Regulation of the Bacteriorhodopsin Photocycle and Proton Pumping in Whole Cells of *Halobacterium salinarium*

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ABSTRACT: Single-turnover kinetics of the bacteriorhodopsin photocycle and proton-pumping capabilities of whole cells were studied. It was found that the $\Delta\tilde{\mu}_{H^+}$ of the cell had a profound influence on the kinetics and components of the cycle. For example, comparing the photocycle in whole cells to that seen in PM preparations, we found that (1) the single-turnover time of the cycle was increased ∼10-fold, (2) the mole fraction of M-fast (at high actinic light) decreased from 50 to 20%, and (3) the time constant for M-slow increased significantly. The level of $\Delta\tilde{\mu}_{H^+}$ was dependent on respiration, ATP formation and breakdown, and the magnitude of a pre-existing K^+ diffusion gradient. The size of the $\Delta\tilde{\mu}_{H^+}$ could be manipulated by additions of HCN, nigericin, and DCCD (*N*,*N*′-dicyclohexylcarbodamide). At higher levels of $\Delta\tilde{\mu}_{H^+}$, further changes in the photocycle were seen. (4) Two slower components of M-decay appeared as major components. (5) The apparent conversion of the M-fast to the O intermediate disappeared. (6) A partial reversal of an early photocycle step occurred. The photocycle of intact cells could be changed to that seen in purple membrane suspensions by the energy-uncoupler CCCP or by lysis of the cells. In fresh whole cells, light-induced proton pumping was not seen until the K^+ diffusion potential was dissipated and proton accumulation facilitated by use of a K^+-H^+ exchanger (nigericin), respiration was inhibited by HCN, and ATP synthesis and breakdown were inhibited by DCCD. In stored cells, the pre-existing K^+ diffusion gradient was diminished through slow diffusion, and only DCCD and HCN were required to elicit proton extrusion.

Early investigations performed with intact cells of *Halobacteria* were focused on proton pumping (*1*−*4*). The photocycle of bR has been most often studied in isolated PM at low ionic strengths. The earliest studies of the photocycle in intact cells and the ability of $\Delta\tilde{\mu}_{H^+}$[1] to slow the cycle were those of Dubrovskii et al. (*5*) and Dancshazy et al. (*6*), where it was shown that the decay half-time of the 412 nm absorbance ($M_{t_{1/2}}$) increased significantly with $\Delta\tilde{\mu}_{H^+}$ (mostly in the form of $\Delta\Psi$). The work was extended using closed envelope vesicles (*7*). This time, the model included two M intermediates, and it was found that the steady-state $\Delta\Psi$ increased as the background level of illumination was increased. Also, the amount of the faster decaying form of M decreased and the slower form increased in both amount and lifetime. The ability of $\Delta\tilde{\mu}_{H^+}$ to influence the kinetics of the photocycle in liposomal systems was demonstrated earlier by Hellingwerf, Westerhoff, and their collaborators (*8*−*10*). In a short review of this phenomenon, Westerhoff and Dancshazy (*11*) attributed the first suggestion that $\Delta\Psi$ could influence photocycle kinetics to Wagner and Hope (*12*).

In this paper, we report studies of the influence of $\Delta\tilde{\mu}_{H^+}$ on the bR photocycle in intact cells employing the spectral multichannel approach (*13*, *14*) and SVD deconvolutions analyzed by least-squares fitting procedures developed in our laboratory (*15*, *16*). The objective is to obtain more detailed information about the modifications of the photocycle seen in nonvesicular PM preparations imposed by the conditions of $\Delta\tilde{\mu}_{H^+}$ which are present in whole cell preparations. We have found that the $\Delta\tilde{\mu}_{H^+}$ in whole cells has a pronounced effect on both the kinetics of the photocycle and the ability to pump protons from the cell interior to the external medium. The slowing in photocycle kinetics was seen to be due to the energy-dependent formation of new and slower forms of the M photocycle intermediate.

MATERIALS AND METHODS

Growth of Cells and Preparation of Samples. Cells of *Halobacterium salinarium* ET1001 were grown as described by Oesterhelt and Stoeckenius (*17*), in a 10 L fermentor, harvested by centrifugation, and resuspended in 100 mL of 4 M NaCl containing 5 mg of DNAase and 1 mM $MgCl_2$. The suspension was stirred at room temperature for 1 h, repelleted by centrifugation at 3000*g* for 10 min, and washed two times in 4 M NaCl by resuspension and centrifugation. The final pellet was then suspended in 4 M NaCl to prepare a stock suspension of cells containing 2.2 mg of BR/mL as determined spectrophotometrically after lysing an aliquot of

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[1] Abbreviations: bR, bacteriorhodopsin protein; BR, bacteriorhodopsin ground state (i.e., non-photoactivated); J−O, photocycle intermediates seen during the decay of photoactivated BR back to the ground-state Br; PM, purple membrane; $\Delta\tilde{\mu}_{H^+}$, electrochemical potential for protons, made up of a chemical component ΔpH and an electrical component $\Delta\Psi$ (i.e., membrane potential); SVD-lsq, singular-value decomposition-based least squares; τ, relaxation time constant, which is the reciprocal of the kinetic constant; DCCD, *N*,*N*′-dicyclohexylcarbodamide; CCCP, carbonyl cyanide *m*-chlorophenoyl hydrazone.

