TRANSMEMBRANE GRADIENT OF K^+ AND Na^+ IONS AS AN ENERGY BUFFER IN *HALOBACTERIUM HALOBIUM* CELLS

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Received 10 August 1981

1. Introduction

A $\Delta\bar{\mu}H^+$-buffering function was proposed for the Na^+/K^+ ion gradient in bacteria [1,2]. It was suggested that upon lowering $\Delta\bar{\mu}H^+$ due to insufficient energy supply, K^+ would diffuse out of the cells down its concentration gradient, forming $\Delta\Psi$. Simultaneously, Na^+ would penetrate into the cells in exchange for H^+ by means of a Na^+/H^+-antiporter, maintaining a ΔpH. Data supporting this hypothesis were obtained in our study of *Escherichia coli* energetics [3,4]. We found that a preformed Na^+/K^+ gradient stabilized the membrane potential and supported motility of an H^+–ATP-synthetase-deficient *uncA* strain of *E. coli* under anaerobic conditions when both respiratory and ATPase mechanisms of $\Delta\bar{\mu}H^+$-generation were not operative.

It may be expected that the $\Delta\bar{\mu}H^+$-buffering role of the Na^+/K^+ gradient is most pronounced in *Halobacteria* whose natural habitat involves very high salt concentrations. In *Halobacterium halobium* cells an ATP decay accompanying transition from aerobiosis to anaerobic conditions in the dark was found to depend on the magnitude of the K^+ gradient [5]. The ATP level decreased much faster in 2.7 M KCl as compared to 0.027 M KCl. It was concluded [5] that in the dark, K^+ extrusion down its concentration gradient forms a membrane potential that supports ATP synthesis. It may be argued, however, that the high extracellular K^+ activates in some way ATP hydrolysis rather than decreases the rate of ATP synthesis by the membrane H^+–ATP-synthetase. It seemed important to discriminate between the above possibilities. Besides, it was interesting to investigate the ability of the Na^+/K^+-gradient to support a function that, unlike ATP synthesis, may operate at comparatively low levels of $\Delta\bar{\mu}H^+$. Bacterial motility that is driven by $\Delta\bar{\mu}H^+$ [6–10] was believed to be such a function.

Here, we have found that the Na^+/K^+ gradient drives net ATP synthesis and is especially efficient in supporting *H. halobium* motility under dark anaerobic conditions.

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide

2. Materials and methods

2.1. *Bacterial growth*

Halobacterium halobium R_1M_1 (kindly supplied by Dr D. Oesterhelt) lacking gas vacuoles and bacterioruberin was grown on a complex medium [11], containing 25% NaCl, 0.2% KCl, 1% $MgSO_4 \cdot 7\ H_2O$, 0.02% $CaCl_2 \cdot 2\ H_2O$, 0.3% Na-citrate and 0.5% peptone Oxoid. The cells were kept at 37°C under white light illumination (1.5 mW/cm^2) and limited aeration to induce rhodopsin synthesis. After a 72 h steady-state phase, the cells were sedimented by centrifugation, washed in the growth medium without peptone and resuspended in one of the following media: 100% Na^+ medium (4270 mM NaCl, 27 mM KCl, 81 mM $MgSO_4$); 100% K^+ medium (1570 mM NaCl, 2700 mM KCl, 81 mM $MgSO_4$); 40% Na^+ or K^+ media which were obtained by appropriate dilution of the 100% media. The exponential phase cells without bacteriorhodopsin were grown in the dark under efficient aerations and harvested 20–30 h after inoculation.

2.2. *Motility rate measurements*

To monitor the motility rate, the bacteriorhodopsin-containing cells were suspended in a 100% Na^+ medium and incubated at 37°C for 24 h. This proce-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

dure sufficiently increased both the portion of motile cells in the population and the motility rate, presumably due to an increase of the number of flagellae per cell. For anaerobic observations, a drop of vaseline oil was placed on a slide and covered with a cover slip. The bacterial suspension (~10^9 cells/ml) was deaerated by being blown through with N_2 and injected into the middle of the vaseline oil chamber by a microsyringe. Observations were made with a Univar (Reichert) microscope under phase contrast. The slide was kept at 37°C on a thermostatted stage, 'Bioterm' Reichert. The cells lacking bacteriorhodopsin were illuminated by white light; the bacteriorhodopsin-containing cells were observed under a low intensity blue light ($\lambda < 490$ nm) that did not induce motility. At the beginning of the experiment, the main motility rate was determined by measuring the rate of 20–30 cells moving along an ocular scale. Then the time required for 10 cells to cross a line dividing the microscope field by half was measured and the rate of motility at a given moment was calculated according to [12].

