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Control of the protonmotive force in *Rhodopseudomonas sphaeroides* in the light and dark and its effect on the initiation of flagellar rotation

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The addition of low concentrations of the uncoupler of CCP (0.01–0.1 μM) to actinically illuminated, photosynthetically grown *Rhodopseudomonas sphaeroides* did not inhibit motility. When CCCP addition was followed by a period of dark, anaerobic incubation the bacteria became nonmotile, and motility was not regained immediately on actinic re-illumination. The length of the delay before the onset of motility on re-illumination was proportional to the concentration of uncoupler added, until at higher concentrations (0.1–5 μM) maximum motility was not regained. Flagellar rotation depends on the protonmotive force, therefore the total pmf and the electrical and chemical components were measured under a variety of environmental conditions. The addition of the uncoupler to dark-incubated bacteria caused the collapse of the respiratory protonmotive force, but had no effect on the rapid reformation of the full protonmotive force on re-illumination. The time-course of protonmotive force generation was very similar to that measured in untreated bacteria and showed little change with increasing concentrations of uncoupler, although the size of the induced protonmotive force was eventually reduced. The ΔpH component of the protonmotive force developed more slowly than the Δψ component, but the time taken for the development of the ΔpH did not increase as the CCCP concentration increased. The delay in motility was longer under conditions where ΔpH was the sole or major component of the protonmotive force. ATP is required for taxis but not motility in bacteria. The addition of CCCP to dark-incubated bacteria caused a rapid fall in intracellular ATP which recovered rapidly on re-illumination. At high uncoupler concentrations the ATP content fell as the protonmotive force was reduced. However, the delay in resumption of motility was observed at CCCP concentrations which did not affect either the protonmotive force or the ATP concentration reached on illumination. There was no delay in recovery of motility when protonmotive force was increased but ATP levels reduced by the addition of the ATPase inhibitor venturicidin. In its proposed that initiation of flagellar rotation involves a protonmotive force dependent modification of the motor and that this modification acts as the on-off switch for the motor.

Introduction

Flagellate bacteria move by rotating semi-rigid helical flagella [1,2], using the proton gradient across the cytoplasmic membrane as the energy source for this rotation [for reviews, see Refs. 1–4]. Flagella can rotate in either a clockwise or an anticlockwise direction, and the control of the frequency of switching the direction of rotation is used to bias the movement of a bacterium in a favourable direction. The protonmotive force across the membrane, in normal conditions, is always in one direction. The change in direction of flagellar rotation probably involves a change in the structure of the protein rings at the base of the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP, tetraphenylphosphoniumbromide; DMO, 5,5-dimethyloxazolidine-2,4-dione.

flagellum so that the unidirectional protonmotive force can rotate these rings in either a clockwise or an anticlockwise direction. In a nongradient environment the change in direction occurs at a regular rate. The binding of chemotactically active chemicals to specific membrane-bound receptors, or a change in protonmotive force across an electron-transfer chain changes the frequency of the switching, biasing the overall movement of the bacterium in a favourable direction [5–7]. There is very little information about either the mechanism of direction changing or the signal from the receptors to the flagella which can alter the frequency of the direction changing.

The purple photosynthetic bacterium *Rhodopseudomonas sphaeroides* when grown anaerobically in the light develops both respiratory and photosynthetic electron-transport chains. It has been shown in the closely related *Rhodopseudomonas capsulata* that the respiratory protonmotive force is much more sensitive to proton ionophores than the photosynthetic protonmotive force [8,9]. The addition of small concentrations of the uncoupler CCCP could therefore be used to remove the protonmotive force generated in the dark by respiration but allow the normal development of the photosynthetic protonmotive force when the bacteria were re-illuminated. This mechanism has been used here to examine the stopping and restarting of the flagellar rotary motor. We present evidence suggesting that although either the electrical or the chemical component of the protonmotive force was the only requirement for continued flagellar rotation once initiated, there was an additional event required for this initiation. The time-course of this event was longer than the time taken for a maximum protonmotive force to develop, but appeared to be dependent on the size of the protonmotive force.

Materials and Methods

Bacteria and growth conditions

Rps. sphaeroides WS8 (a wild-type strain, gift from W. Sistrom) was used throughout this study. It was grown anaerobically in the light at 25°C as previously described [10]. The bacteria were harvested when the maximum percentage of the population was motile, in the early exponential phase. In all experiments both cell number, measured on a Coulter counter, and bacteriochlorophyll content, were measured to ensure that the bacterial population was comparable on different days. After washing twice in the appropriate oxygen-free buffer the bacteria were resuspended in the same buffer.

Buffer composition

Unless otherwise stated the experiments described here were conducted in a 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), 4-morpholineethanesulphonic acid (Mes), glycylglycine buffer which was adjusted to the required pH with NaOH.

In experiments in which the role of different components of the protonmotive force were examined either 100 mM sodium acetate or 5 mM tetraphenyl phosphonium bromide was used to remove the chemical or electrical components as described by Michels et al. [11]. CCCP or venturicidin were added as concentrated solutions in ethanol, controls used ethanol without inhibitors.

Measurement of motility

Bacteria were drawn by capillarity into optically flat microslides (Camlab, U.K.) which were then sealed with vaseline. Motility was observed using a Zeiss photomicroscope and recorded on videotape. The microslides were incubated in the dark when not being examined on the microscope. The times taken for the first bacterium in a field to become motile, for the population to reach 50% of the control and the time taken for the resumption of motility at the same level as the control were recorded. Samples were observed for either 15 min or until maximum motility was regained. The behaviour of the bacteria was analysed from the video recordings.

Measurement of light intensity

Light intensity was measured using a quantum photometer and the intensity used for microscopic investigation and protonmotive force measurement adjusted to a similar intensity.

An assumption had to be made about the functional light intensities. The light intensity at the position of the cuvette, sample holder or micro-

scope slide was measured, and adjusted as near as possible to the same value. The wavelength of actinic light used for measurement of the carotenoid bandshift (approx. 870 nm) could not of course be used for observations. In all cases, therefore, the light intensity used was well above that required to saturate photosynthesis throughout the sample.

Measurement of the carotenoid bandshift

The membrane-bound light harvesting carotenoids undergo an electrochromic absorbance shift when the electrical potential across the cytoplasmic membrane is altered [12]. The size of the shift into either the blue or red end of the spectrum is a very fast indication of any change in the size of $\Delta\psi$.

