Halobacterium halobium Photophosphorylation: Illumination-Dependent Increase in the Adenylate Energy Charge and Phosphorylation Potential†

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ABSTRACT: When *Halobacterium halobium* cells containing bacteriorhodopsin (bR) and an H^+-ATPase are illuminated, both the rate and extent of ATP synthesis are controlled by the light intensity. We measured the intracellular ATP, ADP, and AMP concentrations in the steady state at light intensities up to 100 mW/cm^2 and as a function of illumination time at 2.4 and 25 mW/cm^2. From these data, the adenylate energy charge [EC = ([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP])] was calculated. Both ADP and AMP concentrations decrease as ATP is synthesized. The rate and increase in EC and [ATP] show a similar dependency on illumination intensity. The percentage that each nucleotide contributes to the total pool varies smoothly with EC, indicating an intracellular adenylate kinase reaction: 2ADP $\leftrightarrow$ AMP + ATP, with K_{app} = 0.5. The interaction of three reactions, light absorption by bR, ATP synthesis by the H^+-ATPase, and the adenylate kinase reaction, is necessary and sufficient to define the measured adenosine nucleotide distributions. The steady-state transmembrane potential ($\Delta\psi$) and pH gradient (ΔpH) were measured by using flow dialysis of [^{3}H]triphenylmethylphosphonium ion and [^{14}C]benzoic acid, respectively. We varied $\Delta\psi$ and ΔpH by illumination and by adding the ionophores carbonyl cyanide *m*-chlorophenylhydrazone and triphenyltin chloride. Both in the dark and at high illumination intensities, the intracellular [ATP], EC, and phosphorylation potentials ($\Delta G_p = RT \ln [[ATP]/([ADP][P_i])]$) did not correlate with the measured $\Delta\psi$, ΔpH, or protonmotive force. Under the conditions tested, the steady-state ΔG_p maintained by the H^+-ATPase–adenylate kinase system and the protonmotive force are not in equilibrium in *H. halobium* cells. In fact, we observed light-driven decreases of ΔG_p in the absence of any measurable protonmotive force.

Halobacterium halobium cells containing the light-driven proton pump bacteriorhodopsin (bR)[1] can photophosphorylate ADP to ATP under anaerobic conditions via a DCCD-inhibitable H^+-ATPase (Danon & Stoeckenius, 1974) [for general reviews, see Lanyi (1978) and Stoeckenius et al. (1979)]. This result and the demonstration of light-driven ATP synthesis in proteoliposomes reconstituted with bR and mitochondrial ATPase (Racker & Stoeckenius, 1974) contributed to the general acceptance of Mitchell's chemiosmotic theory (Mitchell, 1966). The theory requires that in coupled membranes the steady-state protonmotive force between the two aqueous phases and the phosphorylation potential are in equilibrium. It has been extensively tested in mitochondria (Slater et al., 1973; Padan & Rottenberg, 1973; Azzone et al., 1978; Holian & Wilson, 1980; Moore & Bonner, 1981), chloroplasts (Ort et al., 1976; Giersch et al., 1980), and bacteria (Casadio et al., 1978; Baccharini-Melandri et al., 1977; Hitchens & Kell, 1982; Maloney & Hansen, 1982). Some observations appear incompatible with the theory, i.e., at least in its most rigorous form which assumes a fixed stoichiometry of protons translocated per ATP formed.

H. halobium cells have the advantages for testing energy-coupling concepts that bR is the best-established example of an electrogenic proton pump and that it occurs in distinct crystalline patches in the coupling membrane, hindering any direct interaction of the proton pump with the ATPase. However, it cannot be excluded that some fraction of the bR molecules may occur as monomers and interact directly with the H^+-ATPase. The *H. halobium* photophosphorylation studies so far indicate (Bogomolni et al., 1976; Bakker et al., 1976; Michel & Oesterhelt, 1976, 1980a,b; Hartmann & Oesterhelt, 1977; Wagner et al., 1978) that strict chemiosmotic coupling appears to hold under some, but not all, conditions (see Discussion). The results presented here extend these findings.

We have measured the adenylate energy charge, phosphorylation potential, and protonmotive force and found that an endogenous adenylate kinase reaction plays an important role in *H. halobium* bioenergetics. Our goal is to accurately determine the chemical energy stored in the adenylate pool under a variety of experimental conditions. This will then serve as a basis for correlating the measured protonmotive force with the phosphorylation potential in *H. halobium* and for testing energy-coupling theories.

Materials and Methods

Growth of Cell Cultures. H. halobium strain R1 was grown in complex medium at 37 °C as described previously (Oest-

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[1] Abbreviations: bR, bacteriorhodopsin; AMP, adenosine 5′-monophosphate sodium salt (Sigma Chemical Co.); ADP, adenosine 5′-diphosphate sodium salt (Sigma Chemical Co.); ATP, adenosine 5′-triphosphate sodium salt (Sigma Chemical Co.); FLE, firefly lantern extract (FLE-50) and D-luciferin (Sigma Chemical Co.); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Sigma Chemical Co.); Mes, 2-(*N*-morpholino)ethanesulfonic acid (Sigma Chemical Co.); menadione, 2-methyl-1,4-naphthoquinone (oil soluble, Sigma Chemical Co.); myokinase, ATP:AMP phosphotransferase (grade III, Sigma Chemical Co.); NADH, nicotinamide adenine dinucleotide (reduced form, Sigma Chemical Co.); pyruvate kinase, ATP:pyruvate 2-*O*-phosphotransferase (type II, Sigma Chemical Co.); [^{14}C]ACS, ^{14}C-labeled acetylsalicylic acid (33.4 mCi/mmol, New England Nuclear); [^{14}C]BA, ^{14}C-labeled benzoic acid (22.3 mCi/mmol, New England Nuclear); [^{14}C]DMO, ^{14}C-labeled dimethyloxazolidine-2,4-dione (46.6 mCi/mmol, New England Nuclear); [^{3}H]TPMP$^+$, ^{3}H-labeled triphenylmethylphosphonium ion, bromide salt (1.13 Ci/mmol, New England Nuclear); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone (K and K Laboratories); DCCD, *N*,*N*′-dicyclohexylcarbodiimide (Pierce Chemical Co.); TPT, triphenyltin chloride (Alfa Products); EC, energy charge; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid; cAMP, adenosine cyclic 3′,5′-phosphate; pmf, protonmotive force; AK, adenylate kinase; MDR, menadione reductase.

