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The effect of pH on proton transport by bacteriorhodopsin

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The pH-dependence of proton motion during the photocycle was investigated by measuring the photoelectric signals due to charge displacement inside bacteriorhodopsin molecules. Measurements were performed on purple membranes oriented in suspension and the kinetics of flash excited electric and light absorption signals was compared. It was found that in the pH range 4.5–8 the photocycle and the successive proton movements have identical kinetics, and do not depend on pH. In the pH range 8–10 both kinetics change, though differently; the charge motion decouples from the photocycle and the photocycle seems to split up into two parallel paths, the photoelectric signal becomes faster. However, the net proton transfer remains the same as at lower pH values. Above pH ≈ 10, the photocycle behaves differently and cannot be described by the parallel pathway model and the net proton displacement drops. The results are explained by the successive titration of two groups (probably tyrosine) participating in proton translocation.

Introduction

Upon light absorption, the bacteriorhodopsin (BR) from *Halobacterium halobium* undergoes a photocycle [1]. During this photocycle it transports protons from the intracellular side to the extracellular medium, converting light into electrochemical energy [2]. The molecular mechanism of proton pumping, however, is not yet known and is the subject of intensive research.

The pumping activity of bacteriorhodopsin has been extensively studied by photoelectric methods in a variety of model systems [3–5]. In a study of Fahr et al. [6], the effect of pH on the kinetics and amplitudes of fast photoelectric signals was investigated. In a previous work we studied the details of proton transfer by flashing light through a suspension of purple membranes oriented by electric field, where we could follow proton motion by measuring the displacement current generated by the simultaneously moving protons. We found that the proton moves across the membrane in discrete steps, and these proton jumps correlate well with (and are therefore coupled to) the successive transitions in the photocycle [7,8]. With plausible assumptions, applying the following reaction scheme for the photocycle: BR → K → L → M → O → BR (where the letters denote the successive intermediates), the relative distances of the proton displacements could also be determined [9].

An important goal of further investigations is to determine the sites through which the proton jumps during the pumping process. Since bacteriorhodopsin is a proton pump, the study of the effect of pH on its functions may yield useful informations about the details of its working. The effect of pH on the spectral properties of bacteriorhodopsin has been studied [10], and changes in the photocycle have also been reported [11,12], with the conclusion that titration of tyrosyl groups results in a

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slower decay of the M form. In a study of the environmental effects on the photoreactions of bacteriorhodopsin, Kalisky et al. [13] measured the yield of the M form in the dependence on pH at low temperatures under continuous illumination. They conclude that formation of a tyrosinate ion is a prerequisite for Schiff-base deprotonation and the formation of the M form. The data published by Hanamoto et al. [14], however, do not confirm this conclusion. On the basis of optical investigations, Kuschmitz et al. [15] concluded that one tyrosyl and four aspartyl groups are involved in the proton transfer. An important observation concerning the effect of pH on the proton transport by bacteriorhodopsin was reported by Renard and Delmelle [16]. They found a strong decrease in the quantum efficiency of light-driven proton extrusion in *H. halobium* cells with increasing pH: at neutral pH values a quantum yield of approx. 0.6 is found, which decreases to approx. 0.3 in alkaline medium (being 0.3 at pH 8). The same authors determined the pH-independent photochemical quantum yield of bacteriorhodopsin [17] and, taking a value of 0.3 [18], concluded a stoichiometry of two protons per cycle below pH 7 and one proton per cycle at pH > 8. Recently, Li et al. [19] found that the proton pumping activity in cells drops at pH values above 7, in agreement with the previous finding. Ort and Parson [20], however, reported a quantum yield for proton release from purple membrane fragments of about 0.4, pH-independent in the pH range 6 to 8.75. In this work we have studied the effect of pH on the photocycle and on the electric signal caused by the movement of protons inside the molecule during the pumping process to obtain further data on the details of proton transport.