2.3. *Measurement of ATP*

A cell suspension (2 ml) containing 1–2 mg protein/ml were placed into a transparent chamber at 25–27°C or 37°C. The cells were illuminated with a 900 W burner with an intensity of 50 mW/cm^2. Light was passed through a heat filter and an orange filter. For ATP determination, 10 μl samples were removed by a microsyringe and injected into 500 μl 0.1 M Tris–acetate, 0.1 mM EDTA (pH 7.75) to bring about immediate cell lysis. ATP was monitored by firefly luciferase (ATP-measuring kit, LKB) according to [13] with a 'Pico-ATP' luminometer (Jobin-Yvon).

Protein was determined following [14].

Valinomycin was from Sigma, DCCD from Serva, peptone (Oxoid), all the other chemicals were of reagent grade.

3. Results

3.1. *ATP synthesis driven by Na^+/K^+ gradient*

Illumination of *H. halobium* cells was found to increase the ATP concentration up to its maximal level (7–10 nmol/mg protein) within 2 min.

Switching off the light led to a drop of the ATP level, which was especially dramatic in the K^+ medium. In the Na^+ medium, the ATP decrease was not so

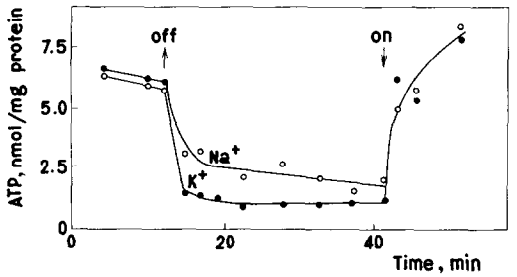

Fig.1. Dependence of the dark anaerobic ATP level on ion composition of the medium. Cells were suspended in 100% Na^+ (–○–) or 100% K^+ (–●–) medium.

pronounced. The ATP concentration was stabilized at a level lower than that in the light but higher than in the dark when the K^+ medium was used (fig.1). This result agrees fairly well with the data in [5].

The lower rate of ATP decay in the Na^+ medium as compared to the K^+ medium can be due either to a slower ATP hydrolysis or to a faster ATP synthesis. In both cases, H^+–ATP-synthetase might be involved, forming $\Delta\bar{\mu}H^+$ at the expense of ATP, which is used to maintain a Na^+/K^+ gradient, or alternatively, forming ATP at the expense of $\Delta\bar{\mu}H^+$ maintained by dissipation of a Na^+/K^+ gradient. These two possibilities may be discriminated between using DCCD, an H^+–ATP-synthetase inhibitor, which must increase the ATP level in the former and decrease it in the latter case.

Addition of DCCD to *H. halobium* cells in the light was shown to decrease the ATP level, but the rate of inhibition was very low, presumably due to slow penetration of the reagent into the cell. In order to increase the permeability, the cells were swollen in a hypotonic 40% medium. The level of ATP in the light was the same in the 40% Na^+ medium and 100% Na^+ medium, in accordance with [5]. In the 40% K^+ medium, the ATP level was lower than in the K^+ (or

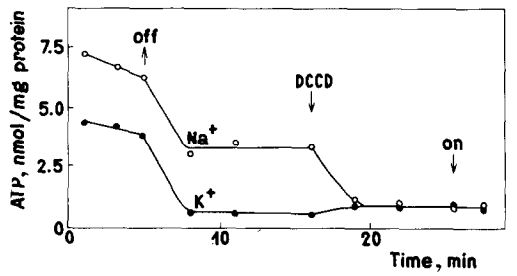

Fig.2. Action of DCCD on the dark anaerobic ATP level. Cells were suspended in 40% Na^+ (–○–) or 40% K^+ (–●–) medium. Addition, 6 × 10^{-4} M DCCD.

