Lehman, W., & Szent-Gyorgyi, A. G. (1975) *J. Gen. Physiol. 66*, 1–30.
Lowey, S., Slaytor, H. S., Weeds, A. G., & Baker, A. (1969) *J. Mol. Biol. 42*, 1–29.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Margossian, S. S., & Lowey, S. (1978) *Biochemistry 17*, 5431–5439.
Marston, S., & Weber, A. (1975) *Biochemistry 14*, 3868–3873.
Moore, P. B., Huxley, H. E., & DeRosier, D. J. (1970) *J. Mol. Biol. 50*, 279–295.
Murray, J. M., & Weber, A. (1980) *Mol. Cell. Biochem.* (in press).
Murray, J. M., Weber, A., & Wegner, A. (1980) in *Regulatory Mechanisms of Muscle Contraction* (Ebashi, S., & Endo, M., Eds.) pp 221–236.
Parry, D. A. D., & Squire, J. M. (1973) *J. Mol. Biol. 75*, 33–55.
Pemrick, S., & Weber, A. (1976) *Biochemistry 15*, 5193–5198.
Phillips, G. N., Jr., Lattman, E. E., Cummins, P., Lee, K. Y., & Cohen, C. (1979) *Nature (London) 278*, 413.
Sekine, T., & Kielley, W. W. (1964) *Biochim. Biophys. Acta 81*, 336–345.
Seymour, J., & O'Brien, E. J. (1980) *Nature (London) 283*, 680–682.
Silverman, R., Eisenberg, E., & Kielley, W. W. (1972) *Nature (London), New Biol. 240*, 207–208.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stewart, M. (1975) *FEBS Lett. 53*, 5–7.
Szent-Gyorgyi, A. G., Szent-Kiralyi, E. M., & Kendrick-Jones, J. (1973) *J. Mol. Biol. 74*, 179–203.
Taussky, H. H., & Schorr, E. (1953) *J. Biol. Chem. 202*, 675.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.
Wakabayashi, T., Huxley, H. E., Amos, L. A., & Klug, A. (1975) *J. Mol. Biol. 93*, 477–497.
Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 53*, 612–673.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.

Environmental Effects on Formation and Photoreaction of the M_{412} Photoproduct of Bacteriorhodopsin: Implications for the Mechanism of Proton Pumping†

Ofra Kalisky, Michael Ottolenghi,* Barry Honig,* and Rafi Korenstein

ABSTRACT: Photochemical studies of the effects of temperature, pH, and dehydration on the formation and back photoreaction of the M_{412} intermediate in the photocycle of light-adapted bacteriorhodopsin (bR_{570}) are carried out. Continuous illumination experiments in the range between −40 and −90 °C indicate that at low temperatures branching occurs at the stage of the L_{550} intermediate in which a back reaction to the parent pigment competes with the formation of M_{412}. At low temperatures the yield of M_{412} is markedly increased at high pH. The effect is attributed to the catalytic action of a protein group of $pK \simeq 10$ on the rate of the $L_{550} \rightarrow M_{412}$ process. Our results, taken together with previous evidence for deprotonation of a tyrosine during the $L_{550} \rightarrow M_{412}$ transition, suggest that the formation of a tyrosinate ion is a *prerequisite* for deprotonation of the Schiff base. A model is proposed in which both the Schiff base and the tyrosine translocate their protons to two acceptor groups, A_1 and A_2, accessible to the outside of the cell through a segment of a proton wire. The model accounts for the observation that up to two photons may be pumped per cycle. The proton-pump mechanism is analyzed in terms of a generalized kinetic scheme for pumping. In contrast to current models for proton pumping which are based on a (primary) light-induced accessibility change of the chromophore (class I models), we introduce a new class (II) of models based exclusively on pK changes. We suggest that in bR_{570} the Schiff base and the tyrosine are accessible to protons on the *outside* surface of the membrane. An analysis of the back photoreaction from M_{412} tends to favor class II models over previous class I models.

In this paper we consider aspects of the molecular mechanism of proton pumping in bacteriorhodopsin, the single protein in the purple membrane of *Halobacterium halobium* [see Stoeckenius et al. (1979) for a comprehensive review]. The chromophore of the light-adapted form of bacteriorhodopsin (bR_{570}) is *all-trans*-retinal bound to the ϵ-amino group of a lysine in the protein in the form of a protonated Schiff base. Absorption of a photon by bR_{570} initiates a photocycle (Figure 1; see Ottolenghi (1980) for a recent review) during the course of which protons are transported from the cytoplasmic side to the outside of the cell.

The $L_{550} \rightarrow M_{412}$ transition appears to play an important role in the proton-pumping mechanism. First, the ejection of protons to the outside of the membrane occurs on a time scale comparable to the rate of M_{412} formation (Lozier et al., 1976;

† From the Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel (O.K. and M.O.), the Department of Physiology and Biophysics, The University of Illinois, Urbana, Illinois 61801 (B.H.), and the Membrane Department, Weizmann Institute of Science, Rehovot, Israel (R.K.). *Received May 16, 1980; revised manuscript received September 24, 1980.* This research was supported in part by the Israel Commission for Basic Research, by the U.S.–Israel Binational Science Foundation, by the National Institutes of Health (EY 03142), and by the National Science Foundation (PCM79-02683 and PCM80-10931).

0006-2960/81/0420-0649$01.00/0

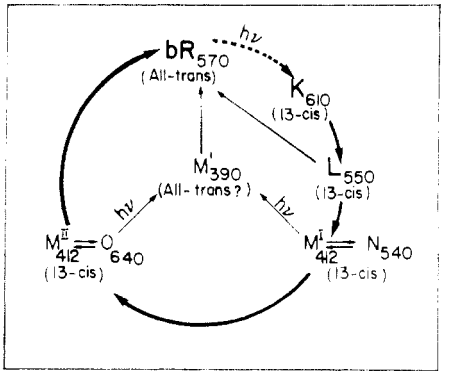


FIGURE 1: Photocycle of light-adapted bacteriorhodopsin, bR_{570}. Adapted from Lozier et al. (1978).

