of pure ester lecithin), a detailed kinetic investigation should be definitive.

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On the Ratio of the Proton and Photochemical Cycles in Bacteriorhodopsin†

D. Kuschmitz and B. Hess*

ABSTRACT: The ratio of protons released per M-412 intermediate formed in illuminated purple membrane sheets of *Halobacterium halobium* varies from 0.3 to 3.0 as a function of the ionic strength and light intensity. The ratio increases with decreasing light intensity. At high ionic strength the illumination time affects the monophasic rate of proton uptake and biphasic rates of the M-412 decay. The influence of the ionic strength on the ratio is discussed on the basis of Gouy–Chapman theory as a consequence of pK_a shifts of light-activated proton binding groups.

Under conditions of low oxygen tension in the presence of light, *Halobacterium halobium* develops a membrane system, the purple membrane (PM),[1] which acts as a light-driven proton pump converting light energy into electrochemical potential energy ($\Delta\mu_{H^+}$) acrosss the bacterial membrane [for summary, see Stoeckenius et al. (1979)]. $\Delta\mu_{H^+}$ can drive ATP synthesis (Oesterhelt, 1974; Danon & Stoeckenius, 1974) and other energy-linked cellular functions (Lanyi, 1978). The energy transduction is due to the function of a single chromoprotein, bacteriorhodopsin, which in a complex of about 10 lipid molecules/protein associates to form the purple membrane in the bacterial plasma membrane (Stoeckenius et al., 1979).

Functional analysis of the isolated purple membrane revealed the occurrence of a photochemical cycle coupled to a release of protons (Oesterhelt & Hess, 1973). This phenomenon also could be observed in the intact bacterial cell (Oesterhelt & Stoeckenius, 1973; Oesterhelt, 1974). It was shown that upon illumination protons are liberated directly from the purple membrane in a time course which correlates with or lags behind the photochemical formation of a stable intermediate absorbing at 412 nm (M-412) (Oesterhelt & Hess, 1973; Lozier et al., 1975; Chance et al., 1975; Lozier

† From the Max-Planck-Institut für Ernährungsphysiologie, Dortmund, D-4600, Federal Republic of Germany. *Received March 5, 1980; revised manuscript received May 1, 1981.*

[1] Abbreviations used: PM, purple membrane; M-412, intermediate absorbing at 412 nm.

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et al., 1976). In the dark, protons are taken up again, and the original state of the purple pigment bacteriorhodopsin is regained (Oesterhelt & Hess, 1973; Oesterhelt, 1974; Lozier et al., 1975; Chance et al., 1975). Extracellular pH changes in bacterial suspensions upon illumination (Oesterhelt & Stoeckenius, 1973) showed a most complex behavior: proton liberation and proton uptake were found to be functions of various external conditions, such as light intensity, pH, and oxygen tension (Bogomolni et al., 1976; Wagner & Hope, 1976; Oesterhelt & Krippahl, 1973). Titration experiments carried out with isolated purple membrane suspensions revealed an approximate stoichiometry of 1:1 between the amount of protons released/or taken up and a full photochemical cycle of bacteriorhodopsoin (Lozier et al., 1975, 1976).

In a more detailed analysis of this relationship, however, ratios in the range from 0.2 up to several hundred were obtained in a comparable pH range, depending on the experimental condtions (Hess & Kuschmitz, 1976, 1978; Kuschmitz & Hess, 1977; Klemperer et al., 1978; Caplan et al, 1978; Bakker & Caplan, 1978; Avi-Dor et al., 1979; Ort & Parson, 1979), indicating that no simple relationship exists between the functioning of the photocycle and the number of protons released. It was also reproted that some of the protons liberated after the onset of illumination are taken up again rapidly during the illumination (Oesterhelt & Hess, 1973). At low pH only net proton uptake is observed (Dencher & Wilms, 1975; Garty et al., 1977). In order to define the conditions for the maximum and minimum ratios, we have analyzed this relationship with respect to its dependence upon ionic strength, light intensity, and illumination time. This paper describes the details of our observations following preliminary reports (Kuschmitz & Hess, 1977; Hess & Kuschmitz, 1978).

Materials and Methods

Growth of *Halobacterium halobium* (mutant R_1M_1) as well as isolation of purple membrane were carried out according to Oesterhelt & Stoeckenius (1974).

Photometric measurements were performed with a "Dortmund" dual wavelength instrument (Oesterhelt & Hess, 1973) (Figure 1a) adjusted at 570 nm for control of light adaptation of bacteriorhodopsin and at 420 nm for the measurement of the concentration of M-412, which was generated under steady illumination with light of 578 nm. The maximum light intensity was 200 W/m^2 as measured with a Tektronix J16 Digitalphotometer.

pH changes were measured fluorometrically with methylumbelliferone as indicator (excitation at 366 nm, emission measured at 465 nm). The measurements were carried out in a 1 × 1 cm all-quartz cuvette placed into a special cuvette holder of the dual-wavelength apparatus directly on top of a Y-shaped light pipe (Figure 1a). The photomultiplier current of the fluorescence beam was measured with a Keithley-153 Microvolt-Ammeter, and the output voltage after passage through a Rockland electronic filter (10 Hz) was recorded with a two-channel Siemens recorder simultaneously connected to the 420-nm measuring beam of the dual-wavelength apparatus.

