# Photosensitive Phosphoproteins in Halobacteria: Regulatory Coupling of Transmembrane Proton Flux and Protein Dephosphorylation

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ABSTRACT A photoregulated reversible protein phosphorylation system controlled by the halobacterial rhodopsins was recently reported. The results presented in this paper identify the initial steps in the pathway from the absorption of light to the photoregulated protein phosphorylation and dephosphorylation reactions. Action spectrum, biochemical, and genetic analyses show that the proton pump bacteriorhodopsin mediates light-induced dephosphorylation of three photoregulated phosphoproteins. Light absorbed by bacteriorhodopsin is used to establish a proton efflux from the cells. The increase in the inwardly directed protonmotive force (pmf) from this efflux induces dephosphorylation of the three phosphoproteins, as demonstrated by the effects of the protonophore CCCP and of artificially imposed transmembrane pH gradients. Upon darkening the cells, cessation of the proton efflux through bacteriorhodopsin causes a decrease in pmf, which induces rephosphorylation of the proteins. Pmf appears to function as a regulator rather than a driving force in this system. Measurements of pmf-driven ATP synthesis in our conditions indicate the regulation of protein phosphorylation by pmf is probably not a consequence of proton flux through the $H^+$ATPase, a known energy coupling structure in these cells. The properties of this system may indicate the existence of a pmf detector which regulates kinase or phosphatase activity; i.e., a regulatory coupling device.

*Halobacterium halobium* cells contain a class of rhodopsinlike, retinal-containing, protein pigments (cf. review in references 1, 2). Two of these pigments have been shown to be integral membrane ion pumps, which use light energy for the electrogenic transport of monovalent cations across the cell membrane. Bacteriorhodopsin, with a maximum absorbance at 565 nm, catalyzes light-driven proton efflux from the cell (3). A second pigment, halorhodopsin, with a maximum absorbance at 588 nm, is responsible for light-driven sodium ion efflux (4, 5, 6, 7). The existence of a third retinal-dependent pigment with an absorption peak at 370 nm has been deduced from the cell's phototactic sensitivities (8, 9, 10). The molecular function of this pigment, named P370, has not been determined, but light absorbed by P370 is said to inhibit photoinduced potassium ion influx (11) and proton efflux (12) in whole cells. The possibility of manipulating ion fluxes by varying the wavelength and intensity of illumination, and the relatively well-characterized ion gradients across halobacterial cell and vesicle membranes, make these cells a promising model system for study of the interaction of electrochemical properties of the cell membrane with cellular biochemistry.

In vivo [$^{32}$P]orthophosphate labeling studies have revealed a photosensitive protein phosphorylation system in *H. halobium* controlled by the halobacterial rhodopsins (13). Exposing whole cells to light induces a rapid dephosphorylation of three specific proteins: a 110,000-dalton protein named LR1, which is associated with membranes after 180,000-*g* centrifugation of sonicated cells, and two soluble proteins, LR2 and LR3, of 83,000 and 62,000 molecular weight, respectively (13, 14). This effect is reversible: darkening the cells results in a rapid rephosphorylation of each of the three light-regulated (LR) proteins (13). In this report, we describe studies which clarify the initial steps in the signaling pathway from photoreception to the LR protein phosphorylation system.

## MATERIALS AND METHODS

Strain $R_1S_2$ (9) with wild-type pigmentation was used throughout, except as indicated in the legend in Fig. 4. Cell culture and preparation, [$^{32}$P]orthophosphate in vivo labeling, whole cell illumination, protein extraction, electrophoresis, and radioautography are described in detail in reference 14. The labeling procedure is to add 25 λ [$^{32}$P]orthophosphate to 1 ml of $2 \times 10^7$ cells suspended in basal salt containing 10 mM HEPES, pH 7.4 (14). After aerobic incubation in the dark for 1 h at 37°C, the cells are used in the experiment. Cell proteins are extracted by acetone precipitation of 200 λ samples of the labeled suspension (14). Cells are maintained aerobic with vigorous stirring throughout the experiments described.

For comparisons between radioautograms of different background densities, densitometer measurements of LR protein bands were normalized to the density of the labeled band at ~40,000 apparent molecular weight (the uppermost member of the doublet, as seen in Fig. 1). The labeling of this band appears to vary in proportion to background.