Ort & Parson, 1978). Second, as indicated by the blue-shifted absorption maximum of M_{412}, the Schiff base loses its proton during the $L_{550} \rightarrow M_{412}$ transition. This fact has been clearly demonstrated in Raman experiments which show that the chromophore of L_{550} is a protonated Schiff base, while that of M_{412} is unprotonated (Lewis et al., 1974; Aton et al., 1977; Marcus & Lewis, 1977; Campion et al., 1977). Evidence demonstrating the movement of an additional proton during the $L_{550} \rightarrow M_{412}$ transition is provided by recent observations of light-induced absorbance changes in the UV, which indicate that a tyrosine residue is deprotonated at this stage (Bogomolni et al., 1978; Hess & Kuschmitz, 1979). The deuterium isotope effect associated with the $L_{550} \rightarrow M_{412}$ transition (Korenstein et al., 1976) is also consistent with proton-dependent processes. Finally, a direct relationship between M_{412} formation and proton pumping is suggested by the observation (Lozier et al., 1978) that the bR_{548} form (present in dark-adapted bacteriorhodopsin and characterized by a 13-cis chromophore), which has no blue-shifted intermediate in its photocycle (Kalisky et al., 1977; Sperling et al., 1977), does not initiate proton pumping.

Below, we report photochemical experiments which investigate the effects of temperature and pH on the M_{412} generation process and the effects of dehydration on its back photoreaction to bR_{570}. These allow us (a) to establish a causal relationship between deprotonation of the chromophore and that of a tyrosine residue, (b) to describe the role of both events in the ejection of protons to the aqueous phase, and (c) to formulate a model for the molecular events which are associated with the generation and photodecay of M_{412}.

A fundamental problem in the elucidation of the function of bacteriorhodopsin is to establish the correlation between the photocycle, as monitored spectroscopically, and the mechanism of proton pumping. A number of workers have made use of the deprotonation of the Schiff base at the M_{412} stage to suggest pumping models ("class I" models) in which the chromophore functions as a "molecular switch" traversing a permeability barrier separating the inside from the outside of the membrane (Stoeckenius et al., 1978; Schulten 1978; Honig, 1978; Lozier et al., 1978). However, evidence that, depending on ionic strength, up to two protons are released per photocycle (Hess & Kuschmitz, 1978; Ort & Parson, 1979) and that two protons may actually be pumped (Bogomolni et al., 1979; Govindjee et al., 1980) suggests that models, which account for only one proton, need to be extended. While appropriate adjustments can in principle be incorporated into class I models, we find it useful to consider a new type of model (designated class II) in which photoisomerization of the chromophore leads to a decrease in the pK of the Schiff base and of a neighboring tyrosine residue, both of which are initially accessible to protons from the *outside* of the membrane. This model, which can account for the stoichiometry of two pumped protons per photocycle, does not involve a change in accessibility (exposure) of the Schiff base prior to its deprotonation. As such, it differs from class I models which are based on a switching mechanism. In order to evaluate the various models, we have correlated spectroscopically defined intermediates in the photocycle with states of the protein defined in a generalized kinetic scheme of proton pumping. On the basis of this analysis, we find that the inhibition of proton pumping by blue light favors models in which pumping is initiated by a pK change on the outside of the membrane.

Experimental Procedures

Experiments in aqueous suspensions of purple membranes from M_1 (*H. halobium*) were carried out in 2:1 glycerol–water mixtures in the presence of 25% NaCl. When working in alkaline systems, we set the pH in the aqueous phase with NaOH prior to the addition of glycerol. Thin layers of the purple membrane were prepared and were equilibrated with different relative humidities as previously described (Korenstein & Hess, 1977). Absorption spectra were recorded on a Cary 14 spectrophotometer with appropriate low-temperature accessories. For illumination of low-temperature systems (light-adapted prior to cooling), a filtered ($\lambda > 500$ nm) tungsten light source was employed.

Double-pulse laser experiments, for the study of the M_{412} back photoreaction, employed a pulsed Xe source for monitoring and for pumping the system to a steady-state population of M_{412}. A synchronized N_2 laser pulse (337 nm, 0.5 mJ, 8 ns) induces the M_{412} photoreaction in a front-face experimental setup previously described (Kalisky et al., 1978). When flash experiments were carried out at temperatures (below −35 °C) at which the M_{412} intermediate is relatively stable, care was taken to illuminate the sample after each laser shot with blue light ($\lambda \simeq 400$ nm). This induces the back photoreaction $M_{412} \rightarrow bR_{570}$, thus regenerating the original solution.

Results and Discussion

(*A*) *Temperature and pH Effects on Yield of* M_{412}. Continuous illumination of purple membrane suspensions was carried out at low temperatures, under conditions in which the L_{550} to M_{412} photointermediates are sufficiently long-lived so as to be detectable by conventional spectrophotometry. Illumination of unbuffered water–glycerol solutions (25% NaCl) of bR_{570} at −90 °C leads to a difference spectrum characteristic of the $bR_{570} \rightarrow L_{550}$ interconversion (Figure 2a). At this temperature such a change in absorbance is stable over a time scale of at least 1 h. When the temperature of the irradiated system is gradually raised in the dark, e.g., to −60 °C, a thermal relaxation takes place leading to recovery of the original bR_{570} absorbance (Figure 2b). It is clear that, in variance with the room-temperature sequence $L_{550} \rightarrow M_{412} \rightarrow bR_{570}$ (Figure 1), the decay of L_{550} below approximately −70 °C is not associated with the generation of the characteristic absorption maximum around 412 nm due to M_{412}. However, when the same bR_{570} sample is irradiated at −45 °C, a stable difference spectrum essentially identical with that of M_{412} at room temperature (Goldschmidt et al., 1976) is observed (Figure 2c). Figure 2d–f demonstrates that basically the same phenomena are observed in thin purple membrane layers, in the absence of glycerol and of high salt concentration. In the thin-layer preparations the effects were independent of the degree of hydration. It is thus evident that at low temperatures the thermal decay of L_{550} leads to the regeneration of bR_{570} in a process which circumvents the M_{412} intermediate.