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erhelt & Stoeckenius, 1974). After 4–5 days' growth with aeration, the cells were harvested and washed twice with and resuspended in basal salt (BS: 4 M NaCl, 27 mM KCl, and 81 mM $MgSO_4$) or high potassium basal salts (K–BS: 3 M KCl, 1.25 M NaCl, and 81 mM $MgSO_4$) at 10 times the harvest titer. The cells were stored at 4 °C in the dark.

Experimental Conditions. The cells were diluted 3–5-fold into the given salt medium buffered with 10 mM Mes (pH 6.0) or Hepes (pH 7.5). To begin all experiments, the cell suspension (~1 mg of protein/mL) was placed in a nitrogen-purged chamber thermostated at 22 °C, illuminated (100 mW/cm^2, 500–700 nm) for 20 min, and then kept in the dark for 20 min. For adenosine nucleotide determination, 50-μL samples were taken in the dark or during the second illumination and rapidly diluted into 2.5 mL of FLE assay buffer (see below) plus 1 mM EDTA. The illumination intensity was adjusted with neutral density filters and measured with a Laboratory Data Control Model 68 Kettering radiant power meter.

The internal water-permeable volume and internal pH were measured by standard techniques (Addanki et al., 1968; Bakker et al., 1976; Michel & Oesterhelt, 1976) except that the cells were centrifuged through a layer of dimethyl phthalate (50 μL; Eastman Organic Chemicals). The internal volume was determined by the distribution of ^3H_2O (1 mCi/g; New England Nuclear) and [^{14}C]sucrose (396 mCi/mmol; New England Nuclear), while the internal pH was measured by using ^3H_2O and a ^{14}C-labeled weak acid (see Table III). Two hundred microliter samples were taken from preilluminated cell suspensions (~5 mg of protein/mL) with the appropriately labeled compounds added 5 min prior to sampling. The samples were layered over the dimethyl phthalate in 0.5-mL microfuge tubes and centrifuged for 2 min (Beckman Model 152 microfuge). A portion of the pellet sliced from the microfuge tube and a 50-μL aliquot of the supernatant were each dispersed in 1 mL of water; 200 μL of these samples, 1 mL of 1% SDS, and 9 mL of Instagel liquid scintillation cocktail (Packard Instrument Co.) were counted in a Beckman LS7000 LSC. The counts were corrected for the sample count spillover between channels.

The transmembrane electric potential ($\Delta\psi$) and pH gradient (ΔpH) in the dark and during illumination of cell suspensions (5–10 mg of protein/mL) were measured by using the flow dialysis assay described previously (Lanyi et al., 1979; Dancshazy et al., 1983).

Biochemical Assays. Cell suspensions were monitored for lysis with the menadione reductase–NADH assay (MDR) (Lanyi, 1972) and used only if the degree of orientation and/or intactness was >95%. Protein content was determined by using the Lowry assay. Bacteriorhodopsin content was measured by the light–dark adaptation assay of cell lysates (Bogomolni et al., 1980). The cells averaged 1.8 nmol of bR/mg of protein (based on six cultures). Inorganic phosphate was determined (Lin & Morales, 1977) by using phosphate standards and experimental samples in distilled water containing 50-fold diluted BS or K–BS. Adenylate kinase activity was measured according to Markland & Wadkins (1966).

Adenosine Nucleotide Measurements. Nucleotide concentrations were measured by using a luminescence biometer (Du Pont) following variations of standard procedures (Kimmich et al., 1975; Holm-Hansen & Karl, 1978). Luciferase enzyme was prepared in the dark by stirring 50 mg of FLE-50 with 5 mL of water for 30 min at 4 °C. The supernatant was collected after centrifugation (12000*g*, 10 min) and 1.0 mM luciferin addition and was stored frozen. Aliquots were thawed, diluted 10-fold into Millipore-filtered assay buffer [10 mM Mops (pH 7.9) + 10 mM $MgSO_4$], kept 1–2 h in the dark at room temperature, and then stored overnight at 4 °C to hydrolyze residual ATP; 100 μL was used per ATP assay. ATP standards (1 mg of ATP/mL) were stored frozen in assay buffer + 1 mM EDTA. Standard curves showing FLE light intensity vs. [ATP] per 10-μL injection were made by using a dilution series prepared in assay buffer plus 50-fold-diluted basal salts. Lysed cell samples containing 0.25–5 pmol of ATP/10-μL injection were assayed. The original salt medium was diluted 500-fold in the final assay mixture, assuring no inhibition of FLE luminescence.

ADP and AMP concentrations were measured by enzymatic conversion to ATP. The length of sample incubation with myokinase and/or pyruvate kinase depended on the specific activity of the kinase enzymes. The conversion of standard nucleotide mixtures showed that ADP and AMP were converted with 95% and 85% efficiencies, respectively (see Figure 1). ATP standards carried through the same procedures showed <5% loss. With no enzymatic conversion, 10-μL samples containing 16 pmol of 5′-ATP, 19 pmol of 5′-ADP, 26 pmol of 5′-AMP, or 25 pmol of cAMP gave relative FLE assay light intensities of 100, 2.03, 0.03, and 0.07, respectively.