Determination of ATP was by the luciferin-luciferase assay with conditions as described in reference 15, except morpholinopropane sulfonic acid (MOPS) buffer was used and bioluminescence was measured with an appropriately encased RCA1P21 photomultiplier tube.

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was from Sigma Chemical Co. (St. Louis, Mo.).

## RESULTS

### *Assay and Characterization of the LR Proteins*

In Fig. 1 are shown the results of in vivo labeling dark-adapted cells with [$^{32}$P]orthophosphate and the effects of illumination and redarkening. No differences are seen between the Coomassie Blue–staining patterns (Fig. 1) of acetone-precipitable proteins from dark-adapted cells (D1), cells illuminated for 5 min (L), and cells that were then darkened for 10 min (D2). The phosphate labeling patterns (Fig. 1) of cells in these three conditions, however, reveal a light-induced loss of phosphate from three specific polypeptides, and a rephosphorylation after redarkening. In addition to the proteins LR1 and LR2 reported previously, Fig. 1 shows a third light-regulated protein (labeled LR3) of ~62,000 apparent molecular weight. The presence of several light-insensitive phosphoproteins near LR3 obscures the dephosphorylation of LR3 in some autoradiograms. Initial chemical characterization of the LR1 and LR2 protein phosphate bonds suggested phosphoserine or phosphothreonine linkages (13), typical products of protein kinase activity.

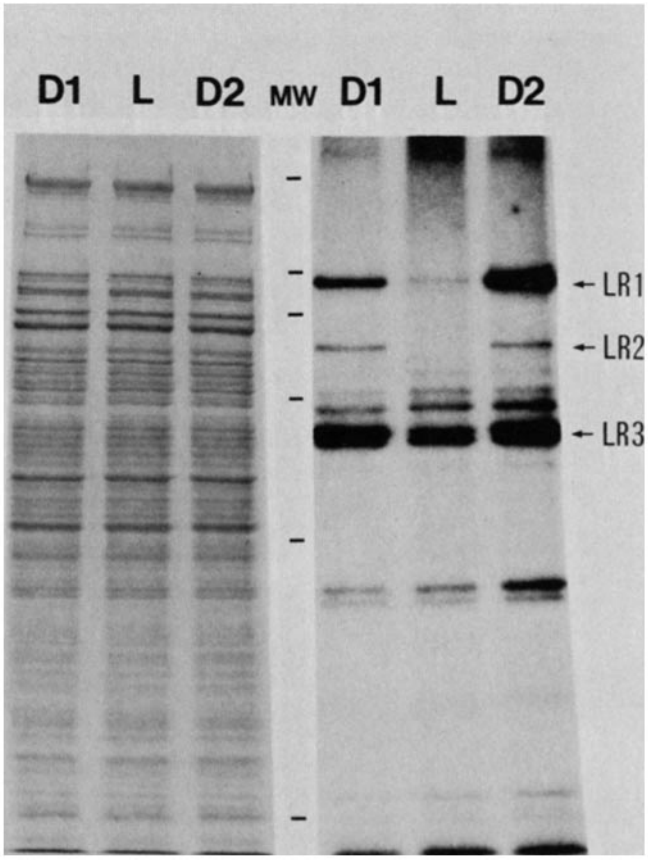


FIGURE 1 Demonstration of three light-regulated proteins. *H. halobium* strain $R_1S_2$ was used in the procedure described. The leftmost three lanes show the Coomassie Blue–stained cell extracts for cells dark-adapted (*D1*), illuminated for 5′ (*L*), then darkened for 10′ (*D2*). The rightmost three lanes show the autoradiogram of the same three gel lanes. Molecular weight standards (*MW*) are from the top: myosin (200,000), β-galactosidase (116,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and bacterioopsin (26,000). *LR1*, *LR2*, and *LR3* designate the three light regulated phosphoproteins.

In all of the measurements described here, the extent of phosphorylation of LR2 and LR3 varies qualitatively in the same way as that of LR1. Since LR1 is the most heavily labeled, it was routinely used for quantitation of extents of LR protein phosphorylation.