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cells in distilled water. The stock suspension was stored at 4 °C. The term "fresh cells" refers to preparations that are less than 1 week old, whereas "aged cells" refers to preparations stored for at least 3 months. Photocycle kinetics, H^+ pumping, and O_2 uptake measurements were performed on cells suspended and incubated overnight in 4 M NaCl, 130 mM K_2SO_4, and 3 mM potassium phosphate at the desired pH.

H^+ pumping and O_2 uptake experiments were performed at pH 7.0. Adjustment to the desired pH was accomplished over a 2 h period by additions of small amounts of 0.1 M KOH or 0.1 M HCl as needed. For kinetic measurements, a suspension of cells containing 10 μg of BR/mL was used, whereas for O_2 uptake and H^+ pumping, 50 μg of BR/mL was used.

Experimental Procedures. Rapid multichannel spectral kinetic measurements and data collection were performed as previously described (*13*, *14*). Data analysis was performed by the SVD-based least-squares procedures (*15*, *16*) termed SVD-lsq in this paper. The rate of O_2 uptake was measured polarographically according to standard techniques (*18*). The rate of proton pumping was measured as previously described (*19*). Illumination for these studies was provided by a 12 V, 100 W quartz halogen bulb (Osram Xenophot HL64625-FCR) mounted in a parabolic reflector (Melles Griot 02RPM001). The position of the filament within the reflector was adjusted to produce a beam with a 2.5 cm diameter at the reaction chamber. Unwanted heat was removed from the beam by passing the light through a 10 cm water filter. To ensure the stability of the light output, the filament was powered by a rechargeable marine battery and the intensity of the light passing through the chamber was continuously monitored by a photodiode (model CD1712, Centronic, Newbury Park, CA) detector with the output displayed on a chart recorder.

RESULTS

Kinetics of the bR Photocycle

As will be shown in the experiments described below, both the kinetics of the photocycle and the pumping of protons are markedly affected by the energy state of the whole cell. Because of this, the kinetics of freshly prepared cells are different from those of cells aged several months at 4 °C.

We present first the kinetics observed in cells that have been stored for ≥3 months because the observed kinetics are easier to understand. It is generally agreed that the major intermediates of the bR photocycle include BR and J to O. In native PM at near-neutral pH, the M consists of two species, one with a τ of ~2 ms (M_f) and one with a τ of ~6 ms (M_s) (*13*). Questions remain about whether there is a single linear sequential photocycle, or one with branches or whether separate cycles may be present. The results discussed in this paper do not depend on these questions. Therefore, no schematic of the photocycle is shown.

Experiments with Aged Cells. Figure 1 shows the influence of external pH on the average turnover time (τ_{av}) for the M intermediates. The parameter τ_{av} is the sum of products of mole fractions and τ for all species of M that are present. Curve 1 is the control for cells incubated overnight at the indicated pHs. The addition of 100 μM DCCD dramatically

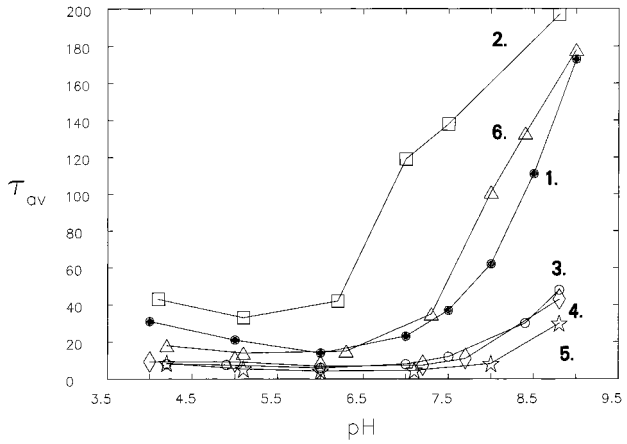

FIGURE 1: Effects of pH and various preincubation conditions on the turnover times of the bR photocycle using aged cells. The cells were stored at 4 °C prior to the experiments whose results are depicted in the figure. τ_{av} is the sum of the products of the mole fraction and τ for all forms of the M intermediate that are present: (1) control cells, incubated overnight and tested the next day at the indicated pH, cells preincubated overnight at the indicated pH in the presence of 100 μM DCCD (2), 10 μM CCCP (3), 100 μM DCCD and 10 μM CCCP (4), and control cells that were lysed prior to measurement by diluting 1:120 with fresh buffer (3 mM potassium phosphate and 130 mM K_2SO_4) and then brought back to 4 M NaCl by the addition of solid NaCl (5) or preincubated with 10 μM nigericin (6).

slowed the photocycle at all pHs (curve 2), whereas the addition of 10 μM CCCP accelerated overall turnover (curve 3). The addition of 10 μM CCCP to the DCCD-treated preparation (i.e., curve 2) completely reversed the slowing of the photocycle caused by DCCD (curve 4). The removal of the "brake" on M turnover seen in the control at the higher pHs can also be accomplished by lysing the cells (curve 5). A simple explanation can account for all of the behavior depicted in these five curves. Above pH 7, $\Delta\tilde{\mu}_{H^+}$ is mainly in the form of $\Delta\Psi$ (*20*) and BR turnover in this pH range leads to a buildup of $\Delta\Psi$ which is known to retard further BR turnover (*5*−*12*). The DCCD level increases in the steady-state level of $\Delta\Psi$ by inhibiting its dissipation in the formation of ATP (*20*), and CCCP increases the rate of turnover by dissipating $\Delta\Psi$ (*20*) even in the presence of DCCD. Cell lysis leads to a further small increase in the rate of turnover, indicating that in the presence of the amount of CCCP that was used, whole cells retained a small residual level of $\Delta\Psi$. The slight effect of 10 μM nigericin on the system (curve 6) is more difficult to explain. Above pH 6.5, there was some increase in τ_{av}. This indicates that although small, some ΔpH was present and converted to an increased $\Delta\Psi$ by nigericin.