Table I: Effect of Experimental Conditions on the Measured Internal Volume of *H. halobium* R1[a]

external salt	pH	additions	determinations	V_i (μL/mg of protein)
basal salt	6.0	none	12	2.24 ± 0.30
		10 μM TPT	2	2.28
	7.5	none	4	2.68 ± 0.27
		32 μM CCCP	2	2.47
K$^+$-basal salt	6.0	none	4	2.54 ± 0.16
	7.5	none	2	2.94
				2.43 ± 0.32[b]

[a] Internal volume was determined by the distribution of ^3H_2O and [^{14}C]sucrose as described under Materials and Methods. All determinations were done on cell suspensions under nitrogen purging at 22 °C using 10 μCi of ^3H_2O and 2 μCi of [^{14}C]sucrose per mL. Results from five separate cell cultures are shown. [b] Average value.

Table II: Intra- and Extracellular Concentrations of ATP and Inorganic Phosphate in *H. halobium* R1

assay (units)	external salt: BS (pH 6.0)	external salt: K-BS (pH 7.5)
MDR (nmol of NADH mg^{-1} min^{-1})[a]		
intact cells	0.8	0.8
lysed cells	44.6	46.0
ATP (mM)[b]		
internal	1.53 ± 0.1	0.45 ± 0.1
external	3×10^{-4}	5×10^{-4}
P_i (mM)[b]		
internal	28.7 ± 4.5	26.9 ± 2.0
external	5×10^{-2}	nd[c]

[a] Cells were lysed by incubation with 0.07% Triton X-100 for 1 h. [b] Internal concentrations were calculated on the basis of an internal volume of 2.4 μL/mg of protein. External concentrations were measured in the supernatant after centrifuging 1 mL of cell suspension at 27000*g* for 15 min. [c] nd, none detected.

Results

Characterization of H. halobium R1 Cells. Tables I–IV give the values for the intactness, internal volume, internal phosphate concentration, internal pH, and DCCD inhibition of photophosphorylation in N_2-purged R1 cells. The data are

Table III: Measurement of the Internal pH of *H. halobium* R1 Using DMO, ASA, and BA

probe[a]	external salt	external pH	addition (μM)	determinations	C_r	internal pH
DMO	BS	7.5		4	0.86	7.50 ± 0.12
	K-BS	7.5		2	1.11	7.59
	BS	6.0		6	1.36	6.61 ± 0.07
	K-BS	6.0		4	1.05	6.09 ± 0.15
ASA	BS	6.0		2	3.22	7.16
			TPT (10)	2	0.83	6.12
	K-BS	6.0		2	5.10	7.03
			CCCP (32)	2	2.00	6.52
BA	BS	6.0		2	3.01	7.12
			CCCP (32)	2	1.25	6.56
	K-BS	6.0		2	7.07	7.19
			TPT (10)	2	1.14	6.12
BA	BS	6.2		12	3.53	7.40 ± 0.09
			TPT (10)	8	0.78	6.18 ± 0.15

[a] Internal pH was determined by the distribution of ^3H_2O and the ^{14}C-labeled weak acid probe as described under Materials and Methods. All determinations were done on cell suspensions under nitrogen purging at 22 °C using 10 μCi of ^3H_2O and 1 μCi of ^{14}C-labeled weak acid per mL. Final probe concentrations were 22 μM DMO, 33 μM ASA, or 44 μM BA.

Table IV: Effect of DCCD Treatment on Photophosphorylation and L-Arginine-Driven Substrate-Level Phosphorylation in *H. halobium* R1

expt[a]	DCCD	ATP (μg/mg of protein)[b] dark	light	dark	L-Arg
A	−	1.23	4.35	1.23	4.35
B	−	0.90			3.55
C	+	0.45	0.71	0.38	3.8
D	+	0.49			3.7

[a] The ATP content of cells suspended in basal salts + 67 mM Mes (pH 6.0) was determined as described under Materials and Methods. Where indicated, the cells were pretreated with 25 μM DCCD at 0 °C for 24 h as described by Michel & Oesterhelt (1980a). [b] The indicated incubations were sequential, each for 20 min. The final L-arginine concentration was 0.18%. Each value is the average of triplicate samples.

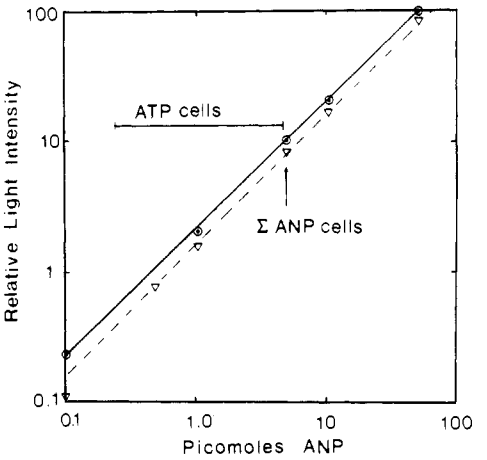

FIGURE 1: FLE luminescence assay calibration curve for ATP (⊙) and enzymatically converted ATP + ADP + AMP (▽) standards. Samples containing serial dilutions of ATP or an equimolar mixture of ATP, ADP, and AMP were treated with myokinase and pyruvate kinase and then assayed as described under Materials and Methods. Each point is the average of three determinations. Note the myokinase–pyruvate kinase conversion efficiency is <100% and must be taken into consideration when calculating the adenosine nucleotide contents of the samples. Also shown are the assayed quantities for the range of ATP values found in the lysed cell samples, ATP_{cells}, and a typical value for the total nucleotide pool, $\sum ANP_{cells}$ (see text).

presented here to provide a comparison with other reports (Bakker et al., 1976; Michel & Oesterhelt, 1976, 1980a) and are used to calculate the internal adenosine nucleotide concentrations, phosphorylation potentials, and protonmotive force values given below.

