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Effect of pH on the stoichiometry of light-induced proton release and uptake from purple membrane suspensions

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Purple membrane fragments from *Halobacterium halobium* are used to investigate the pH variations induced by a steady illumination of bacteriorhodopsin. The pH changes are measured fluorometrically as a function of the pH of the suspensions. The light-induced accumulation of the M_{412} intermediate is recorded in similar experimental conditions. The two sets of data are used to determine the pH influence on the H^+/M_{412} ratio. Results are compared with those obtained with intact cells.

Introduction

Bacteriorhodopsin is a retinal-containing protein found in the purple membrane of *Halobacterium halobium*. Upon illumination, the chromophore undergoes a photochemical cycle which leads to a translocation of protons across the cell membrane [1,2]. Numerous experiments were performed with various model systems in order to correlate the intermediate formation and the proton transfer mechanism.

Several groups have shown that in intact cells each photocycle liberates two H^+ in the extra-cellular medium at least at acidic and neutral pH values [3–5]. At alkaline pH levels, only one H^+ seems to be released [5].

With purple membrane suspensions, Lozier et al. [6] found an approximate stoichiometry of 1 : 1 between the number of mole of H^+ released and the molar concentration of a stable intermediate adsorbing at 412 nm. In more recent studies, ratios ranging from 0.2 to several hundreds were measured depending upon the experimental conditions [7–11].

The aim of the present paper is to analyze to what extent a comparison can be drawn between the findings made with intact cells and those which relate to purple membrane suspensions. We will provide experimental evidences tending to demonstrate that the two systems are not directly comparable.

We report on pH changes measured during steady illumination of purple membrane suspensions. The pH changes were detected fluorometrically using methylumbelliferone as an indicator. Fluorescence variations were measured as a function of the pH of the medium. The steady-state fluorescence intensity was correlated with the actual concentration of M_{412}. From the two sets of data, a computation of the H^+/M_{412} ratio could be made. Since the measurements described in the present paper were carried out using experimental conditions similar to those previously adopted for studying intact bacteria [5], it will be significant to compare the two sets of results.

Materials and Methods

Purple membrane fragments from *Halobacterium halobium* (strain R_1M_1) were isolated as

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previously described [12]. Our experiments were performed with bacteriorhodopsin concentration of $1.14 \cdot 10^{-5}$ M. This concentration was determined at 568 nm with a Perkin-Elmer 559 spectrophotometer ($\varepsilon = 63\,000\ M^{-1} \cdot cm^{-1}$). The NaCl concentration was 2 or 4 M.

The pH changes were measured fluorometrically using methylumbelliferone as an indicator (excitation at 366 nm, emission measured at 465 nm). Its concentration was $1.06 \cdot 10^{-5}$ M. Fluorescence was measured with a modified Perkin-Elmer MPF44 spectrofluorimeter. The excitation beam was provided by a 300 W xenon lamp (Varian VIX 300 UV) and filtered through an infrared filter (Schott KG1). The spectrofluorimeter photomultiplier was protected from diffused actinic light with a interference filter (Schott AL454).

We verified that in our experimental conditions, methylumbelliferone fulfills the criteria required for a good pH indicator [8]. The possible interaction between the indicator and the membranes was tested according to the procedure proposed by Chance and Scarpa [13]. Purple membrane sheets do not modify significantly the titration curve of the indicator. Indeed, the pK value which is equal to 7.4 in salt solution (2 M NaCl) increases by 0.1 unit in the presence of membranes. Another control was performed as follows: after centrifugation of unbuffered samples ($48\,300 \times g$, 50 min), 100% of the pH indicator were recovered in the supernatant. These controls were performed between pH 6.5 and 8.5. In buffered suspensions, the yield was 95–100% after the first centrifugation. However, after resuspending the pellet in the same volume of buffer, the remaining amount of indicator was recovered in the supernatant after a second centrifugation. The fluorescence variations were calibrated by adding 5-μl aliquots of 10^{-4} and 10^{-3} M HCl solutions.

The fluorescence measurements were corrected to take into account the filtering effects associated with the formation of M_{412}. Acidification and alkalinization of the medium were determined from the differences of the fluorescence intensities between unbuffered and buffered membrane suspensions.

A 900 W xenon lamp (Osram) was used to induce the pH variations. The parallel light beam was passed through a $CuSO_4$ solution (2%, w/v) and through a cut-off filter (Schott OG530). The incident light intensity was 650 W/m^2 between 510 and 700 nm. The 500-μl samples were introduced into thermostated quartz cells. Experiments were performed at 23 ± 0.3°C.

