ELECTRIC-FIELD INDUCED p*K*-CHANGES IN BACTERIORHODOPSIN

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1. Introduction

Bacteriorhodopsin, the only protein of the purple membranes of halobacteria [1], acts as a light-driven proton pump, translocating protons from the inner to the outer side of the plasma membrane [2]. The light-induced asymmetric proton distribution contributes to the membrane potential by up to 10–40 mV [3]. Assuming a membrane thickness of 50 Å, this potential difference corresponds to an electric field strength of $2–8 \times 10^6$ V/m. Since the protein is polyionic it is possible that photocycle and proton transport are affected by a direct action of the membrane electric field on the conformation of bacteriorhodopsin.

An electric field ($1–30 \times 10^5$ V/m and 1–30 μs duration) causes a cycle of structural changes within the bacteriorhodopsin of purple membrane suspensions [4]. The data suggest an induced-dipole mechanism which involves a restricted rotational displacement of the chromophore by an angle of $\geqslant 20°$C toward the membrane normal [4].

We show here that the field-induced structural changes in purple membrane involve alterations of the p*K*-values of at least two types of proton binding sites of bacteriorhodopsin. The comparison of the time course of the pH changes indicates that the electric field-induced p*K*-shifts are opposite in direction to the light-induced p*K*-changes. This observation suggests a possible negative feedback of the increased electric membrane field on the proton transporting conformations of bacteriorhodopsin.

2. Experimental

2.1. *Materials*

Purple membranes isolated from M1 strain of *Halobacterium halobium* were a gift of Professor D. Oesterhelt. Bacteriorhodopsin concentration, [bR], in purple membrane suspensions was determined on the basis of the extinction coefficient of $\epsilon = 63\,000$ $M^{-1} \cdot cm^{-1}$ at 570 nm [5].

The pH indicator bromothymolblue (3′,3″-dibromothymolsulfonephthalein, p$K \simeq 6.8$ at 293 K and low ionic strength), HCl and NaOH used for the titration were Merck products.

The optical density per/cm purple membrane suspensions for the electric-field-induced pH change measurements was adjusted to 0.33 at 570 nm ([bR] = 5.1×10^{-6} M) in reflux--multiple distilled water (of conductivity 0.9 μS at 293 K). At this low protein concentration the contribution of light scattering is negligible and the optical density equals the absorbance. The pH of the suspension was adjusted with NaOH to pH 6.70 ± 0.05, unless otherwise noted. The concentration of bromothymolblue, [In], was 2.0×10^{-5} M.

2.2. *Spectroscopic acid–base titration*

The pH measurements were performed with a glass electrode connected to a Radiometer pHM62. The optical density measurements and titrations were carried out in a thermostated cell at 293 K with a Cary 118 spectrophotometer.

2.3. *Electric field-induced pH changes*

The electro-optical measurements were performed with an electric relaxation spectrometer developed by Schallreuter and Neumann [6]. A collimated light from a 100 W halogen lamp passed through a Schoeffel-monochromator and a Nicoll polarizer. The wavelength was fixed at 615 nm (the absorption peak of free indicator) and the polarizer was set at 55°C with respect to the electric field so that the pure electric dichroism of bacteriorhodopsin was eliminated [4].

Rectangular electric pulses up to 2.3×10^6 V/m with pulse durations of 10–170 μs were applied to

the sample solution. The light transmitted through the sample cell was detected with a photodiode, amplified and displayed on an oscilloscope. The change in the absorbance $\Delta A_{55°}$, caused by the electric field is calculated from the light transmittance change $\Delta I_{55°}$:

$$\Delta A_{55°} = -\log [1 + (\Delta I_{55°}/I] \quad (1)$$

where I is the transmitted light in the absence of the electric field. The pH change has been estimated from $\Delta A_{55°}$ at 615 nm. The relationship of the proton concentration to the absorbance is given in the next section.

All electric field experiments were carried out at 293 K. The temperature increase, due to Joule heating, is negligible under the present conditions of low ionic strength, $I_c \cong 1$ mM [4].