The internal volume (V_i), defined as the internal water space in cell pellets, was calculated from the differential distribution of ^3H_2O and [^{14}C]sucrose (see Materials and Methods). V_i was determined for each separate cell culture and the measured value used for calculations of the pmf or internal pH. Table I gives the V_i values obtained under the conditions used in our work. On the basis of 26 determinations, an average V_i value of 2.4 μL/mg of protein was found. Effects of the external salt composition and pH or the addition of ionophores were within experimental errors. Internal volumes of 2.5 μL/mg of protein (Bakker et al., 1976) and 3.2 μL/mg of protein (Wagner et al., 1978) have been reported.

H. halobium has a membrane-bound, NADH-dependent menadione reductase (MDR) accessible only from the cytoplasm. MDR serves as a convenient marker enzyme for damage to the permeability barrier (Lanyi, 1972). Cell cultures were used only if they were >95% intact by this assay. In Table II, the internal ATP and inorganic phosphate (P_i) concentrations are given for cells suspended in BS (pH 6.0) and K–BS (pH 7.5). The internal ATP concentration is at least 1000-fold higher than the external ATP concentration. P_i is also concentrated inside the cells and has a value of ~28 mM independent of the external conditions. This phosphate concentration is 3–5 times greater than the total adenylate pool size (see Figure 2). Michel & Oesterhelt (1980a,b) reported a value of ~50 mM P_i both in the dark and after 10-min illumination; this difference in concentration is probably due to growth conditions. Table II demonstrates that the *H. halobium* cell is a closed system in this respect. Thus, the problem of nucleotide exchange with the external volume does not affect the interpretation of energy-coupling results.

The internal pH of *H. halobium* R1 cells, and therefore the transmembrane pH gradient (ΔpH), has been measured by using the uptake of the weak acid dimethyloxazolidine-2,4-dione (DMO) (Bakker et al., 1976; Michel & Oesterhelt, 1976). Both groups found that the internal pH was constant at 7–7.5 for external pH below 7.5, although the measurement was inaccurate for ΔpH values <0.4 (Michel & Oesterhelt, 1980a). As shown in Table III, when R1 cells were suspended in either BS or K–BS at pH 7.5, the internal pH was 7.5 measured with DMO. However, at external pH 6.0, the internal pH was found to be 6.6 in BS and 6.1 in K–BS. A priori, there is no reason to expect this pattern of changes in the internal pH. Also given in Table III are the values for

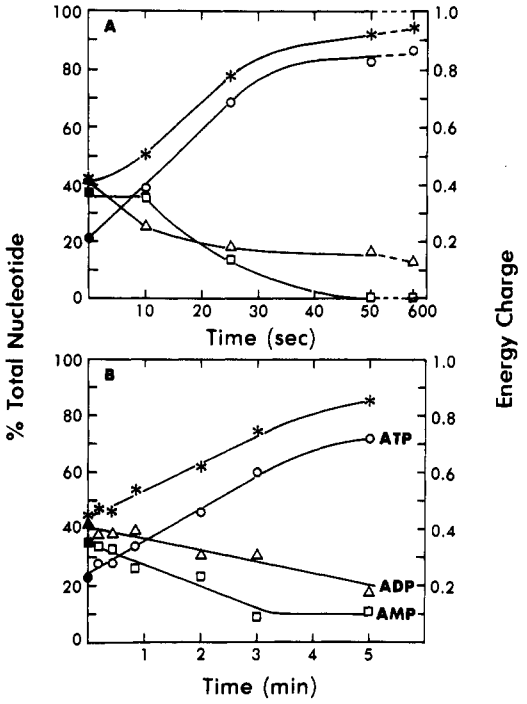

FIGURE 2: Changes in the adenosine nucleotide pool distribution vs. time. Anaerobic *H. halobium* R1 cell suspensions in BS + 10 mM Mes (pH 6.0) were illuminated at (A) 25 or (B) 2.4 mW/cm^2 and samples taken at the times indicated. The ATP (○), ADP (△), and AMP (□) contents were determined as described under Materials and Methods. The time zero values (closed symbols) are the average of triplicate samples taken prior to illumination. The values are expressed as the percentage that each nucleotide contributes to the total pool. The total internal nucleotide pool concentration was 8.9 ± 0.5 mM based on an internal volume of 2.5 μL/mg of protein. The corresponding energy charge (*) values were calculated by using eq 1.

the ratio of the DMO concentration in the total centrifuge pellet water to that of the extracellular water (C_r). C_r monitors the amount of labeled weak acid taken up by the cells (Addanki et al., 1968). In all cases, the C_r values are low for DMO, thus leading to the measurement inaccuracy mentioned above.

Other weak acid probes (acetate, benzoate, acetylsalicylate) with lower pK_a values were tested since they should give larger C_r accumulation ratios for the same pH gradient (Lanyi et al., 1979). Attempts to measure the internal pH of intact cells with acetate led to very inconsistent results (data not shown), probably due to cellular metabolism (Aitken & Brown, 1969). Benzoic acid and acetylsalicylic acid gave more consistent results and larger C_r values than DMO. For cells in BS at pH 6.2, the internal pH was 7.4 ± 0.1 pH units when measured with benzoic acid. Both benzoic and acetylsalicylic acids gave the same value of internal pH 7.1 whether the cells were suspended in BS or K–BS at pH 6.0. The ionophore triphenyltin chloride (TPT) collapses the pH gradient (Table III) by electroneutrally exchanging Cl^- and OH^- across the membrane (Selwyn et al., 1970). Since there is no chloride gradient in *H. halobium* (Ginzburg, 1978), the high chloride concentration should allow the proton/hydroxyl gradient to be dissipated as we observed. At high concentration, the uncoupler CCCP lowers the pH gradient by ~0.5 pH unit but does not completely collapse it.

