after addition of 0.1 M NaCl. B: Cl^- dependence of the amplitude (○) and τ (●) of the main 'positive' electrogenic phase. Cl^--free hR sheets were incorporated at 0.5 M N_2SO_4, pH 6.8.

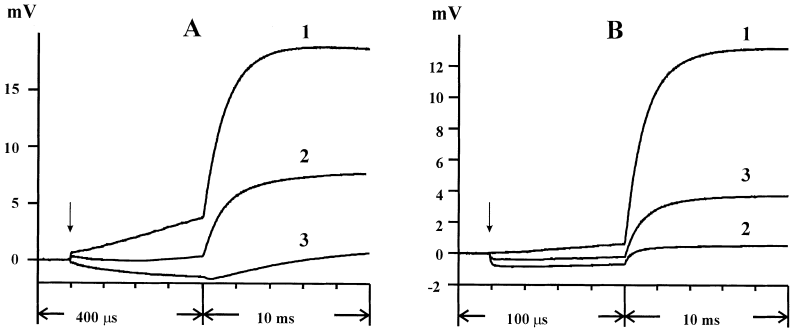

Fig. 3. A: pH dependence of the photovoltage response of the Cl^--binding hR sheets incorporated at 0.1 M NaCl. 1, pH 6; 2, pH 4.5; 3, pH 3. B: Effect of continuous illumination on the laser flash-induced electric response of hR sheets at 5 mM NaCl, pH 6.8. 1, flash-induced response without continuous illumination; 2, flash-induced response under continuous illumination; 3, flash-induced response 1 min after switching off the continuous illumination.

forms is revealed. The polarity of the chloride-dependent phase indicates that the membrane sheets with the red-shifted hR form are adsorbed onto the planar membrane by their extracellular surface similar to hR sheets at high NaCl concentration, whereas the blue-shifted membrane sheets are absorbed by their cytoplasmic surface.

The difference between the photoresponses of chloride-binding and chloride-free forms resembles that of bR purple neutral and blue acid form, respectively. Acidification of the bR suspension results in protonation of Asp-85 and formation of the blue acid form incapable of proton transport. The fast ($\tau < 0.1$ μs) photovoltage response of the blue form does not exceed 10% of the overall photoresponse of the purple neutral form associated with transmembrane proton translocation, and has opposite polarity ('negative' phase) [9,18–20]. The similar fast phase of the purple neutral form photoresponse also has the opposite direction and its amplitude is about one half of the photoresponse of the corresponding blue acid form. Thus, one may conclude that the photoresponse of the blue form is due to charge redistribution after retinal isomerization, and negatively charged Asp-85 in neutral form partially compensates this change. Probably, the same retinal isomerization leads to the photoresponse of the chloride-free hR form, and the greater mobility of chloride ion in comparison with Asp-85 in bR provides the full electrical compensation of this process in the chloride-binding form.

It is interesting that in spite of the absence of an Asp-85 analogue in hR, acidification induces changes in the photoresponse of the chloride-binding form corresponding to changes in the photoresponse of bR purple neutral form. In both cases, the amplitudes of the 'positive' phases decrease while the amplitude of the 'negative' phase increases in the bR photoresponse or becomes visible in the hR photoresponse at low pH (Fig. 3A). However, in hR, unlike bR, the following pH increase fails to fully restore the amplitude of the 'positive' photoresponse. Acidification also shifts the spectrum of the chloride-binding form of hR toward the blue. The spectrum of this new form of hR is quite similar to the spectrum of the chloride-free form at low ionic strength. One may conclude that acidification inhibits the chloride binding to hR.