The M_{412} concentration variations induced by the steady illumination, were measured at 412 nm using a Perkin-Elmer spectrophotometer. The actinic light beam was identical to the one previously described. The spectrophotometer photomultiplier was protected with two superposed interference filters (Agfa-Gevaert 405). A differential molar absorption coefficient, $\Delta\varepsilon_{420} = 23\,000\ M^{-1} \cdot cm^{-1}$ was used in the M_{412} measurements [14].

Results

Fig. 1 shows the methylumbelliferone fluorescence variations induced by bacteriorhodopsin illumination at 568 nm. The measurements were carried out at pH 6.70, 7.07 and 8.20 with unbuffered (A) and buffered (B) suspensions. The fluorescence changes observed with buffered suspension, are due to internal filtering effects associated with the accumulation of the intermediate M_{412} (see Materials and Methods). Since the filtering effects are more important when the pH of the medium becomes more alkaline, it indicates that the concentration of M_{412} increases accordingly.

The fluorescence changes observed with unbuffered suspensions are clearly biphasic at pH 6.70 and 7.07. By contrast, at pH 8.28, the fluorescence intensity increases very rapidly and the effect seems to be monophasic.

The rapid fluorescence rises detected at pH 6.70 and 7.07 correspond to instantaneous alkalinizations of the mediums. At pH 6.70, this rapid uptake of protons by the membrane sheets is followed by a slower fluorescence change which is associated with a release of protons. This slow process has a half-time of about 6 s. It concerns some 25% of the protons initially taken up during the rapid phase.

By contrast, at pH 7.07, the rapid rise of the fluorescence intensity is followed by a slower increase associated with a slow uptake of protons by the membrane sheets. In our experimental conditions, the transition between the slow H^+ release

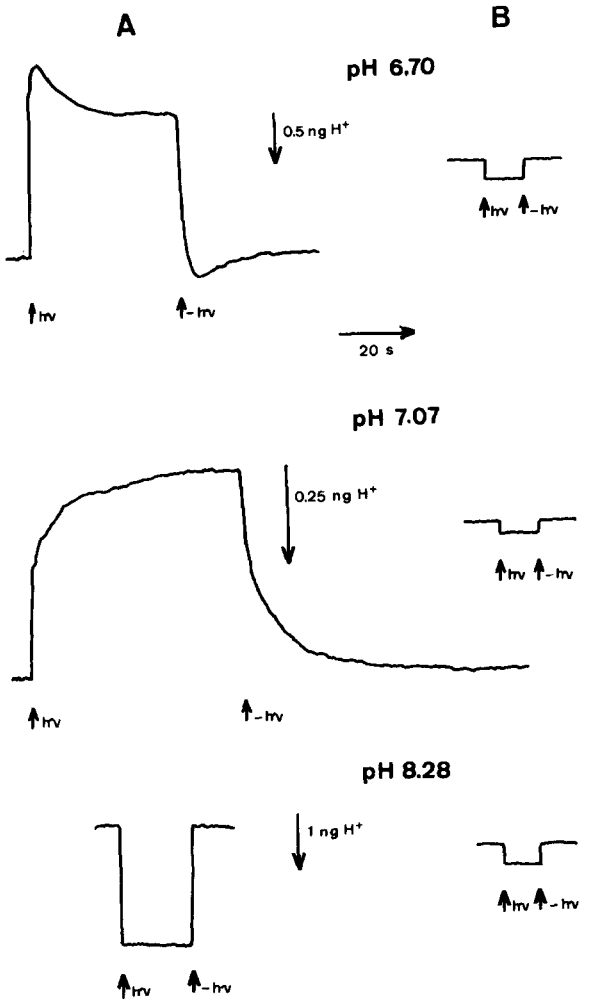

Fig. 1. Light-induced fluorescence variations of methylumbelliferone in purple membrane suspensions in the absence (A) and in the presence (B) of 66 mM phosphate buffer. The variations at pH 6.70 were recorded with a sensitivity 2-fold higher than at pH 7.07 and 8.28. Calibrations take into account modifications of the buffer capacity of the suspensions.

and the slow H^+ uptake occurs at pH 6.9.

Fig. 1A shows that at the onset of illumination slow variations of the fluorescence intensity are also detected at pH 6.70 and 7.07. The amplitudes of the light-induced fluorescence variations are plotted in Fig. 2 versus the pH of the membrane suspensions. The results concern the steady fluorescence intensities measured at the end of the continuous illumination period. The proton capture is maximum at pH 6.9. It should be stressed,