When *H. halobium* cells containing bR are suspended in basal salts without nutrients and incubated under N_2 in the dark, the ATP level falls to a low value. The cells are able to maintain this ATP level for long periods (Hartmann & Oesterhelt, 1977; Danon & Stoeckenius, 1974; Arshavsky et al., 1981). If the cells are then aerated or illuminated with wavelengths which excite bR, the ATP level rises rapidly by 2–5-fold depending on the conditions and the cell culture. In agreement with Oesterhelt's group (Hartmann & Oesterhelt, 1977; Michel & Oesterhelt, 1980b), we found that, as a function of light intensity (band-pass 500–700 nm), (a) the photophosphorylation rate is linear up to 25 mW/cm^2 and then saturates at ~0.15 nmol of ATP^{-1} (mg of protein)$^{-1}$ (data not shown) and (b) the extent of ATP synthesis saturates between 3 and 10 mW/cm^2 depending on the cell culture (see Figures 3 and 5). ATP synthesis driven by respiration or illumination can be inhibited by DCCD (Danon & Stoeckenius, 1974; Michel & Oesterhelt, 1980a) (Table IV). It has been reported that under anaerobic conditions the uptake of L-arginine can drive substrate-level ATP synthesis independent of the H^+-ATPase (Hartmann et al., 1980). As shown in Table IV, for untreated cells the extent of ATP synthesis is the same whether driven by light or L-arginine. When the cells are pretreated with DCCD, photophosphorylation is inhibited while L-arginine-driven ATP synthesis is unaffected. Because amino acids are the main carbon source for *H. halobium*, the large stimulation of substrate-level phosphorylation by L-arginine indicates that basal metabolism is low under these experimental conditions.

Table V: Localization of bR, MDR, and AK Activities in the Membrane and Cytoplasmic Fractions of Freeze-Thawed *H. halobium* R1

sample[a]	bR (μg/mg of protein)	MDR[b] sp act.	AK[b] sp act.	AK[b] total act.
P-1	81	28	44	11
S-1	44	5	217	88
P-2	237	21	83	5
S-2	<1	<2	314	86
S-2D	nd[c]	nd	342	63

[a] P-1 and S-1 are the pellet and supernatant fractions obtained by centrifuging broken cells at 27000*g* for 45 min. S-1 was then centrifuged at 250000*g* for 90 min to give P-2 and S-2. S-2 was dialyzed overnight at 0 °C to give S-2D. All samples were prepared and assayed in K-BS + 20 mM Tris (pH 7.5). [b] The lowest detectable activities for bR, MDR, and AK assays were 1 μg/mg of protein, 2 nmol of NADH min^{-1} (mg of protein)$^{-1}$, and 5 nmol of NADH min^{-1} (mg of protein)$^{-1}$, respectively. [c] nd, not determined.

Endogenous Adenylate Kinase Activity and Light-Dependent Changes in the Adenosine Nucleotide Pool. An endogenous adenylate kinase (AK) reaction plays an important role in *H. halobium* photophosphorylation. This AK activity is mainly localized in the cytoplasm (Table V). R1 cells were broken by freeze–thawing in liquid nitrogen. The membrane and cytoplasmic fractions were separated by centrifugation using both bR and menadione reductase activities as markers for the membrane fraction. Of the total adenylate kinase activity, ~15% sediments with the membrane fractions while ~85% remains in the supernatant. It is not clear whether the AK activity in the membrane fraction arises from a membrane-bound protein or is an artifact; 90% of the soluble AK activity could be inhibited by P^1,P^5-bis(5′-adenosyl)pentaphosphate, a specific inhibitor of adenylate kinase (Lienhard & Secemski, 1973).

Figure 2 shows the changes in AMP, ADP, ATP, and the adenylate energy charge (EC) levels for anaerobic R1 cells illuminated at 25 (A) and 2.4 mW/cm^2 (B). The energy charge (Atkinson, 1968) is defined as

$$EC = \frac{[ATP] + [ADP]/2}{[ATP] + [ADP] + [AMP]} \quad (1)$$

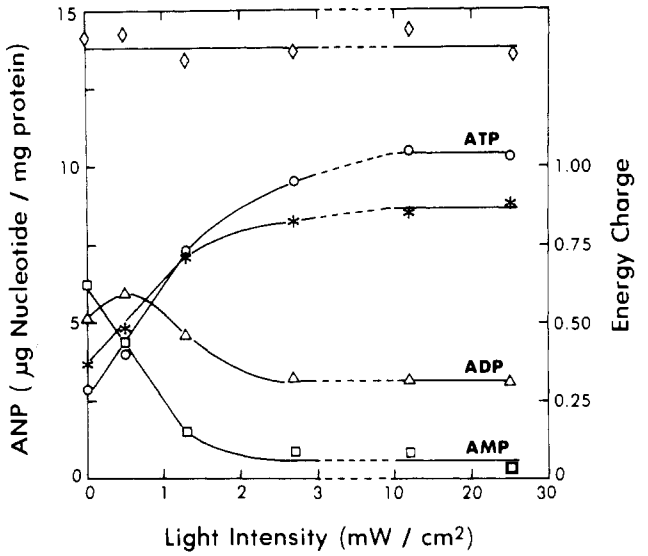

FIGURE 3: Light intensity dependence of the steady-state adenosine nucleotide pool distribution. Anaerobic cell suspensions in BS + 10 mM Mes (pH 6.0) were illuminated at the given light intensity for 15–30 min and triplicate samples taken. The averaged ATP (○), ADP (△), AMP (□), total nucleotide pool (◇), and adenylate energy charge (*) values are shown.