The absorbance/cm A, of the pH indicator is given by:

$$A = \epsilon_{In} \cdot [In^-] + \epsilon_{InH} \cdot [InH] \quad (2)$$

where ϵ_{In} and ϵ_{InH} are the molar extinction coefficients and $[In^-]$ and $[InH]$ are the concentrations of free and protonated indicator, respectively; A varies with pH according to:

$$InH \rightleftarrows In^- + H^+ \quad (3)$$

At $\lambda = 615$ nm, $\epsilon_{InH} \ll \epsilon_{In}$. With:

$$[In^-] = [In]_T - [InH] \quad (4)$$

where $[In]_T$ is the total concentration of the indicator and:

$$K = \frac{[H^+]\,[In^-]}{[InH]} \quad (5)$$

we obtain for the pH range around the pK-value:

$$A_{615} \cong \frac{\epsilon_{In} \cdot [In]_T}{1 + [H^+]/K} \quad (6)$$

Equation (6) shows that an increase in $[H^+]$, i.e., a decrease in pH, causes a decrease in A_{615}.

3. Results

Fig.1 shows the absorbance spectra of a mixture of purple membrane and bromothymolblue over

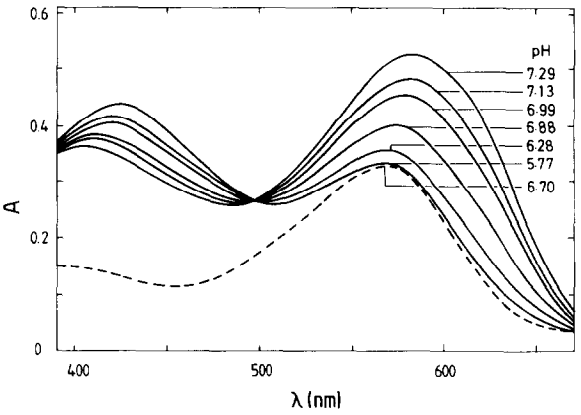

Fig.1. Absorption spectra, A (λ), of a purple membrane suspension (– – –) and a mixture of purple membrane and bromothymolblue at various pH-values (——) at 293 K; [bR] = 5.1×10^{-6} M; [In] = 2.0×10^{-5} M.

pH 6.0–7.5, together with a spectrum without bromothymolblue as a reference: the spectrum of the purple membrane suspension is independent of pH. The maintenance of the isosbestic point of bromothymolblue at 497 nm in the presence of purple membrane indicates that there is apparently no interaction between purple membrane and bromothymolblue in this pH range.

The data of spectroscopic acid–base titrations at 615 nm are shown in fig.2. The absorbance contribution due to the purple membrane was subtracted. The titrations were done both by adding NaOH and by

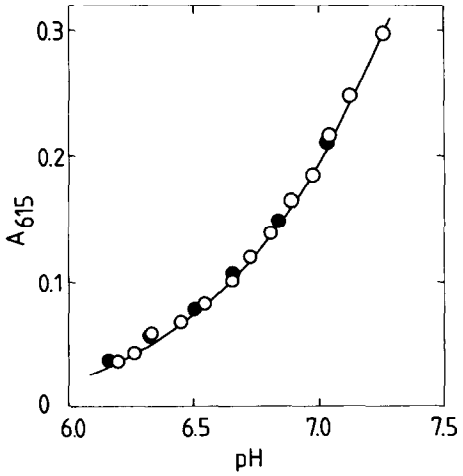

Fig.2. Absorbance/cm at 615 nm, A_{615}, of bromothymolblue (BT) in the presence of purple membranes (PM) as a function of pH at 293 K; $A_{615} = A$ (PM + BT) – A (PM); (○) addition of NaOH; (●) addition of HCl.