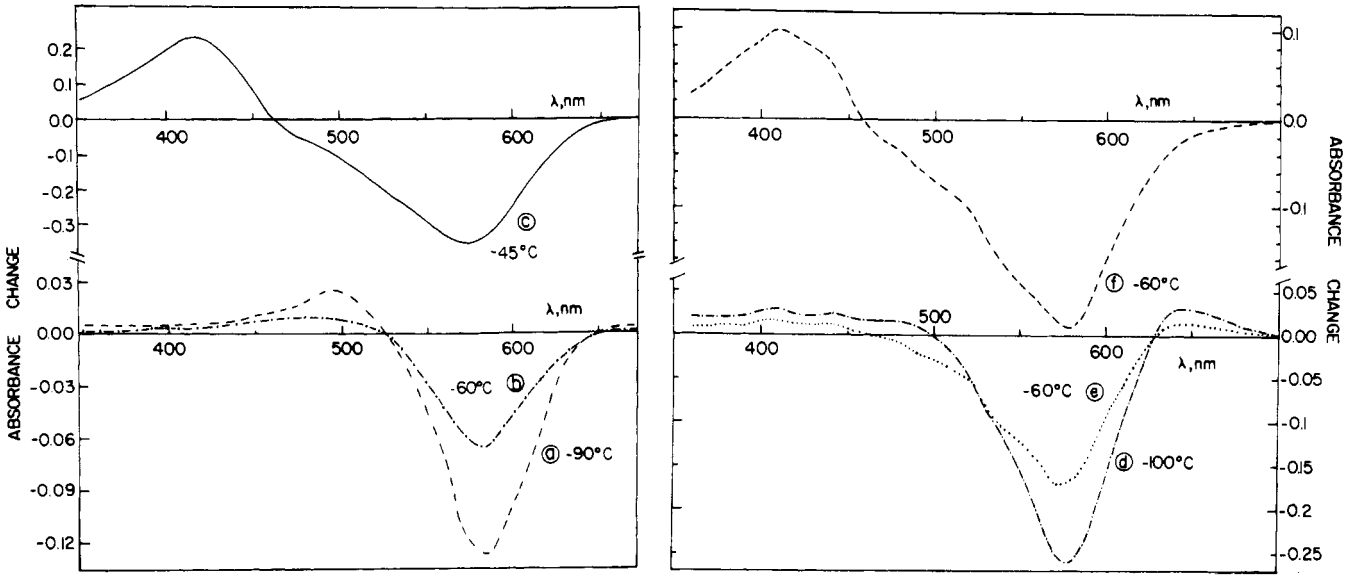
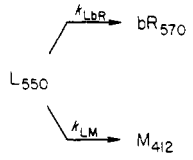


FIGURE 2: Difference spectra after continuous illumination ($\lambda > 500$ nm) of bR_{570} at low temperatures. (Left) Purple membranes in water–glycerol (1:2) with 25% NaCl. (a) After illumination with $\lambda > 500$ nm at −90 °C. (b) After warming up (a) to −60 °C in the dark. (c) After direct illumination of a similar sample at −45 °C. (Right) Purple membrane layers equilibrated with 94% relative humidity. (d) Photostationary state obtained after irradiation with $\lambda = 578$-nm light at −100 °C. (e) After warming up of (d) to −60 °C. (f) Photostationary state obtained after direct irradiation of a similar sample at −60 °C.

Moreover, our results indicate that these phenomena are purely temperature effects and are not associated with environmental changes such as degree of hydration or the presence of glycerol and/or salt.

These conclusions are consistent with a simple mechanism involving braching at L_{550}:



Accordingly, the temperature dependence of the yield of M_{412} (ϕ_M) is accounted for by a temperature effect on the ratio k_{LM}/k_{LbR}, so that below −60 °C $k_{LbR} \gg k_{LM}$, while at room temperature $k_{LM} \gg k_{LbR}$. A quantitative analysis of the yield of M_{412} for temperature between room temperature and −60 °C shows that the kinetics of M_{412} formation is also dependent on branching processes prior to the formation of L_{550} (O. Kalisky and M. Ottolenghi, unpublished experiments).

In order to obtain further insights as to the mechanism of M_{412} formation, we carried out continuous illumination experiments at −90 °C in water–glycerol solutions of bR_{570}, varying the pH in the aqueous phase prior to mixing with glycerol. Figure 3 shows that in alkaline systems, M_{412}, rather than L_{550}, is the stable photoproduct at −90 °C and that a titrationlike curve, with a break around pH 9.5, is obtained for the relative yield of M_{412} produced at −90 °C. Since in neutral solutions at −90 °C L_{550} is stable for hours, while in alkaline systems the M_{412} generation process takes place within less than 1 s, it is evident that at high pH, k_{LM} increases by at least 4 orders of magnitude.

(B) Environmental Effects of the Back Photoreaction of M_{412}. Optical excitation of intermediates in the photocycle of bR_{570} induces photoreactions which lead to the recovery of the original pigment [see Stoeckenius et al. (1979) and Ottolenghi (1980) for recent reviews]. Using double-pulsed excitation methods at room temperature, we have shown (Kalisky et al., 1978) that the back photoreaction from M_{412} is initiated by the generation of a blue-shifted photoproduct, M'_{390}. The latter undergoes an exponential thermal decay