The carotenoid bandshift was measured using an Aminco dual-wavelength spectrophotometer with the wavelength pair 510–523 nm. Actinic illumination was at 90° to the measuring beam using 871 nm filter and a variable light intensity source.

Samples were incubated in the spectrophotometer for at least 30 min in the dark under a stream of nitrogen before the experiments were started. Inhibitors, or ethanol as controls, were added directly to the stirred cuvette and any change in membrane potential followed. Samples were drawn into optical flat capillary tubes after each treatment for parallel microscopic examination.

Measurement of $\Delta\psi$ by ion distribution

In many previous studies it has been found that the carotenoid bandshift consistently gave higher values for the $\Delta\psi$ component of the protonmotive force than did ion-distribution techniques [12,13]. The reasons for this are speculative and may involve the measurement of charge separation as well as bulk potential changes by the carotenoids.

The $\Delta\psi$ component of the protonmotive force was therefore examined using cation distribution in addition to the carotenoid bandshift. This was measured in conditions when all components of the protonmotive force were present, and also with the ΔpH component reduced.

The distribution of [^{3}H]tetraphenylphosphoniumbromide [TPP^+] was followed by the method described by Khan and Macnab [14]. TPP^+ was used as it crosses the outer membrane of Gram negative bacteria and has a faster equilibration rate than other cations.

Bacteria (3 ml, approx. 10^9 ml^{-1}, measured accurately on a Coulter counter) were incubated with 1.25 μCi ^{3}[H]TPP^+ per ml in the cuvette of the dual-wavelength spectrophotometer to allow measurements of the carotenoid bandshift, TPP^+ uptake and motile behaviour on the same sample. All three parameters were measured on the same sample to allow for the possibility that the number of molecules of CCCP per cell was critical for the uncoupling effect. Samples (50 μl) were taken at intervals and added directly to 2 ml ice-cold experimental buffer and vacuum filtered onto 0.6 μm pore polycarbonate membranes and washed with a further 2 ml cold buffer. The buffer had previously been sparged with nitrogen and filtration was done under a stream of nitrogen. The filters were air-dried and the radioactivity counted.

The $\Delta\psi$ was calculated using the Nernst equation after correction for radioactivity bound to the filter and the outside of the bacteria by repeating the procedure with heat killed bacteria.

Measurement of the chemical potential

The ΔpH component of the protonmotive force was measured by flow dialysis [15]. 0.8 ml bacterial suspension was added to the top-stirred compartment of the flow dialysis apparatus and incubated under nitrogen, the compartment was separated from the lower compartment by 24 nm Visking tubing. Dimethyl oxylidine (^{14}[C], 0.5 μCi per ml) was added to the upper chamber. After allowing for equilibration, samples (50 μl) were collected from the lower chamber using a fraction collector, directly into scintillation vials. Fresh suspensions were used for each concentration of uncoupler. After equilibration samples were taken with time before and after illumination. The experiment was repeated with both heat-killed cells and totally uncoupled cells to ensure measurement of collapsed protonmotive force. The two measurements of binding to inactive cells gave different results. It was therefore impossible from these experiments to give absolute values for ΔpH but the relative changes and the time-course of ΔpH development were unaffected by this problem.

It should be noted that the measurement of

both the $\Delta\psi$ and ΔpH by ion distribution techniques relies on a measurement of distribution in whole cells without a protonmotive force. In both methods heat-killed cells and cells treated with 10 μM uncoupler gave different values for distribution at zero protonmotive force. Also in both cases at low protonmotive force values the calculated potential showed considerable variation, because of the use of the Nernst equation for calculations. Therefore, as mentioned above, absolute values may be inaccurate even at high values of protonmotive force, but relative values, compared to the bandshift were accurate. At potentials calculated to be below 30 mV any inherent error would be amplified, and we therefore consider the size of potential shown by the carotenoid bandshift to be closer to reality.

Measurement of intracellular volume

The intracellular volume of *Rps. sphaeroides* was measured by sucrose exclusion [16]. After resuspension in 5 ml 10 mM Hepes buffer at pH 7.0 0.2 μCi ^{14}[C] sucrose and 2.5 μCi ^{3}[H] water were added to the samples. Samples of 1 ml were centrifuged and the radioactivity in 0.1 ml aliquots of the supernatant measured. After careful removal of all the supernatant the pellets were resuspended in isotope-free buffer and again centrifuged and the radioactivity in the supernatant again measured. After correction for cross-channel counting the intracellular volume was calculated as the difference between the ratios of ^{3}[H] and ^{14}[C] from the two centrifugations. The volume per cell was $4.8 \cdot 10^{-10}$ μl.

Intracellular ATP measurements

The phosphatase enzymes of many bacteria are very resistant to normal killing procedures, and early attempts to isolate ATP by precipitation at -10°C suggested that phosphatases in this strain of *Rps. sphaeroides* remained active close to this temperature.

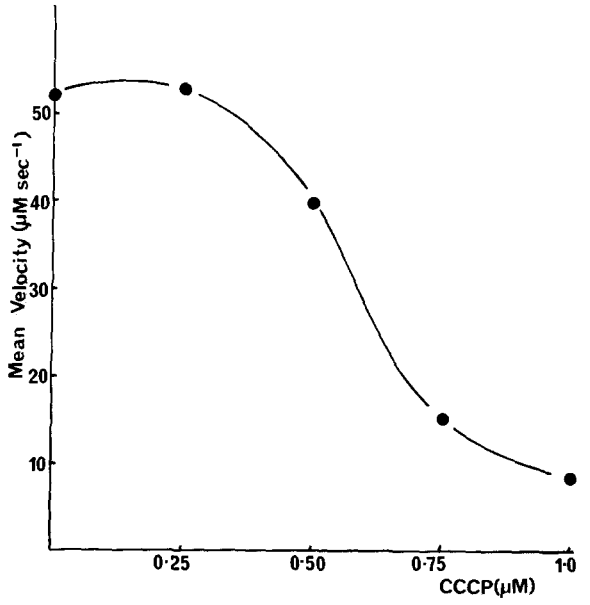
The bacteria were therefore broken and the protein precipitated at temperatures maintained below -10°C. The bacteria, in the appropriate buffer, were kept anaerobic in a syringe in the path of a variable-intensity light source. Aliquots were injected at intervals into baths of liquid nitrogen. The bacteria were ground under liquid nitrogen and then ground with an equal volume of 20% trichloroacetic acid in 40% ethanol. The mixture was allowed to thaw slightly, then reground under nitrogen and slowly rethawed. The temperature remained below -10°C for at least 20 min after which time the precipitate was removed by centrifugation. The trichloroacetic acid was removed from the supernatant by five washings with 4 volumes of water-saturated ether and, after removal of the residual ether, the ATP in the samples measured using a constant light signal luciferase assay (Boehringer).