Materials and Methods

Purple membrane fragments were isolated from *H. halobium* strain ET100 according to Oesterhelt and Stoeckenius [21]. The measurements were carried out on a salt-free water suspension of purple membranes. The pH was set by adding small quantities of HCl or NaOH to the sample; it was measured with a conventional pH meter (OP-264/1, Radelkis, Hungary) before the measurements. The bacteriorhodopsin concentration was set to $1.6 \cdot 10^{-4}$ mol/l. The sample with the pH set was filled in a 1 mm cuvette, with platinized platinum electrodes immersed in the solution. The distance between electrodes was 7 mm. The electrodes were used to orient the sample by a DC electric field and to measure the current transients generated by the exciting flash. The experimental arrangement was the same as in Ref. 7. The orienting voltage was 10 V, 50 kΩ load resistance was used. Typical sample resistance was 50–80 kΩ, which changed somewhat by varying the pH. The orienting field was switched on the sample for 2.5 s, and the exciting flash was triggered 50 ms after switching off the electric field. The remaining small voltage due to electrode polarization was compensated. This procedure of establishing a field-free measurement was needed to avoid the current due to the transiently liberated charges [6]. The orientation of the purple membranes was well preserved due to the slow relaxation (about 200 ms) at all pH values.

The orientation of the sample was checked by measuring the change in linear dichroism during oritentation. The voltage of 10 V resulted in near-saturating oritentation; it changed a little, however, with pH (possibly due to changes in the dipole moment of the purple membranes). The nonsaturating orientation was further decreased (about 20%) by the relaxation after switching off the field. The amplitudes of the measured electric signals are proportional to the orientation (the kinetics are not influenced because the rate of relaxation is slow compared to those in the electric signals); the results of different experiments were compared after appropriate normalization.

The exciting flash came from a Zeiss FL3A dye laser with dye Rhodamine 6-G (wavelength, 580 nm; duration, 1 μs; flash energy, 30 mJ). The electrical band width of the measuring system (together with the amplifier) was 3 MHz, the time resolution of the experiments was limited by the laser flash to 1 μs. Both the electric and the absorption signals (measured simultaneously) were stored in a 10 MHz transient digitizer (KFKI, NEO 200-B, Hungary) and later transferred to a computer for analysis. The measurements were carried out at 22°C.

Results

Fig. 1 shows the typical responses at pH 6.6. In Fig. 1a and b the electric signals following the laser flash are seen, Fig. 1c, d and e depict the corresponding absorption kinetic signals. The first, large component of the electric signal corresponding to the all-*trans*-13-*cis* isomerization of the retinal chromophore [8] follows the laser flash. It is followed by an exponential component of negative amplitude simultaneously with the K → L step. (This is more apparent at lower temperatures [8].) Two components with positive amplitude follow: one coupled to the L → M step, the other to the M → O → BR step. Quantitative analysis is detailed in Refs. 8 and 9. For the ratios of the proton displacements (d_i) the following results were obtained:

$$\frac{d_{\text{BR-L}}}{d_{\text{L-M}}} = -0.28 \pm 10\%;$$

$$\frac{d_{\text{BR-L}}}{d_{\text{M-BR}}} = -0.034 \pm 10\%;$$

$$\frac{d_{\text{L-M}}}{d_{\text{M-BR}}} = 0.12 \pm 10\%$$

In the pH range 4.5–8, practically no pH-dependence is seen. However, increasing the pH to about 8–9 produces dramatic changes both in the photocycle, as measured by absorption kinetics,