where the amounts are expressed in mole fractions of the total adenylate pool. EC measures both the adenosine nucleotide distribution within the total pool and the amount of phosphate bond energy stored in the pool. At 2.4 mW/cm² illumination, the ATP content and EC increase with time, approaching the steady-state level in ~5 min. At 25 mW/cm², the changes in the nucleotide distribution are similar, but the steady-state levels are reached in <1 min. If light-driven proton pumping and ATP synthesis were the only reactions taking place, AMP should remain relatively unchanged, i.e.

$$ADP + nH^+_{out} + P_i \leftrightarrow ATP + nH^+_{in} + H_2O \quad (2a)$$

$$\Delta G_{ATPase} = \Delta G^{\circ\prime} + RT \ln \frac{[ATP]}{[ADP][P_i]} \quad (2b)$$

and

$$\frac{\Delta\tilde{\mu}_{H^+}}{F} = \frac{\Delta G^{\circ\prime}}{nF} + \frac{RT}{nF} \ln \frac{[ATP]}{[ADP][P_i]} \quad (2c)$$

where $\Delta\tilde{\mu}_{H^+}/F$ is the driving force applied to the H^+-ATPase via proton pumping by bR. However, the rapid drop in AMP levels indicates that at least one other intracellular reaction significantly affects the nucleotide distribution in the steady state.

The endogenous adenylate kinase reaction

$$2ADP \leftrightarrow AMP + ATP \quad (3a)$$

$$\Delta G_{AK} = \Delta G^{\circ\prime} + RT \ln \frac{[ATP][AMP]}{[ADP]^2} \quad (3b)$$

could account for this. Since the total adenylate pool size was constant under all conditions tested (see Figures 2 and 3), an AK reaction in equilibrium should be apparent from the nucleotide distribution. According to Atkinson & Chapman (1979), the EC equals half the number of anhydride-bound phosphate groups per adenosine so that at a constant pool size the EC monitors the level of phosphorylation activity in the system. In the presence of AK, the concentrations of ATP, ADP, and AMP should be near the equilibrium defined by eq 3a. However, while the relative contribution of each nucleotide to the pool will vary with the EC, the AK reaction

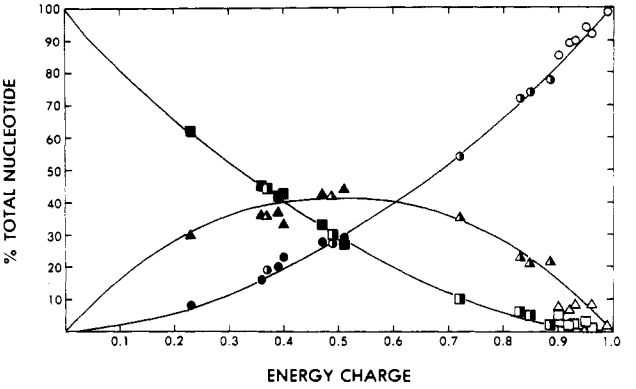

FIGURE 4: Percentage contributions of ATP (circles), ADP (triangles), and AMP (squares) to the total nucleotide pool vs. the adenylate energy charge. The lines are drawn for the adenylate pool distribution expected for an adenylate kinase reaction in equilibrium (K_{app} = 0.5). The half-filled symbols from left to right are the light intensity dependence data shown in Figure 3 for 0–25 mW/cm². The filled and open symbols are data from the dark and illuminated samples, respectively, indicated in Table VI.

does not change the energy charge. Any process which drives phosphorylation of ADP to ATP will increase the EC, but the relative nucleotide distribution will remain in AK-mediated equilibrium in the steady state.

Under the conditions used here, the steady-state levels of ADP converted to ATP are controlled by the illumination intensity (Figure 3). The distribution of nucleotides changes in a complicated manner as the light intensity increases from 0 to 10 mW/cm² while the EC increases monotonically from 0.4 to 0.9. Figure 4 shows these data (half-filled symbols) plotted as the percentage of total nucleotide vs. the EC. The lines are the calculated distributions expected for an AK reaction with K_{app} = 0.5. This plot shows that the steady-state adenylate distribution is controlled by both the phosphorylation activity of the bR-driven H^+-ATPase (eq 2c) and the intracellular AK reaction (eq 3a). A similar result was obtained in intact chloroplasts (Giersch et al., 1980). Since the EC can be driven to its maximum value (Figure 4 and Table VI), we may assume that metabolic utilization of ATP under the experimental conditions used here is low. Thus, the interaction of three reactions, light absorption by bR, ATP synthesis by the H^+-ATPase, and the AK reaction, is necessary and sufficient to define the adenosine nucleotide distributions measured in *H. halobium*.

Relationship of the Adenylate Energy Charge and Phosphorylation Potential to the Measured Protonmotive Force. The coupled bR, H^+-ATPase, and AK reactions provide an experimental framework for examining energy coupling between the transmembrane protonmotive force and photophosphorylation in *H. halobium*. Before testing this relationship, we will discuss the antagonistic effects of the light intensity and ionophore concentration on photophosphorylation. As shown in Figure 5 and reported by Hartmann & Oesterhelt (1977), at a given light intensity the steady-state ATP level is lowered by increasing CCCP concentrations, but as the light intensity increases, the inhibition is reduced. Similar results were obtained for the effect of light intensity and TPT concentration (data not shown). The rates of photophosphorylation are not completely restored by high light intensities in either case. Light intensities >100 mW/cm² can overcome the inhibition of photophosphorylation but cannot prevent the collapse of $\Delta\psi$ and ΔpH by CCCP and TPT (see Table VI).