Results

The effect of CCCP on motility

The effect of increasing concentrations of CCCP on velocity. There was no effect on either the number of motile bacteria nor on their velocity when concentrations of CCCP betwen 0.01 and 0.1 μM were added to *Rps. sphaeroides* under continuing actinic illumination. As the concentration of CCCP was increased from 0.5 to 10 μM the speed of swimming was reduced as previously reported [17] (Fig. 1). The reduction in velocity was correlated with the maximum size of protonmotive force obtained with that concentration of uncoupler. Fig. 2 shows the reduction in the size of membrane potential obtained, as measured by the carotenoid bandshift, with increasing concentrations of uncoupler and with saturating actinic illumination. The addition of 0.01 μM CCCP to bacteria incubated in the dark caused a decrease in absorbance as the protonmotive force maintained either by residual respiration or reversed ATPase activity in the dark was collapsed. The initial size of this dark protonmotive force was dependent on the length of anaerobic, dark incubation. Subsequent illumination allowed complete reformation of the $\Delta\psi$ to the pretreatment level, i.e., previous dark plus illuminated $\Delta\psi$. Switching off the light led to a rapid fall to zero $\Delta\psi$, as measured by the final totally uncoupled level.

Although the velocity of swimming decreased with increasing uncoupler concentrations, motility still continued when the protonmotive force was decreased to about 5% of the control, less than 10 mV measured by the carotenoid bandshift. The effect of CCCP was examined both by using a



different bacterial suspension for each uncoupler concentration and also by increasing the concentration gradually in one suspension. This was to allow for either increasing sensitivity or any recovery that may occur after prolonged contact with the uncoupler.

Delay in onset of motility. When the addition of CCCP (over 0.01 μM) to light-incubated bacteria was followed by a period of dark incubation, motility was lost. It was also lost if CCCP was added directly to anaerobic dark-incubated bacteria. When illuminated with actinic light

Fig. 1. Mean velocity of *Rps. sphaeroides* treated during actinic illumination with CCCP. Bacteria were suspended in 10 mM Hepes/Mes/glycylglycine buffer at pH 7.0 as in Materials and Methods. CCCP was added as concentrated solution in ethanol to bacteria under illumination and the average velocity of 50 bacteria calculated at each concentration.

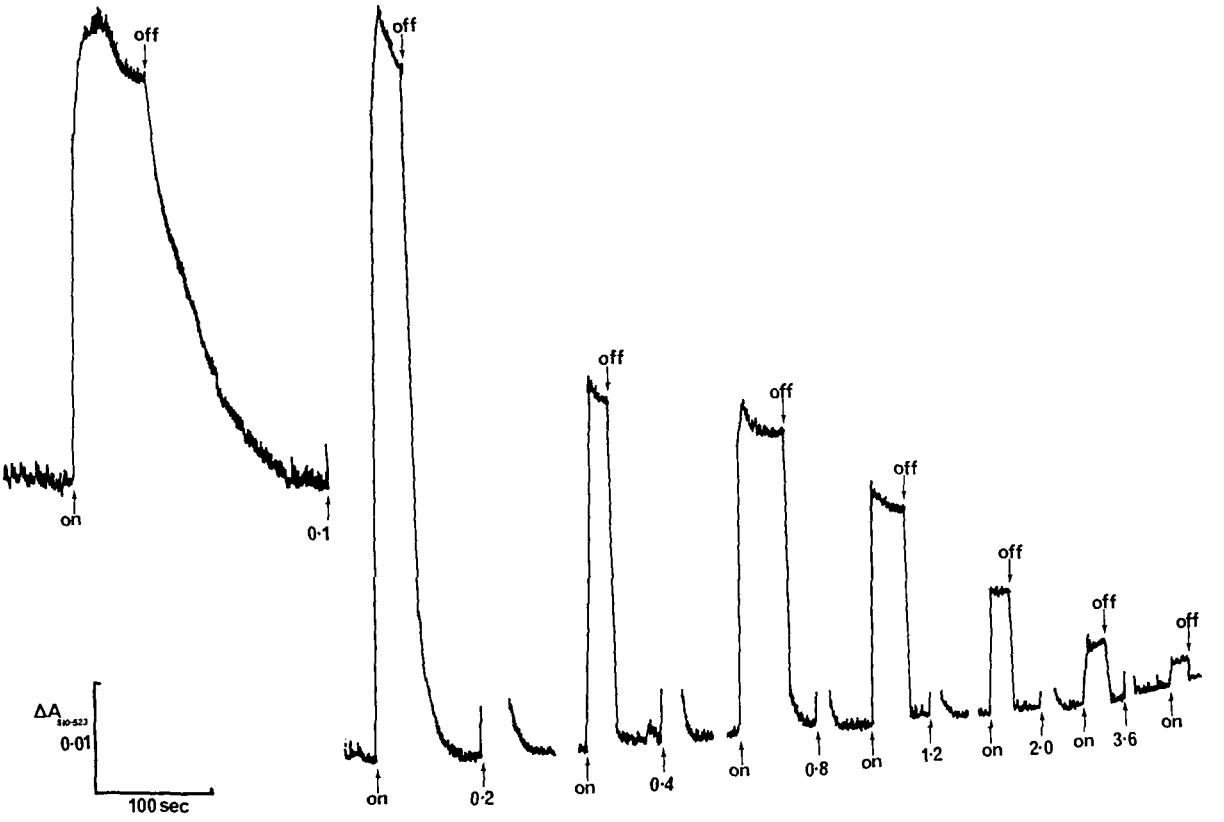


Fig. 2. Change in the carotenoid bandshift with increasing CCCP concentrations. The carotenoid bandshift was measured as described in Materials and Methods. Equilibration for 5 min was allowed after each addition of CCCP (μM final concentrations) before illumination.

motility resumed, but with a delay. The length of the delay (Fig. 3) was dependent on the concentration of CCCP added, the pH of the buffer and the light intensity used for reillumination, varying from a few seconds to many minutes. Above 0.2 μM CCCP 100% motility was not recovered. The delay was also dependent on a period of dark incubation. The maximum delay was achieved after about 4 min dark incubation and remained constant for a specific set of conditions on one suspension of bacteria for many hours (up to 48 h was followed). When maximum motility was regained after reillumination, another period of 4 min dark incubation was required to reimpose the delay. The resumption of motility by the total population after reillumination was gradual; the population did not start swimming synchronously, but one or two would suddenly start to swim after which there would be a rapid increase in the number of motile bacteria up to a maximum. The individual bacteria swam immediately at their maximum velocity.