The data in Figure 1 do not reveal the full complexity of the underlying changes in the photocycle that are responsible for the changes in the τ_{av} parameter. In Tables 1−6, spectral and kinetic data acquired in the six experiments described in Figure 1 are analyzed by SVD-lsq using models with two, three, and four components. The table numbers correspond to the curve numbers shown in Figure 1. Using statistical criteria of standard errors and dependency values (*15*, *16*), it was determined which of these models is consistent with each set of acquired data. In Table 1, it is seen that for the control experiment (Figure 1, curve 1), near neutrality (pH 6−7.5), only two components of M are involved. In this pH range, the rise in τ_{av} with increasing pH (Figure 1, curve 1)

Table 1: Quantitative Parameters of Photocycle Kinetics (No Additions)[a]

	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0
Time Constant (τ) (ms)								
average	31	21	14	23	37	62	111	173
component 1	3.6	2.7	2.4	2.9	2.4	2.0	2.2	3.3
component 2	17	14	19	28	43	44	65	76
component 3	58	39	–	–	–	83	158	237
component 4	–	–	–	–	–	–	–	–
Mole Percent[b]								
component 1	27	34	29	20	18	14	12	13
component 2	30	25	72	80	81	27	31	20
component 3	44	42	–	–	–	60	58	66
component 4	–	–	–	–	–	–	–	–
O-Formation Parameters								
$\%A_{640}$[c]	57	64	91	100	61	9	12	0
M_f-pos[d] (nm)	665	670	650	670	673	578	583	566
Turnover (milli OD)[e]								
total M	2.2	2.3	2.7	2.6	2.8	2.8	2.8	2.1
BR	4.1	4.3	4.6	4.9	5.1	4.9	5.4	3.8

[a] Table numbers correspond to the curve numbers shown in Figure 1. Therefore, all data in Tables 1−6 were obtained with aged cells. [b] Mole percents are based on the total amount of M present being 100%. [c] $\%A\Delta 640 = 100 \times (A_{640})/(A_{640} + A_{569})$. [d] The wavelength position of the maximum positive absorbance in the SVD-derived difference spectrum for M_f. [e] Single-turnover change in milli OD at 412 (M) or 569 nm (BR).

Table 2: Quantitative Parameters of Photocycle Kinetics (Preincubation with 100 μM DCCD)[a]

	pH 4.1	pH 5.1	pH 6.2	pH 7.0	pH 7.5	pH 8.8
Time Constant (τ) (ms)						
average	43	33	42	119	138	197
component 1	2.3	2.9	2.0	2.1	2.3	3.3
component 2	8.9	9.5	8.7	7.9	11	10
component 3	24	42	53	78	55	48
component 4	79	95	123	212	209	269
Mole Percent						
component 1	21	31	26	20	17	12
component 2	15	3	14	13	11	1
component 3	17	56	47	19	15	17
comonent 4	47	9	13	48	61	70
O-Formation Parameters						
$\%A_{640}$	49	53	7	0	0	0
M_f-pos (nm)	670	676	569	560	569	566
Turnover (milli OD)						
total M	2.7	3.1	3.5	3.1	3.1	3.1
BR	5.2	6.1	6.8	5.5	5.7	4.9

[a] See the footnotes of Table 1.

Table 3: Quantitative Parameters of Photocycle Kinetics (Preincubation with 10 μM CCCP)[a]

	pH 4.2	pH 4.9	pH 6.0	pH 7.0	pH 7.5	pH 8.4	pH 8.8
Time Constant (τ) (ms)							
average	8.1	7.6	6.0	8.0	12	30	48
component 1	2.7	3.3	3.0	3.2	3.0	2.5	3.1
component 2	8.3	10	11	13	21	50	81
component 3	16	45	–	–	–	–	–
component 4	–	–	–	–	–	–	–
Mole Percent							
component 1	29	56	62	54	51	42	42
component 2	54	41	38	46	49	58	58
component 3	17	4	–	–	–	–	–
component 4	–	–	–	–	–	–	–
O-Formation Parameters							
$\%A_{640}$	100	84	88	85	52	16	0
M_f-pos (nm)	647	644	647	653	650	575	575
Turnover (milli OD)							
total M	2.7	3.7	2.7	2.6	2.4	2.8	2.2
BR	5.5	6.2	4.7	4.7	4.8	4.7	4.1

[a] See the footnotes of Table 1.