Na^+) 100% solution. We attribute this to partial lysis of cells in the 40% K^+ medium. DCCD appeared to rapidly inhibit light-driven ATP synthesis in both 40% Na^+ and 40% K^+. After placing cells in the dark under anaerobic conditions (fig.2), ATP rapidly decreased in the 40% K^+ medium and became stabilized at ~1/2 of the light level in the 40% Na^+ medium. Addition of DCCD caused a rapid decrease in the ATP concentration in the Na^+ medium to the level of ATP in the K^+ medium. DCCD had no effect on ATP concentration in the K^+ medium. Subsequent illumination failed to increase the ATP level, indicating complete inhibition of the H^+–ATP-synthetase.

It may be then concluded that stabilization of the ATP level in the Na^+ medium is due to net ATP synthesis by H^+–ATP-synthetase.

Duration of ATP synthesis in the dark varied in different experiments, but was ⩽3 h. Addition of DCCD to the cells in the dark after this period did not influence the ATP level.

Valinomycin, like DCCD, was found to be active only in the 40% medium. Addition of valinomycin to *H. halobium* cells in the Na^+ medium simultaneously with switching off the light essentially decreased the initial rate of ATP decay (fig.3). However, the situation changed by 30 min. Now the ATP level in the presence of valinomycin appeared to be lower than in the control (without the ionophore) sample. After 80 min in the dark, valinomycin was added to the control cells, bringing about a significant increase in the ATP level that was, however, unstable and spontaneously decreased several minutes later (fig.3).

3.2. *Motility of* H. halobium *in the absence of exogenous energy sources*

Bacteria from steady state phase were placed into an anerobic chamber and illuminated by weak blue light that did not energize the cells. In the K^+ medium, motility vanished within 15 min, whereas in the Na^+ medium it slowly decreased and was still apparent after 4 h in the dark. In both cases, subsequent illumination by yellow light increased the motility rate to the initial level (fig.4).

Motility was even more effectively supported by a Na^+/K^+-gradient in exponentially grown cells. Grown under aerobic conditions, these cells lacked bacteriorhodopsin. In the K^+ medium, oxygen exhaustion immediately paralyzed bacteria. In the Na^+ medium, however, the bacteria were still motile after 9 h incubation under anaerobic conditions (fig.5).

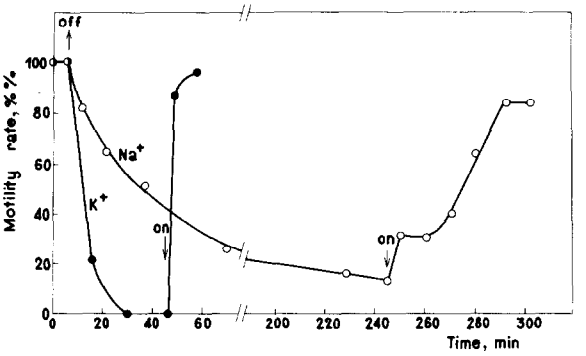

Fig.4. Dependence of *H. halobium* motility on the ion composition of the medium under anaerobic conditions in the dark. Cells were suspended in 100% Na^+ (–○–) or 100% K^+ (–●–) medium. Maximal motility rate was ~3 μm/s in both media. Changes in yellow light (λ > 520 nm) are indicated by arrows.

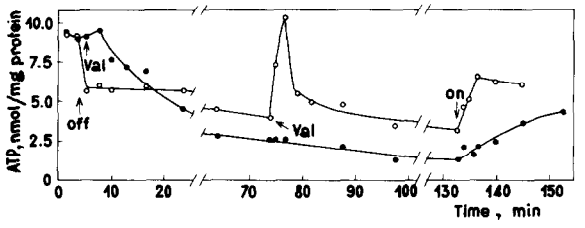

Fig.3. Changes in the dark anaerobic ATP level induced by valinomycin. Cells were suspended in 40% Na^+ medium. Valinomycin (Val) was added as indicated by arrows to a final concentration of 10^{-5} M at 4 min (–●–) or 73 min (–○–) incubation.

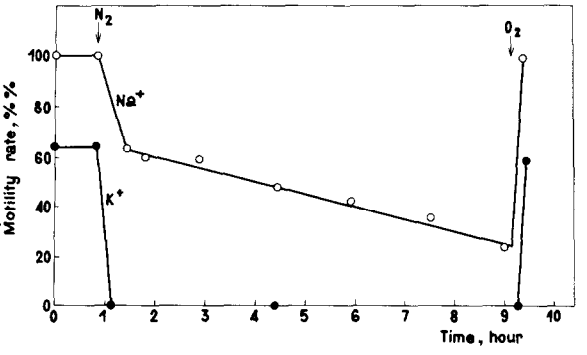

Fig.5. Motility of *H. halobium* lacking bacteriorhodopsin. Cells were grown under extensive aeration to the exponential phase. At indicated times the suspensions were deaerated. Aeration was provided by temporary opening the chamber. 100% Na^+ (–○–) or 100% K^+ (–●–) media were used.