The criterion for energy coupling between the driving force and the driven H^+-ATPase reaction is given by eq 2c. The

Table VI: Effects of External Salt, pH, and Illumination on the Adenylate Energy Charge, Phosphorylation Potential, and Protonmotive Force of *H. halobium* R1[a]

expt	external salt	external pH	light	addition	Δψ (mV)	ΔpH (mV)	pmf[b] (mV)	EC[c]	59 log [[ATP]/([ADP]·[Pi])][d] (mV)	$\Delta\tilde{\mu}_{H^+}/F$[e] (mV)
A	BS	6.0	−		−68	−66	−134	0.51*	79	135
			+		−78	−74	−152	0.99*	182	170
			+	CCCP	<−15	−64	−64	0.77†	141	156
			−	CCCP	<−15	−64	−64	0.28	67	131
B	BS	6.0	−	TPT	−62	<−15	−62	0.36*	72	118
			+	TPT	−96	<−15	−96	0.92*	147	158
			+	TPT + CCCP	−40	<−15	−40	0.98	145	142
			−	TPT + CCCP	<−15	<−15	<−15	0.42	92	125
C	BS	7.5	−					0.48*	81	136
			+					0.95*	178	168
			+	CCCP				0.84	126	151
			−	CCCP				0.29	62	130
D	K-BS	6.0	−		−74	−56	−130	0.40*	80	136
			+		−92	−72	−164	0.96*	152	160
			+	CCCP				0.90	127	151
			−	CCCP				0.32	69	132
E	K-BS	6.0	−	TPT	<−15	<−15	<−15	0.39*	74	119
			+	TPT	−85	<−15	−85	0.93*	152	145
			+	TPT + CCCP	<−15	<−15	<−15	0.96	150	144
			−	TPT + CCCP	<−15	<−15	<−15	0.50	85	122
F	K-BS	7.5	−					0.23*	58	128
			+					0.90*	143	157
			+	CCCP				0.83	128	152
			−	CCCP				0.23	71	133

[a] Ionophores were added at 10 μM final concentration 15–20 min prior to the measurement. The illumination source and intensity (120 mW/cm²) were the same in both pmf and nucleotide assays. Each nucleotide value is the average of triplicate samples. The cell suspensions were 5.7 and 1.7 mg of protein/mL in the pmf and nucleotide assays, respectively. [b] Δψ and ΔpH = −59 mV × ΔpH units were measured by flow dialysis. A value of <−15 mV means that the determination was below the resolution of the technique. [c] The nucleotide distributions for data marked with asterisks are plotted in Figure 4. The adenosine nucleotide distribution for the daggered determination did not fit the AK equilibrium relationship shown in Figure 4. [d] An internal phosphate concentration of 30 mM (see Table II) was used. [e] Calculated by using eq 2c and assuming $\Delta G^{\circ\prime}$ = 7.5 kcal/mol for experiments A, C, D, and F or 6.5 kcal/mol for experiments B and E (Rosing & Slater, 1972) and a proton/ATP stoichiometry of n = 3 (Bogomolni et al., 1976).

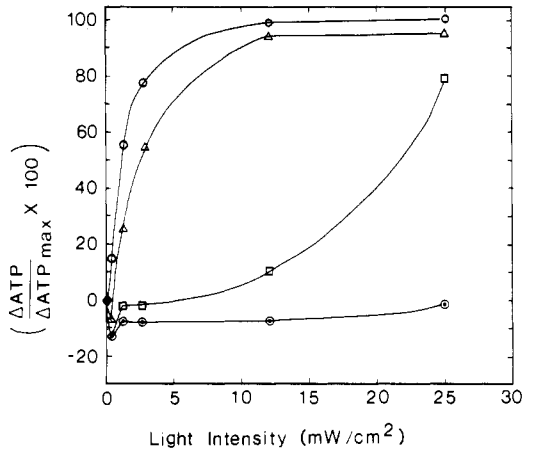

FIGURE 5: Effect of the protonophore CCCP on the light intensity dependence of the steady-state ATP increase. Anaerobic cell suspensions in BS + 10 mM Mes (pH 6.0) were continuously illuminated at the given light intensity. Triplicate samples were taken for ATP determination before (○) and 15 min after each addition: (△) 0.3 μM; (□) 3 μM; (⊙) 30 μM CCCP. Addition of equal amounts of ethanol had no effect on the ATP levels. ΔATP_{max} = 12.7 μg of ATP/mg of protein.

chemiosmotic hypothesis (Mitchell, 1966; Nichols, 1982) predicts that eq 2c should hold under steady-state conditions and that the driving force is the protonmotive force:

$$-\frac{\Delta\tilde{\mu}_{H^+}}{F} = \text{pmf} = \Delta\psi - 2.3\frac{RT}{F}\Delta\text{pH} \qquad (4)$$

Therefore, the pmf should be in one to one equilibrium with the right-hand side of eq 2c. This relationship is tested in Table VI. The components of the pmf, Δψ and ΔpH, were measured by using flow dialysis of the lipophilic cation [^{3}H]TPMP$^+$ and the weak acid [^{14}C]BA (also see Table III), respectively. Binding of TPMP$^+$ to the cells is significant (Michel & Oesterhelt, 1980a) but has been corrected for in the measurements given (Dancshazy et al., 1983); BA does not bind significantly (S. L. Helgerson et al., unpublished results). The pmf measured by the distribution of the probe molecules refers to the extra- and intracellular bulk phases. The energy charge and phosphorylation potential were measured as described above. The nucleotide ratios for the EC determinations in Table VI showed that the total nucleotide pool size was constant in all cases and that the nucleotide distributions were in AK-mediated equilibrium in all but one case (see Table VI, footnote *c*). The experiments were performed on cells from the same culture in separate flow dialysis and adenosine nucleotide assays.