Table 4: Quantitative Parameters of Photocycle Kinetics (Preincubation with 100 μM DCCD and 20 μM CCCP)[a]

	pH 4.0	pH 5.0	pH 6.0	pH 7.2	pH 7.7	pH 8.8
Time Constant (τ) (ms)						
average	9.3	9.3	6.9	7.4	11	43
component 1	3.7	3.2	2.8	3.5	3.2	2.7
component 2	12	15	11	12	19	31
component 3	–	–	–	–	–	95
component 4	–	–	–	–	–	–
Mole Percent						
component 1	32	47	50	52	52	38
component 2	68	53	50	48	48	26
component 3	–	–	–	–	–	36
component 4	–	–	–	–	–	–
O-Formation Parameters						
$\%A_{640}$	90	74	91	100	54	12
M_f-pos (nm)	664	647	647	644	644	575
Turnover (milli OD)						
total M	4.2	4.0	4.2	4.5	5.2	4.3
BR	6.7	7.3	7.7	7.9	7.7	6.5

[a] See the footnotes of Table 1.

is explained by the increase in the mole percent and τ of the slower component (2). At higher pHs, the marked rise in τ_{av} is ascribed to a new M species with an extremely high τ (component 3) which becomes the major component. The rise in τ_{av} in the lower pH range also results from a new slower M component. The faster M component at pH 7 represents 20% of total M, compared to about 50% for the faster component seen in PM at the same level of actinic light. The mole percent of the faster M species steadily increases to 60% as the level of laser intensity is decreased by use of a series of neutral density filters with optical densities of 0.2, 0.4, 0.6, 0.8, and 1.0 (data not shown). The "O-formation parameters" show the extent of M_f to O conversion previously described in the PM system (*13*). In the SVD-derived difference spectrum, the predominant positive peak which accompanies the disappearance of M_f is seen near 640 nm (i.e., O intermediate). The position of the positive peak which accompanies the disappearance of M_s is near 569 nm (i.e., ground-state BR). The fraction of combined 569 and 640 nm absorbance that is in the A_{640} component is shown in row 10 of Table 1, and the wavelength position of the maximum absorbance is shown in row 11 of Table 1. It appears that for pH ranges below 8, M_f decay is accompanied by the formation of O which appears to be red-shifted in the whole cell. The bottom two rows of the table show the amounts of M and BR turnover (in units of milli OD) for each experiment listed in the table. Maximum turnovers were in the pH range of 6−8.5.

In Table 2 (+DCCD), the correlation of increased τ_{av} with newer, slower forms of M is extended. At all the pHs that were tested, four species of M are present. The extremely high τ_{av} values are ascribed to the markedly increased τ values and high mole percents of the two new M species that are seen. It is also seen that for pH ranges above 5, there is no evidence of concomitant formation of O ac-

Table 5: Quantitative Parameters of Photocycle Kinetics (Lysed Cells)[a]

	pH 4.2	pH 5.1	pH 6.0	pH 7.1	pH 8.0	pH 8.8
Time Constant (τ) (ms)						
average	8.5	5.4	4.3	4.7	8.3	30
component 1	2.3	3.3	2.3	2.7	2.1	1.8
component 2	8.2	6.5	5.1	7.1	15	13
component 3	22	–	–	–	–	62
component 4	–	–	–	–	–	–
Mole Percent						
component 1	8	33	27	53	51	42
component 2	87	67	73	47	49	14
component 3	5	–	–	–	–	45
component 4	–	–	–	–	–	–
O-Formation Parameters						
$\%A_{640}$	*b*	100	100	100	42	0
M_f-pos (nm)	644	647	644	644	569	560
Turnover (milli OD)						
total M	2.6	3.0	4.4	3.9	3.3	3.4
BR	5.3	6.2	7.5	7.6	6.4	6.3

[a] See the footnotes of Table 1. [b] Could not be determined because A_{569} was negative.

Table 6: Quantitative Parameters of Photocycle Kinetics (Preincubation with 10 μM Nigericin)[a]

	pH 4.2	pH 5.1	pH 6.3	pH 7.3	pH 8.0	pH 8.4	pH 9.0
Time Constant (τ) (ms)							
average	18	14	15	35	101	133	178
component 1	2.0	3.5	2.6	2.7	2.0	1.5	2.5
component 2	8.8	13	13	29	17	28	36
component 3	22	38	63	78	55	150	233
component 4	103	–	–	–	146	379	–
Mole Percent							
component 1	14	42	32	22	13	13	18
component 2	43	39	59	54	10	9	7
component 3	39	19	14	23	15	72	75
component 4	6	–	–	–	62	6	–
O-Formation Parameters							
$\%A_{640}$	100	88	83	53	16	0	0
M_f-pos (nm)	662	647	647	615	615	569	577
Turnover (milli OD)							
total M	2.2	2.5	2.7	2.6	2.8	3.3	2.1
BR	3.9	4.6	4.9	4.9	4.6	5.1	3.2

[a] See the footnotes of Table 1.

companying M_f decay (cf. O-formation parameters). In the presence of CCCP (Tables 3 and 4), the new slower forms of M are markedly diminished or eliminated and the apparent decay of M_f to O is reestablished. The same behavior as evoked by CCCP can be attained by simply lysing the whole cells (Table 5). This observation serves a useful purpose as a monitor for cell lysis. At neutrality in the lysed preparation, at least 50% of the M intermediate present exhibits a τ near 7 ms. In the control case (Table 1), no M component with a τ near 7 ms is present, indicating no detectable cell lysis upon storage. The same trends which accounted for an increase in τ_{av} discussed above (new and slower forms of M) also account for the small changes seen in the case with nigericen (Table 6) compared to the control.