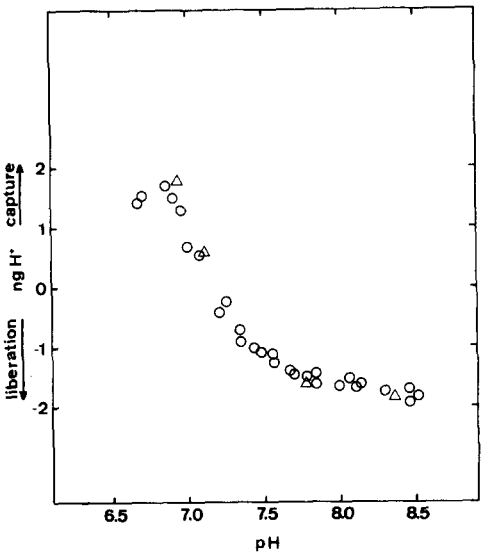

Fig. 2. Effect of pH on the number of protons released or taken up upon illumination (510–700 nm) of purple membrane suspensions. (○, 2 M NaCl; △, 4 M NaCl).

however, that this maximum would disappear if the variations of fluorescence intensity were measured at the end of the rapid phase.

Fig. 2 demonstrates that the light-induced transfer of protons is determined by the pH of the suspension. Below pH 7.1, the effect corresponds to an uptake of H^+, whereas an H^+ release is detected at pH levels higher than 7.1. At pH 8.5, 1.74 ng H^+ is liberated, which corresponds to 0.31 H^+/molecule of bacteriorhodopsin. Similar results were observed at 2 and 4 M NaCl.

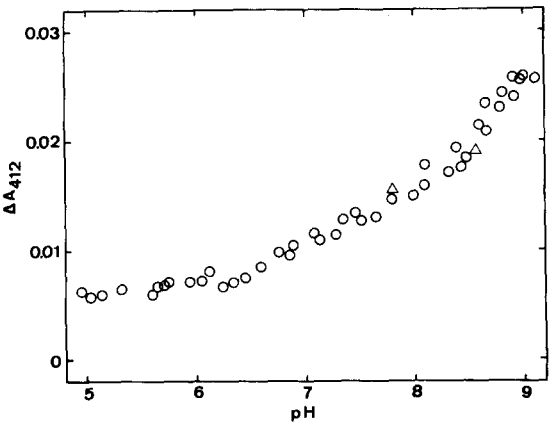

Fig. 3. Effect of pH on the light-induced variations of the M_{412} concentration (○, 2 M NaCl; △, 4 M NaCl).

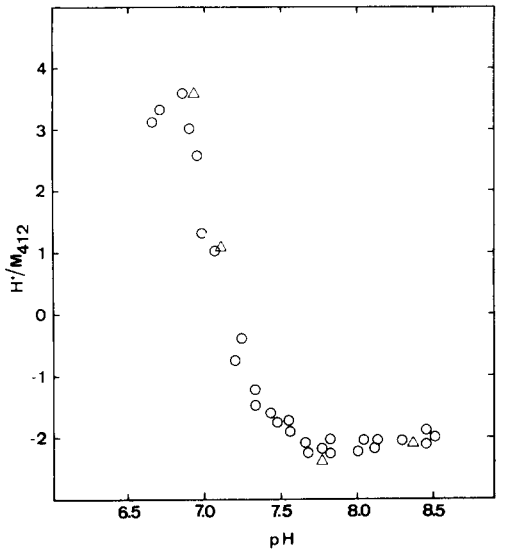

Fig. 4. Effect of pH on the H^+/M_{412} ratio. The negative values correspond to a release of protons.

Fig. 3 shows that the accumulation of the M_{412} intermediate is more pronounced at high pH values. This effect was already underlined in Fig. 1B. Ionic strength does not influence significantly the steady-state concentration of M_{412}. The M_{412} concentration remains constant during the illumination period. This contrasts with the pH variations observed in identical experimental conditions (Fig. 1).

From the data shown in Fig. 2 and 3, the number of H^+ transferred per molecule of M_{412} formed can be computed. The corresponding results are given in Fig. 4. At pH 6.9, the uptake is maximum. It amounts to 3.6 H^+/M_{412}. From pH 7.6 up to pH 8.5, about two protons are released per molecule of M_{412} formed. These computations are based on a differential molar absorption coefficient for M_{412} of 23000 $M^{-1} \cdot cm^{-1}$ [8,15]. Some discrepancy exists in the literature concerning the value of this coefficient [8,15,16]. If one assumes that $\Delta\varepsilon = 45000$ [16], the maximum uptake would correspond to 7.0 protons. On the other hand, 3.9 protons would be released from pH 7.6 up to 8.5.

Discussion

Fluorescence changes of the optical pH indicator provide a sensitive experimental method for measuring small pH variations induced by light in purple membrane suspensions. The light-induced variations of the fluorescence intensity are strongly pH dependent (Figs. 1 and 2). They correspond to either an uptake or a release of protons by the membrane sheets. This suggests that at least two functional residues of the bacteriorhodopsin molecule are involved in the light-induced pH changes: one residue could determine the uptake of protons, the other could be responsible for the proton release. Actually, it has been suggested that the effect of pH on the proton transfer might correspond to light-induced modifications of the pK values of the corresponding residues [17].