### *Bacteriorhodopsin Is the Photoreceptor Mediating LR Protein Photosensitivity*

Inhibitor studies indicated that the photosensitivity of the LR phosphoproteins is retinal-dependent (13). We have investigated the photosensitivity further to determine which retinal pigment regulates the LR phosphoproteins. Several measurements provide compelling evidence that specifically bacteriorhodopsin is the photoreceptor: (*a*) Bacteriorhodopsin content in well-aerated logarithmic phase cells is minimal. As cell density increases in late logarithmic phase, bacteriorhodopsin synthesis is triggered, presumably by the decrease in oxygen tension, and the cellular content of the pigment rapidly increases (16). As shown in Fig. 2, the photosensitivity of LR1 dephosphorylation develops concomitantly with bacteriorhodopsin during culture growth. (*b*) The three known retinal pigments in halobacteria differ in their absorption spectra. Bacteriorhodopsin and halorhodopsin both absorb relatively long wavelength light, with absorption maxima at 565 nm and 588 nm, respectively. P370, the repellent phototaxis receptor, absorbs maximally in the UV and at 370 nm. The action spectrum of dephosphorylation of LR1 shown in Fig. 3 is consistent with either bacteriorhodopsin or halorhodopsin, but not with P370. (*c*) Phosphorylated LR1 proteins, in a mutant which lacks bacteriorhodopsin, but contains halorhodopsin, are insensitive to light, even after relatively long exposures (Fig. 4), indicating halorhodopsin is not a photoreceptor for

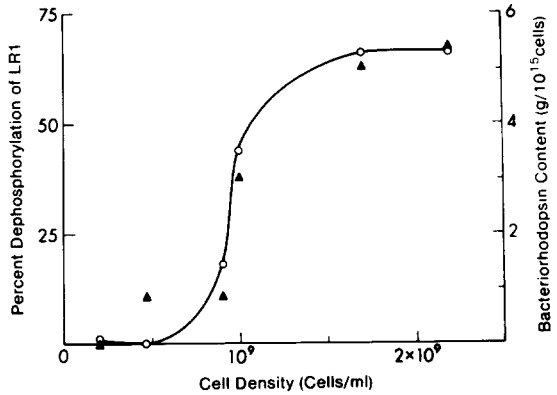


FIGURE 2 LR1 phosphorylation photosensitivity vs. cellular bacteriorhodopsin content (▲) during culture growth. Cell cultures were grown as described (14) to the indicated cell densities. Percent dephosphorylation of LR1 in vivo (○) was assessed by densitometry of radioautographic exposures of $^{32}$P-labeled LR1 isolated on SDS polyacrylamide gels (14). Illumination of each cell suspension was for 5 min at $5 \times 10^5$ ergs/cm$^2$ with orange light (Corning 3-69 long-pass filter). Bacteriorhodopsin content was determined by the light/dark adaptation method described in reference 30.

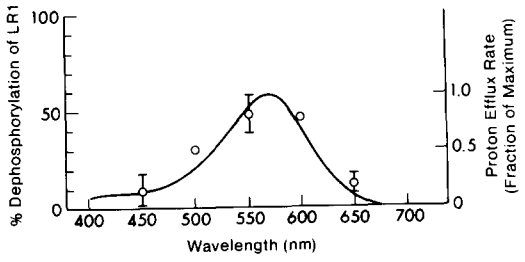


FIGURE 3 Action spectrum of LR1 dephosphorylation. Percent dephosphorylation of the LR1 protein was determined as in Fig. 2. Illumination of cell suspensions was at 2.3 × $10^5$ ergs/cm$^2$ s for 5 min defined by 40-nm bandpass filters with peak transmission at the wavelength indicated on the abscissa. The proton efflux action spectrum is redrawn from reference 30. The length of the error bars is two standard errors of the mean of two independent measurements.

LR protein dephosphorylation under these conditions. It should be noted that under our conditions halorhodopsin does not establish a significant light-induced increase in membrane potential in the bacteriorhodopsin-deficient mutant used here (S. Helgerson, personal communication).

We conclude bacteriorhodopsin mediates the photosensitivity of the LR protein phosphorylation system.