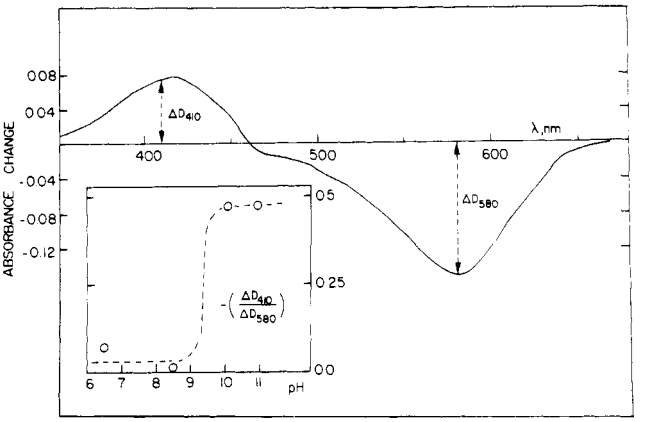


FIGURE 3: Effect of pH on photolysis of bR_{570} (2:1) glycerol–water with 25% NaCl) at −90 °C. The difference spectrum was obtained after continuous illumination of the sample at pH 10.8 as described in the text. The insert represents the transition from the phenomena observed at relatively low pH (as shown in Figure 2) to those of alkaline pH of the present figure.

Table I: Effects of Hydration and of Added Guanidine Hydrochloride (8 M at pH 8) on the Half-Lives ($\tau_{1/2}$) of the Decay of M_{412} and of Its Photoproduct, M'_{390} [a]

system (22 °C)	$\tau_{1/2}$ ($M'_{390} \rightarrow bR'_{570}$) (ns)	$\tau_{1/2}$ ($M_{412} \rightarrow bR_{570}$) (s)
H_2O	150 ± 20	~5 [c,d]
45% rh [b]	165 ± 20	0.2–10 [d]
8% rh [b]	160 ± 20	0.9–28 [d]
8 M Gdn·HCl	155 ± 20	3–10 [e]

[a] H_2O represents membrane suspensions in water. The values reported for M_{412} reflect the nonexponential nature of its decay. [b] rh = relative humidity. [c] In milliseconds. [d] Korenstein & Hess (1977). [e] Korenstein et al. (1979).

regenerating the characteristic spectrum of bR_{570}. The $M'_{390} \rightarrow bR_{570}$ process is characterized by a half-life of ~150 ns and is thus faster by a factor of $\sim 10^5$ than the decay of M_{412}. The rate of the latter reaction is known to be affected by a variety of environmental factors [see Stoeckenius et al. (1978); Ottolenghi, 1980]. It is especially sensitive to the degree of

membrane hydration (Korenstein & Hess, 1977) and to the presence of guanidine hydrochloride (Pettei et al., 1977; Korenstein et al., 1979).

In the present work we have applied the double-pulse excitation method for measuring the dependence of the M'_{390} decay rate on hydration as well as on the presence of guanidine. Table I shows that in contrast to the dramatic environmental effects on the decay of M_{412}, the decay of M'_{390} is insensitive to the protein conformational changes induced by guanidine (Korenstein et al., 1979) as well as to the absence of the external aqueous phase. While the guanidine effects merely show that the decay of M_{412} and M'_{390} follow totally different routes, it is the hydration experiments which bear directly on the nature of the M_{412} photoreaction. This point will be discussed below in relation to the molecular aspects of the proton pump.

(C) Molecular Mechanism of M_{412} Formation and Proton Ejection. It is useful to first summarize the molecular events that are associated with the formation of M_{412}. We have seen above that there is a branching reaction at the L_{550} stage such that at low temperature the formation of M_{412} is inhibited and L_{550} reverts thermally to bR_{570}. At low temperatures, the branching ratio is extremely sensitive to pH. The effect corresponds to the catalytic function of a group with p$K \simeq$ 10 which, upon losing a proton, dramatically enhances the rate of the $L_{550} \rightarrow M_{412}$ process. It seems reasonable to suggest that deprotonation of the same group also takes place during the photocycle prior to the formation of M_{412} and that it is the inhibition of this deprotonation that accounts for the lack of formation of M_{412} at low temperatures.

A number of other results support this conclusion. For example, Ehrenberg & Lewis (1978) and Lewis et al. (1978) reported a titration-like pH dependence [almost identical in shape with that of Figure 3 (inset)] for the rate of appearance of the unprotonated C=N stretching frequency at 1619 cm^{-1} as well as for that of the M_{412} C=C stretching mode at 1566 cm^{-1}. The effect observed in the Raman experiments is much smaller than the one reported here, and, since it corresponds to room temperature where the $L_{550} \rightarrow M_{412}$ transition predominates, it does not affect the yield of M_{412}.

Lewis et al. (1978) interpreted their data in terms of a group of p$K \simeq$ 10 which directly controls the rate of Schiff base deprotonation. A model was proposed in which the group is identified as a lysine residue in close interaction with the Schiff base. However, a number of arguments lead us to propose an alternative explanation for the pH effects on M_{412} formation. (a) Since the lysine residue in the model of Lewis and co-workers (Lewis et al., 1978; Marcus & Lewis, 1978) is assumed to be unprotonated in bR_{570} at neutral pH it could not be responsible for the observed titration curve; (b) the identification of the interacting group as a lysine is based on a comparison of the relative effects of ^{15}N enrichment on the C=N (Schiff base) stretching frequency of bacteriorhodopsin and model compounds (Marcus et al., 1979). However, the frequency shifts observed are actually quite similar (16 cm^{-1} for bR_{570} and 13 cm^{-1} for model protonated Schiff bases) and are in the range expected from a simple mass effect (Aton et al., 1980). Thus, it seems unnecessary to invoke an interaction of the Schiff base with an additional nitrogen-containing group in bR_{570}.

