

Initiation of flagellar rotation in *Rhodopseudomonas sphaeroides*

Evidence for the direct interaction of anionic uncouplers with the flagellar motor

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Received 22 April 1985

The collapse of the proton-motive force (pmf) in dark-incubated *Rps. sphaeroides* by the addition of low concentrations of anionic uncouplers caused a loss of motility. When added to bacteria under continuous illumination, the same uncoupler concentration had no effect on motility. Re-illumination of the non-motile, dark-incubated bacteria caused a very fast increase in the pmf, but there was a delay before pmf-driven flagellar rotation was initiated. The length of the delay, which could be many minutes, was shown to be dependent on the type of uncoupler used, the ΔpH component of the pmf and to be independent of the overall size of the pmf or the temperature. It is concluded that the delay in reinitiation of motility was due to the direct interaction of anionic uncouplers with sites on the flagellar motor proteins.

Chemiosmosis Motility Uncoupling agent Photosynthesis Respiration

1. INTRODUCTION

Flagellate bacteria move by rotating semi-rigid helical flagella [1]. This rotation is driven by a 'motor' complex embedded in the cytoplasmic membrane. It has been shown experimentally that the motor is a chemiosmotic machine driven by the pmf [2,3]. A number of models for the operation of the rotary motor have been developed [3–7]. These models propose that the motor rotation is driven by the movement of protons down the proton gradient. Interaction between the protons and the proteins of the motor turns the electrochemical energy of the gradient into the mechanical energy of rotation. The absence of a deuterium effect and

the temperature independence of the system suggest a simple, interactive process [5].

In the purple photosynthetic bacteria the pmf required for motor rotation can be generated by photosynthetic electron transport in the light or by respiratory electron transport or ATP hydrolysis in the dark. Although the maximum pmf generated is similar in the dark and light, the rate of pmf generation in the dark is much lower than in the light. This makes it possible to select concentrations of uncouplers which reduce the pmf to zero in the dark but allow full potential development in the light. Using the appropriate concentration of the uncoupler CCCP, we have previously shown that following a period in the dark at zero pmf, motility stopped and did not immediately restart on re-application of a pmf by illumination [8]. The length of the delay in re-initiation of motility varied with the concentration of CCCP. We suggested that this delay may have been caused by the disassociation of the proteins in the flagellar motor

Abbreviations: Mes, 4-morpholineethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; S13, 5-chloro-3-*t*-butyl-2'-nitro-salicylanilide; pmf, proton-motive force

involved in controlling the proton flow through the motor, the reformation of the proton pore requiring the re-imposition of a proton gradient. If this hypothesis were correct the delay in re-initiation should be dependent on the size of the re-imposed pmf, not the specific uncoupler used and the time taken for motility to restart might be expected to be temperature dependent if it involved the conformational change of a protein into a functional state.

We have now investigated these effects further and find that the anionic uncouplers S13 and DNP behaved in a similar manner to CCCP, but the dissipation of the pmf at alkaline pH values by valinomycin and potassium did not cause a delay. The length of the delay with the protonophores was dependent on the specific uncoupler used, not on the size of pmf it allowed to reform. We found that the length of the delay was also pH dependent but temperature independent. These results suggest a direct interaction of anionic uncouplers with the flagellar motor.

2. MATERIALS AND METHODS

Rhodospseudomonas sphaeroides WS8 (wild-type strain from W. Sistrom) was grown to early exponential phases in 100-ml filled bottles at 25°C under illumination as described [9]. After harvesting the bacteria were resuspended in 20 mM Hepes, Mes, glycylglycine buffer at the required pH.

The membrane potential was measured by the electrochromic carotenoid bandshift using an Aminco DW2 dual-wavelength spectrophotometer using the wavelength pair 510–523 nm.