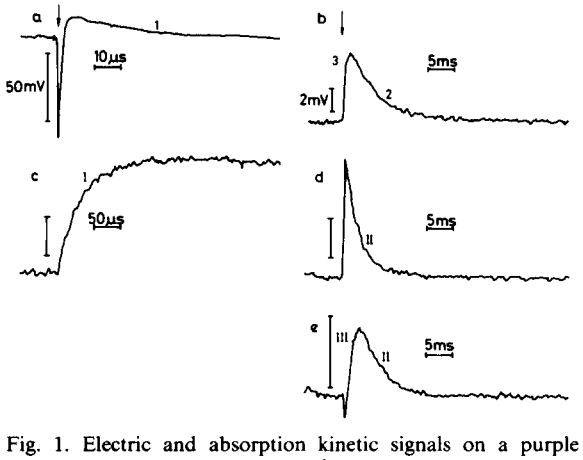

Fig. 1. Electric and absorption kinetic signals on a purple membrane suspension of $1.6 \cdot 10^{-4}$ mol/l concentration measured at pH 6.6 at different time resolutions. The arrow indicates the exciting laser flash. (a, b) Photoelectric signal. Sample resistance; $R_s = 75$ kΩ. Load resistance: $R_L = 50$ kΩ. (c, d) Absorption kinetic signals measured at $\lambda = 405$ nm. The vertical bar indicates an absorption change of 10%. (e) Absorption kinetic signal measured at $\lambda = 660$ nm.

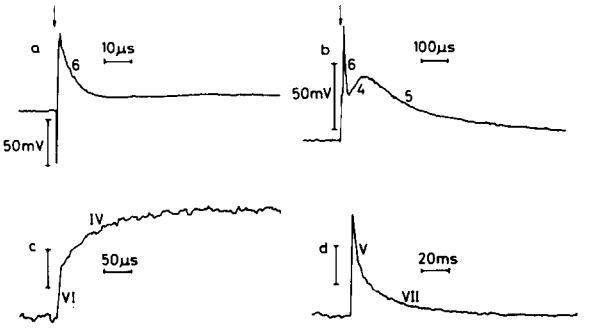

Fig. 2. Electric and absorption kinetic signals on a purple membrane suspension of $1.6 \cdot 10^{-4}$ mol/l concentration measured at pH 10.2 at different time resolutions. (a, b) Photoelectric signal. Sample resistance: $R_s = 53$ kΩ. Load resistance: $R_L = 50$ kΩ. (c, d) Absorption kinetic signals measured at $\lambda = 405$ nm. The vertical bar indicates an absorption change of 10%.

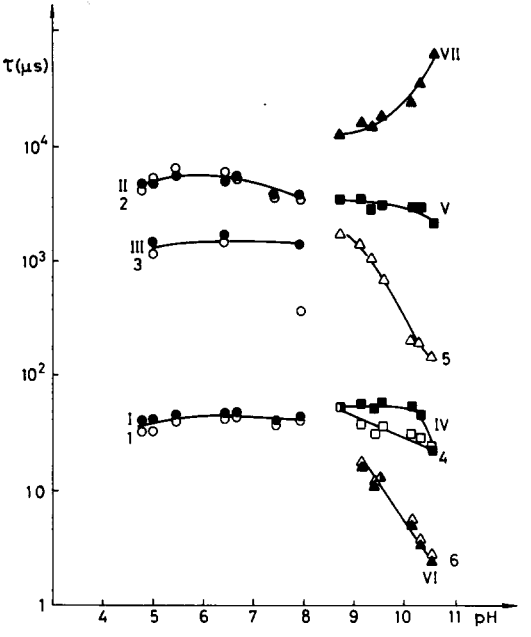

Fig. 3. The dependence of the time-constants of the photoelectric and absorption kinetic signals on pH. The rate constants are assigned to the different components of the signals according to the notaitons in Fig. 1 and 2 as follows. ●, absorption kinetic data at pH < 8, components I, II (405 nm) and III (660 nm); ■, absorption kinetic data at pH > 8, components IV and V (405 nm); ▲, absorption kinetic data at pH > 8, components VI and VII (405 nm); ○, photoelectric data at pH < 8, components 1, 2 and 3; □, photoelectric data at pH > 8, component 4; △, photoelectric data at pH > 8, components 5 and 6.