## Protonmotive Force Regulates LR Protein Phosphorylation

Bacteriorhodopsin is a light-driven proton pump which translocates protons from the interior of the cell to the extracellular environment (3). If this proton flux driven by bacteriorhodopsin mediates the photoregulation of the protein phosphorylation system, one might expect the proton ionophore CCCP to prevent light-induced dephosphorylation by uncoupling this proton flux from the protein phosphorylation system. In fact, CCCP added to cells before illumination prevents the dephosphorylation of LR1 by light (Fig. 5). Furthermore, CCCP added after illumination reverses the effect of light by inducing rephosphorylation of LR1 (Fig. 5). We conclude the protein ejection by bacteriorhodopsin, through an unknown coupling pathway, induces the LR protein dephosphorylation.

*A priori* one expects two immediate consequences of proton ejection by bacteriorhodopsin: (*a*) an increase in internal pH ($pH_{int}$) (which may or may not be significant, depending on the cytoplasmic buffering capacity); and (*b*) an increase in membrane potential ($\Delta\psi$), since proton ejection by bacteriorhodopsin results in a net transfer of charge across the membrane. It is known that in *H. halobium*, the steady-state values of both $pH_{int}$ and $\Delta\psi$ show a pronounced dependence on external pH ($pH_{ext}$) (17, 18). For example, $\Delta\psi$ is maximal (~130 mV) above $pH_{ext}$ = 7.5, and has less than half this value at $pH_{ext}$ = 6.0. Internal pH is highly dependent on $pH_{ext}$ as well; $pH_{int}$ is constant at 7.4 when $pH_{ext}$ is between 5.5 and 7.4; but once above $pH_{ext}$ = 7.4, then $pH_{int}$ = $pH_{ext}$. If $\Delta\psi$ or $pH_{int}$ (or $\Delta pH$) regulate the phosphorylation system, then we should see a pronounced dependence of the extent of LR phosphorylation on $pH_{ext}$. As shown in Fig. 6, LR1 is phosphorylated to similar extents over a broad range of $pH_{ext}$. Furthermore, light-induced LR1 dephosphorylation occurs at all $pH_{ext}$ tested. Since the influence of light on $\Delta\psi$ and $pH_{int}$ (or $\Delta pH$) has been shown to be much smaller than the influence of $pH_{ext}$ on these parameters (17, 18) the data of Fig. 6 excludes regulation of LR protein phosphorylation by any one of these parameters. The one related parameter which is constant in the dark over a broad range of $pH_{ext}$, and is increased by proton ejection by bacteriorhodopsin at essentially all $pH_{ext}$, is the transmembrane electrochemical potential difference of the proton ($\Delta\tilde{\mu}_{H^+}$), or protonmotive force (pmf) as defined by Mitchell (19). Pmf is the sum of electrical ($\Delta\psi$) and chemical ($\Delta pH$) components and is relatively independent of $pH_{ext}$ because of the opposing variation of $\Delta\psi$ and $\Delta pH$.

A further test of pmf regulation of LR protein phosphorylation is provided by a pH jump experiment as first applied by Jagendorf and Uribe (20). Applied to halobacteria, the rationale of this experiment is that a sudden acidification of the cell suspension will cause an increase in pmf. This increase is transient, because diffusion of protons into the cell will decrease $\Delta\psi$ until the original value of pmf is restored. We subjected labeled cells in the dark to an abrupt acidification from $pH_{ext}$ = 7.5 to $pH_{ext}$ = 5.5. We observe dephosphorylation of the three LR proteins as shown in Fig. 7, and the dephosphorylation is indeed transient, correlating with the expected behavior of pmf. From these results we conclude that pmf regulates LR protein phosphorylation and a change in pmf is the membrane signal mediating photosensitivity of the LR phosphoproteins.

## Relationship of LR Protein Regulation to ATP Synthesis

In previous in vivo studies illumination of bacteriorhodopsin resulted in significant increases in cellular ATP content (reviewed in reference 1). It is generally accepted that, as expected from chemiosmotic theory, these increases in ATP result from proton influx through a proton-translocating ATPase driven in reverse. Therefore, the ATP increases would be expected to correlate with pmf, the force driving protons into the cell. One possibility is that this ATP increase is the signal for decreasing the extent of phosphorylation of the LR proteins. Note that a simple substrate role of ATP in a protein kinase reaction cannot account for the LR protein phosphorylation changes because of the direction of the effect: conditions which increase ATP decrease LR protein phosphorylation.