Pulsed illuminations were performed down to 20 ms with a commercial photoshutter (opening and closing times 2.4 ms); the dual-wavelength electronic circuit for the measurement of the absorbance and fluorescence changes was used. The output signals were recorded in a transient recorder (Biomation 802). The temperature was adjusted around 0 °C and controlled with equipment from L'Air Liquide.

Calculations were carried out with a molar absorption coefficient, ϵ, of 63 000 mol^{-1} cm^{-1} for bR-570 (Oesterhelt & Hess, 1973) and a differential molar absorption coefficient,

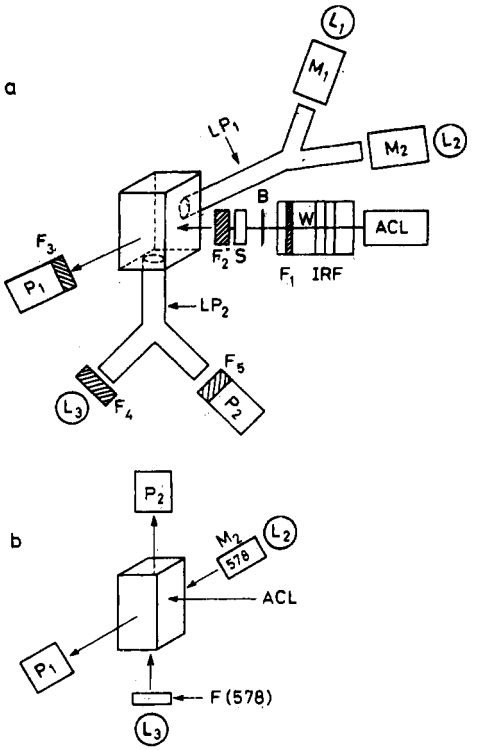

FIGURE 1: (a) (Photometric analysis) Light of two 400-W tungsten–halogen lamps (L_1, L_2) selected by means of two monochromators (M_1, M_2) (double monochromators of Bausch & Lomb) and chopped by rotating disks (not shown) at 1 kHz (Oesterhelt & Hess, 1973) is passed through light pipes (LP_1) onto the side of the cuvette toward a photomultiplier (P_1) equipped with a guard filter [420 ± 30 nm (Schott)] for measurement of the transmitted light. (Fluorometric analysis) Light of a 200-W xenon–mercury lamp (L_3) selected through a 366-nm Eppendorf filter (F_4) is guided through the light pipe (LP_2) onto the bottom of the cuvette for fluorescence excitation, the emission being accepted through the second arm of the pipe and passed toward a photomultiplier (P_2) through an interference filter (F_5) of 465 nm (Schott). Actinic light was obtained by a 900-W xenon arc (ACL) (Zeis-Ikon), passed through infrared filters (IRF), a water bath (w), and a 500 nm cutoff filter (F_1) (Schott) followed by an iris diaphragm (B), a collecting lens (ommited in the scheme), a photoshutter (S), and an 578-nm Eppendorf filter (F_2) focussed onto the side of the cuvette. (b) Experimental setup for measuring the light-effective volume (for explanation, see text).

$\Delta\epsilon_{420}$, of 23 000 mol^{-1} cm^{-1} for M-412 (Lozier et al., 1975, 1976).

Because the measuring cuvette was not totally illuminated by the actinic light, the light-effective volume out of a total volume of 2.05 mL, giving the nanomoles of M-412 formed in the illuminated state, had to be determined. This was performed as indicated schematically in Figure 1b by measuring the absorption difference of a purple membrane suspension in basal salt ether (Oesterhelt & Hess, 1973) upon 578-nm illumination in the horizontal direction (ΔE_h) with the actinic pathlength (d_h) being 1 cm and simultaneously in the vertical direction (ΔE_v) where the actinic light pathlength is not known. The average vertical actinic light pathlength (d_v) is given by the ratio between the vertical and horizontal absorption change ($d_v = \Delta E_v d_h / \Delta E_h$). The average value in a 1 × 1 cm cuvette was 1.12 cm, giving an average light effective volume of 1.12 mL. The nanomoles of released protons was determined in the total volume (2.05 mL) by calibration of the pH indicator response with known amounts of acid and base (see below).

Titration of the pH Indicator. For exclusion of interactions between the pH indicator and the purple membrane, the fluorescence intensity of 50 μM methylumbelliferone (in 20 mM potassium phosphate at 2 °C) was titrated in the presence

and absence of purple membrane. The presence of 12 μM bR quenched the methylumbelliferone fluorescence by about 20% but did not affect the pK_a of the titration curves significantly. The pK_a was 7.4 in the absence of the purple membrane and 7.5 in the presence. The quenching is due to light absorption by bacteriorhodopsin (intermolecular filtering; see also below). The pK_a of methylumbelliferone is not changed by the addition of 2 M NaCl. Binding experiments of methylumbelliferone to purple membrane (equimolar concentrations of 10 μM) gave the following results: After centrifugation 100 ± 3% methylumbelliferone was recovered in the supernatant in 20 mM potassium phosphate, pH 6, 7.5, and 9. In buffered (20 mM potassium phosphate, pH 7.5) and unbuffered (adjusted to pH 7.5) suspensions with increasing concentrations of NaCl (up to 4 M) 95–100% of the indicator were recovered in the supernatant, and the remaining up to 5% was found after only one washing of the purple membrane pellet with the same volume of suspension medium. Only with 50 mM Tris buffer (pH 7.5) in the presence of 2 M KCl and 2 M NaCl 86% and 81% methylumbelliferone, respectively, was recovered in the first supernatant. Thus, according to the principles given by Chance & Scarpa (1972), methylumbelliferone fullfills the criteria of a useful pH indicator in this system [see also Chance et al. (1975)].