Additions were made to the stirred, anaerobic cuvette as small volumes of concentrated solutions in ethanol, controls were ethanol without the inhibitor. Bacteria were stimulated with actinic light at 870 nm at 90° to the measuring beam using a variable light source. Bacteria were withdrawn at intervals from the cuvette of the spectrophotometer for microscopic examination. They were drawn by capillarity into 0.1 mm light path microslides (Camlabs, England) and the capillaries sealed. After a minimum of 15 min dark incubation they were examined using a Zeiss photomicroscope, and the time taken for the first cell in a field to regain motility and the time taken

to reach 50 and 100% of the motility of the untreated control population was measured.

The effect of temperature was determined using a temperature controlled stage fitted to the microscope. Capillaries were incubated at different temperatures in the dark for at least 15 min. The time taken to regain motility was followed with different concentrations of uncoupler at each temperature. A different sample was used for each treatment and temperature.

3. RESULTS

In *Rps. sphaeroides* changes in the membrane potential can be determined by measuring changes in the electrochromic bandshift of the membrane bound carotenoids, a rapid, non-invasive technique [10].

Fig.1 shows the changes in the potential under anaerobic conditions, on illumination and on the addition of uncouplers. CCCP and S13 had qualitatively similar effects on the membrane potential. DNP and the potassium ionophore valinomycin had similar effects but required high concentrations, probably because of problems crossing the outer membrane of the bacteria. In all cases there were concentrations which reduced the membrane potential in the dark but allowed re-establishment of a full membrane potential on illumination. At high concentrations both the dark and light membrane potentials were reduced.

The membrane potential in the dark could be reduced by either the protonophores or the cyclic polypeptide potassium ionophore valinomycin resulting in loss of motility. Fig.1 shows the time taken for the bacteria to become motile following re-imposition of a membrane potential by actinic illumination. Ionophore concentrations were chosen to give equivalent membrane potentials for each of the different ionophores. When individual bacteria regained motility, they immediately swam at the velocity observed for bacteria kept under continuous illumination at the appropriate pmf, i.e. they did not initially swim slowly and increase velocity but swam immediately at high speed. The 3 anionic proton ionophores all caused a delay before motility resumed, but there was no delay in the presence of valinomycin, even when acetate was present to remove the Δ pH component of the pmf. The length of the delay varied not with the

