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# PHOTOPHOSPHORYLATION IN CELL ENVELOPE VESICLES FROM HALOBACTERIUM HALOBIUM

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SUMMARY

We have prepared vesicles from cell envelope membranes of Halobacterium halobium strains $R_1$ and ET-15 which are able to synthesize ATP in response to illumination. This photophosphorylation is inhibited by dicyclohexylcarbodiimide (DCCD) and by phloretin. ATP synthesis in L vesicles from the $R_1$ strain (which contain bacteriorhodopsin) is inhibited by the protonophore 1799 but not by valinomycin. In M vesicles from the $R_1$ strain and in ET-15 vesicles (both contain halorhodopsin) photophosphorylation is inhibited by both 1799 and valinomycin. These data are consistent with the idea that light-driven ATP synthesis can be coupled to the electrochemical $H^+$ gradient generated by bacteriorhodopsin or by halorhodopsin through the membrane potential component of protonmotive force.

When grown with illumination and at low oxygen tension, Halobacterium halobium $R_1$ cells synthesize large amounts of the purple membrane protein, bacteriorhodopsin (1). Bacteriorhodopsin (bR)[1] is a retinyl protein mediating light-driven $H^+$ efflux from whole cells and from cell envelope vesicles (2). H. halobium also synthesizes the pigment halorhodopsin (hR), which effects light-driven, electrogenic efflux of $Na^+$ from intact cells and from cell envelope vesicles (4,5). Proton uptake then occurs as a passive electrophoretic response to the membrane potential generated by $Na^+$ extrusion. Thus cells of the $R_1$ strain have two light-dependent mechanisms for generating protonmotive force (pmf):

[1]Abbreviations: 1799, bis(hexafluoroacetonyl)acetone; ANP, total adenylate (AMP+ADP+ATP); bR, bacteriorhodopsin; hR, halorhodopsin; DCCD, dicyclohexylcarbodiimide; MES, 2-(N-morpholino)ethanesulfonic acid; PEI, polyethyleneimine; pmf, protonmotive force; $\Delta pH$, transmembrane pH gradient; $\Delta\mu_{Na^+}$, transmembrane $Na^+$ concentration gradient; and $\Delta\Psi$, membrane potential.

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(i) directly, by ejection of $H^+$ by bR to form a pH gradient and $\Delta\Psi$; and (ii) indirectly, from $\Delta\Psi$ created by the primary ejection of $Na^+$ by hR, as we have shown elsewhere (5,6).

When whole cells of H. halobium $R_1$ which contain purple membrane are illuminated, their ATP content rises. This photophosphorylation can be accounted for on the basis of coupling of a $H^+$-ATPase to light-driven proton pumping by bR (7). Yet strains in which bR is deficient or lacking altogether also synthesize ATP in response to illumination, presumably as a result of light-driven $Na^+$ extrusion by hR present in such cells (4). It is difficult to determine whether ATP is synthesized in these cells in response to pmf alone or in response to $\Delta\mu_{Na^+}$ as well, i.e., whether a $Na^+$-ATPase is present in addition to a $H^+$-ATPase (7). To address this question we have developed a technique for the assay of ATPase function in envelope membrane vesicles. This permits us to examine the roles of various electrochemical gradients in ATP synthesis and hydrolysis in vesicles from $R_1$ and mutant strains. The work presented here is part of a study of photophosphorylation in various vesicle preparations. We conclude that a $H^+$-ATPase alone is sufficient to account for the properties of light-driven ATP synthesis observed in all vesicle preparations.

## MATERIALS AND METHODS

Halobacterium halobium ET-15 (a $bR^-$, $hR^+$ strain (16) obtained from H. J. Weber, UC at Berkeley) and $R_1$ cells were grown under illumination as previously described (8). Vesicles were prepared by sonication with subsequent purification by differential centrifugation onto a cushion of 4M NaCl, 17% sodium tartrate (9). Rapidly sedimenting vesicles (L vesicles) from strain $R_1$ contain predominantly bR while vesicles sedimenting more slowly (M vesicles) contain predominantly hR (5). The slowly and rapidly sedimenting vesicle fractions prepared from the $bR^-$ strain ET-15 were pooled (ET-15 vesicles).

Vesicles were loaded by osmotic shock as previously described (8,9). All experiments were carried out at 31°C. Vesicle suspensions were illuminated with a Sylvania 300W ELH lamp. Light was passed through 3 cm 1% $CuSO_4$ and a Corning 3-69 cutoff filter (8). Intensity was determined at the focus with a YSI radiometer. Vesicle samples were collected on nitrocellulose filters (0.45 μM pore size) and washed with cold 2 mM $P_i$, 3 M NaCl at pH 6.0 (8). Filters were extracted in chilled

0.5 mM $NaH_2AsO_4$ (for luciferase assay) or in 0.2 mM $P_i$, 0.2 mM ADP, 0.2 mM ATP at pH 7.5 (for assay of $P_i$ fixation). Extracts were quenched at 100°C for three minutes.