The last column in Table VI, $\Delta\tilde{\mu}_{H^+}/F$, is the apparent driving force calculated from the measured phosphorylation potentials by using eq 2c. $\Delta G^{\circ\prime}$ was assumed to be 6.5 or 7.5 kcal/mol (Rosing & Slater, 1972) (see footnote *e*). Fogt & Rechnitz (1974) have calculated that high metal ion concentrations should not greatly affect $\Delta G^{\circ\prime}$. An H$^+$/ATP stoichiometry n = 3, estimated from the initial proton inflow during photophosphorylation (Bogomolni et al., 1976), was used. Oesterhelt's group (Hartmann & Oesterhelt, 1977) has criticized this measurement and estimates a value of 4.9 ± 1.6 on the basis of the linear relationship between Δψ and the phosphorylation potential under illumination at pH 8 (Michel & Oesterhelt, 1980b). However, they found that n = 3 fit their data for an equilibrium between the pmf and the phosphory-

lation potential in the dark for pH 6–8.5 (Michel & Oesterhelt, 1980a). The absolute value of a fixed n does not qualitatively affect the results given here.

The calculated values for the driving force, $\Delta\tilde{\mu}_{H^+}/F$, are remarkably constant. For internal pH 7, $\Delta\tilde{\mu}_{H^+}/F$ was 133 ± 3 and 158 ± 7 mV in the dark and light, respectively, independent of the external medium or ionophores. For internal pH 6, $\Delta\tilde{\mu}_{H^+}/F$ averaged 121 and 145 mV in the dark and light, respectively. Both sets of measurements show an increase of ~25 mV upon illumination. There is, however, no strict correlation between the calculated $\Delta\tilde{\mu}_{H^+}/F$ values and the measured pmf values shown.

Discussion

We have measured the chemical potential energy stored in the adenylate pool of photophosphorylating *H. halobium* cells and shown that two parameters, the adenylate energy charge and the phosphorylation potential, characterize the adenylate pool. The endogenous adenylate kinase reaction (Table V) catalyzes the steady-state adenosine nucleotide redistribution, so that at a constant adenylate pool size the EC value uniquely defines the distribution (Figure 4). Thus, the EC provides an independent criterion for evaluating the accuracy of the ATP/ADP ratio. From this ratio and the internal phosphate concentration, we calculated the intracellular phosphorylation potentials and using eq 2c converted them to driving force values, i.e., to a measure of the energy applied to the H^+-ATPase to produce the measured ΔG_p. The values chosen for the proton to ATP stoichiometry (n) and the standard free energy of hydrolysis ($\Delta G^{\circ\prime}$) are discussed above. The results indicate that the driving force is relatively constant at 133 mV in the dark and at 158 mV under illumination independent of the external salt medium, pH, or added ionophores (Table VI). When the chemical potential energy of the adenylate pool is expressed as these driving force values, the variation in the measurements is <5% in all cases.

Michel & Oesterhelt (1980b) have reported that in the presence of the uncoupler CCCP (4 μM) photophosphorylation can occur with no concurrent increase in $\Delta\psi$. The experiment was done in basal salts at pH 8.2 so that ΔpH was zero. $\Delta\psi$ was apparently not completely collapsed by CCCP. Thus, the experiment showed that ATP could be synthesized in the light with no increase in the pmf. The present report extends this finding. Both $\Delta\psi$ and ΔpH can be collapsed to low levels (<−15 mV) while the increase in the phosphorylation potential is unaffected at high illumination intensities (Table VI). The same authors reported (Michel & Oesterhelt, 1980a) that in the dark there was an apparent equilibrium between the phosphorylation potential and the pmf. However, under the conditions tested here, we found that once again the pmf can be collapsed with no significant decrease in ΔG_p. If the ATPase has an energy-controlled inhibitor subunit as in mitochondria (Gomez-Puyou et al., 1979), this latter result would be trivial. In the dark, ΔG_p would be a function of the basal metabolism and the action of AK to buffer changes in the adenylate pool (Atkinson & Chapman, 1979).

Evidence against strict chemiosmotic coupling between ΔG_p and the pmf (as measured by the distribution of probes between the internal and external bulk phases) has been reported in chloroplasts, mitochondria, and bacteria (see the introduction for references). Giersch et al. (1980) have found that the pmf and ΔG_p are far from equilibrium in intact chloroplasts and that ΔpH could be decreased by 50% with no decrease in ΔG_p. In mitochondria, $\Delta\psi$ could be decreased by 50% with little effect on the rate of phosphorylation (Padan & Rottenberg, 1973). Wilson has found that while there is no definite coupling between ΔG_p and the pmf in mitochondria, the rate of respiration does strictly correlate with ΔG_p (Holian & Wilson, 1980; Wilson & Forman, 1982). A similar correlation may hold here since the bR proton-pumping rate should be linear with intensity at low illumination levels (Figure 3).

Several alternative energy-coupling mechanisms have been suggested. Synthesis of ATP driven by the pmf in localized regions of the membrane (local anhydrous proton pools or microchemiosmotic theory) has been proposed (Williams, 1962, 1975; Azzone et al., 1978) and criticized (Mitchell, 1977; Westerhoff et al., 1981). Energy coupling via direct conduction along the membrane surface by specific (Baker et al., 1982; Haines, 1983) and nonspecific pathways (Kell, 1978; Hong & Junge, 1983) has also been proposed. A sliding H^+/ATP stoichiometry could also explain the results; this, however, becomes less and less likely as the measured pmf approaches zero. There are not enough data at present to evaluate these alternative mechanisms in *H. halobium* cells. The primary goal of this report was to characterize an experimental system for testing such mechanisms. We have now selected another *H. halobium* strain in which photophosphorylation is driven only by bacteriorhodopsin (S. L. Helgerson et al., unpublished results) and any potential energetic contribution from the electrogenic chloride pump halorhodopsin (Mukohata & Kaji, 1981; Schobert & Lanyi, 1982) can be excluded. We are using this strain for a more rigorous evaluation of the conditions which uncouple the pmf and ΔG_p (Helgerson & Stoeckenius, 1983) based on the relationship of the bR proton-pumping rate, the phosphorylation potential, and the protonmotive force under static head conditions (Rottenberg, 1979).

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Registry No. ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; AK, 9013-02-9; ATPase, 9000-83-3.

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