Experiments with Fresh Cells. Whereas the decay kinetics of stored cell controls (i.e., no additions) at pH 7 were described best with two components (Table 1), freshly prepared control cells required four components (Figure 2). Both preparations showed M decay in two components with τ values near 2 and 24 ms. The component with a τ of 137

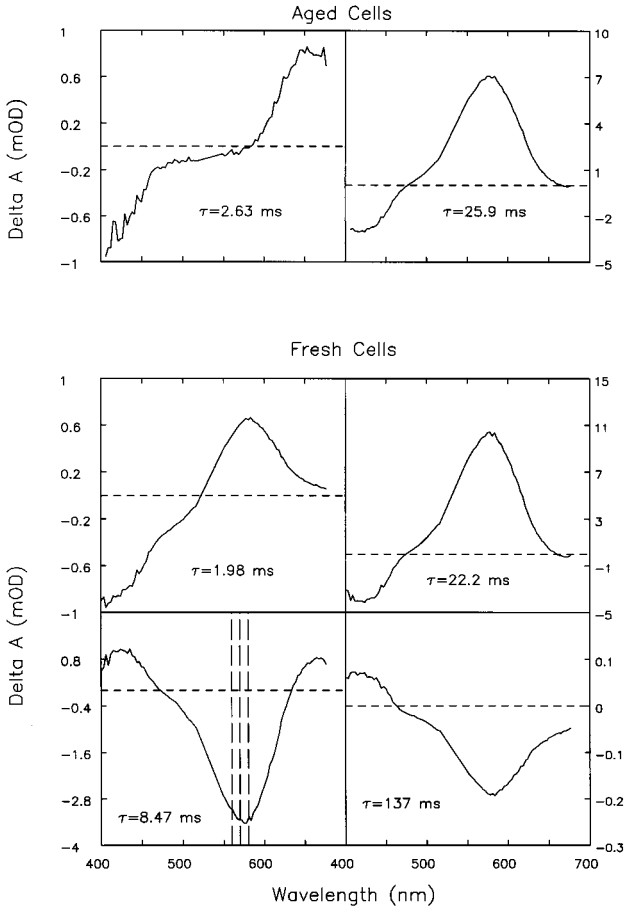

FIGURE 2: Kinetic difference spectra, obtained by SVD-lsq, for the photocycles of controls using either fresh or aged cells at pH 7.0. The relative amount of each transition in milli OD units is indicated by the magnitude shown on the *Y*-axis. The three, long dashed vertical lines in the lower left-hand panel are drawn at 560, 570, and 580 nm.

ms in the fresh cell preparation accounted for only about 1% of the total change in M. We do not want to speculate about any possible significance it may have. The major change between the two preparations is in the appearance of an additional component with a τ of 8.5 ms, showing an apparent anomalous partial reversal of the photocycle. That is, after the peak formation of M is attained, only M decay and BR formation are normally seen. However, in the freshly prepared cells, the step at a τ of 8.5 ms showed the formation of M and O intermediates accompanied by either BR or N disappearance. If there were a simple N to M reversal, the negative peak in the difference spectrum should be centered near 560 nm. However, it is seen that the negative peak occurred nearer to 570 nm. It is impossible to rule out some N to M reversal, but clearly, a major amount of the disappearing species must be BR.

The stoichiometries for M turnover involved in the photocycles seen in fresh and aged cells support the view of a partial photocycle back reaction. With the aged cells, M intermediate decay amounted to a change of 3.75 mOD units, that is, 0.75 mOD at a τ of 2.63 ms and 3 mOD at 25.9 ms. With the same quantity of fresh cells, the decay of M amounted to a change of 4.75 mOD units, 0.85 at a τ of 1.98 ms and 3.9 at 22.2 ms. The additional 1 mOD of decay seen in the fresh cells comes from the 1 mOD unit of M formation seen in the step at 8.47 ms. Because of the

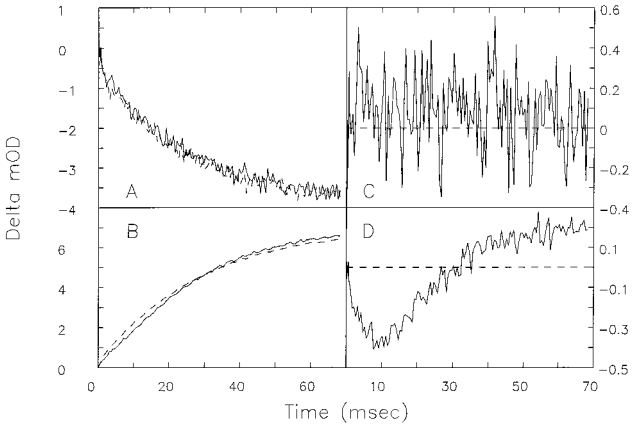

FIGURE 3: Kinetics for decays of the M intermediate at 412 nm (A) and recoveries of BR at 569 nm (B) in fresh (solid lines) and aged cells (dashed lines). These data are from the experiments whose results are depicted in Figure 2. Differences (fresh minus stored cells) in the decays of the M intermediate are shown in panel C, and recoveries of BR are shown in panel D. These are the differences for the curves shown, respectively, in panels A and B.

unexpectedness of such a finding, additional confirmation of the phenomenon was sought from the raw (i.e., not analyzed by the SVD-lsq procedure) data. Figure 3 shows the actual absorbance curves for M decay (panel A) and BR recovery (panel B) in the two experiments shown in Figure 2. No normalization of the data was performed other than setting all absorbances at time zero to zero. Consistent with the SVD-lsq results, it is seen that in the first 10 ms, the fresh cells (relative to the stored cells) showed an enhanced amount of M intermediate and a decreased amount of BR. These differences are more clearly illustrated in panels C and D of Figure 3. The high level of noise in the M decay curves is due to the fact that the difference is on the order of a fraction of 1 mOD on a starting background of 820 mOD. Nonetheless, the difference curve is clearly above the zero line. The difference is more evident in the BR recovery curves where the maximum change is 4 mOD on a background of 750 mOD. These differences between fresh and stored cells are seen only in the control cases (no additions or pretreatments). The fresh and stored cells exhibit the same kinetics, when all energy stores are depleted by pretreatment with CCCP or when maximum $\Delta\Psi$ is formed by pretreatment with DCCD.