Fig. 3B shows that a 'negative' photoresponse may be observed at neutral pH under some specific conditions. Contin-

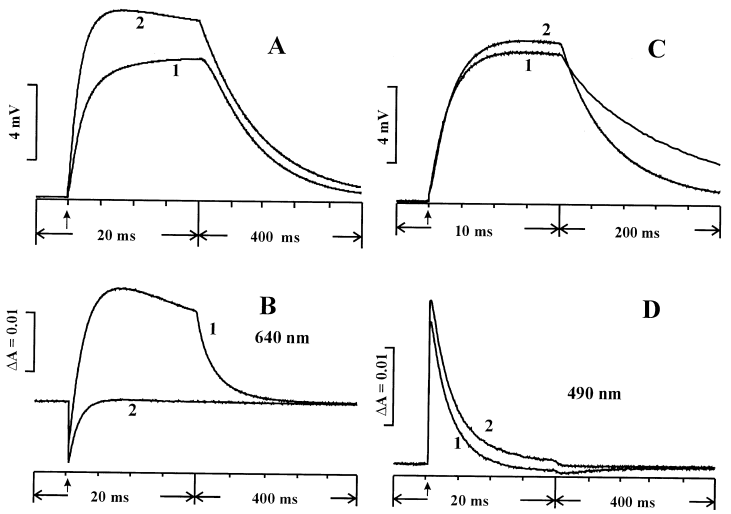

Fig. 4. A: Photovoltage response of hR just after addition of 2 M NaCl. The hR sheets were incorporated at 10 mM NaCl, pH 6.8. 2, photoresponse of the same sample in 30 min incubation. B: Flash-induced optical changes at 640 nm in hR sheet suspension. 1, 0.1 M NaCl, pH 6.8; 2, 2 M NaCl, pH 6.8. C: 1, photovoltage response of hR sheets incorporated at 0.1 M NaCl, pH 6.8. 2, photoresponse of the same sample 1 h after addition of 2 M NaCl. D: Flash-induced optical changes at 490 nm in hR sheet suspension. 1, 0.1 M NaCl, pH 6.8; 2, 2 M NaCl, pH 6.8.

uous illumination of the planar membrane induces generation of a transmembrane potential difference of 50–100 mV which is quite stable at 0.1 M NaCl but discharges at low (2–5 mM NaCl) concentration even during illumination. In the latter case, laser flash, added during the continuous illumination, generates only a 'negative' signal. The 'positive' signal is gradually restored after switching off the light. This effect is possibly due to Cl^- exhaustion in the water cavity between the hR sheet and the planar membrane leading to the formation of the chloride-free form of hR.

The fast chloride-dependent 'positive' phase can be ascribed to the $K \Rightarrow L$ transition in the hR photocycle. According to Gerscher et al. [21], in contrast to the parent state, the hR520 (L) intermediate reveals a closer proximity of the bound anion to the Schiff base. Our data indicate that this process of L intermediate formation is accompanied by a Cl^- shift in the cytoplasmic direction.

The nature of the main Cl^--dependent phase is not so clear. Its rate coincides with the $L \Rightarrow N$ transition in the photocycle scheme suggested by Varo et al. [16] (see Section 1). Computer analysis reveals an additional component with a small 'negative' amplitude and with $\tau \approx 100$–200 μs. This component is supposed to be a lag phase which may indicate that either some electrically silent process precedes the main electrogenic phase, or the transition with $\tau \approx 100$–200 μs is electrogenic and follows the electrically silent process with $\tau \approx 1$–2 ms. The latter possibility is in line with Varo's scheme which suggests that Cl^- release takes place during the $N \Rightarrow O$ transition at a rate considerably faster than that of the $L \Rightarrow N$ transition. Nevertheless, the following experiments indicating a poor correlation of the photovoltage kinetics with this scheme make the former explanation much more probable.