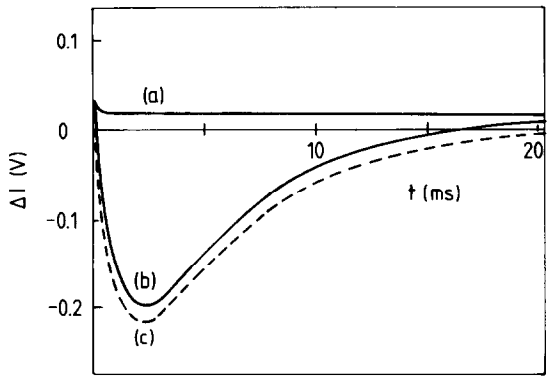

Fig.3. Light intensity changes, ΔI at 615 nm (55° polarization angle), of a purple membrane suspension ([bR] = 5.1×10^{-6} M) caused by a rectangular electric pulse of $E = 1.54 \times 10^6$ V/m applied at $t = 0$ for 170 μs; initial pH 6.70; 293 K; the light intensity I at $t = 0$ is 3.0 V: (a) in the absence of bromothymolblue (optical effect of bacteriorhodopsin alone); (b) in the presence of bromothymolblue ([In] = 2.0×10^{-5} M); (c) calculated intensity change, difference (b) – (a), indicating the pH-changes caused by the protein.

adding HCl; no hysteresis was observed. This curve is used for a calibration of field induced absorbance changes in terms of pH changes.

Fig.3(b) shows a typical optical signal of the mixture of purple membrane and bromothymolblue caused by the electric impulse. Fig.3(a) is the optical signal in the absence of bromothymolblue under the same condition, which is known to reflect field-induced conformational changes of the bacteriorhodopsin [4]. The bromothymolblue solution alone yields no optical

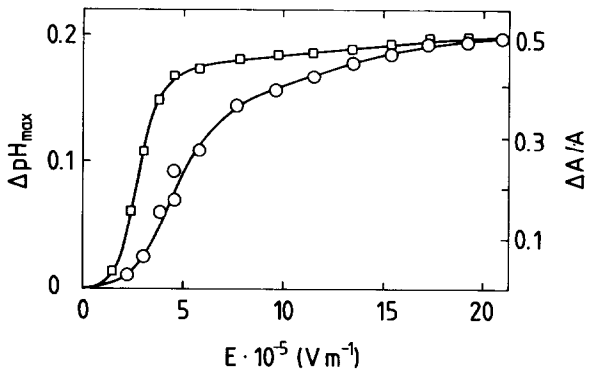

Fig.4. Electric field strength dependence of the maximum pH change, ΔpH_{max} (○) (pulse length $\Delta t_E = 170$ μs, $\lambda = 615$ nm) and the steady state electric dichroism $\Delta A/A$ (□) (Δt_E = 10–80 μs, $\lambda = 565$ nm) [4], respectively; experimental conditions as in fig.3.

change in the electric field. The difference in the absorbance of the purple membrane suspension in the presence and the absence of bromothymolblue, however, shows a larger net optical change caused by a pH change (fig.3(c)). After switching off the field, the pH of the suspension first increases (reflecting proton uptake by purple membranes), reaches a maximum at ~2 ms, followed by a pH-decrease to the original value. The maximum pH change, ΔpH_{max}, under the experimental conditions specified in fig.3 is 0.19. It is found that ΔpH_{max} increases with decreasing the initial pH of the suspension over pH 6.0–7.0. For the initial pH 6.7, no net pH change was detected during applying the field for 10–170 μs. However, ΔpH_{max} after removing the field increased with increasing pulse duration.

The kinetics can be analyzed as a sum of at least two exponentials, the amplitudes of which have opposite signs to each other. The relaxation times of the fast and the slow components were 1 ms and 5 ms, respectively, independent of the field strength and of the pulse duration.

The field strength dependence of ΔpH_{max} for the same pulse duration of 170 μs is shown in fig.4. For a comparison, the field strength dependence of the electric dichroism [4] is also shown: ΔpH_{max} increases with increasing the electric field; the electric dichroism increases also, but in a different manner.

4. Discussion

Since the transient pH increase of the medium must reflect pK changes in the purple membrane, the observations described above can be interpreted in terms of field-induced structural changes of bacteriorhodopsin. The structural conversions reflect changes in the proton affinity of at least 2 types of proton binding sites. Fig.5 shows a model which rationalizes the observed transient pH changes caused by the electric impulse.