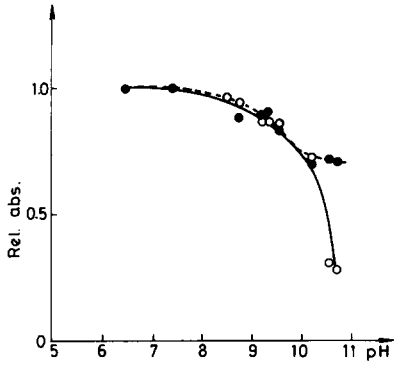

Fig. 4. The dependence of the amplitude ratios of the slow and fast components in the rise and decay of the absorption kinetic signals, respectively, at 405 nm on pH. O, ———, the data corresponding to the rise of the signal (ΔA_{max}: amplitude of the exponent fitted tothe respective components of the absorption kinetic curve):

$$\frac{\Delta A_{max,slow}}{\Delta A_{max,slow} + \Delta A_{max,fast}}$$

●, – – –, the data corresponding to the decay of the signal:

$$\frac{\Delta A_{max,fast}}{\Delta A_{max,fast} + \Delta A_{max,slow}}$$

In both cases the sum $\Delta A_{max,fast} + \Delta A_{max,slow}$ is independent of pH.

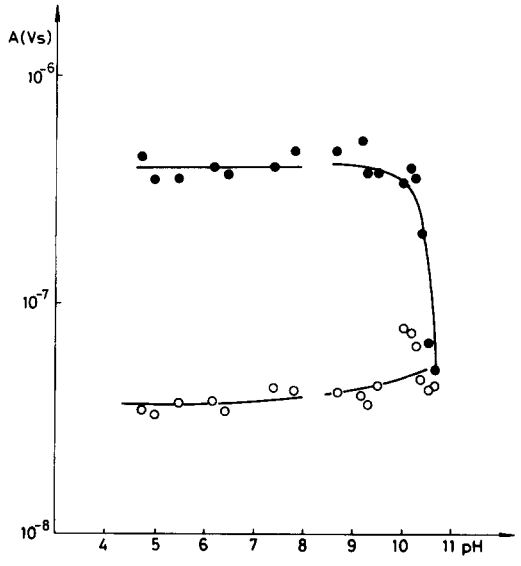

Fig. 5. The dependence of the areas of the components of the electric signals on pH. The data were normalized to the amplitude of the first negative step. O, the area of the first positive component of the electric signal, that corresponds to the L → M transition (components 1 and 6); ●, the area of the slow components of the electrical signals (components 2,3 and 4,5).

and in the photoelectric signals. To illustrate this, in Fig. 2c,d we show the rise and decay of the M form (at this pH no O form can be observed), and in Fig. 2a,b the corresponding photoelectric signals, both measured at pH 10.2. It can be seen that both the rise and the decay of the M form consist of two components of comparable amplitudes, and that the photoelectric signal completely changes its character.

Fig. 3 summarizes the exponential time constants characteristic for the absorption kinetic and photoelectric signals in the dependence of pH. It is very interesting to follow the course of the curves. Until pH ≈ 8 all the absorption and electric signals are coupled and change little with pH. At pH 7.5 the signals corresponding to the O → BR transition separate, and above pH 8 the rise and decay of component M separate into two components. The coupling of the two signal types does not hold for most components. It is worth noting that the total amplitude of the change in absorbance at 405 nm is independent of pH in the range under study. In Fig. 4 are plotted the relative amplitudes of the slow and fast components for rise and decay, respectively. It is interesting to observe that until pH ≈ 10 the relative contribution of the slow rise follows that of the fast decay. Above pH ≈ 10 the contribution of the slow component of the rise becomes much smaller than that of the fast decay.

The area under the electric signal curve is also informative because it is proportional to the charge displacement, d [7,8]. Fig. 5 shows the areas of the successive components. They were normalized to the first negative step (which proved to be pH-independent), eliminating the differences due to possible variations in orientation at different pH values.

Discussion

It is seen from these results that in the range of pH 4–8 the behaviour of the system is unaltered. This range contains also the values of bacteriorhodopsin's normal operating conditions, supporting our previous description. Studying the system under extreme conditions may, nevertheless, help in understanding the details of how bacteriorhodopsin functions.