Estimation of H^+/bR Ratios. Protonation and deprotonation reactions of the purple membrane were analyzed in unbuffered suspensions of the purple membrane in water or NaCl or KCl solutions (up to 4 M) containing 10 μM methylubelliferone. The methylumbelliferone was titrated to half-maximal fluorescence intensity, corresponding to a bulk pH around 7.5, by addition of small amounts of either NaOH and KOH. Before or after the pH adjustment of the system the purple membrane was light adapted by constant illumination with 578-nm light until the adsorption at 570 nm remained constant.

Compensation of Intermolecular Filtering Effects. For compensation for intermolecular filtering effects due to the absorption of M-412 at 366 nm developing upon illumination, in each experiment the sample was first calibrated by addition of 10 nmol of HCl per 2.05-mL cuvette volume. Then, the experiment was carried out in the presence of 50 mM Tris buffer, pH 7.5 (Figure 2). In control experiments it was established that the addition of 10 nmol of HCl in the dark and addition of 10 nmol of KOH or NaOH in the light gave the same amplitude of fluorescence change. The buffering did not affect the light-induced absorption change at 420 nm, but it decreased the fluorescence changes. At high salt concentrations about 10% of the fluorescence change observed in the absence of the buffer remained in the presence of the buffer (Figure 2a). We interpret this as the unspecific change due to the intermolecular filtering effect. At low salt concentrations, the unspecific fluorescence change was about 50% of the change seen without the buffer (Figure 2b). The unspecific fluorescence (ΔF_{buffer}) change was linear with ΔE_{420} over the total intensity range of actinic illumination at low as well as high ionic strength (not shown). The fluorescence change, specific for an acidification (alkalinization) of the medium, ΔF_{H^+}, is defined as the difference between ΔF_{total} and ΔF_{buffer}.

Results

pH Change during Light and Dark Reaction. Upon illumination of a purple membrane suspension in 2 M NaCl (Figure 2a) with 578-nm light, the fluorescence intensity of methylumbelliferone decreases, indicating an acidification of the medium. The trace shows an overshoot, indicating an equilibration phase of roughly 30 s during which some of the

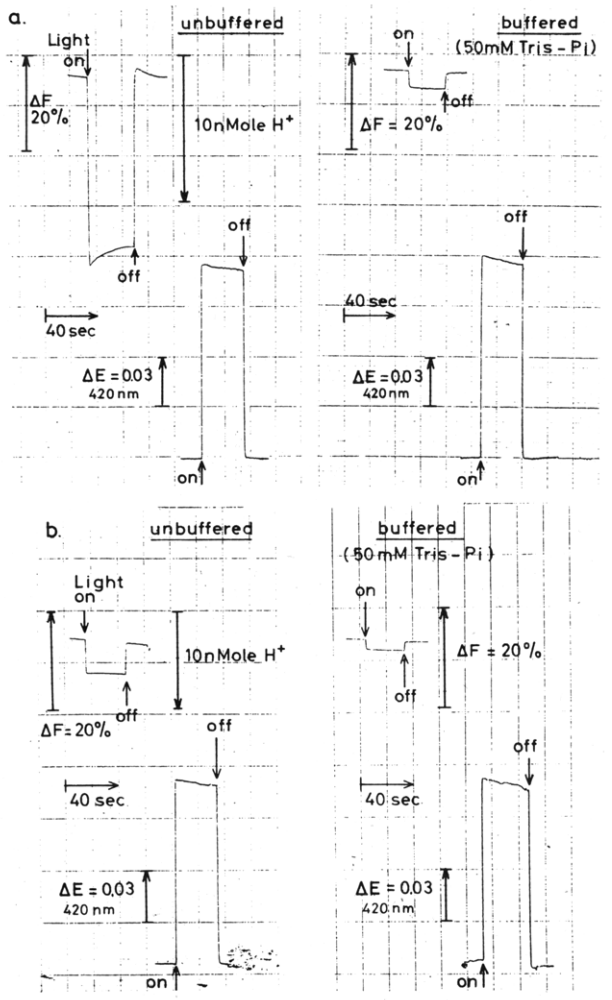

FIGURE 2: Light-induced proton release and M-412 formation of unbuffered (adjusted to pH 7.5 by fluorescence) and buffered (50 mM Tris–phosphate, pH 7.5) purple membrane suspension (11 μM bacteriorhodopsin, 10 μM methylumbelliferone) in 2 NaCl (a) and water (b); temperature 0.5–1.0 °C; light intensity 200 W/m^2.