Respiration by Whole Cells. Fresh cells using endogenous substrates respire with a rate of uptake of ~450 μmol of O_2 min^{-1} (mg of bR)$^{-1}$. The addition of CCCP does not enhance the rate of uptake, and therefore, these cells behave like typical bacteria (*21*). The supply of endogenous substrate is so large that it required 10 days of continuous stirring to reduce the O_2 uptake rate to zero. The cells that were stored for 3 months still exhibited a rate of uptake of ~280 μmol of O_2 min^{-1} (mg of bR)$^{-1}$. Overnight incubation with 10 mM CN^- inhibited ~90% of the respiration; 5 mM inhibited ~77%. This sensitivity to cyanide is similar to that seen in *Escherichia coli* (*22*).

Proton Pumping with Fresh Cells

Figures 4 and 5 show proton release and uptake by fresh whole cells subjected to different treatments with ionophores and inhibitors. An upward trend indicates alkalinization and a downward trend acidification of the external medium. The control shown in Figure 4 was incubated overnight at room temperature with no additions, while the other preparations whose results are depicted in the figure were incubated in the presence of various substances as indicated. The arrows denote the times when either light (i.e., "*hν*") was turned on or off, or when an addition was made to the medium. There are two important general observations for all of the cases shown in Figure 4. (1) There is a continual trend toward alkalinization throughout the incubation, even in the dark. (2) The initial illumination did not lead to any quantitatively significant proton release. The first observation can be explained by the existence of a strong K^+ gradient due to ~3 M inside versus ~0.3 M outside (*23*−*26*). The slow diffusion of K^+ from the inside to the outside is partially offset by the movement of H^+ from the outside to the inside. The second observation is consistent with the findings of Bakker et al. (*20*), who found that above neutral pH, $\Delta\tilde{\mu}_{H^+}$ is almost entirely in the form of $\Delta\Psi$. The earliest proton movements and the K^+ diffusion potential generate a sufficient $\Delta\Psi$ to inhibit any further extrusion of protons. In

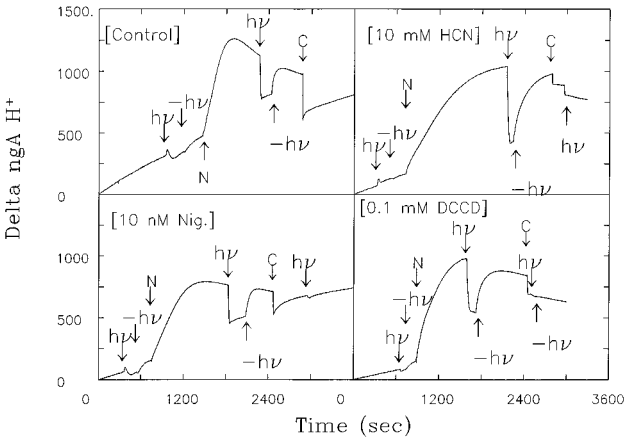

FIGURE 4: Proton pumping by fresh cells. All preparations were incubated at pH 7.0 overnight at room temperature with either no additions (Control) or exposed to the agent shown in brackets. The next day, proton release to the external medium was monitored after illumination (*hν*), light-off (−*hν*), and addition of 20 μM nigericin (N) or 25 μM CCCP (C). The *Y*-axis is scaled for changes in ngA H^+ in the external medium. An upward trend indicates alkalinization and a downward trend acidification.

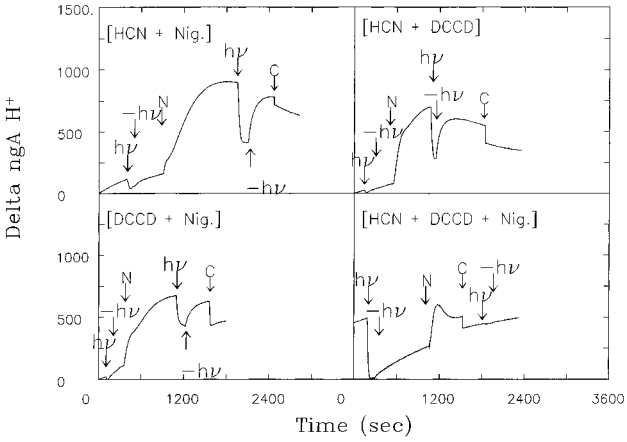

FIGURE 5: Proton pumping by fresh cells. All preparations were incubated at pH 7.0 overnight at room temperature in the presence of the combination of agents listed in the brackets. All other details are the same as described in the legend of Figure 4.