It was mentioned above that the time constant of the main Cl^--dependent component does not depend on NaCl concentration. Moreover, we did not observe any additional slow 'positive' components in the photoresponse in the wide range of NaCl concentration. However, according to Varo's scheme, the existence of slow electrogenic components is expected for the following reasons. First, if Cl^- uptake is electrogenic, then the process of O relaxation should be electrogenic. Second, inasmuch as the scheme includes the reversible reactions $K \Leftrightarrow L \Leftrightarrow N \Leftrightarrow O$, electrogenicity of the Cl^- release process suggests electrogenicity of O decay even if the uptake process is not electrogenic. The rate of O decay depends on the chloride concentration: for example, at 0.1 M NaCl its time constants are about 25 ms (50%) and 60 ms (50%). Nevertheless, such components were not revealed in the photoresponse. One might assume that the absence of these components is due to the existence of the passive discharge component with comparable rate constants. However, according to our data, this explanation is hardly probable. Addition of 2 M NaCl to the sample incubated at 10 mM NaCl results in the acceleration of discharge to 130 ms (Fig. 4A, curve 1). Nevertheless, computer analysis shows three 'positive' components with $\tau \approx 1.4$ ms (50%), 5 ms (16%) and 26 ms (34%). Thus, the slow components can be revealed in spite of the very fast discharge. Prolonged incubation of the sample results in the disappearance of the slow components (Fig. 4A, curve 2). It is supposed that the reason for the appearance of the slow components after addition of 2 M NaCl is the formation of a sodium diffusion potential on the membrane sheets, providing the positive charging of the internal water cavity of the adsorbed sheets and inducing deceleration of the main Cl^--dependent component whose polarity coincides with the polarity of the diffusion potential.

An additional experiment was carried out in order to test the electrogenicity of the slow phases of the photocycle. An increase in NaCl concentration from 0.1 M up to 2 M inducing a significant acceleration of the O decay results in the disappearance of the slow components in the optical density relaxation at 570 nm (not shown) as well as the disappearance of the O intermediate (Fig. 4B). One may conclude that this should lead to an acceleration of all electrogenic events associated with the O intermediate decay leading to their completion in the millisecond time domain. In turn, the increase in NaCl concentration should enhance the amplitude of the photovoltage response. However, the photoresponse amplitude (Fig. 4C) increases by no more than 10% only (even taking into account some acceleration of the photoresponse decay). Note that the NaCl concentration increase leads to a comparable enhancement in L(hR520) intermediate formation measuring at 490 nm (Fig. 4D). Thus, the overall photopotential increase is probably due to the increase in the Cl^--binding hR form. It can be suggested that the Cl^- uptake is electrically silent. In any case, the data obtained are not in line with Varo's scheme [16,17] with respect to the reversibility of the $K \Leftrightarrow L \Leftrightarrow N \Leftrightarrow O$ transitions. It is noteworthy that, according to our data, the Cl^- release from the Schiff base on the extracellular surface is accompanied by a significant electrogenic event both in the bR acid purple form and in the bR D85S mutant [9]. Taking into account the similarity of the bR and hR structures [22], the data obtained are in contradiction with the possible non-electrogenic character of the analogous process in hR.

The following interpretation of the above data seems probable. The main Cl^--dependent phase in the photovoltage response comprises two events: (i) Cl^- release from the Schiff base through the cytoplasmic half-channel and (ii) subsequent Cl^- uptake through the extracellular half-channel. The rate of Cl^- release does not depend on the Cl^- concentration as the rate of Schiff base deprotonation in the bR remains constant within the wide pH range. The following Cl^- uptake process is considerable faster than the Cl^- release, and therefore, is not revealed as a separate kinetic component. Consequently, an intermediate (i.e. Cl^--free form) coupled with these conversions is not revealed for kinetic reasons. We suggest that the O intermediate belongs to a photocycle of some hR form incapable of chloride transport (13-*cis*-hR or a special kind of all-*trans*-hR). We believe that Cl^- participates in the photoconversion of this form, but this process is electrically silent. It is interesting that the revision of the photocycle of the hR from *Halobacterium salinarium* by Varo et al. [23] led to a similar conclusion. The authors excluded the O intermediates from the photocycle of the chloride-binding all-*trans*-hR. As a result, this revised photocycle includes the reaction scheme $hR \Rightarrow K \Leftrightarrow L_1 \Leftrightarrow L_2 \Leftrightarrow N \Rightarrow hR$ whose kinetic parameters do not depend on Cl^- concentration. Our data are qualitatively in line with this photocycle scheme, rather than with the scheme proposed for the *N. pharaonis* hR.