Measurement of components of the protonmotive force

Membrane potential. The size of the electrical component of the protonmotive force and the rise-time of the potential were measured using two different techniques. The carotenoid bandshift uses the electrochromic absorbance shift of the endogenous membrane bound light-harvesting carotenoids. The bandshift in whole cells can be approximately calibrated by reference to a valinomycin/K^+ standard curve from chromatophores [12]. This method always gives higher values for the membrane potential than does the distribution of labelled cations, such as TPP^+. The possible reasons for the discrepancy have been reviewed elsewhere in several publications [12,13]. However, for the purposes of this study the absolute value of the membrane potential was unimportant; it was the size relative to the controls and the time-course of development which were relevant. Both the carotenoid bandshifts and TPP^+ distribution were

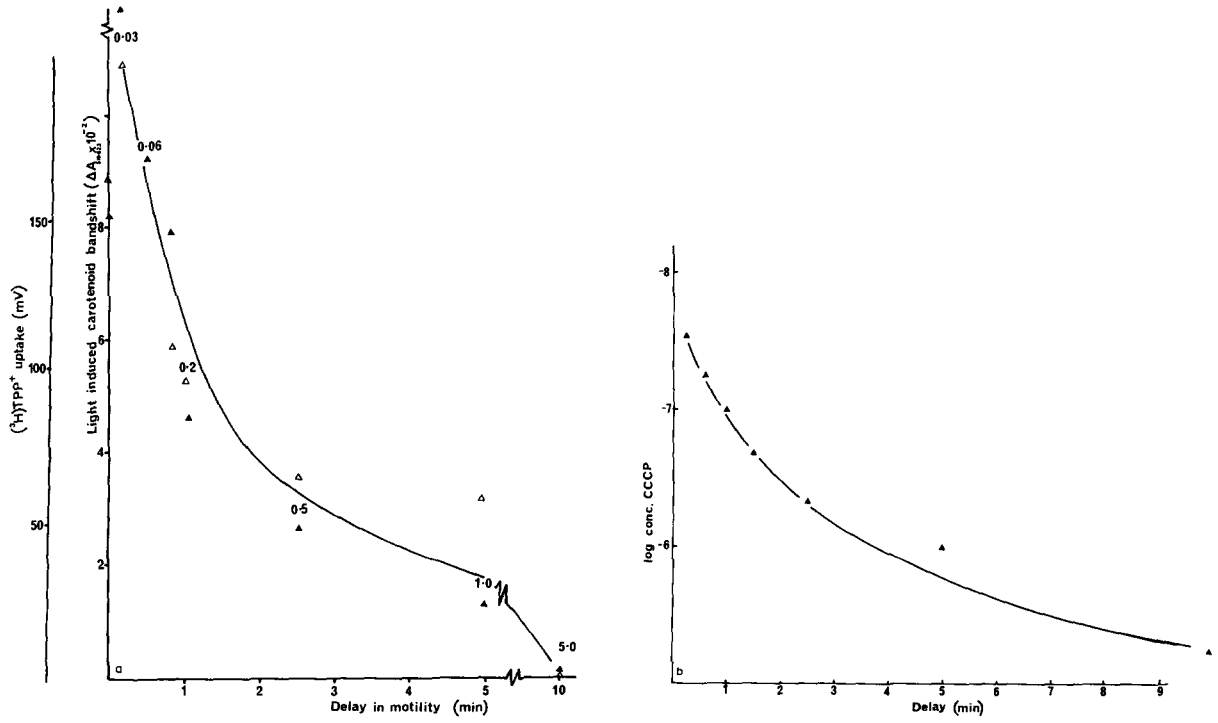


Fig. 3. (a) Delay in onset of motility and size of $\Delta\psi$ with increasing CCCP concentrations. $\Delta\psi$ was measured both by carotenoid bandshift and ^{3}[H]TPP^+ uptake. *Rps. sphaeroides* was suspended in 10 mM Hepes/Mes/glycylglycine buffer (pH 7.5) and the carotenoid bandsfhit (▲———▲) and ^{3}[H]TPP uptake (△———△) measured on the same sample with increasing concentrations of CCCP. Each point represents CCCP (μM). (b) Delay before 50% control motility regained (pH 7.0).

followed and both methods gave the same overall picture. Over the range 0.01–0.1 μM CCCP the size of the light-induced Δψ remained high (when compared to the control potential at that pH) (Figs. 2 and 4). The apparent increase in the light-induced membrane potential measured by the bandshift on the addition of CCCP was the result of the collapse of the baseline membrane potential, maintained by dark respiration. The maximum remained unchanged (Figs. 2 and 4). Studies have shown [8,9] that respiratory potentials are more sensitive to uncouplers than photosynthetically generated potentials. The respiratory potential therefore collapsed after treatment with small concentrations of uncoupler to dark-incubated cells, as shown by both the carotenoid bandshift and TPP^+ distribution, but the maximum potential was still developed upon actinic illumination.

There was an increasing delay in time taken for the onset of motility after reillumination as the concentration of CCCP was increased, although the size of the membrane potential remained high and the rate of development measured by either the carotenoid bandshift or TPP^+ distribution showed a rise to beyond the critical value for flagellar rotation within the resolution time of the system. As the concentration was increased beyond 0.1 μM the maximum size of the membrane potential measured decreased, but not until the potential was below 80 mV (TPP^+ uptake) did the speed of motility and the percentage of bacteria regaining motility start to be affected. The time taken to reach the maximum potential was not measurably altered between untreated and treated bacteria, under 200 ms when measured by the carotenoid bandshift, and under 60 s using TPP^+ uptake.

Although the size of the membrane potential was comparable with that measured in untreated bacteria and developed within the time-scale of the first possible measurement, the bacteria did not start to move until several seconds or minutes after the development of the potential.