To explain the phenomena at high pH, let us first discuss the results of the absorption kinetic experiments. As shown, in the alkaline region, both in the rise and decay the M form exhibits biphasic kinetics with comparable amplitudes. Until pH ≈ 10 the weights of the two components in the rise and decay changed with pH smoothly and in the same way, i.e., the amplitude ratio of the fast rise is equal with that of the slow decay, and vice versa. Kinetically, this can be explained in the following way: in this range in the photocycle two M forms are present in independent parallel reaction paths, one with appearance and decay not too different from the normal case, and one having a fast rise and slow decay – this indicates that a branching in the photocycle exists. Recently, Mathew et al. [21] succeeded in showing the spectral differences of two M forms, the one with the fast decay having an absorption maximum at 412 nm and the slow one at 405 nm, however, at pH 7.5 and high (3 M) salt concentration. For kinetics reasons, two L forms must exist (only in this case do we see two exponentials in the rise of M) therefore the branching of the cycle occurs at the K form at least. The data agree with previous observations [12,14,19] and extend them in relating (a) the fast rise with slow decay and (b) slow rise with fast decay. The titration of processes (a) and (b) coincides with the titration of the rise in M in Ref. 14. It may now be stated more firmly on the basis of Ref. 14 that the ionization state of a tyrosine near to the Schiff-base region influences the entropy factor of the rise and decay of the M form. The slow rise and the fast decay correspond to a tyrosine neighbour and the fast rise and the slow decay to a tyrosinate ion.

Above pH 10, the dependence displays a different trend: the amplitude ratios of the absorption change lose their equality. In this case a simple model with independent parallel paths can not be applied. Our data are not sufficient for selection from the possible more complicated kinetic models; at this point we say only that in this pH range a new change occurs in the photocycle, resulting in a different pattern of pathways.

Turning now to the discussion of the electric signals, the correlation of their time constants with those of the absorption signals in the pH range 4.5–8 confirms and extends our previous results [7–9]. We may therefore state that the cycle of charge motion and the photocycle as observed by light absorption are coupled at physiological pH.

At pH > 8 the coupling of the two cycles changes. While the components of the M rise find their electric correspondents (curves IV-4, and more expressed IV-6, in Fig. 3), the electric signal with the long lifetime (curve 5) is much faster than any component in the decay of M (curves V, VII). The dramatic changes in the kinetics do not affect the area of the electric signal until pH ≈ 10 (Fig. 5). We interpret these data as a clear demonstration of the constancy of the stoichiometry in the pH range 4.5–10. This statement agrees with the result of Ort and Parson [20] and extends the range of its validity and contradicts the results in Refs. 16, 19. The reason for the disagreement remains unclear. It has to be pointed out, however, that in our method only the charges moving inside the bacteriorhodopsin are sensed, and additional effects like emission of Bohr protons or trapping of pumped protons at the cell surfaces, which certainly appear in pH measuring methods and which may depend on pH, do not contribute.

On the basis of the data presented, the following conclusions may be drawn. When pH is increased, two groups – having an important functional role in the photocycle – are successively titrated in bacteriorhodopsin. First – as has already been reported [11] – a tyrosine residue probably becomes deprotonated, which, in proteins, has an expected pK of 9.6–10 [23]. The effect of this new charged group on the photocycle is to change the cycle's kinetics. Also, the kinetics of the proton transfer change greatly: the steps are accelerated and they are no longer coupled to the photocycle. However, the sum of the areas of the components is always positive, indicating a true charge translocation, and is constant in the pH range of 4.5–10, substantiating a pH-independent stoichiometry [24].

Titrating the second group, the photocycle is changed again, with different ratios of the fast and slow components in the rise and decay of the M form. In this case the net proton transfer ceases, although small, internal proton movements can still be observed. This means that the protonated state of this second group is vital for the pumping process.

This second group may also be a tyrosine in a different moiety with higher apparent pK, as suggested in Ref. 12. The pK of 10.5 for a tyrosine deprotonation reported in Ref. 11 also supports this conclusion.

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