Although, on the basis of its pK, lysine remains a plausible candidate for the group that catalyzes the formation of M_{412}, recent laser photolysis experiments suggest that tyrosine (Tyr), which also has a pK of $\sim$10, is a more likely candidate. Bogomolni et al. (1978) observed UV absorbance changes (between 240 and 320 nm) during the photocycle which closely corresponded to a $Tyr-Tyr^-$ difference spectrum. (At least one tyrosine moiety is titratable in bR in the dark.) They concluded that a Tyr residue is deprotonated prior to the generation of M_{412}. The same conclusions have been recently confirmed by Hess & Kuschmitz (1979) who have been able to resolve the tyrosinate generation process, placing it after the formation of L_{550} but prior to that of M_{412}. The fact that deprotonation of a tyrosine precedes the $L_{550} \rightarrow M_{412}$ transition leads us to suggest that it is this Tyr residue which is also responsible for the pH effect on the M_{412} yield at low temperatures and on the M_{412} formation rate at room temperature as detected in Raman experiments. It seems reasonable to conclude that the deprotonation of a tyrosine residue is a prerequisite for the formation of M_{412}.

The simplest way of accounting for this conclusion is to postulate that the Schiff base proton is transferred to the ionized tyrosine during the formation of M_{412}. However, the observation (Bogomolni et al., 1978; Hess & Kuschmitz, 1979) that reprotonation of the tyrosine occurs only during the decay of M_{412} precludes this possibility. It appears then that two protons, that of the Schiff base and that of the tyrosine, are translocated during the formation of M_{412}. It is of interest to consider the fate of these protons. The rate of tyrosine deprotonation (Bogomolni et al., 1978; Hess & Kuschmitz, 1979) is substantially faster than the rates reported for the appearance of protons in the aqueous phase (Lozier et al., 1976; Dencher & Wilms, 1975; Lozier et al., 1979; Ort & Parson, 1978). Thus, there must be an internal acceptor group, A_2, that is protonated as a consequence of tyrosinate formation. There is evidence indicating that a second internal group, A_1, serves as an acceptor for the Schiff base proton. First, the yield of M_{412} (in which the Schiff base is deprotonated) is unaffected by the absence of the aqueous phase (Korenstein & Hess, 1977). Second, at room temperature, M_{412} formation is significantly faster than the release of protons into the aqueous phase (Lozier et al., 1976, 1979). (Experiments carried out by Ort and Parson indicate that this situation holds only above pH 8. In the pH range 6–8 the rate constant reported for M_{412} formation is somewhat smaller than that of proton release. See also footnote 1.)

It is interesting to consider the possible relation between the internal displacement of protons discussed above and the proton-pumping mechanism. The assumption that both protons are ultimately released (or displaced two other protons) into the aqueous phase would be consistent with the observation that, depending on environmental conditions, up to two protons are released per photocycle (Hess & Kuschmitz, 1978; Ort & Parson, 1979; Govindjee et al., 1980). A simple model consistent with the direct involvement of the Schiff base and tyrosine protons in the proton pump [where it is assumed that two protons are pumped per photocycle (Bogomolni et al., 1979; Govindjee et al., 1980)] is shown in Figure 4. The primary photochemical event induces a change in the proton affinity of the tyronsine which as a result transfers its proton to an acceptor group A_2. The reduction in the pK of the tyrosine may result from the approach of a positively charged group. As depicted in the model, the negatively charged tyrosinate subsequently catalyzes the transfer of the Schiff base proton to another acceptor group, A_1. The actual mechanism of proton transfer from the chromophore might involve an intermediate step in which the proton is first bound to the tyrosine which subsequently transfers it over some distance, via a conformational change. Other mechanisms for Tyr^--catalyzed proton transfer are obviously possible.

The release of protons to the aqueous phase takes place on a time scale of $\sim 10^{-4}$ s (Lozier et al., 1976; Ort & Parsons, 1978). The rate of proton release by A_1H and A_2H is determined by the rate constant k_d in the equilibrium

$$A_iH \underset{k_r}{\overset{k_d}{\rightleftharpoons}} A_i^- + H^+$$

For an acid A_iH in a homogeneous aqueous solution $k_d = k_r 10^{-pK_a} \simeq 10^{10} 10^{-pK_a}$, where K_a is the dissociation constant of A_iH. Since the photoinduced deprotonation rate of the membrane in bR_{570} is on the order of 10^4 s^{-1}, it is evident that the pK of the releasing groups, A_1 and A_2, cannot exceed ~ 6. Moreover, if A_1 and A_2 are traps for protons originating from the tyrosine and the Schiff base, their proton affinity (at least during the transfer step) must be significantly higher than those of the donating groups. this implies that during the photocycle the pK's of both the tyrosine and the Schiff base are reduced (from ~ 10 and above 12, respectively) to ~ 5. [While this may at first appear surprising, it should be recalled that the pK of the Schiff base of bacteriorhodopsin is shifted by at least 6 units relative to those (Favrot et al., 1978) of protonated Schiff bases in solution.]

The mechanism shown in Figure 4 accounts for a maximum stoichiometry of two protons pumped per photocycle. It is proposed as a general working hypothesis and does not deal with detailed mechanistic questions such as which of the two protons is translocated under conditions of a stoichiometry of one or how it is possible to account for noninteger stoichiometries. The mechanism readily accounts for all observations where Schiff base deprotonation precedes proton release into the aqueous phase. These include the results of Ort & Parson (1978) at pH >8 and the report of Lozier et al. (1979) for pH 7 and 7.8. However, in apparent contradiction with the report of Lozier and co-workers, Ort and Parson find that at pH <8, proton release is somewhat faster than M_{412} formation. This result cannot be accounted for by the scheme of Figure 4 unless an additional group is introduced which, as the tyrosine, releases its proton prior to Schiff base deprotonation.[1]

(*D*) *Implications of the M_{412} Photoreaction.* Our analysis so far has implicated the involvement of four groups that participate in the proton-ejection mechanism: the chromophore, a tyrosine, and the two acceptor groups, A_1 and A_2. However, the results summarized in Table I suggest that additional proton binding groups are involved in the pumping mechanism. This conclusion is based on the observation that the yield and decay rate of the M'_{390} photoproduct of M_{412} in highly dehydrated systems are identical with those observed in aqueous membrane suspensions. In the dehydrated systems, the Schiff base cannot be reprotonated from the aqueous phase. Absorbed water as a proton source for reprotonation can also be excluded on the basis of the lack of any relative humidity effect on the decay of M'_{390}. Thus, there must be an internal donor to the Schiff base in M'_{390} which, after losing its proton, is reprotonated from the medium in a subsequent step.