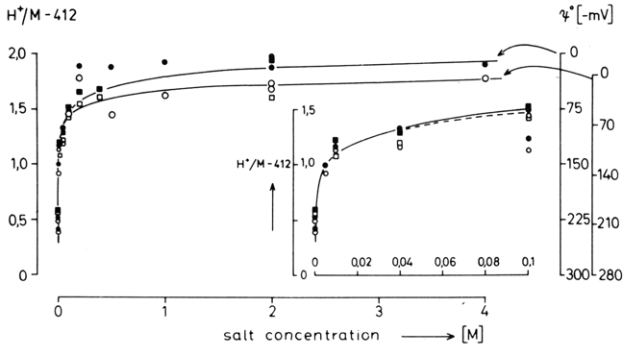

FIGURE 3: Ratio between proton release and M-412 formation as a function of ionic strength in NaCl (●) or KCl (■) on the basis of the maximum values obtained and in NaCl (○) or KCl (□) on the basis of the steady-state values. (For conditions, see Figure 2). The lines represent surface potentials (right-hand scale) calculated with charge densities of 0.005 (left scale) and 0.0025 e/Å^2 (right scale).

released protons are taken up again. This observation confirms the earlier report (Oesterhelt & Hess, 1973) that approximately 33% of the initially released protons are rebound in a secondary reaction during the illumination. On switching off the light, an overshoot toward alkalinization is recorded, demonstrating that a slow portion of the initally incorporated protons are released again in a slower secondary reaction. The extent of the overshoots in the dark and the light (approxi-

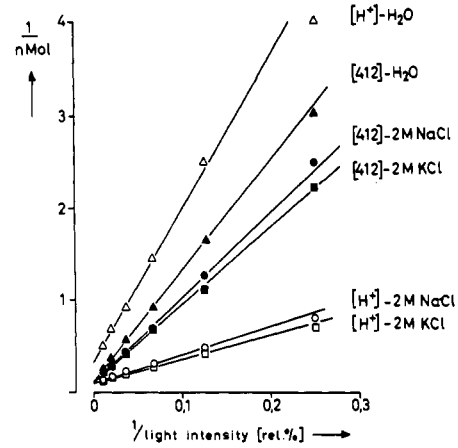

FIGURE 4: Double-reciprocal plots of the light-induced proton release and M-412 formation versus light intensity (conditions as in Figure 2).

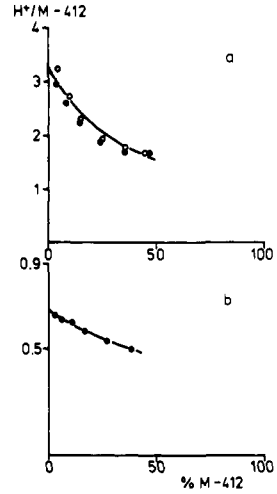

FIGURE 5: Ratio between maximum proton release and M-412 formation as a function of M-412 occupancy. Purple membrane suspensions: (a) 2 M NaCl (●) or 2 M KCl (○); (b) water (conditions as in Figure 2).

mately 10% in Figure 2a) sometimes reaches values up to 30% of the initial fluorescence change (see below and Figure 3) with equilibration times up to 120 s. Although no systematic dependence of the overshoots has been found so far, the amplitudes appear to be smaller at low ionic strength. The overshoots cannot be due to a binding-release process of the indicator itself because they are not observed in the presence of the buffer. Furthermore, it should be mentioned that overshoots are also observed in the experiments carried out with a glass electrode [see above, Oesterhelt & Hess (1973), and Rott & Avi-Dor (1977)].

In the light-dependent formation of M-412, a maximum of 5% overshoot is seen in the dark–light transition, whereas in the reversed transition no overshoot is observed. This result shows that the pH kinetics do not correlate with the M-412 kinetics.

The reaction of the purple membrane suspended in water is illustrated in Figure 2b. While the absorbance changes at 420 nm upon illumination are nearly the same as those seen in the presence of high salt (Figure 2a), the specific fluorescence change is considerably smaller. In addition the calibration with 10 nmol of HCl gives a 33% smaller response than calibration in the presence of salt. This difference in the calibration response is due to the ionic strength dependence of the buffer capacity of the purple membrane. At pH 7.5, the buffer capaicty is higher in water than in high salt solution (unpublished experiments).

H^+/M-412 Ratio as a Function of Ionic Strength. The ratio between the amount of H^+ released and M-412 formed under steady illumination can be computed from the specific fluorescence change, and ΔE_{420}. Our results, shown in Figure 3, demonstrate the strong influence of ionic strength on this ratio (Hess & Kuschmitz, 1976, 1978; Kuschmitz & Hess, 1977; Ort & Parson, 1979; Avi-Dor et al., 1979). At pH 7.5 the H^+/M-412 ratio is approximately 0.3–0.5 in water suspensions of purple membrane. The ratio increases with increasing NaCl or KCl concentrations. At high ionic strength, the maximum amplitude of the proton liberation gives a H^+/M-412 ratio of 1.9, and the light-induced steady state gives a ratio of 1.7. Within experimental error, no difference between potassium and NaCl ions was detected. This suggests that the phenomenon of variable H^+/M-412 ratios can be related to the ionic strength alone.