Previous in vivo studies of the effect of illumination on ATP content in *H. halobium* have used cells in anaerobic conditions and partially depleted of internal energy reserves to maximize the effect of light (1). In our protein phosphorylation measurements reported here, the cells, freshly harvested from a growth-supporting medium, have not been depleted of their internal energy reserves. Furthermore, we routinely measure light-induced LR protein dephosphorylation under conditions of continuous vigorous aeration of the cells. As one would expect, we do not see the large changes in ATP content reported in the anaerobic studies. For a sudden shift from dark to light which completely dephosphorylates LR1 we typically measure increases in ATP content between 15% and 25% at 5′ after the onset of illumination. Is this ATP increase the signal for LR protein dephosphorylation? Two observations argue strongly against such a signaling role for ATP: (*a*) ATP content varies by much more than 25% in the dark in differing conditions and yet no corresponding variation in extent of LR protein phosphorylation is observed. For example, the amount of time the cells incubate at their maximum culture density has a great effect on cellular ATP content, as shown in Fig. 8*a*. The increase in ATP from illumination shown in Fig. 8*a* is small compared to the differences between cultures, yet illumination dephosphorylates the LR proteins to similar extents in the three cultures as shown in Figure 8*b*. (*b*) The second obser-

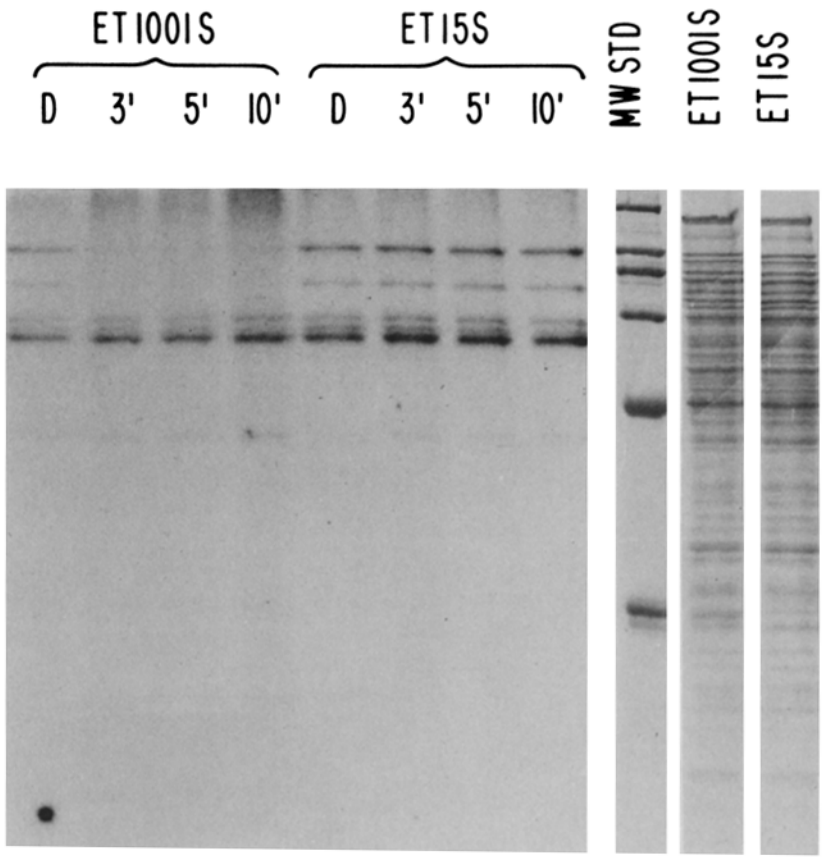


FIGURE 4 Comparison of the bacteriorhodopsin-deficient strain ET15S and the bacteriorhodopsin-containing strain ET1001S. The strains are individual isolates from pigment mutants selected and characterized by H. J. Weber (University of California, San Francisco). ET1001S contains bacteriorhodopsin, but lacks carotenoids. ET15S lacks both carotenoids and bacteriorhodopsin, but contains the other retinal pigments. Preparation of cells, illumination, and radioautography were as described (14). The Coomassie Blue-staining pattern of the two strains are shown to the right of the molecular weight standards (*MW STD*) in this composite. Incorporation of $^{32}P$ in whole cell proteins is shown to the left of the *MW STD* lane for both strains. *MWSD* as in Fig. 1.