Such a sequence is also consistent with the ~ 150-ns regeneration time of the protonated Schiff base (i.e., the decay of M'_{390}) which is far too fast to be associated with a process

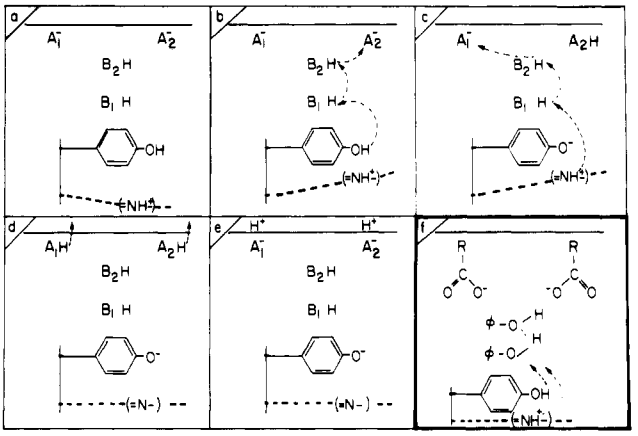


FIGURE 4: Schematic molecular description of the steps (a–e) leading to the photoejection of up to two protons to the outside of the purple membrane. The upper horizontal line represents the external aqueous surface. (f) shows a suggested molecular structure for the groups A_1^-, B_2^-, B_1H, and B_2H. The dashed lines are the retinal chromophore. (a) Before isomerization; (b and c) after isomerization; (d–f) in a relaxed position.

involving proton uptake from the solution (excluding two-dimensional diffusion of protons on the surface of the membrane). [At pH ~ 7, the rate of the latter process cannot exceed $k_r[H^+]$ ($\sim 10^3$ s^{-1}).] The internal proton donor cannot be A_1 or A_2 which, by definition, become deprotonated in aqueous membrane suspensions. Thus, we postulate the existence of an additional group, B_1H, which supplies a proton to the Schiff base during the back reaction from M_{412}. Evidently, the 570-nm absorption restored after the decay of M_{412} cannot be due to the original bR_{570} state but rather to a species, bR'_{570}, in which only the Schiff base, but not B_1, is protonated. At present, no data is available as to the rate of reprotonation of the tyrosinate during the back photoreaction from M_{412}. The data when available will indicate whether another group (B_2H, arbitrarily included in Figure 4) needs to be invoked to account for reprotonation of the tyrosine from an internal source. An additional point still awaiting clarification is the time scale of the final relaxation to the original bR_{570} state in the M_{412} photoreaction. According to our analysis, the latter process is described by the sequence

$$M_{412} \xrightarrow{h\nu} M'_{390} \xrightarrow{k_1} bR'_{570} \xrightarrow{k_2} bR_{570}$$

where k_1 represents reprotonation of the Schiff base from the internal source B_1H ($\tau_{1/2} = 150$ ns). Since bR'_{570} and bR_{570} differ in the state of protonation of the protein, but not of the chromophore, they are probably indistinguishable by optical spectroscopy in the visible range. Time-resolved pH changes in the back photoreaction of M_{412} may lead to a determination of k_2.

A number of considerations bear on the location and identification of the group B_1H, and these lead us to the specific molecular model shown in Figure 4f. (a) B_1H must connect the Schiff base and the outside of the membrane. The alternative possibility, that B_1H is connected to the inside, may be excluded by the effect of blue light on the efficiency of proton pumping. Thus, Karvaly & Danschazy (1977) have shown that the selective illumination within the blue (412-nm) absorption band of M_{412} inhibits proton transport across the membrane. Using double-pulse laser techniques, we have also shown (Lozier et al., 1978) that proton pumping is inhibited even when irradiation of M_{412} follows the release of protons to the external medium. It is therefore evident that the back photoreaction from M_{412} induces uptake of protons from the *outside* of the membrane.

[1] It may be possible, however, that the results of Ort and Parson at pH <8 (at 3.4 °C) reflect primarily the rapid release of the tyrosine proton. Under their experimental conditions (Ort & Parson, 1978), ~ 1.5 protons are released per photocycle (Ort & Parson, 1979). Attributing one released proton to the tyrosine would imply that approximately one-third of the effect is due to protons originating from the Schiff base which, according to Figure 4, cannot precede M_{412} formation. It would be of interest to test, by a detailed deconvolution analysis, the existence of a slower component in the proton-ejection kinetics.

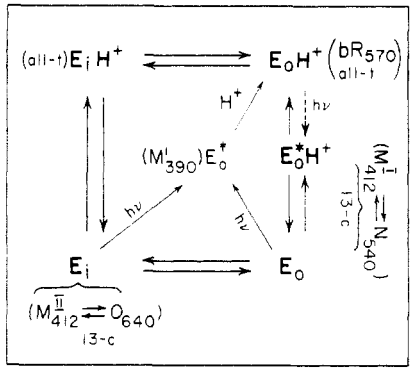


FIGURE 5: Class II model for the mechanism of proton pumping in bacteriorhodopsin. The spectroscopically defined states of the photocycle are associated with the kinetically defined species. One kinetic form (e.g., E_i) may correspond to more than one photocycle intermediate since the various E's are in general composite states that are the only forms detectable in steady-state transport measurements (Stein & Honig, 1977). See text for definition of indices.

(b) In placing B_1H between the Schiff base and A_1^- and A_2^-, we have made the simplifying assumption that the pathway for uptake of protons in the back photoreaction

$$M_{412} \xrightarrow{h\nu} bR_{570}$$

is identical with the pathway of ejection of protons during the photocycle.

(c) The dual requirement that B_1H mediates the transfer of a proton from the Schiff base in L_{550} to A_1^-, yet serves as an internal proton donor to the nitrogen of M'_{390}, can best be met by a hopping mechanism (proton wire) of the type suggested by Nagle & Morowitz (1978). A short segment of such a wire has been incorporated in the model given in Figure 4.