H^+/M-412 Ratio as a Function of Light Intensity. The dependence of the H^+/M-412 ratio on the light intensity was followed by attenuating the light intensity with calibrated gray filters. In Figure 4 the reciprocal concentrations of M-412 and released protons are plotted against the reciprocal light intensity. Straight lines (r^2 = 0.996–0.999) are obtained from M-412 [in agreement with earlier results; Hess & Osterhelt (1975); Hess & Kuschmitz (1979)] and for the liberated protons. While at pH 7.5 the slopes of the M-412 lines are only slightly different at low and high ionic strength, the slopes of the lines representing the liberated protons are functions of the ionic strength. The linear relationship shown in Figure 4 allows an extrapolation to light saturation, giving H^+/M-412 ratios of ~1 and ~0.33 for high and low ionic strength, respectively. On the other hand, the H^+/M-412 ratio increases progressively the less bR is present in the M-412 form (Figure 5), reaching a maximum ratio of ~3 and ~0.67 (high and low ionic strength, respectively) at a light intensity which leads to approximately 3% M-412 occupancy.

H^+/M-412 Ratio as a Function of Illumination Time. For analysis of the H^+/M-412 ratio during pre-steady-state time, the illumination period was varied. Figure 6 shows that within approximately 300 ms a steady-state level of M-412 and methylumbelliferone fluorescence is reached. Whereas Figure 6a illustrates the transitions for high ionic strength conditions, Figure 6b displays low ionic strength conditions (see below). The results of these experiments are summarized in Figure 7 and demonstrate that at low ionic strength the H^+/M-412 ratio as calculated from peak amplitudes is independent of illumination time (0.51 ± 0.02) over the time range studied but at high ionic strength the H^+/M-412 ratio decreases to 1.1–1.3 at 20-ms illumination time. The liberation of at least part of the light-sensitive protons evidently lags behind the M-412 steady state onset at high ionic strength.

Thus, ionic strength controls not only the amount of protons released but also the kinetics (compare parts a and b of Figure 6). This is confirmed by analysis of the rates of M-412 decay and proton uptake. At low ionic strength we could not detect any difference between the kinetics of proton uptake and the M-412 kinetics at the different illumination times. The kinetics followed single exponentials with half-times of 69 ± 4 and 70 ± 2 ms, respectively, [for microsecond flash experiments, see Lozier et al. (1976)]. At high ionic strength, however, the proton uptake becomes slower with decreasing illumination time while the M-412 decay becomes faster (Figure 8). Also, the M-412 decay is composed of two exponentials, the ratio of which changes in favor of the fast exponential with shorter illumination; in contrast, the proton uptake always follows a single exponential (Ort & Parson, 1978).

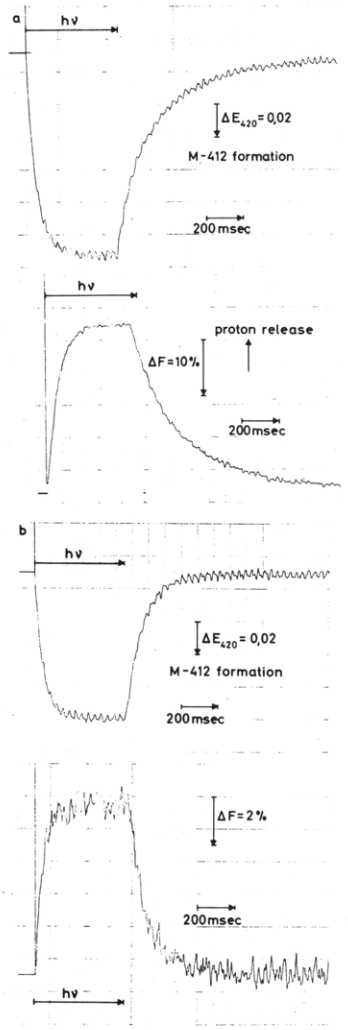

FIGURE 6: Kinetics of proton release and reuptake as well as M-412 formation and decay at an illumination time of 0.5 s. Purple membrane suspension: (a) 2 M NaCl; (b) water (conditions are in Figure 2).

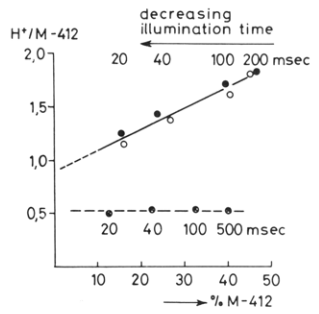

FIGURE 7: Ratio between proton release per M-412 formation as a function of illumination time. Purple membrane suspensions in water (⊗), 2 M NaCl (●), or 2 M KCl (○).

When the amplitudes of the M-412 decay components are related to the total M-412 amplitude reached in the light steady state, it is seen (Figure 9) that the amplitude of the fast components remains constant while that of the slow component increases with increasing illumination time. The amplitude of the slow component is linearly related to the H^+/M-412 ratio (Figure 9, insert).