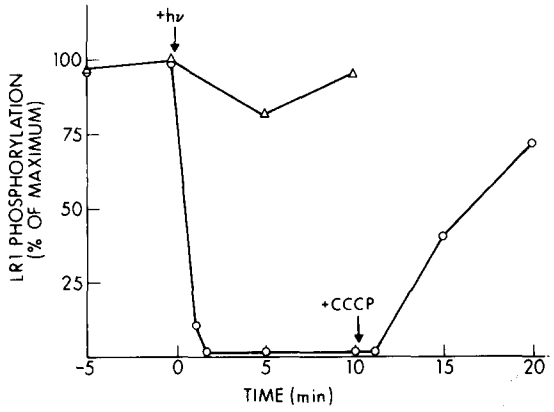


FIGURE 5 Effect of CCCP on photoinduced LR1 dephosphorylation. Aliquots from cell suspensions were taken at various times before and after illumination was begun at $t = 0$. The extent of phosphorylation of the LR1 protein was determined as in Fig. 2. To a first 1-ml cell suspension (△), 2 μl 10 mM CCCP in ethanol were added immediately before illumination. To a second 1-ml cell suspension (○), 2 μl 10 mM CCCP in ethanol were added immediately after the 10 min illumination sample was taken. Addition of ethanol alone does not affect LR1 phosphorylation.

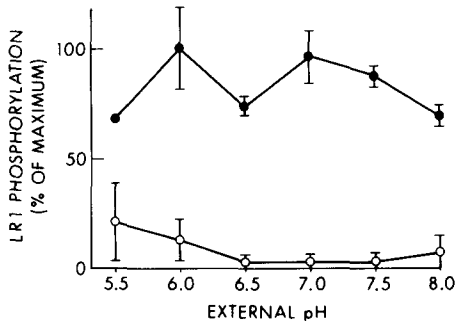


FIGURE 6 Photosensitivity of phosphorylation at various external pH. LR1 phosphorylation was assessed as in Fig. 2 for dark-adapted cells (●) and cells illuminated (○) as in Fig. 2. After the in vivo labeling procedure (14), the pH of the cell suspension was adjusted to the pH noted on the abscissa. Cells were incubated for 1 h at the indicated pH before sampling.

vation arguing against ATP signaling is that even the relatively small light-induced increases in ATP shown in Fig. 8 *a* can be virtually eliminated by the manner in which the light is delivered to the cells, yet dephosphorylation of LR proteins still occurs. The measurements in Fig. 9 show that the initially increased ATP level resulting from a sudden shift from dark to light is not maintained: after 10 min in the light the ATP level had fallen to a value only ~7% higher than the initial dark value. In the experiment of Fig. 9, dephosphorylation of LR1 was complete (97% dephosphorylated) 1 min after the onset of illumination and LR1 remained dephosphorylated during the subsequent slow decline of ATP level in the light (98% dephosphorylated at 10 min). This overshoot in ATP content resulting from an abrupt step increase in light intensity (Fig. 9)