most other systems that have been described, the electroneutral exchange of K^+ for H^+ is promoted by the addition of valinomycin. We find that valinomycin does not promote proton efflux with the whole cells. This is most likely due to the large existing K^+ gradient described above. Valinomycin would be expected to enhance the diffusion potential for K^+, leading to a strong opposing voltage gradient for H^+ movement. In addition, the charge-neutralizing influx of K^+ would be against the large concentration gradient. A small amount of nigericin was added to promote this electroneutral exchange. As can be seen in all four cases shown in Figure 4, the addition of nigericin ("N") evoked a marked enhancement of the alkalinization of the external medium, showing its ability to promote K^+-H^+ exchange. It is also seen in all cases that the addition of nigericin promoted the expected proton release upon illumination. It is important to note that the extent of the light-induced proton release following nigericin addition was limited by the amount of protons taken in as a result of its addition. The end of the proton release returned the pH trace to about the expected level for the continued rise seen in the absence of illumination and nigericin. This important phenomenon has been previously noted (*20*). CCCP ("C" in Figure 4) was added at the end to determine the level of external pH when both internal and external compartments are equilibrated. Overnight preincubation with HCN and DCCD was used to inhibit and reduce any pre-existing background of $\Delta\tilde{\mu}_{H^+}$ supported by respiration or ATP breakdown (*20*). Preincubation with nigericin was used to facilitate electroneutral exchanges of H^+ and K^+, to diminish the large K^+ diffusion potential, and to accumulate an internal supply of H^+ via K^+-H^+ exchange. If the background of $\Delta\tilde{\mu}_{H^+}$ is abolished and the electroneutral exchange is facilitated, we should expect to see an immediate acidification of the external medium upon illumination. A small step in this direction was obtained by preincubation of the cells with both HCN and nigericin (Figure 5), where a small acidification was seen upon illumination. Under these conditions, two phases in the nigericin-induced alkalinization are seen. In fact, this biphasic response is also clearly seen in the other two cases where two of the three components were used. This two-phase feature, however, is not examined further in this paper. The most dramatic result of the pretreatments is seen when all three additions were made (Figure 5, bottom right panel). There was an immediate marked acidification upon illumination. In contrast to the other cases where only two additions were made, the concentration of H^+ released to the external medium lowered the external pH to below that at the start of the experiment. It thus appears that to obtain significant, initial light-induced proton extrusion from whole cells, any pre-existing $\Delta\tilde{\mu}_{H^+}$ must be dissipated by incubation both with HCN and with DCCD, there must be a prior accumulation of protons driven by the existing K^+ gradient, and the membrane must be capable of significant electroneutral H^+-K^+ exchange. In addition to the release of the existing $\Delta\tilde{\mu}_{H^+}$ by preincubation with all three components, the pre-existing K^+ gradient was dissipated. This was evident both from the absence of a general trend toward alkalinization during the course of the incubation and from a minimal degree of alkalinization upon addition of nigericin in the dark (not shown). Finally, the reasons for overnight preincubation should be clarified further. The uninhibited cells maintain a level of $\Delta\tilde{\mu}_{H^+}$ due both to continuing respiration and the breakdown of endogenous ATP. When both of these processes are inhibited, time must be allowed for the decay of the pre-existing $\Delta\tilde{\mu}_{H^+}$. In addition, the ability of nigericin to enhance the level of electroneutral H^+-K^+ exchange, leading to a significant internal accumulation of protons, is slow. Overnight incubation with 10 nm nigericin is much more effective in this regard than the direct use of 1 μM nigericin.

Proton Pumping with Stored Cells. The same experiments depicted in Figures 4 and 5 were conducted with stored cells, and several striking differences were seen (data summarized, but not shown). (1) The slow drift toward alkalinization which was attributed to a K^+ gradient-driven K^+-H^+ exchange was markedly reduced. (2) The response of the system to light-on and light-off events, as well as to all additions, was faster with the stored cells than with the fresh cells. (3) The magnitude of light-induced proton translocation was generally smaller in stored than fresh cells. (4) The effect of preincubation with DCCD was quite different in the two cases. With fresh cells, incubated with DCCD alone, illumination did not result in any significant release of protons, whereas with aged cells it did. The effects of preincubation with DCCD in the presence of HCN were even more dramatically different in terms of light-induced proton pumping in the aged and fresh cells. Specifically, the magnitude of proton pumping was much greater with the stored cells than with the fresh. All of these differences in behavior we have observed both with respect to proton pumping and photocycle kinetics in aged and fresh cells can be explained by losses of the pre-existing K^+ gradient (see ref *26*), respiratory substrates, ATP levels, permeability barriers to ions upon aging, and the process of internalization of H^+ driven by electroneutral K^+-H^+ exchange.

Another striking difference in the behavior of aged and fresh cells is seen after overnight preincubation with all three components, HCN, DCCD, and nigericin. As shown above for the case with fresh cells, there was a diminution of the pre-existing K^+ gradient as evidenced by the lack of response to addition of nigericin in the dark. With these cells, a strong light-induced pumping of protons was seen. In the case of the aged cells, there was even less alkaline response to addition of nigericin in the dark and virtually no light-induced proton pumping to the external medium. These observations are consistent with the view that the prior accumulation of external H^+ driven by K^+ efflux is essential for light-induced proton efflux. The decreased level of the K^+ gradient in the aged cells was unable to cause any significant prior uptake of medium protons, and therefore, very little light-induced H^+ translocation was observed.