Development of the chemical component of the protonmotive force. Previous work has suggested that the two components of the photosynthetically derived protonmotive force have different rates of development, the electrical potential developed within milliseconds and then fell slightly over about 1 min as the chemical component developed, keeping the overall size of the protonmotive force approximately constant, but the percentage contribution of the chemical and electrical components changing over the first few minutes. The final size of the contribution of each component depended on the pH and composition of the suspending medium [11]. When measured using DMO flow dialysis this result was confirmed. The chemical potential appeared to be somewhat slower than the electrical potential in reaching maximum, although it is impossible to distinguish from these experiments whether the delayed response was caused by Δ pH per se or an indirect effect of the measuring molecule DMO on the ΔpH components. The length of time taken to reach this maximum, however, did not increase as the concentration of uncoupler was increased and the size of the chemical potential developed to a value above the critical value for flagellar rotation within 30 s (Fig. 5).

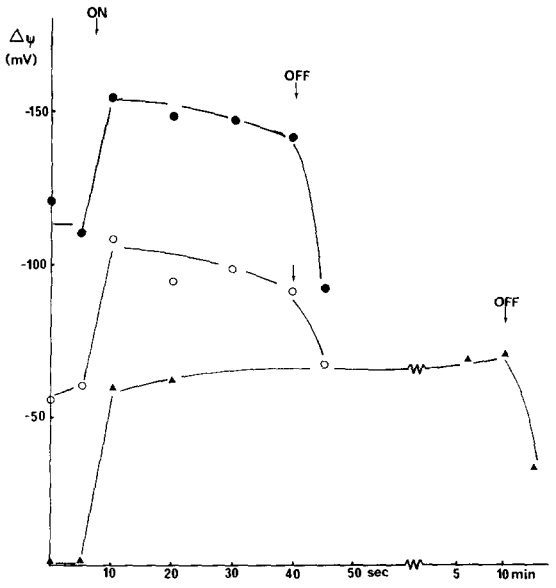


Fig. 4. Time-course of Δψ increase measured by [^{3}H]-TPP uptake at different concentrations of CCCP. *Rps. sphaeroides* was suspended in 10 mM Hepes/Mes/glycylglycine (pH 7.5) as described in Materials and Methods. ●———●, no addition; ○———○, 0.5 μM CCCP; ▲———▲, 1 μM CCCP.

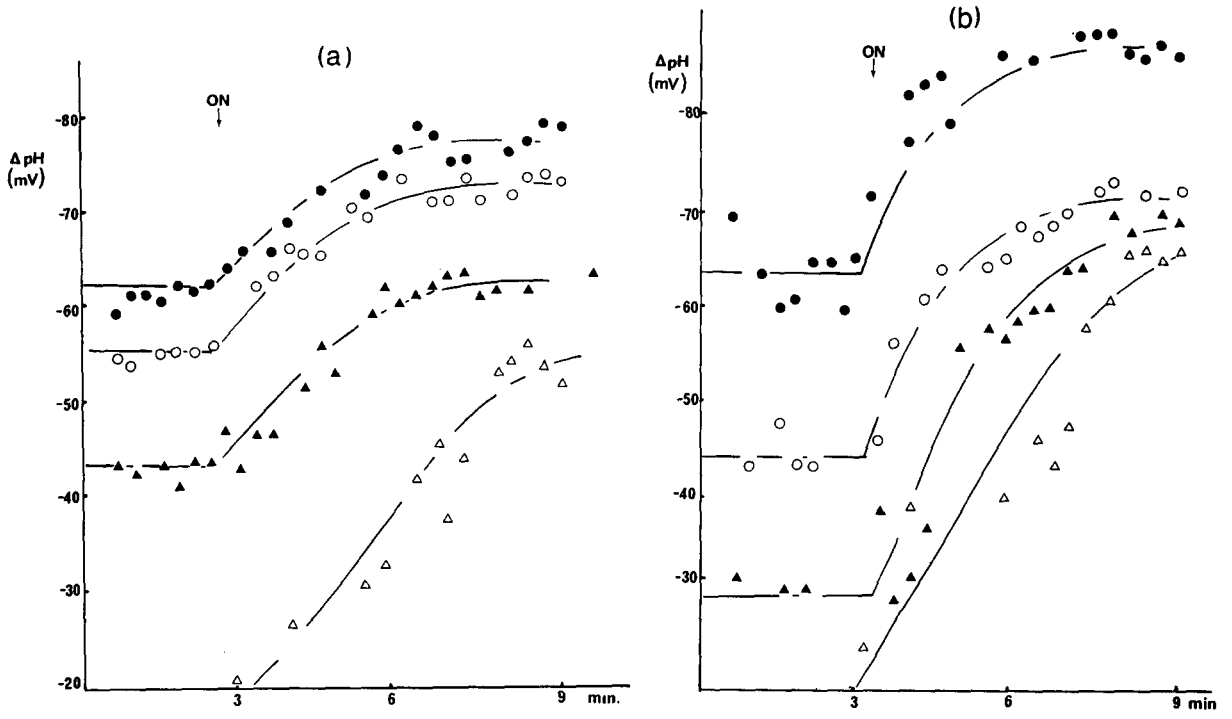


Fig. 5. Time-course of ΔpH increase measured by [^{14}C]DMO uptake illumination at different concentrations of CCCP. (a) pH 6.5, (b) pH 6.0. ●———●, no addition; ○———○, 0.02 μM, ▲———▲, 0.2 μM; △———△, 1 μM. No ΔpH could be accurately measured at pH values over 7.0.

Effect of medium composition and pH on the protonmotive force and the onset of motility

Because the time-course for the development of the two components of the protonmotive force was apparently different, the delay in the onset of motility after dark-uncoupler treatment was examined in media in which one component was decreased. The delay in resumption of motility was also investigated in the absence of uncoupler. One component of the protonmotive force was removed by the addition of either acetate (to remove ΔpH) or a permeant cation (to remove $\Delta\psi$), and the role of the other component was investigated by the addition of increasing concentrations of an inhibitor of the remaining component.

The particular concentrations of inhibitor used to remove the components of the protonmotive force were chosen as they had been previously shown to be effective in whole cells [11] and because, when added to illuminated bacteria, there was no significant effect on motility at pH values where the decreased component was not the major contributor to the protonmotive force. At these concentrations TPP^+ abolished the carotenoid bandshift, and acetate, in the presence of potassium ions, enhanced the bandshift at acid pH values.