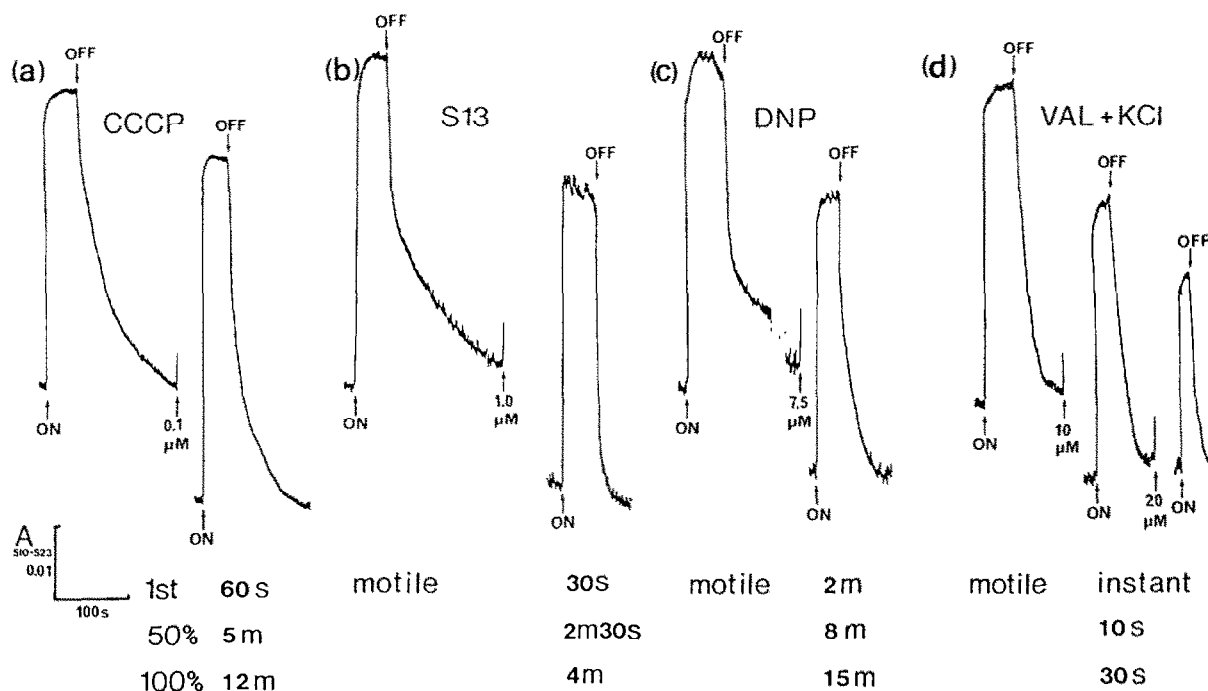


Fig.1. The light-induced carotenoid bandshift of whole cells of *Rps. sphaeroides* before and after inhibitor addition. The concentration of inhibitor was calculated to remove the dark membrane potential but allow reformation of a potential on re-illumination. The time taken for motility to re-initiate and to reach control levels after re-illumination is given under each treatment.

size of the membrane potential created on re-illumination but varied with the concentration of the specific uncoupler used.

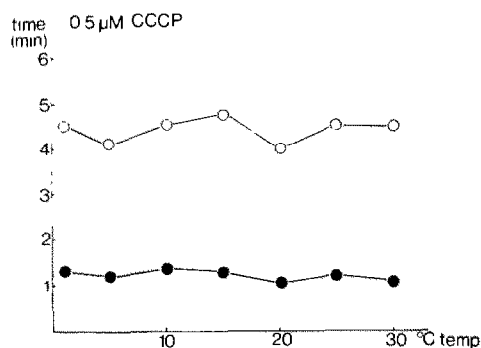


Fig.2. Delay in initiation of motility in bacteria treated in the dark with 0.5 μ M CCCP at temperatures from 2 to 30°C. A different sample of bacteria from a single population treated with the uncoupler was used for each temperature measurement. (●—●) Time taken for first cell in a field to swim, (○—○) time taken for population in a field to reach 50% of control motility.

Fig.2 shows the length of that delay for a specific concentration of CCCP at temperatures from 2 to 30°C. If the delay was the result of a conformational change, the delay would be expected to be reduced at high temperatures; however, the delay remained constant over the whole temperature range for any specific concentration of uncoupler.

The delay in re-initiation of motility was always longer at acid pH values than at alkaline values (table 1). To investigate whether this was a result of the pK of the uncouplers or reflected a difference in the role of the different component of the pmf in the initiation of flagellar rotation the Δ pH component of the pmf was reduced by the addition of potassium acetate to bacteria at pH 6.5. The total pmf remained similar as the $\Delta\psi$ component increased in response to the decreased Δ pH and presence of K⁺. Table 1 shows that the delay in motility at pH 6.5 with both Δ pH and $\Delta\psi$ components of the pmf was longer than the delay found at the same pH but with the Δ pH compo-

Table 1

Time taken for motility to start, reach 50% and 100% of control motility after re-illumination of bacteria treated with 0.75 μ M DNP in the dark

pH	Time taken for motility		
	1st	50%	100%
6.5	2 min	8 min	15 min
7.0	55 s	2 min 45 s	5 min
7.5	4 s	45 s	2 min
6.5 + Ac	10 s	1 min 30 s	5 min

The total pmf in all cases was approximately the same. The sample at pH 6.5 was treated with potassium acetate at pH 6.5 to increase the $\Delta\psi$ component of the pmf and decrease the Δ pH, pH and $\Delta\psi$ were measured before and after acetate addition

nent removed. The delay at pH 6.5 with an increased $\Delta\psi$ was similar to the length of delay for the same concentration of uncoupler at pH 7.5.