(*E*) *On the Mechanism of Proton Pumping.* (*1*) *Generalized Kinetic Models for Proton Pumping.* We have demonstrated above that large light-induced p*K* changes are an important component of the bacteriorhodopsin photocycle. In order to understand the possible mechanistic role of these effects, it is useful to consider a generalized kinetic scheme for a proton pump (Honig, 1978). Figure 5 shows a four-step kinetic scheme for such a pump. The index i denotes a state of the protein (E) in which a proton to be transported can be exchanged with the inside of the membrane, while the index o denotes accessibility from the outside. The transformation between E_i and E_o (or E_iH^+ and E_oH^+) involves a conformational change between two states of the unprotonated (or protonated) carrier. It is obvious that two such states of bacteriorhodopsin exist since it would otherwise be impossible to accomplish the vectorial transport of protons. It should be pointed out that more states of the protein are possible, but those depicted in Figure 5 are the minimum required for a pump and, as such, allow a straightforward kinetic analysis of the system (Stein & Honig, 1977; Honig, 1978).

In the dark all forms are in thermal equilibrium so that the rates of all forward and back reactions are equal. It is only required that light accelerate the rate constants of any clockwise step in Figure 5 relative to its reverse rate constant in order to pump protons in the direction i → o. In principle, the stable form of the carrier, bR_{570}, could correspond to any of the four kinetic forms depicted in the figure. However, the observation that proton release precedes proton uptake (Lozier et al., 1976) implies that bR_{570} corresponds to a protonated form, either E_iH^+ or E_oH^+ (Stein & Honig, 1977). It should be noted that although the model assumes that only one proton is pumped per full cycle, it can be readily generalized to a higher stoichiometry.

There are two limiting cases (or "classes" of mechanisms) which describe the role of light in driving the proton pump. In one (class I), the effect of the photochemistry is to drive the protein from state E_iH^+ to E_oH^+, i.e., to change the exposure (accessibility) of a titratable group from one side of the membrane to the other. This mechanism need not involve any p*K* changes of the titratable group. [However, these may affect the efficiency of the pump (Honig, 1978).] Alternatively, it is possible to envisage a pump which is uniquely based on a light-induced p*K* change (class II). That is, the role of light is to facilitate deprotonation on the outside of the cell by accelerating the step $E_oH^+ \rightarrow E_o + H^+$. Obviously, combinations of the two classes of mechanisms are also possible.

On the basis of the discussion of the previous section, it was suggested that during the course of the photocycle, large p*K* changes occur for two groups, the Schiff base and a nearby tyrosine. It is possible that such changes accompany a change in exposure of these groups in keeping with the basic requirement of the first class of mechanisms discussed above. A second possibility is that there are no changes in accessibility of either group and that the effect of light is to induce a protein conformational change leading to a decrease in the p*K* of both groups which are originally exposed to the *outside* of the membrane.

(*2*) *Two Classes of Pumping Mechanisms in bR.* On the basis of the evidence that the primary photochemical event in bacteriorhodopsin is an isomerization of the chromophore [see e.g. Honig et al. (1979)], a number of workers have proposed a pumping mechanism in which photoisomerization changes the exposure of the Schiff base proton from the inside to the outside of the membrane (Stoeckenius et al., 1978; Schulten & Tavan, 1978; Honig, 1978). (In mechanisms of this type, the photoisomerization corresponds to the kinetic step $E_iH^+ \rightarrow E_oH^+$.) [All of these (class I) models have also incorporated p*K* changes between E_iH^+ and E_oH^+.] However, the evidence discussed above for the decrease in the p*K*'s of the Schiff base and the tyrosine during the photocycle makes it tempting to consider a new class of models (class II), based *entirely* on light-induced p*K* changes.

A *representative* class II model, where for simplicity only one proton is pumped, is shown in Figure 5. Here, bR_{570} corresponds to E_oH^+ so that initially the transport system is accessible from the outside. The form $E_o{}^*H^+$ is a state of the protein in which the p*K* on the exterior has been reduced following a photoisomerization of the chromophore, as shown in Figure 5, and E_o is formed following deprotonation. The nature of the $E_o \rightarrow E_i$ transition may be deduced from kinetic patterns associated with the M_{412} and K_{640} intermediates. It has been pointed out previously (Lozier et al., 1978) that the decay at 412 nm exhibits two kinetic stages, one matching the growing-in of O_{640} and one matching its decay. These patterns were interpreted, as shown in Figure 1, by a mechanism in which an initially generated form of M_{412} (M^I_{412}) decays into a second form, M^{II}_{412}, which is rapidly equilibrated with O_{640}. [The existence of two consecutive forms of M_{412} has previously been suggested by Hess & Kuschmitz (1977) and incorporated in the model of Schulten & Tavan (1978).] In the present model, the $M^I_{412} \rightarrow M^{II}_{412}$ step is identified with an unspecified (protein or chromophore) conformational change which is associated with an accesibility shift from the outside ($E_o = M^I_{412}$) to the inside ($E_i \equiv M^{II}_{412}$). The decay of O_{640} corresponds in this model to reisomerization of the chromophore, which may be facilitated by the low barrier to thermal isomerization of the Schiff base in its protonated form (Schulten & Tavan, 1978). This restores the p*K* of the Schiff base to its initial

value. Uptake of protons from the inside ($E_i + H^+ \rightarrow E_iH^+$) and rearrangement of the protein to its original conformation, restoring the initial accessibility ($E_iH^+ \rightarrow E_oH^+$), now complete the cycle.