There was no delay in the onset of motility if either the electrical or chemical component of the protonmotive force was removed, as long as the other component was maintained. The addition of CCCP to the dark-incubated bacteria in the presence of either TPP^+ or acetate caused a delay in the onset of motility on illumination similar to that described above. At low pH values, where ΔpH was a major component, the delay in onset of motility was longer than at alkaline pH values or in environments where ΔpH was removed by the addition of acetate (Table I).

Table I shows the length of delay at different pH values when there was a complete protonmotive force and when the ΔpH component was

TABLE I

THE EFFECT ON THE DELAY OF ONSET OF MOTILITY AFTER ACTINIC ILLUMINATION OF REDUCING THE ΔpH COMPONENT OF THE PROTONMOTIVE FORCE BY CHANGING THE pH OF THE MEDIUM OR ADDITION OF EITHER SODIUM OR POTASSIUM ACETATE

(A and B) $1.4 \cdot 10^9$ cells/ml in Mes/Hepes/glycylglycine buffer were adjusted to the required pH by NaOH. Acetate was added as 0.1 ml 3 M sodium acetate, corrected to the required pH to 3 ml cells incubated in dual-wavelength spectrophotometer. CCCP was added as concentrated solution in ethanol sequentially to one sample. A new bacterial suspension was used for each pH and for measurements with and without acetate. (C) $1.1 \cdot 10^9$ cells/ml suspended and treated as above, but 0.1 ml 3 M potassium acetate was added.

The bandshift is measured as arbitrary absorbance units using the wavelength pair $A_{510-523}$ and saturating illumination. The baseline for the bandshift with no additions includes the uncoupler-sensitive dark-respiratory potential, the size of the bandshift with no additions increases with length of anaerobic incubation and is therefore, without the addition of respiratory inhibitors, not a measure of the absolute $\Delta\psi$. The apparent increase in CCCP addition is as in Fig. 2. The result of the loss of the dark $\Delta\psi$ during dark inhibition reflects the total $\Delta\psi$ in the untreated bacteria.

– means that the motility did not reach control values with 15 min observation; ND means not determined.

(A) Without sodium acetate

CCCP (M)	Bandshift	First motile	100%
pH 6.5			
0	0.041	motile	
10^{-8}	0.043	2s	1 min 15 s
$2 \cdot 10^{-8}$	0.048	15 s	5 min
$4 \cdot 10^{-8}$	0.045	60 s	–
$8 \cdot 10^{-8}$	0.041	2 min 15 s	–
$1.6 \cdot 10^{-7}$	0.028	1 min 25 s	
$5.6 \cdot 10^{-7}$	0.015	non motile	
pH 7.0			
0	0.03	motile	
10^{-8}	0.036	motile	
$2 \cdot 10^{-8}$	0.043	17 s	1 min 30 s
$4 \cdot 10^{-8}$	0.041	70 s	5 min
$8 \cdot 10^{-8}$	0.039	3 min	–
$1.6 \cdot 10^{-7}$	0.033	5 min	–
$5.6 \cdot 10^{-7}$	0.034	non motile	
pH 7.5			
0	0.031	motile	
10^{-8}	0.037	10 s	2 min 30 s
$2 \cdot 10^{-8}$	0.042	10 s	5 min
$4 \cdot 10^{-8}$	0.046	40 s	6 min
$8 \cdot 10^{-8}$	0.044	50 s	15 min
$1.6 \cdot 10^{-7}$	0.034	1 min 40	–
$5.6 \cdot 10^{-7}$		ND	

(B) With sodium acetate

CCCP (M)	Bandshift	First motile	Approx. 100%
pH 6.5			
0	0.046	motile	
10^{-8}	0.044	10 s	3 min
$2 \cdot 10^{-8}$	0.045	15 s	4 min
$4 \cdot 10^{-8}$	0.044	40 s	7 min
$8 \cdot 10^{-8}$	0.038	45 s	–
$1.6 \cdot 10^{-7}$	0.024	4 min 5 s	–
$5.6 \cdot 10^{-7}$	0.010	non motile	–

TABLE I (continued)

CCCP (M)	Bandshift	First motile	Approx. 100%
pH 7.0			
0	0.03	motile	
10^{-8}	0.038	15 s	2 min
$2 \cdot 10^{-8}$	0.040	25 s	2 min 30 s
$4 \cdot 10^{-8}$	0.041	50 s	4 min
$8 \cdot 10^{-8}$	0.038	1 min	4 min
$1.6 \cdot 10^{-7}$	0.037	45 s	7 min
$5.6 \cdot 10^{-7}$	0.012	50 s	15 min
pH 7.5			
0	0.044	motile	
10^{-8}	0.043	20 s	1 min 30 s
$2 \cdot 10^{-8}$	0.044	20 s	2 min
$4 \cdot 10^{-8}$	0.045	30 s	3 min 10 s
$8 \cdot 10^{-8}$	0.042	20 s	3 min 30 s
$1.6 \cdot 10^{-7}$	0.032	35 s	4 min 30 s
$5.6 \cdot 10^{-7}$	0.014	1 min 35 s	5 min

(C) With potassium acetate

CCCP	Bandshift	First motile	Approx. 100%
pH 6.5			
0	0.056 [a]	motile	
10^{-8}	0.058	20 s	1 min 30 s
$2 \cdot 10^{-8}$	0.058	20 s	2 min
$4 \cdot 10^{-8}$	0.057	35 s	2 min 30 s
$8 \cdot 10^{-8}$	0.043	40 s	5 min
$1.6 \cdot 10^{-7}$	0.026	50 s	10 min
$5.6 \cdot 10^{-7}$	0.014	1 min 20 s	–

pH 7.0 and pH 7.5: not determined

[a] Without potassium acetate: 0.026.

removed by the addition of acetate. If acetate was added as the sodium salt to washed bacteria, there was no compensating increase in the $\Delta\psi$ value as measured by the bandshift, but if added as the potassium salt there was an increase in the size of the light-induced bandshift at pH 6.5, presumably because the reduced ΔpH component was replaced by an increased $\Delta\psi$. In all cases it was evident that the delay was longer in bacteria with both components of the protonmotive force than when $\Delta\psi$ was the major component. For example, at pH 7.5, where $\Delta\psi$ was the major component of the protonmotive force, complete recovery of motility in the presence of 0.04 μM CCCP took 6 min after illumination; but under the same conditions, but at pH 6.5 where both components of the protonmotive force were present, there was no complete recovery after 15 min observation. If the ΔpH component were removed by the addition of sodium acetate at pH 6.5, motility was normal after about 7 min illumination, comparable to the delay at pH 7.5 with a complete protonmotive force. If sodium acetate were replaced by potassium acetate, the delay at pH 6.5 decreased still further to 2 min. The increase in $\Delta\psi$ apparently allowed by the presence of K^+ appeared to reduce the length of the delay. The overall size of $\Delta\psi$ was therefore important in determining the length of the delay before resumption of motility.