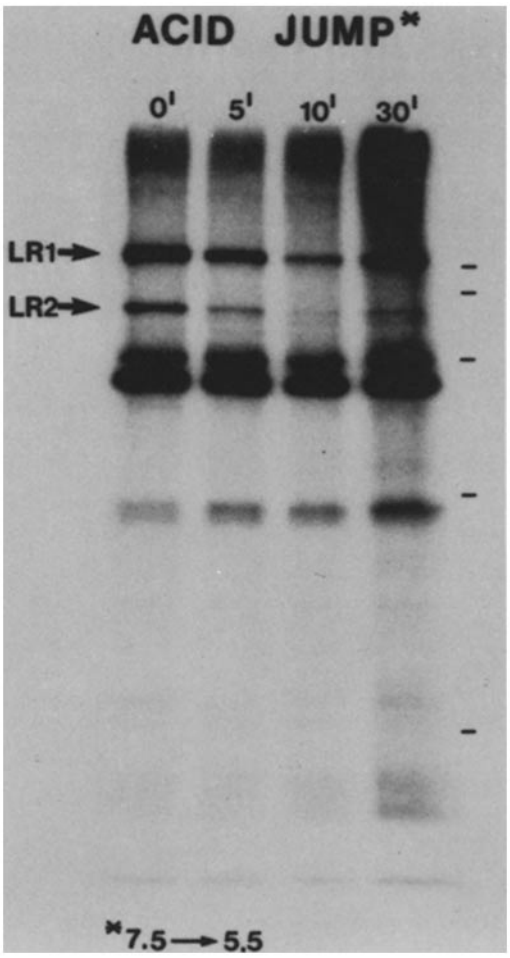


FIGURE 7 Transient dephosphorylation by a pH jump. Dark-adapted cells at pH 7.5 were exposed to a sudden acidification of their suspension buffer to pH 5.5 by addition of 0.1 N HCl in basal salt (2.5 μl to 1 ml suspension), vigorously stirred as previously described (14). Samples were taken as described (14) at the indicated number of minutes after HCl addition. Molecular weight standards (-) as in Fig. 1, except myosin is not indicated. The positions of *LR1* and *LR2* are indicated by arrows; *LR3* is not indicated but its transient dephosphorylation is also evident in the figure.

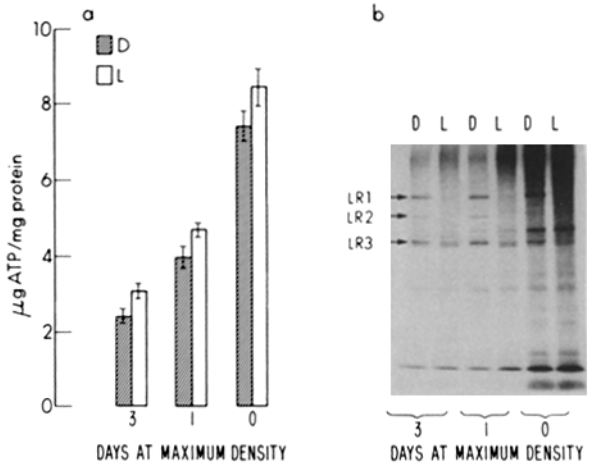


FIGURE 8 ATP content and protein phosphorylation in cultures of differing ages. Cells inoculated and grown as described in reference 14 were used at the indicated number of days after which maximum cell density was reached (the maximum density of $2.8 \times 10^9$ cells/ml occurs 3 d after inoculation). (*a*) ATP content and (*b*) the LR protein labeling pattern were assessed as described in Materials and Methods for dark-adapted cells (*D*) and cells illuminated as in Fig. 4 for 5 min (*L*). The length of the error bars in *a* is 2 standard errors of the mean. Cellular protein content was the same in the three cultures (7.0 mg protein/$10^{10}$ cells).

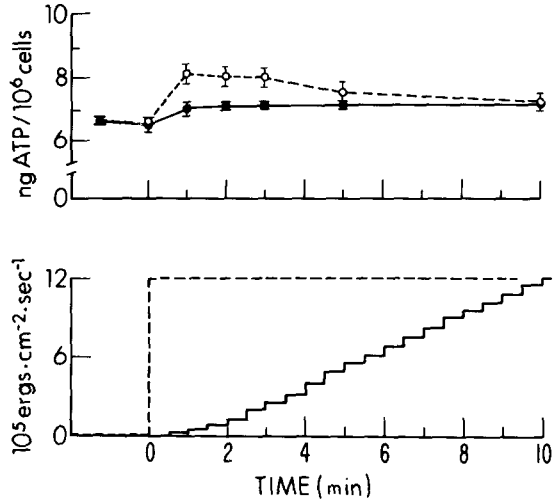


FIGURE 9 Photophosphorylation overshoots the steady state level after a sudden increase in light intensity. Cellular ATP content was measured in a cell suspension exposed to a sudden increase in intensity (dotted lines and open circle points) or to a light ramp of gradually increasing intensity (solid lines and filled circle points) of orange light (14). The light ramp was delivered by gradually opening a diaphragm inserted in the light beam.

is not required for the dephosphorylation of LR proteins. As also shown in Fig. 9, if the light intensity increase is delivered gradually over time, the ATP content climbs to the new steady-state level in the light without overshooting the final steady-state level. At 10 min, at the end of the light ramp, LR1 was essentially completely dephosphorylated (96% dephosphorylated).