ATP was assayed by the luciferin/luciferase method of Kimmich et al. (10) slightly modified for use with a Packard refrigerated liquid scintillation spectrometer. Internal standards were used throughout. AMP and ADP were converted to ATP for total adenylate (ANP) determination according to Kimmich et al. (10) except that kinase reactions were terminated by a 2 min, 95°C quench in situ. Extract ATP values were corrected for buffer carry-over on the basis of ANP assays run in parallel.

$^{32}P_i$ incorporation into ATP was determined by ion-exchange over PEI cellulose according to Magnusson et al. (11). Protein was assayed according to Lowry et al. (12) with lysozyme as standard.

$^{32}P_i$ was obtained as the sodium salt from New England Nuclear. MES, ADP, PEI cellulose, ATP and firefly tail extract were from Sigma Chemical Co. 1799 was supplied by P. Heytler of DuPont. Inorganic chemicals used were reagent grade.

## RESULTS

As shown in figure 1, ATP is synthesized in response to illumination in L vesicles, where ion fluxes are driven by bR (figure 1A); in M vesicles, where ion fluxes are driven by hR (figure 1B); and in ET-15 vesicles, which lack bR entirely (figure 1C). Control experiments (data not shown) demonstrate that the ATP formed is within the vesicles, not accessible to the bulk medium. The appearance of ATP in response to light is paralleled by incorporation of $^{32}P_i$ into nucleoside triphosphate (figure 2).

It should be noted that the scales of photophosphorylation shown in figure 1 are different for each vesicle type; nearly 40-fold more ATP is synthesized per mg of protein in illuminated L vesicles than in M vesicles. This is due at least in part to differences in "loading efficiencies" among vesicle types. Under identical loading conditions there is an order of magnitude difference in the amount of entrapped adenylate in L and in M vesicles (16 vs. 1.4 nmol/mg protein, respectively). Hence about 8% of the ADP in L vesicles and about 2.5% of the ADP in M vesicles can be phosphorylated to ATP upon illumination. Whether differences in loading are due to differences in internal volume,

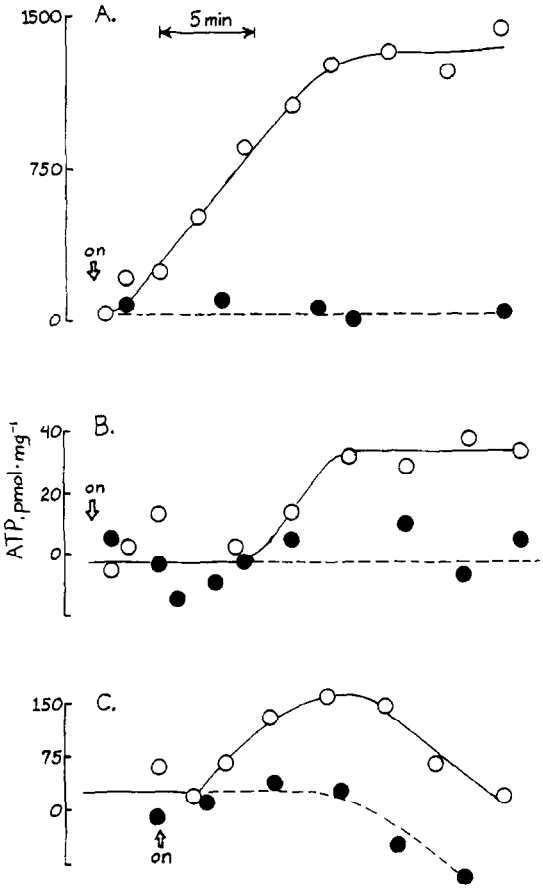


Figure 1: Photophosphorylation in (A)L vesicles from strain $R_1$; (B)M vesicles from strain $R_1$; and (C) vesicles from strain ET-15. Vesicles were loaded with 1.8 M NaCl, 0.9 M KCl, 0.05 M $MgCl_2$, 20 mM $P_i$, 10 mM MES, 3 mM ADP at pH 6.0 and assayed in the same buffer except that extravesicular ADP was 1.5 mM (A,B) or 0.8 mM (C). Protein concentrations were (A) 0.28, (B) 0.51 and (C) 0.59 mg/ml. (●) Dark controls; (○) vesicles illuminated at 2.5 x $10^6$ erg $cm^{-1}$ $s^{-1}$ from the time indicated by the open arrow. Data for M and ET-15 vesicles are presented as two-point moving averages for clarity. Apparently negative values are artifacts of a sloping baseline.

different filter retention, incomplete buffer exchange after osmotic shock, or to some other factor has not been determined.