The decrease in the length of the delay as the pH was changed from 6.5 to 7.5 in the presence of sodium acetate, which removed the ΔpH but did

not allow a compensating increase in $\Delta\psi$, suggested that the activity of CCCP may increase at low pH values.

Intracellular concentrations of ATP

There is evidence that ATP is required for a tactic response [19,20]; changing the direction of flagellar rotation may involve a mechanism similar to that involved in stopping and restarting flagellar rotation. We therefore examined the intracellular concentration of ATP after the addition of CCCP and reillumination. The time-scale of formation and final concentrations of ATP with and without CCCP treatment were measured at different pH values, at different actinic light intensities, and with the components of the protonmotive force controlled.

Fig. 6 shows the intracellular concentration of ATP with increasing concentrations of CCCP and Fig. 7 shows the time taken for that concentration to be reached after reillumination. Bacteria treated with CCCP incubated anaerobically in the dark had reduced intracellular levels of ATP; however, when illuminated, the ATP content rose to its maximum concentration within the sampling time. Although the final intracellular content showed a linear decrease as the protonmotive force decreased, the time taken to reach this concentration remained within the sampling time. The intracellular concentration in most cases was higher after treatment and illumination than in bacteria treated with the ATPase inhibitor venturicidin [24]. Treatment with venturicidin caused no delay in the onset of motility, although the tactic responses were lost [21].

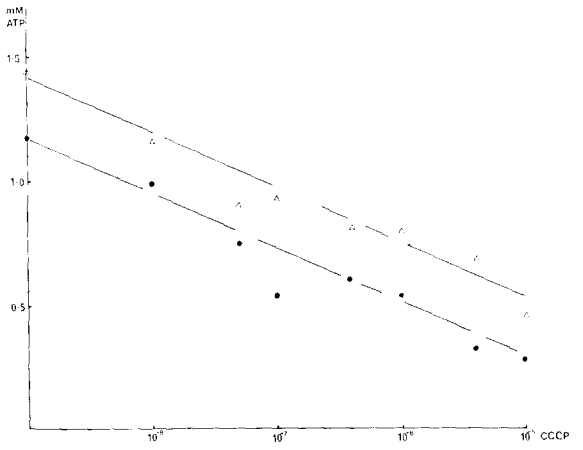


Fig. 6. Decrease in the final intracellular concentration of ATP after 5 min, constant illumination in the presence of increasing concentrations of CCCP. Each set of points represents a separate experiment.

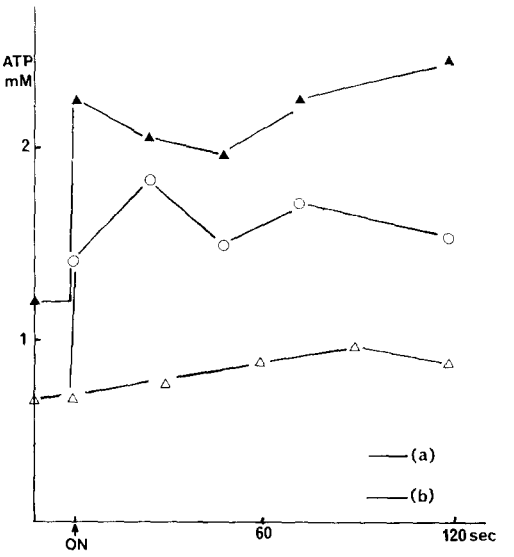


Fig. 7. Time-course in increase in intracellular ATP on illumination after CCCP treatment measured by 'constant-light' luciferase assay as described in Materials and Methods. △——△, no addition; ○——○, 0.05 μM CCCP; △——△, 0.5 μM. (a) represents the final concentration of ATP after 2 min illumination in the presence of 2.5 $\mu g \cdot ml^{-1}$ venturicidin; (b) idem, in the presence of 10 μM CCCP. The bacteria were incubated in the dark for 30 min in the presence of venturicidin to ensure maximum inhibition.

Discussion

There is now little doubt that bacterial flagella rotate as a result of the protonmotive force drawing protons across the cytoplasmic membrane between or close to two protein rings at the base of the flagellum. Under natural conditions this gradient is unidirectional, but this unidirectional gradient can cause rotation of flagella in either a clockwise or an anticlockwise direction [4]. It appears that the rotation of the rings is caused by simple coupling between the protons and the proteins in the rings [22]. Evidence from peritrichously flagellate bacteria suggests that tumbling events are not

caused by all the flagella changing from anticlockwise to clockwise rotation at the same instant, but is the result of a critical number rotating in a clockwise direction at one time [3]. The mechanism causing direction changing is therefore not likely to be a general membrane-linked event. Control of the frequency of direction changing is fundamental to taxis. In chemotaxis the binding of a favourable chemical causes suppression of the switch to clockwise rotation. In phototaxis a change in the protonmotive force across the photosynthetic electron-transport chain causes a change in tumble frequency. Part of the direction-changing event must involve stopping and restarting the flagellar motor, usually in the opposite direction. For this reason we examined some of the properties involved in restarting flagellar rotation once stopped.

When grown anaerobically in the light *Rps. sphaeroides* develops both the components of cyclic photosynthetic electron transport and of respiratory electron transport [8]. These bacteria are therefore capable of developing a protonmotive force by respiring in the dark or using photosynthesis in the light. Respiration was inhibited when the bacteria were actinically illuminated, probably because the faster turnover rate of the photosynthetic electron transport inhibited the respiratory electron-transport pathway [9]. The rate of photosynthetic electron transport appeared to be many times that of respiratory electron transport in these bacteria, making the respiratory protonmotive force more sensitive to uncouplers than the photosynthetic one [9]. It was therefore possible to add a concentration of uncoupler to dark-incubated, light-grown bacteria which would collapse the protonmotive force and thus stop flagella rotation, but would not prevent the redevelopment of a maximum protonmotive force when the bacteria were again actinically illuminated. The bacteria should under these conditions be non-motile when incubated in the dark, and regain motility as the protonmotive force redeveloped.