Discussion

Mechanisms for the proton translocation function of the purple membrane were suggested largely on the basis of the retinal photocycle of bacteriorhodopsin by a number of authors (Schreckenbach et al., 1977; Stoeckenius, 1978; Koslov &

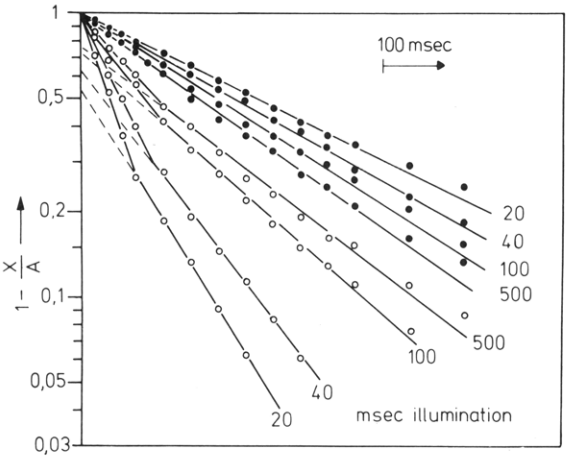

FIGURE 8: First-order plots of proton uptake (●) and M-412 decay (○) kinetics as a function of illumination time; purple membrane suspension in 2 M NaCl. $A = A_{t=0}$ = amplitude at time zero, $X = A_{t=0} - A_t$ (A_t = amplitude at time t).

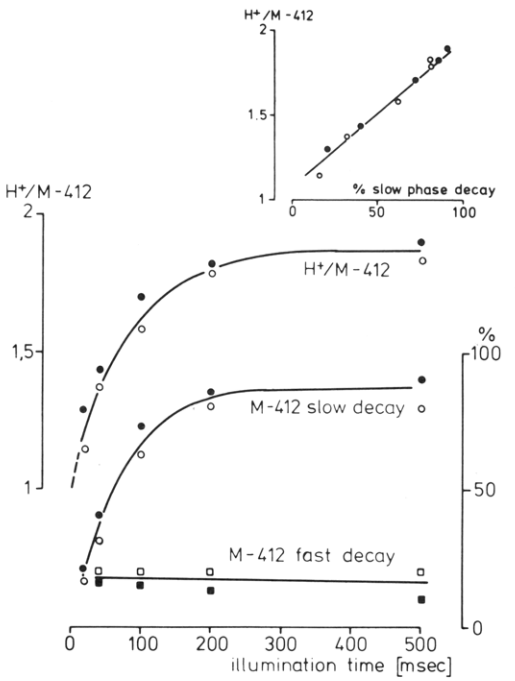

FIGURE 9: Ratio between proton release per M-412 formation and fast and slow decay amplitude of M-412 decay as a function of illumination time: (○, □) 2 M KCl (●, ■) 2 M NaCl. The total M-412 amplitude at 500-ms illumination is set to 100%. (Insert: plot of the H^+/M-412 ratio vs. the slow phase amplitude of M-412 decay.

Skulachev, 1977; Schulten & Tavan, 1977). The retinal–Schiff-base linkage which is protonated in the dark and deprotonated in the photocycle intermediate M-412 (Lewis et al., 1974; Stockburger et al., 1979) is considered to switch the proton actively from one proton diffusion pathway to a second one, both connecting the two bulk phases adjacent to the inner and outer membrane sites with the retinal switch. Proton transfer is suggested to occur through chains of hydrogen bonds according to mechanisms proposed by Nagle & Morowitz (1978) and Dunker & Marvin (1978).

For such a mechanism, a 1:1 ratio between the photoactivated retinal chromophore and the transported proton is expected, independent of bulk conditions such as a relatively large range of pH and ionic strength. In open membrane sheets the proton translocation process is short circuited. Yet, protons transferred across the membrane must still be stoichiometrically related to the number of bacteriorhodopsin molecules

being in the deprotonated M-412 state if the "switch" model holds. If no such simple relationships are detected, the possibility of superimposed protonation–deprotonation reactions must be raised. Indeed, our results obtained in open membrane sheets at pH 7.5 under continuous illumination showed variable ratios not in accord with the straightforward predictions of the "switching model", although some conditions hold where a 1:1 ratio is observed.

On the other hand, the following observations, both obtained under transient flash illumination and at low ionic strength, seem to agree with a 1:1 ratio. Lozier et al. (1976) calculated this ratio from experiments with purple membrane suspensions in water at pH 7.8 by correcting the ratio of 0.7 derived from maximum amplitudes (low M-412 occupancy: 2.25%) for the finding that protons were released with a slower rate than M-412 was formed and protons were taken up already (with a slightly slower rate than M-412 decay) before they had completely left the purple membrane (extrapolation to zero time). Ort & Parson (1979) estimated at pH 7.8 and 14.6 mM KCl a quantum yield for the deprotonation reaction of 0.25 which is similar to the quantum yield of 0.25–0.3 described for M-412 formation (Becher & Ebrey, 1976; Goldschmidt et al., 1976, 1977). However, the deprotonation quantum yield increased to 0.43 at 400 mM KCl, giving nearly a 2:1 ratio between the quantum yields of deprotonation and M-412 formation, and, moreover, the quantum yield decreased below 0.2 with increasing laser flash intensity. Although the latter result is qualitatively in agreement with our finding that H^+/M-412 ratio decreases with increasing light intensity (increasing M-412 occupancy in the light steady state), the authors discussed their results as a consequence of cross excitation at high light intensity of the K intermediate of the bacteriorhodopsin photocycle which does not lead to M-412 (M-412 state was not defined in the experiments of Ort and Parson) but directly to the bR-570 state (Lozier et al., 1975).