DCCD and phloretin are inhibitors of photophosphorylation in intact cells (7,13). These inhibitors are effective in vesicles as well, as the data presented in figure 3 show. Phloretin at 100 μM inhibits the rate of ATP synthesis by 87%. DCCD has a comparable effect after 10 min preincubation (figure 3).

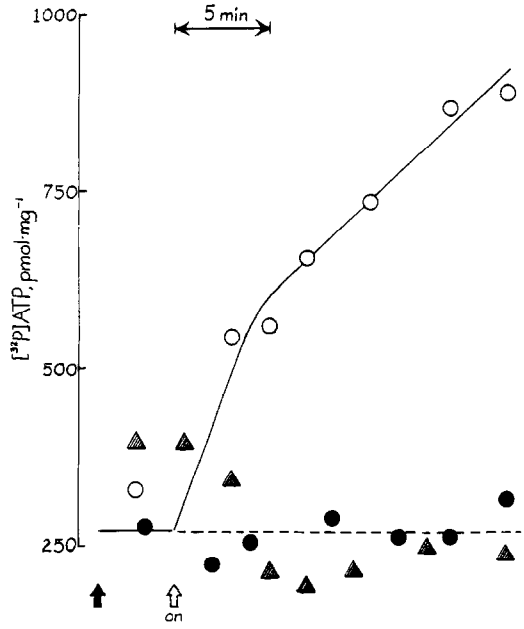


Figure 2: $^{32}P_i$ fixation in illuminated L vesicles. Vesicles were loaded and assayed as described for Figure 1 except that 96 μCi $^{32}P_i$ was included at 12 mCi/mmol and extravesicular ADP was 3 mM. Protein concentration was 1.0 mg/ml. (●) Dark control; (○) illuminated; (▲) illuminated in the presence of 15 μM 1799 (added at filled arrow). Illuminations were begun as indicated by the open arrow. Data are shown as two-point moving averages for clarity.

It has been shown (14) that 1799 can reduce protonmotive force in vesicles from H. halobium to near zero. In L vesicles the protonophore short-circuits $H^+$-pumping by bR, dissipating ΔpH and ΔΨ (14) and preventing formation of $\Delta\mu_{Na}+$ via the $Na^+/H^+$ antiporter (15). As shown in figures 2 and 4A, photophosphorylation is not observed in L vesicles when 1799 is present to short-circuit bR. In hR-containing vesicles, however, protonophores have only a modest effect on ΔΨ and $\Delta\mu_{Na}+$ because $Na^+$ efflux is the primary light-driven event. Protonophores dissipate pmf by allowing the formation of a reversed $H^+$ gradient equal in magnitude to ΔΨ (5). Yet addition of 1799 reduces photophosphorylation below detectable levels in M and ET-15 vesicles (figure 4B, C). This indicates that the observed ATP synthesis must be driven by pmf, created indirectly through electrogenic ejection of $Na^+$ by hR.

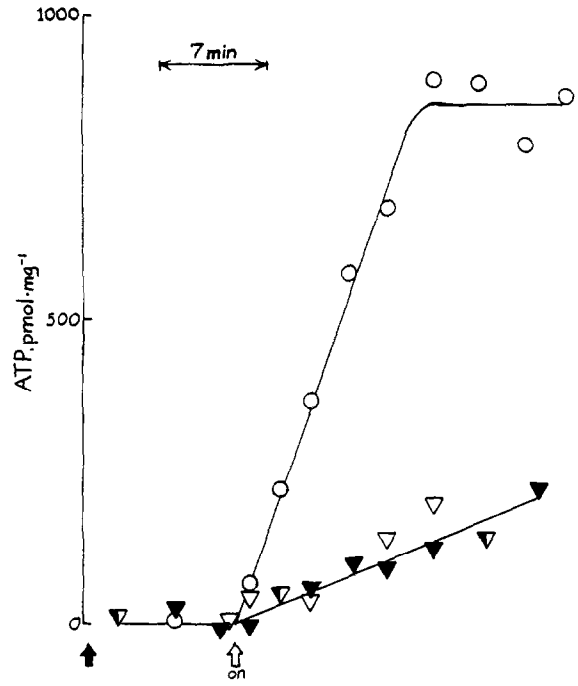


Figure 3: Inhibition of ATP synthesis in illuminated L vesicles by DCCD and by phloretin. Vesicles were loaded as described for Figure 1 except that extravesicular ADP was 3 mM. Protein concentration was 0.36 mg/ml. (○) No addition; (▽) 10 μM DCCD; (▼) 100 μM phloretin. Additions were made at the filled arrow and illumination begun as indicated by the open arrow.