The results presented here confirm this. When CCCP was added in low concentrations to bacteria incubated in the light, continued normal motility was observed which stopped after a few minutes dark incubation. This concentration of uncoupler therefore had no effect on continued flagellar rotation as long as a protonmotive force continued to be generated by photosynthetic electron transport. Measurement of the components of the protonmotive force by various methods also showed that low concentrations of uncoupler had no effect on the measured components in the light, but they collapsed rapidly when incubated in the dark. Reillumination resulted in a very fast redevelopment of the $\Delta\psi$ component of the protonmotive force, within the measuring time of the system, but a delay in the onset of flagellar rotation. The delay increased as the concentration of uncoupler was increased, and the consequent maximum $\Delta\psi$ reduced. Addition of increasing concentrations of uncoupler to illuminated bacteria resulted in a gradual reduction in swimming velocity after about 0.5 μM, but no observable effect between 0.01 and 0.2 μM.

These results show that photosynthetic bacteria were able to continue swimming using a very small protonmotive force, as previously suggested [17]. When the uncoupler was added to actinically illuminated bacteria they continued to swim, although with a reduced velocity, until the $\Delta\psi$ measured by the carotenoid bandshift was less than 5% of the maximum, suggesting an ability to continue rotation at below 10 mV.

After a period of dark incubation in the presence of uncoupler the resumption of motility on reillumination was not synchronous in the population, but individual bacteria would show different delays, although once motility was reinitiated there was not a gradual increase in velocity shown by individual bacteria; rather, they swam at maximum velocity. This variation in the length of the delay between bacteria could reflect either an uneven distribution of uncoupler molecules between bacteria cells, making some more uncoupled than others, or a difference in activity or concentration of a component required for flagellar rotation.

The delay in motility was shorter if $\Delta\psi$ was the major component of the protonmotive force,i.e., at alkaline pH values or in the presence of acetate. The problems involved in completely removing the $\Delta\psi$ component of the protonmotive force in photosynthetic bacteria, and measuring the true size of the ΔpH, make evaluation of this result difficult. It is possible, although difficult, to reconcile with the known properties of the flagellar motor [22]

that the electrical component of the protonmotive force is more efficient at initiating flagellar rotation than the chemical component. It is possible, however, that a protonmotive force of critical size must be maintained across the cytoplasmic membrane for a specific time for the flagellar motor to function, and the increased time observed in environments in which the ΔpH component of the protonmotive force was important reflected the slower rise of the ΔpH.

The difference in delay times at acid and alkaline pH values may reflect the mechanism of action of CCCP as a weak acid. As can be seen from the results a small change in CCCP concentration could have a measurable effect on the length of the motility delay. At acid pH values there may be decreased ionisation of CCCP and an increased ability to cross the cytoplasmic membrane. It would therefore be difficult to distinguish between an increased delay caused by more active CCCP molecules per cell or an increased ΔpH.

These results also suggest that the method of flow dialysis with DMO for measuring changes in ΔpH may give artificially long times for development of the ΔpH in whole bacteria. Although at high values of CCCP the delay in the development of motility was longer than the time measured for maximum ΔpH development, at zero or very low CCCP concentrations the time-course for ΔpH development to the maximum level, similar to that measured at high CCCP values, was longer than the time taken for some bacteria to start moving in conditions where the $\Delta\psi$ component was small. This suggests that a critical ΔpH value was reached before any substantial increase was measured by DMO distribution. For this reason we concentrated on the measurement and manipulation of the $\Delta\psi$ component of the protonmotive force.

Measurements of the $\Delta\psi$ component showed that the protonmotive force redeveloped to a value well above that required for motility many seconds, or in some cases many minutes, before motility resumed after illumination. We therefore looked for an additional protonmotive-force-dependent component which could also be involved in flagellar rotation. It has been shown that, although not the primary driving force for flagellar rotation, ATP is required for chemotaxis [18,19]. Chemotaxis involves the controlling of the frequency of direction changing of the flagella to bias the overall movement of bacteria in a favourable direction. This process may therefore involve a mechanism similar to the initiation of rotation, a stopping and restarting of rotation in another direction; we therefore measured the intracellular concentration of ATP in the presence of uncoupler. There appeared to be a linear relationship between the final intracellular ATP concentration and the protonmotive force developed at particular CCCP concentrations. However, not only was the final ATP concentration reached within the sampling time of the experiment, but bacteria treated with venturicidin to inhibit ATP formation via the ATPase showed no delay in the onset of motility. In the presence of venturicidin the addition of CCCP had the same effect on the delay as it had in the absence of the ATPase inhibitor. There was no evidence that ATP itself or an ATP-dependent reaction was required for the initiation of flagellar rotation.

These experiments confirm that the bacterial flagellar motor can be driven by either the electrical or chemical component of the protonmotive force. The generation of a protonmotive force was not, however, sufficient to initiate motor rotation immediately if the protonmotive force had been held at or close to zero for several minutes. The results presented here show that reinitiation of motor rotation after a period of reduced protonmotive force requires an additional protonmotive-force-dependent event.

This protonmotive-force-dependent event could be envisaged as a chemical motor modification occurring at low frequency. The increased delay with increasing uncoupler concentrations would suggest a number of modification sites per motor. Motor reversal in control circumstances would require modification of these sites, and a change in frequency of reversals with chemotaxis would be caused by the changing activity of a specific site-modifying enzyme. Total inactivation by the loss of protonmotive force would require multiple modifications for reinitiation, and thus the delay in motility. Attempts to identify a possible chemical modification have, however, been unsuccessful and the lack of involvement of ATP in the process suggests that modifications involving phosphoryla-

tion or methylation are unlikely.

The control of initiation of flagellar rotation may therefore involve a physical interaction of protons and specific channel proteins. The formation of a functional proton-conducting channel in or attached to the flagellar motor may require the physical interaction of protons with one or more proteins which constitute the pore. If the protonmotive force is decreased to zero, the physical integrity of the channels may be lost, either by conformational changes within the channel proteins or physical movement of one of the proteins from the complex. Motility would not be regained until the protonmotive force had been reconstituted at a critical level for enough time to allow reformation of the proton-conducting channel.

Total inactivation of the motor by either a physical or a chemical change to the structure could be a mechanism for preventing protonmotive force leakage through the proton-conducting channels of the flagellar motor under extremely adverse conditions.

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