Large deviations from 1:1 H^+/M-412 ratio were found by various authors under light steady-state conditions (Klemperer et al., 1978; Caplan et al., 1978; Eisenbach et al., 1978). These discrepancies are due to the degree of light saturation. Furthermore, different results are also obtained by physiochemical modification of the membrane (Avi-Dor et al., 1979; Bakker & Caplan, 1978).

In agreement with Fischer [cited in Hartmann et al. (1977)], we found upon extrapolation to light saturation a ratio of unity at higher ionic strength. In addition, it is important to note that our experiments yielded at low ionic strength a H^+/M-412 ratio clearly well below unity and at high ionic strength, clearly above. The finding of the H^+/M-412 ratio being greater or smaller than unity indicates secondary proton reactions in addition to a deprotonation and reprotonation of bR-570. In the case of a ratio smaller than unity, protons released from bR-570 must be rebound; in the other case, additional protons must originate from other parts of bacteriorhodopsin.[2] Since it is known that conformational changes of the protein moiety accompany the photocycle of bacteriorhodopsin, as indicated by fluorescence and absorption changes in the aromatic region of the bacteriorhodopsin spectrum (Oesterhelt & Hess, 1973; Bogomolni et al., 1978; Konishi & Packer, 1978; Hess & Kuschmitz, 1979; Rafferty, 1979) as well as by titration experiments (Hess et al., 1978), we suggest that such light-induced conformational changes affect the reactivity of proton binding groups of various amino acid residues as defined by their local pK.

[2] Such protons are commonly called Bohr protons.

Thus, a pK shift of one or several proton binding groups from a value below the pH of the medium in the dark state to a value above the pH of the medium in the light state, by which protons released from bR-570 are immediately rebound, must take place, yielding low H^+/M-412 ratios. A pK shift to the opposite direction would lead to an additional proton release. Furthermore, both types of pK shift might occur in different groups.

If the amino acid residues taking part in such conformational changes are readily accessible to the medium, an influence of ionic strength on their reactivity must be expected. Indeed, this influence is recorded in our experiments.

On the basis of this reasoning, the sensitivity of the H^+/M-412 ratio toward ionic strength allows us to apply the Gouy–Chapman theory in a global sense, implying both sides of the membrane. This leads to an estimation of the upper boundary of an apparent average surface potential of approximately 220 mV at low ionic strength for both sides of the membrane according to McLaughlin, (1977). This is indicated in Figure 3 (see right ordinates) and demonstrates that the classical relationship of Gouy–Chapman, in a first approximation, fits our observation (see Figure 3). For this calculation a density of one charge per 200 Å^2 and 400 Å^2 as upper and lower boundaries for both sides were assumed (Blaurock, 1975; Unwinn & Henderson, 1975; Kates & Kushwaha, 1978; Ovchinnikov et al., 1979). Because the apparent pK's of proton binding groups of charged membranes are determined by the surface potential (Goldstein et al., 1964), the influence of ionic strength on such groups during the photocycle must be observed as illustrated here [see also Hess et al., (1978)].

Although the nature of acidic and basic groups involved as well as their respective pK's are currently not known, the result of our kinetic experiments allow us to discuss three different proton pools. Besides the proton pools located at the two purple membrane surfaces (Neugebauer et al., 1978) being a function of ionic strength and pH (Debye–Stern layer) (Kell, (1979), the proton pool in the active site is participating (proton located at the Schiff base, tyrosine, and other groups).

In addition, we suggest a third proton pool which is activated by the photocycle in a conformation cycle, resulting in pK changes of proton binding groups [see also Garty et al., (1977); Caplan et al. (1978)]. This pool is accessible to the bulk medium, as indicated by the ionic strength influence. If the relaxation of this pool is slower than the relaxation of the photocycle (M-412 decay), the pool can be exhausted by several photocycle turnovers until a dynamic equilibrium is reached between this pool and the turnover of the photocycle. Then, the dynamics of the pool might become rate limiting for the photocycle. The kinetic result obtained in the light steady-state onset (pre steady state) clearly shows this behavior (see Figure 8). Except for a 10–20% fast phase, M-412 slow-phase decay is similar to the rate of proton uptake in the light steady state. In the pre steady state, however, with shorter illumination time the M-412 decay becomes progressively faster [see also Korenstein et al. (1978)] the less protons have left the purple membrane, but the proton uptake becomes slower (Hess, 1976). The latter observation corroborates with the analysis of photopotentials (Trissl & Montal, 1977) and of light-induced conductivity changes (Slifkin et al., 1979) compared to the M-412 kinetics. This suggests that the M-412 component is reprotonated from this pool in a fast reaction until the pool is exhausted and regenerated from the medium in the light-induced conformational cycle.