In our experimental conditions, the transition between the uptake and the release of protons occurs at pH 7.1. Takeuchi et al. [17] observed this transition at pH 5.7 at 10°C. However, Garty et al. [18] reported that the pH of transition increases significantly with temperature. The pH effect on the proton binding was also measured by Renthal [19] in various experimental conditions. The maximum acidification was observed between pH 9 and 10. At 3 M KCl and 30°C, the number of protons liberated increases slightly between pH 8.0 and 8.5. This observation is in agreement with our results.

The M_{412} concentration increases monotonically when the pH of the medium becomes more alkaline (Fig. 3). This effect contrasts with the pH changes observed in identical experimental conditions (Figs. 1 and 2). The different behaviours suggest again that more than one proton pool is involved in the complex pH variations.

We observed that the H^+/M_{412} ratio varies from +3.6 at pH 6.9 to −2.0 at pH 7.5. This finding contrasts strongly with the results of Caplan et al. [9] who reported a value of 500 at pH 6.9. The discrepancy cannot be explained by the fact that these investigators used a differential molar absorption coefficient $\Delta\varepsilon = 30000$ $M^{-1} \cdot cm^{-1}$ which is larger than ours [10]. It should be stressed, however, that our results are in reasonable agreement with more recent data reported by Kuschmitz and Hess [8] and by Stoeckenius et al. [15].

The molecular basis for the change in the H^+/M_{412} ratio remains unexplained. Several investigators have shown that specific residues such

as lysine [17], tyrosine [20–22] and carboxylic groups [23] release or absorb protons upon illumination. It would be interesting to investigate the effect of pH on these reactions.

Another approach would be to correlate the proton uptake and release with the formation of the intermediates. For example, it has been shown recently [24,25] that O_{660} plays a key role in the proton-transfer mechanism, and that the O_{660} concentration becomes very small at pH values higher than 8.0.

In membrane suspensions, the light-induced pH changes cannot be associated with a net transport of protons across the membrane sheets, since both faces are exposed to the same medium. In that respect, membrane sheets and intact bacteria are not directly comparable. In membrane sheets, the light-induced pH changes imply either stoichiometric or Bohr protons. Stoichiometric protons can be defined as those which are liberated due to the accumulation of M_{412}. It is indeed well known that the Schiff base is unprotonated in the M_{412} intermediate, whereas the chromophore is protonated in the bacteriorhodopsin$_{568}$ form. It has also been shown that a tyrosine residue of the bacteriorhodopsin molecule becomes deprotonated during the L_{550}–M_{412} transition [20–22]. Bohr protons have been defined as protons released or captured by the protein following light-induced conformational changes.

At pH 6.9, 3.6 protons are captured per molecule of M_{412} formed. This value is relatively high. It seems to imply Bohr protons, more especially because stoichiometric protons are probably liberated at this pH. On the contrary, at pH 7.5 and higher, two protons are liberated per molecule of M_{412}. This release could imply either stoichiometric and/or Bohr protons. It should be noticed that the data shown in Fig. 4 would vary with the value adopted for the differential molar absorption coefficient of the M_{412} intermediate. However, the trend of the discussion does not depend upon this numerical value.

A direct comparison between the results described in the present work and those reported on intact bacteria seems inappropriate. Bogomolni et al. [3] determined the quantum efficiency for proton ejection from *Halobacterium halobium* and found values ranging from 0.4 to 0.7. Renard and Delmelle [5] measured a quantum yield equal to 0.64 at pH 5.9, the value decreasing progressively to 0.28 at alkaline pH values. Govindjee et al. [4] studied flash-induced proton release in bacteria, and measured an H^+/M_{412} ratio of about 2.

In our experiments with intact cells [5], pH changes were induced by light fluxes which were about 20-fold weaker than in the experiments reported in the present work. Dancshazy et al. [26] have shown that the kinetics of M_{412} become slower when the membrane potential increases in cells. It seems, however, reasonable to assume that the steady-state concentration of M_{412} is smaller in intact cells than in purple membrane suspensions. This should reduce accordingly the concentration of stoichiometric protons. On the other hand, the pH changes measured in intact bacteria were recorded on a slow time-scale with a glass electrode. In these conditions, Bohr protons are probably not measured, since they are released and captured during each photocycle. Hence, intact cells and membrane sheets are not directly comparable.

It has been underlined that the pH changes measured in intact cells with glass electrodes do not prove unquestionably that the protons are really pumped across the cell membrane. It is felt, however, that the results presented here strongly support such an interpretation.

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