4. DISCUSSION

These experiments show that bacterial flagellar motor rotation was stopped when the transmembrane pmf was reduced to 0 mV by uncouplers. Application of a pmf as a result of photosynthetic electron transport resulted in restarting flagellar rotation at maximum rate, but with a delay. Direct microscopic observation of the flagella during the period of the delay showed that they were completely stationary during the delay (unpublished). The fact that motility when it started in individual bacteria was always at high velocity suggests that the delay was not caused by the sequential incorporation of force generating units reported by Block and Berg [11] for paralysed mutants of *Escherichia coli*.

The time taken to restart the flagellar motor was not, as had been expected, directly dependent on the size of the imposed pmf but was dependent both on the pmf and the type of uncoupler used to reduce the pmf to zero during dark incubation. When the potassium ionophore valinomycin, which has no external charge, was used there was no appreciable delay in restarting the motor. When the anionic proton ionophores CCCP, S13 or DNP were used however, there was a long delay before

motility was started. The results suggest that the uncouplers had two effects. The removal of the pmf by addition to bacteria in the dark resulted in the stopping of the pmf-driven flagellar motor. When this was the only result of the inhibitor, motility started immediately, as in the presence of valinomycin. The proton ionophores, however, produced a secondary inhibition which caused a long delay in restarting motor rotation. It is likely that this delay was caused by the direct interaction of the uncouplers with the motor as the length of the delay depended on the specific uncoupler used. As the length of the delay was temperature independent, the inhibition probably involved a simple ionic interaction with the motor and not protein conformational changes, enzyme activation or lipid fluidity. The length of the delay was concentration dependent and as *Rps. sphaeroides* has only one flagellum per cell this may indicate that there are multiple binding sites on each motor. The fact that these sites were only inhibited when the bacteria were held at 0 mV, i.e. when the flagellar motors were stopped, suggests that the sites had a high affinity for the uncouplers but that the uncouplers were excluded from the sites of inhibition during active motor rotation.

The delay was always longer when the chemical component of the pmf was part of the overall pmf and shorter, although present, if the Δ pH was replaced by the electrical component. Observation of dark-incubated, starved bacteria showed that there was a very short delay in motility in these cells when a pmf was re-imposed, and it was always longer at acid pH values.

A possible mechanism to account for the above results would be as follows. While a pmf is applied across the cytoplasmic membrane, the ion pore in the flagellar motor is open, exposing the ionic sites on the motor to high concentrations of ions (H^+/OH^-) preventing the binding of inhibitors to these sites. When the pmf is abolished the pore may close, leaving the ion binding sites in a hydrophobic environment. Under these conditions the anionic inhibitors are able to bind to anion binding sites on the motor. When a pmf is re-applied across the membrane the pore re-opens. It re-opens in response to the $\Delta\psi$ component of the pmf rather than the Δ pH component, suggesting a voltage-dependent ion channel. The uncoupler bound to the anion binding site on the motor is

now re-exposed to the extracellular medium but the dissociation of the uncoupler is slow and rotation is inhibited until all the binding sites are free. The binding sites could be non-specific with inhibition due to steric effect. However, the inhibition of motility by lipophilic anionic uncouplers may be due to binding to specific anion binding sites within the motor proteins. Most models for flagellar rotation involve the biased transfer of protons via specific charged sites on two different components of the motor, a rotor and a stator, but the observation that anionic uncouplers inhibit rotation may suggest that the driving ion could be OH^- rather than H^+ .

ACKNOWLEDGEMENTS

J.P.A. is a Lister Institute Advanced Fellow. We would like to thank Professor J.M. Palmer (Imperial College, London) for the gift of S13.

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