Due to the fact that at physiological pH and ionic strength the pH of the bulk as well as of the membrane surface (where the Gouy–Chapman layer is negligible under these conditions) is several orders of magnitude smaller than the bacteriorhodopsin concentration in the purple membrane,[3] the conformation proton pool suggested here might well fulfill the function of a readily light-activated pool to furnish protons for the flux through the membrane. This function might be important for the rapid generation of an electrical potential across the membrane in one or a few photocycle turnovers. This is suggested because of the observation that a membrane potential of 300 mV is generated in 100 ms at only 1% saturation of the photocycle turnover in intact cell (Hartmann et al., 1977).

While the manuscript was in revision, Govindjee et al. (1980), using *p*-nitrophenol as pH indicator and extrapolating to zero time, reported H^+/M-412 ratios in sheets between 0.64 (10 mM KCl) and 1.8 (500 mM KCl); in vesicles and whole bacterial cells the ratio was 1.97–2.3. Also the quantum efficiency for proton pumping was found to be about twice the quantum efficiency of the photocycle (Bogomolni et al., 1980). Furthermore we have found a light-induced activation of proton binding groups connected to the M-412 intermediate with pK_a's of about 7 and 8.5 at high and low ionic strength, respectively (Kuschmitz & Hess, 1980).

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X-ray Diffraction and Calorimetric Study of Anhydrous and Hydrated *N*-Palmitoylgalactosylsphingosine (Cerebroside)†

M. J. Ruocco, D. Atkinson, D. M. Small, R. P. Skarjune, E. Oldfield, and G. G. Shipley*

ABSTRACT: Differential scanning calorimetry and X-ray diffraction of anhydrous and hydrated *N*-palmitoylgalactosylsphingosine (NPGS) show evidence of complex polymorphic behavior and interconversions between stable and metastable structural forms. Anhydrous NPGS exhibits three lamellar crystal forms (A, B, and B′) at temperatures below 143 °C and a liquid-crystal form between 143 and 180 °C before melting to an isotropic liquid at 180 °C. The crystal B → liquid-crystal transition is accompanied by an enthalpy change, ΔH, of 11.2 kcal/mol of NPGS, while a relatively small enthalpy change (ΔH = 0.8 kcal/mol) marks the liquid-crystal → liquid transition. The A and B′ crystal forms do not hydrate readily at room temperature. When heated, crystal form A in the presence of water undergoes an exothermic transition at 52 °C to produce a thermodynamically stable hydrated crystal E form. X-ray diffraction shows that this stable bilayer crystal form has a highly ordered hydrocarbon chain packing arrangement; melting to the bilayer liquid-crystal form occurs at 82 °C with a large enthalpy change, ΔH = 17.5 kcal/mol of NPGS. A complex liquid-crystal → crystal transition is observed on cooling; the cooling rate independent exotherm involves the transition of the hydrated liquid crystal to an intermediate metastable crystal form identical with anhydrous crystal form A. The subsequent cooling rate dependent step involves the conversion of the metastable crystal form A to the stable crystal form E. We suggest that hydrated crystal form E is stabilized by both a highly ordered chain packing mode and a lateral intermolecular hydrogen bonding network involving the sphingosine backbone, the galactosyl group, and interbilayer water molecules. Although disruption of both the specific hydrocarbon chain packing and H-bonding networks occurs at the high enthalpy transition to the bilayer liquid-crystal Lα form, these two types of interactions are not reestablished simultaneously on cooling. First, recrystallization of the hydrocarbon chains accompanies removal of water from the lipid interface, leading to "dehydrated" metastable crystal form A. This is followed by a time-dependent, temperature-dependent hydration process which allows a rearrangement of the hydrogen-bonding matrix. Alterations in the NPGS–NPGS and NPGS–water interactions accompany further changes in the hydrocarbon chain packing and lead to the formation of the stable E form.

Sphingolipids, including glycosphingolipids and sphingomyelin, are derived from the long-chain, aliphatic base sphingosine and differ structurally from the glycerol-based phospholipids and glycolipids. Glycosphingolipids are present in most animal cell membranes, albeit usually in relatively small amounts. Specific examples of this lipid class include globosides, which occur in tissues of the kidney, heart, and the reticuloendothelial system, and gangliosides, which occur in a number of tissues acting as receptors for hormones and toxins (Fishman & Brady, 1976). Some tissues, however, contain relatively large amounts of glycosphingolipids; for example, the myelin sheath of the central and peripheral nervous system has as its major (20% dry weight) polar lipid *N*-acyl-1-*O*-β-D-galactosylsphingosine or galactocerebroside (Johnson et al., 1948; Lapetina et al., 1968). The structural and/or functional role of this glycosphingolipid in the myelin membrane remains to be established.

Pronounced glycosphingolipid accumulation in various tissues (brain, liver, spleen, etc.) can result as a consequence of

† From the Biophysics Institute, Departments of Medicine and Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118 (M.J.R., D.A., D.M.S., and G.G.S.), and the School of Chemical Sciences, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801 (R.S. and E.O.). *Received March 24, 1981.* This investigation was supported by Research Grants HL-18623, HL-07291, and HL-19481 from the National Institutes of Health and Research Grant PCM 76-01491 from the National Science Foundation. D.A. is an Established Investigator of the American Heart Association.

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