At pH 6.70 ± 0.05 the field-induced pK shifts are such that the release of protons from a site 1 (pK_1) is by chance cancelled by uptake of protons from a site 2 (pK_2); under different conditions of the initial pH a small net pH change is observed. When the field is switched off, the re-uptake of protons to site 1 is faster than the re-release of protons from site 2. In order to model these observations a minimum reaction scheme is given by:

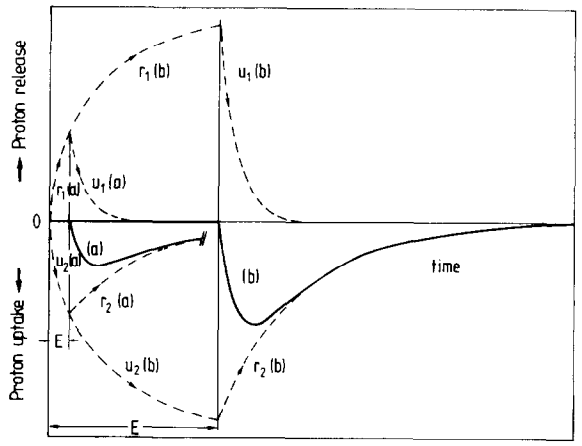

Fig.5. Analysis of the observed transient pH-changes induced by electric umpulses of the same field strength but at two different pulse durations: (a) short; (b) longer pulse. Decomposition of the observed curves (a) and (b) (——) into uptake (u) and release (r) contributions (– – –) sites 1 and 2. For instance, in case (a), $r_1(a)$ is the H^+ release in the field and $u_1(a)$ is the H^+ re-uptake after switching-off the field, both for protonation-site 1. The curves $r_1(a)$ and $u_1(a)$ plus the respective curves $u_2(a)$ and $r_2(a)$ for site 2 result in the actually measured curve (a).

$$B_1H \rightleftharpoons B'_1H \rightleftharpoons B'_1{}^- + H^+ \qquad (7)$$

$$B_2{}^- + H^+ \rightleftharpoons B'_2{}^- + H^+ \rightleftharpoons B'_2H$$

where the subscripts 1 and 2 denote sites 1 and 2, respectively, and B and B′ symbolize the different conformations of the sites, preferred in the absence and the presence of the field, respectively. The difference in the kinetics not only accounts for the appearence of the ΔpH_{max}, but also results in a dependence of ΔpH_{max} on the pulse length and the field strength. For instance, the extent of the proton release from site 1 as well as of the uptake to site 2 become larger during a longer pulse as shown in fig.5; at the field strength applied the conformational changes which lead to the pK shifts are still proceeding at >100 μs.

As shown in [4], the electric dichroism reaches a steady state within 6–10 μs, suggesting that a saturation of the rotation of retinal chromophore is established in this time range. Therefore, the electric dichroism due to the retinal chromophore indicates only the rapid parts of the field-induced conformational changes. The difference in field strength dependence between the extent of the dichroism and of the pH change reflects that these indicators for conformational changes are not equivalent.

The conformational changes responsible for the pK shifts could involve small transverse displacements of some parts of the bacteriorhodopsin chain, as suggested by data of enzymatic digestion of the proton-pumping state [7]: some parts of the bacteriorhodopsin molecule are more exposed out of the membrane, whereas others are hidden in the membrane layer.

It is instructive to compare the light-flash-induced pH changes of the purple membrane suspension with our electric field results. According to [8], the pH of the purple membrane suspension first decreased after the light-flash, and then returned to the original pH (~6.62). The kinetics were also analyzed in terms of two exponentials, the amplitudes of which have the opposite sign to each other; the relaxation times were 1.5 ms and 8 ms in 10 mM KCl, (pH-indicator, p-nitrophenol; [In] = 2.5×10^{-5} M, [bR] = 2×10^{-6} M). The time constants of the flash-induced pH-changes are practically the same as the electric field-induced pH change. However, the sequence of pH-increase and pH-decrease is opposite compared to the field-induced pH-changes. It is therefore possible that the increase in the membrane potential which results from proton pumping exerts a negative feed back (reducing proton transport) via an electric field effect directly on the structure of bacteriorhodopsin.

Acknowledgement

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