DISCUSSION

Near neutral pH, the kinetics of a PM preparation are explained by a photocycle containing two forms of M intermediate, one, M_f, whose decay is accompanied by a rise in the level of O intermediate and exhibits a time constant of ~2 ms and the other, M_s, with a time constant near 5 ms and whose decay is accompanied by the formation of ground-state BR (*13*). The average τ for total M turnover is ~4 ms (*27*). In this work, we find that the photocycle in whole cells of *H. salinarium* near neutral pH also exhibits two forms of M intermediate with τ values near 3 and 30 ms and an

average τ of ~20 ms. As was seen in the PM preparations, the faster form appears to decay to the O intermediate, but its relative proportion of the total M is smaller [~0.2 in whole cells (this work) compared to ~0.5 in PM (*27*)]. The kinetics of the photocycle in whole cells can be transformed to those in PM abruptly by lysing or in a slow continuous fashion by titration with the energy uncoupler, CCCP, to concentrations above 10 μM. The kinetics of BR turnover in whole cells can be slowed further by addition of DCCD, which causes an increase in $\Delta\Psi$, or by raising the external pH. The slowing in turnover is due to the appearance of new, slower forms of the M intermediate and to shifting the magnitudes of mole fractions of the different forms of M to the slower species. It is worth noting that the same slowing in turnover due to the formation of new slower forms of M intermediate can be obtained by disruption of lipid−protein interactions by brief exposure of PM to dilute Triton X-100 (*27*, *28*). It is a possibility worth considering that the mechanism for slowing the photocycle by $\Delta\Psi$ may also involve lipid−protein interactions. The overall results obtained in these studies with the various inhibitors and uncouplers support the view that the existence of a $\Delta\Psi$ retards processes which lead to the formation of $\Delta\Psi$. This is analogous to the well-established phenomenon of respiratory control in tightly coupled electron transport systems and the retardation of M decay seen in BR liposomal systems (*9*−*11*). The inhibitory effect of $\Delta\tilde{\mu}_{H^+}$ on the forward kinetics of the photocycle may be even more extensive than currently thought. The data (Figures 2 and 3) show that at sufficiently high levels of stored energy, as seen in fresh cells, there is an apparent partial reversal of the forward cycle. How an existing $\Delta\tilde{\mu}_{H^+}$ is able to modify the kinetics leading to its formation poses an important question. Although the answer is not known at present, there seem to be some obvious possibilities: the electrostatic repulsion of an existing field will retard the further movement of charges, the $\Delta\Psi$ may cause protein conformational changes that can effect forward and/or reverse kinetic constants, and the polarization of essential bound water molecules may by altered.

A primary linkage between the bR photocycle and the energy state of the cell is the light-induced pumping of protons to the cell exterior, a process which itself is regulated by its end product, $\Delta\tilde{\mu}_{H^+}$. We have studied light-induced, proton translocation in this system as an indicator of the energy state of the intact cell and its modification by standard inhibitors and uncouplers of energy conversion. The intact cells do not respond to illumination by acidification of the external medium as one would expect. However, by use of conditions which lead to dissipation of a strong K^+ diffusion potential (outside positive), inhibition of respiration by HCN, inhibition of ATP synthesis and breakdown by DCCD, and enhancement of K^+-H^+ exchange by nigericin, the expected light-induced medium acidification is revealed. The preexisting energy-induced states exist at high levels in fresh whole cells. The proton pumping experiments show that upon storage for long periods of time, the K^+ gradient is discharged through an exchange with external univalent cations, including H^+ and Na^+. ATP is slowly consumed to pump protons, which are subsequently driven inward by the K^+ exchange process. We and others have noted that the protons translocated to the external medium upon illumination are those which were previously internalized by the K^+-H^+ exchange process (*20*). It has also been noted here and previously that the first response of control cells to illumination is a DCCD-sensitive alkalinization of the medium, which can be attributed to the $\Delta\Psi$-driven uptake of protons by the ATP-forming enzyme (*20*, *26*). In the case of aged cells, because of the depletion of the ATP pool, the reaction is energetically poised for ATP synthesis. In the absence of DCCD in aged cells, the net light-induced external H^+ change is alkalinization due to ATP synthesis. In the presence of DCCD, previously accumulated internal protons are available for net acidification as seen in Figure 5. DCCD slows the kinetics of the photocycle because it elevates $\Delta\Psi$. In the absence of DCCD, the steady-state level of $\Delta\Psi$ that is formed both from the bR and from respiration-driven proton pumps is decreased by ATP synthesis.

Conclusions. The PM preparation is a valid system for studying the kinetics, pathways, proton-release, and proton-binding steps of the basic photocycle. In the intact cell, however, the performance of the photocycle is under strict control exerted by the overall energy state, which itself is dependent on respiration, ATP levels, and a strong K^+ diffusion potential. This control affects not only the multiturnover process of proton pumping but also the kinetics for a single turnover of the cycle. The earliest steps of charge separation dramatically influence the kinetics of the later steps. Thus, the addition of CCCP, or disruption of the whole cells by lysis, results in single-turnover kinetics that are much faster than those which occur in the intact energized cell. From this work, it is clear that the photocycle in the intact energized cell is mechanistically different from that in isolated PM, particularly in the number of M intermediates involved. All photocycle schemes so far proposed are based on the cycle as observed in the PM near pH 7, which consists of only two M species. A real challenge for the future is to consider a scheme (or schemes) that will account for the additional intermediates and apparent partial reversal seen under conditions of extreme pHs and high-energy states of the cell. An additional regulatory influence, which operates in both the PM and whole cell preparations, is that the intensity level of actinic light alters the relative amounts of fast and slow forms of M intermediates that participate in the photocycle and hence the overall turnover rate.

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