The model presented above (Figure 5) accounts for the blue-light effect in reducing the pumping efficiency of bR_{570} in a very simple way. It seems clear that the mechanism for the back photoreaction from M_{412}, which has an unprotonated 13-cis chromophore (Pettei et al., 1977; Tsuda et al., 1980), to bR_{570}, which has a protonated all-trans chromophore, involves a primary cis → trans photoisomerization about the 13–14 double bond. This generates the all-trans (unprotonated) species M'_{390} which undergoes a fast reprotonation to bR_{570}. Since the back photoreaction occurs whether M^{I}_{412} or M^{II}_{412} is excited (Kalisky et al., 1978; O. Kalisky and M. Ottolenghi, unpublished results), it is necessary to account for the inhibition of pumping (Lozier et al., 1978) from either of the two states. Since it is the initial trans–cis isomerization which triggers the p*K* changes of groups exposed to the outside of the membrane as well as the subsequent conformational changes of the protein ($E_o \rightarrow E_i$), it is expected that a cis → trans isomerization would lead to a reversal of the conformational changes of the protein (i.e., $E_i \rightarrow E_o$) and to the uptake of a proton from the outside of the membrane. Accordingly (see Figure 5), we identify M'_{390} with a state of the protein E_o^+ in which the back (13-cis → trans) isomerization of the chromophore has restored accessibility to the outside. Reprotonation of the Schiff base from the internal source, BH, followed by reprotonation of the protein from the outside, regenerates the original state E_oH^+ (bR_{570}).

A similar analysis, when applied to previously suggested class I models [e.g. Honig (1978) and Schulten & Tavan, (1978)], indicates that difficulties are encountered when attempting to explain the blue-light effect (e.g., the back photoreaction of M_{412} would be expected to enhance rather than inhibit pumping). Thus, while models of this type can by no means be ruled out, the blue-light effect does seem to favor the new class II mechanisms (at least over the class I mechanisms that have been published). In any case, we feel that it is important to use the blue-light effect as a criterion in the evaluation of different models.

Acknowledgments

We are grateful to Drs. T. Ebrey, F. Tokunaga, L. Eisenstein, and P. Ormoz for valuable discussions.

References

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995.

Aton, B., Doukas, A. G., Narva, D., Callender, R. H., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 79.

Bogomolni, R. A., Stubbs, L., & Lanyi, J. K. (1978) *Biochemistry 17*, 1037.

Bogomolni, R. A., Lozier, R. H., Sivorinovsky, G., & Stoeckenius, W. (1979) *Biophys. J. 25*, Abstr. W-AM-Po101.

Campion, A., El-Sayed, M. A., & Terner, J. (1977) *Biophys. J. 20*, 369.

Dencher, N., & Wilms, M. (1975) *Biophys. Struct. Mech. 1*, 259.

Ehrenberg, B., & Lewis, A. (1978) *Biochem. Biophys. Res. Commun. 82*, 1154.

Favrot, J., Sandorfy, C., & Vocelle, D. (1978) *Photochem. Photobiol. 28*, 271.

Goldschmidt, C. R., Ottolenghi, M., & Korenstein, R. (1976) *Biophys. J. 16*, 839.

Govindjee, R., Ebrey, T., & Crofts, A. R. (1980) *Biophys. J. 30*, 231.

Hess, B., & Kuschmitz, D. (1977) *FEBS Lett. 74*, 20.

Hess, B., & Kuschmitz, D. (1978) *Front. Biol. Energ.*, [*Pap. Int. Symp.*] *1*, 257–264.

Hess, B., & Kuschmitz, D. (1979) *FEBS Lett. 100*, 334.

Honig, B. (1978) *Dev. Halophilic Microorg. 1*, 109–121.

Honig, B., Ebrey, T., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503.

Kalisky, O., Goldschmidt, C. R., & Ottolenghi, M. (1977) *Biophys. J. 19*, 185.

Kalisky, O., Lachish, U., & Ottolenghi, M. (1978) *Photochem. Photobiol. 28*, 261.

Karvaly, B., & Dancshazy, Z. (1977) *FEBS Lett. 76*, 36.

Korenstein, R., & Hess, B. (1977) *Nature (London) 270*, 284.

Korenstein, R., Sherman, W. V., & Caplan, S. R. (1976) *Biophys. Struct. Mech. 2*, 267.

Korenstein, R., Hess, B., & Marcus, M. (1979) *FEBS Lett. 102*, 155.

Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462.

Lewis, A., Marcus, M. A., Ehrenberg, B., & Crespi, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4642.

Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S.-B., & Stoeckenius, W. (1976) *Biochim. Biophys. Acta 440*, 545.

Lozier, R. H., Niederberger, W., Ottolenghi, M., Sivorinovsky, G., & Stoeckenius, W. (1978) *Dev. Halophilic Microorg. 1*, 123–141.

Lozier, R. H., Niv, H., Hwang, S. B., Havel, D., Bogomolni, R. A., & Stoeckenius, W. (1979) *Biophys. J. 25*, 77a.

Marcus, M. A., & Lewis, A. (1977) *Science (Washington, D.C.) 195*, 1328.

Marcus, M. A., & Lewis, A. (1978) *Biochemistry 17*, 4722.

Marcus, M. A., Lemley, A. T., & Lewis, A. (1979) *J. Raman Spectrosc. 8*, 22.

Nagle, J. F., & Morowitz, H. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 298.

Ort, D. R., & Parson, W. W. (1978) *J. Biol. Chem. 253*, 6158.

Ort, D. R., & Parson, W. W. (1979) *Biophys. J. 25*, 341.

Ottolenghi, M. (1980) *Adv. Photochem. 12*, 97.

Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, K., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955.

Schulten, K. (1978) *Dev. Halophilic Microorg. 1*, 331–335.

Schulten, K., & Tavan, P. (1978) *Nature (London) 272*, 85.

Sperling, W., Karl, P., Rafferty, Ch. N., & Dencher, N. A. (1977) *Biophys. Struct. Mech. 3*, 79.

Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1978) *Biochim. Biophys. Acta 505*, 215.

Stein, W., & Honig, B. (1977) *Mol. Cell. Biochem. 15*, 27.

Tsuda, M., Glaccum, M., Nelson, B., & Ebrey, T. G. (1980) *Nature (London) 287*, 351.