Acknowledgements: We are grateful to Prof. J. Lanyi and Prof. R. Needleman for providing the halobacterial strain and to Alexey Semenov, Mahir Mamedov and Alexey Radionov for critical reading of the manuscript. This work was supported by the Russian Foundation for Basic Research (Grant 97-04-49749) and INTAS (Grant 93-2852).

References

[1] Lanyi, J.K. (1986) Annu. Rev. Biochim. Biophys. Chem. 15, 11–28.
[2] Lanyi, J.K. (1990) Physiol. Rev. 70, 319–330.
[3] Oesterhelt, D., Tittor, J. and Bamberg, E. (1992) J. Bioenerg. Biomembr. 24, 181–191.
[4] Oesterhelt, D. (1995) Israel J. Chemistry 35, 475–494.
[5] Der, A., Szaraz, S., Toth-Boconadi, R., Tokaji, Z., Keszthelyi, L. and Stoeckenius, W. (1991) Proc. Natl. Acad. Sci. USA 88, 4751–4755.
[6] Der, A., Toth-Boconadi, R. and Keszthelyi, L. (1989) FEBS Lett. 259, 24–26.
[7] Sasaki, J., Brown, L.S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995) Science 269, 73–75.
[8] Brown, L.S., Needleman, R. and Lanyi, J.K. (1996) Biochemistry 35, 16048–16054.
[9] Kalaidzidis, I.V. and Kaulen, A.D. (1997) FEBS Lett. 418, 239–242.
[10] Bamberg, E., Tittor, J. and Oesterhelt, D. (1993) Proc. Natl. Acad. Sci. USA 90, 639–643.
[11] Varo, G., Brown, L.S., Needleman, R. and Lanyi, J.K. (1996) Biochemistry 35, 6604–6611.
[12] Bamberg, E., Hegemann, P. and Oesterhelt, D. (1984) Biochemistry 23, 6216–6221.
[13] Hegemann, P., Oesterhelt, D. and Bamberg, E. (1985) Biochim. Biophys. Acta 819, 195–205.
[14] Dèr, A., Fendler, K., Keszthleyi, L. and Bamberg, E. (1985) FEBS Lett. 187, 233–236.
[15] Schaft, B. and Engelhard, M. (1994) Biochemistry 33, 6387–6393.
[16] Varo, G., Brown, L.S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995) Biochemistry 34, 14490–14499.
[17] Varo, G., Needleman, R. and Lanyi, J.K. (1995) Biochemistry 34, 14500–14507.
[18] Drachev, L.A., Kaulen, A.D. and Skulachev, V.P. (1978) FEBS Lett. 87, 161–167.
[19] Drachev, L.A., Kaulen, A.D., Khitrina, L.V. and Skulachev, V.P. (1981) Eur. J. Biochem. 117, 461–470.
[20] Skulachev, V.P., Drachev, L.A., Kaulen, A.D., Khitrina, L.V., Zorina, V.V. and Danshina, S.V. (1987) in: Retinal Proteins. Proceedings of an International Conference (Ovchinnikov, Yu.A., Ed.), pp. 531–552, VNU Science, Utrecht.
[21] Gerscher, S., Mylrajan, M., Hildebrandt, P., Baron, M.-H., Müller, R. and Engelhard, M. (1997) Biochemistry 36, 11012–11020.
[22] Havelka, W.A., Henderson, R.A., Heymann, J.A.W. and Oesterhelt, D. (1993) J. Mol. Biol. 234, 837–846.
[23] Varo, G., Zimányi, L., Fan, X., Sun, L., Needleman, R. and Lanyi, J.K. (1995) Biophys. J. 68, 2062–2072.