From these measurements it seems very unlikely that cellular ATP regulates the LR protein phosphorylation system. If cellular ATP functions as a signal for LR protein dephosphorylation, the phosphorylation system would need to sense ATP with a considerable resolution; i.e., the ATP signal would require amplification.

## DISCUSSION

The results presented above identify the initial steps in the pathway from the absorption of light to the photoregulated protein phosphorylation and dephosphorylation reactions in *H. halobium*. Light absorbed by bacteriorhodopsin is used to establish a proton efflux from the cells. The increase in the inwardly directed pmf from this efflux induces dephosphorylation of the LR phosphoproteins. Upon darkening the cells, cessation of the proton efflux through bacteriorhodopsin causes a decrease in pmf, which induces rephosphorylation of the LR proteins.

In accord with Mitchell's chemiosmotic hypothesis, energy stored in the membrane as pmf has been shown to be the driving force for diverse energy-dependent processes: ATP synthesis by membrane bound ATPases, metabolite transport by bacterial membrane permeases, and mechanical movement by the bacterial flagellar motor (cf. review in reference 21). In these cases proton flux is energetically coupled to an energy-

dependent reaction in which work is obviously performed. No such energy dependence is expected for protein dephosphorylation, since protein phosphatases can catalyze dephosphorylation without energy input. Furthermore, protein phosphorylation is an energy-dependent reaction, yet LR protein phosphorylation is induced by a decline in pmf. Therefore pmf appears to function as a regulator, rather than a driving force, for the LR protein phosphorylation system.

In view of the importance of pmf to the cellular economy, one might expect this fundamental energetic parameter to serve a role as a regulator of cellular processes, in addition to its energization function. There is some recent evidence for regulation of specific processes by pmf: in separate in vivo studies of *Escherichia coli* metabolism, pmf has been implicated as a regulator of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) degradation (22) and cAMP synthesis (23). Physiological studies indicate that perturbations of pmf regulate swimming behavior of aerotactic bacteria (24) and that by a different mechanism pmf exerts steady-state regulatory effects on the flagellar motor (25). The existence of specific pmf sensors in tactic bacteria has been addressed theoretically by Glagolev (26) and experimentally in studies of halobacterial sensory reception by Baryshev et al. (27). In no case has a regulatory pathway which confers sensitivity of a specific reaction to pmf been elucidated.

Regulatory effects of pmf on intracellular processes might result from pmf control of intracellular ion or metabolite concentrations; i.e., regulation mediated by transport systems coupled to proton flux. Another possibility is that pmf acts by inhibiting pmf-generating structures in the membrane. For example, in halobacteria photoinduced pmf inhibits respiration (3, 28). Inhibition of respiratory proton ejection could generate an accumulation of a reduced electron transport intermediate which controls LR protein phosphorylation. If this were the case, the halobacterial protein phosphorylation photoregulation may have interesting analogies with the recently reported control of chloroplast protein phosphorylation by the redox state of plastiquinone (29).

A basic question is: Are the regulatory functions of pmf consequences of proton flux through known energetic coupling channels (e.g. through permeases or the $H^+$ ATPase) or do distinct regulatory coupling channels exist? In particular, it is important to determine whether pmf-driven ATP synthesis is responsible for pmf regulation, since ATP is a known allosteric regulator of cellular reactions. In the cases of pmf regulation of the flagellar motor (24, 25), ppGpp breakdown (22), cAMP synthesis (23), and the LR protein phosphorylation system described here, ATP levels, *per se*, do not appear to mediate the regulation. A potentially interesting aspect of these systems is that they may indicate the existence of proton channels which function specifically in regulatory coupling, i.e., pmf detectors which control specific enzymatic activities. The existence of such structures has not been proven, but is an intriguing possibility.

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