Valinomycin in $K^+$ buffer has very different effects on pmf supported by bR than on pmf supported by hR (5,6). In L vesicles valinomycin stimulates $H^+$ pumping by bR because it dissipates $\Delta\Psi$ (18). Hence there is little change in net pmf. As expected, valinomycin does not inhibit photophosphorylation in L vesicles (figure 4A). In M and ET-15 vesicles, $\Delta\Psi$ also is dissipated by valinomycin but since there is no compensating increase in $\Delta$pH, pmf is abolished as well (14). Hence photophosphorylation in M and ET-15 vesicles is blocked by valinomycin (figure 4B,C).

## DISCUSSION

A considerable literature has accumulated on photophosphorylation in Halobacterium halobium cells (7,17). The data are consistent with a

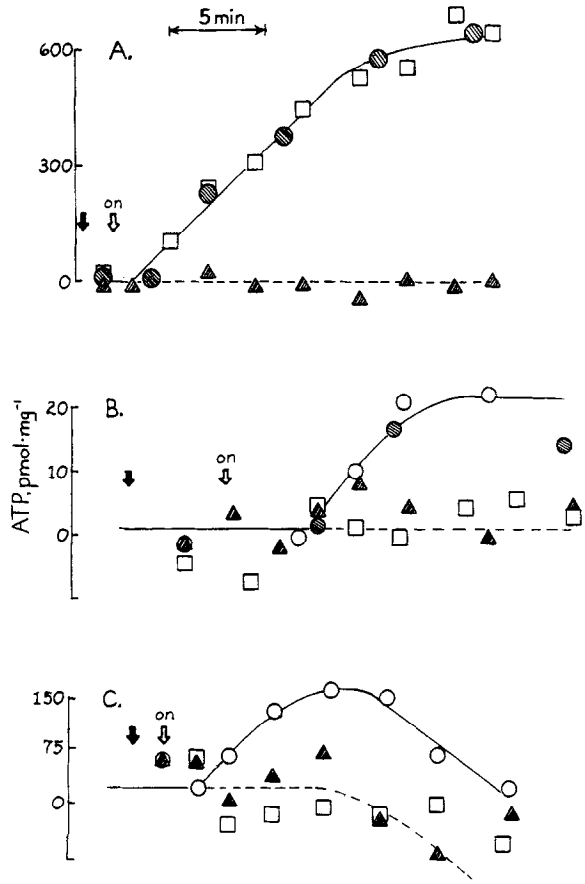


Figure 4: Effects of ionophores on photophosphorylation in (A) L vesicles, (B) M vesicles, and (C) ET-15 vesicles. Loading and assay were as described for Figure 1 except that extravesicular ADP was 3 mM in (A). Protein concentrations were (A) 0.36, (B) 0.55 and (C) 0.59 mg/ml. (○) No additions; (●) 0.1 % methanol; (□) 1 μM valinomycin; (▲) 1 μM 1799. Additions were made at the filled arrows and illumination begun as indicated by the open arrows. The broken lines show the time courses of dark controls. Data are shown as two-point moving averages in B and C for clarity.

chemiosmotic interpretation of coupling of ATP synthesis to electrochemical $H^+$ gradients. Photophosphorylation also occurs in $bR^-$ strains (3,4), but the exact nature of the driving force involved has not been determined.

The data presented here show that photophosphorylation can occur in L and M vesicles (8,9) from H. halobium $R_1$ ($bR^+$, $hR^+$) and in vesicles from strain ET-15 ($bR^-$, $hR^+$) (16). The ATP synthesis observed involves

$P_i$ fixation (figure 2) and so is not simply an adenylate kinase activity.

Photophosphorylation coupled to hR (M and ET-15 vesicles) and to bR (L vesicles) is inhibited by 1799. It is inhibited by valinomycin only when coupled to hR (figure 4B,C). Taken together these effects show that: (i) pmf is a necessary and sufficient conditon for photophosphorylation; and (ii) neither $\Delta\Psi$ nor $\Delta\mu_{Na}+$ is sufficient. Therefore coupling of ATP synthesis to light-driven ion fluxes is predominantly via $H^+$. At the present time we cannot exclude some involvement of other ions, e.g., $K^+$ (17).

For all vesicle preparations assayed, there is a lag between the start of illumination and the onset of ATP synthesis (figure 1). For L and ET-15 vesicles, the delay is only 1 to 3 min but for M vesicles it is about 10 min. The reason for the different lag times is not known; redistribution of $Na^+$, $K^+$ and $H^+$ may be involved. Other studies (R.D. Clark and R.E. MacDonald, manuscript in preparation) show that intravesicular hydrolysis of ATP is optimal near pH 6.5 and is stimulated by $K^+$. Mukohata and Kaji have recently suggested (3) that intracellular acidification prevents $bR^-$, $hR^+$ strains of H. halobium from sustaining high ATP levels under prolonged illumination. We have evidence that internal acidification probably also limits hR-driven ATP synthesis in vesicles under the experimental conditions employed in our study.

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