4. Discussion

ATP synthesis driven by ΔpK in bacterial cells preloaded with K^+ then placed in a K^+-free medium with valinomycin has been observed [15–17]. The hypothesis of a $\Delta\bar{\mu}H^+$-buffering function of the Na^+/K^+-gradient presupposed that ATP may be synthesized during the dissipation of Na^+ and K^+ gradients in the absence of added ionophores, i.e., by means of an endogenous K^+ uniporter and Na^+/H^+ antiporter [1,2].

In agreement, a stabilization of the ATP level was demonstrated [5] in *H. halobium* cells during a post-illumination period if the cells were incubated in a Na^+-rich, K^+-poor medium, i.e., if there were high Na^+/K^+ gradients between the cytoplasm and the outer solution. In a K^+-rich, Na^+-poor medium the ATP decay was very fast, which could be due to a higher rate of hydrolysis of pre-synthesized ATP or, alternatively, to a lower net ATP synthesis rate. Enhanced ATP hydrolysis in a K^+ medium might have been a result of a K^+ electrophoresis at the expense of energy of a pre-formed ATP: ATP $\rightarrow \Delta\Psi \rightarrow \Delta pK$ (see [18]), whereas lower ATP synthesis should have been a consequence of the lack of ΔpK and the absence of the $\Delta pK \rightarrow \Delta\Psi \rightarrow$ ATP energy transduction. A similar reasoning can be applied to transduction the ΔpNa component of the Na^+/K^+ gradient.

Our data show that the ATP level stabilization in the Na^+ medium is due to a net ATP formation mediated by H^+–ATP-synthetase since DCCD addition immediately decreases the ATP concentration in the Na^+ medium down to the level observed in the K^+ medium. As to the ATP amount in cells incubated in the K^+ medium, it hardly changed after the DCCD treatment (fig.2). It is clear that in terms of the enhanced ATP hydrolysis concept, DCCD had to increase the ATP level in the K^+ medium up to that in the Na^+ medium, the latter being unaffected.

Moreover, the above data on the DCCD effect can be discussed with respect to the possible involvement of a K^+-ATPase. This DCCD-resistant enzyme inducible under K^+-poor conditions in *E. coli* [19,20], if existing in *H. halobium*, should in principle be responsible for net ATP synthesis coupled with K^+ efflux. In this case, however, DCCD had to be without effect in both the Na^+- and K^+-medium.

A DCCD-induced decrease in the dark, anaerobic ATP level in *H. halobium* was first observed in [21], where either CCCP or DCCD accelerated the ATP decay. Rapid ATP exhaustion in the presence of an uncoupler was interpreted as being due to activation of the H^+-ATPase. In [21] the DCCD effect remained a puzzle as the Na^+/K^+ gradient as an energy source was not known and the role of ionic composition of the medium in the action of DCCD was not analyzed.

Discussing the DCCD experiments, we would like to mention an important technical point. To observe the effect of DCCD as well as of valinomycin, we dilute a 100% medium down to 40%. Such treatment breaks down a barrier preventing the cytoplasmic membrane from being attacked by the above compounds.

This observation is consistent with our finding that in a 100% medium, DCCD inhibits photophosphorylation only slightly [22], and with [5] where monaktin was used instead of valinomycin which did not penetrate into the cells.

A study of the rate of ATP decay in the presence of valinomycin (fig.3) provides an insight into the strategy that is used by the cell when energy is supplied by a Na^+/K^+-gradient. During the first 20 min in the dark, the ATP pool decreases more slowly with than without valinomycin. However, on longer dark incubation, valinomycin proved unfavourable. In fact, an endogenous system regulates the activity of the ATP-stabilizing mechanism in such a way that, in the Na^+ medium, cessation of illumination decreases the ATP level rather quickly to a certain, relatively stable, value which is lower than in the light, but higher than in the K^+ medium. In the presence of valinomycin, on the contrary, a permanent decrease in the ATP pool occurs, so that complete exhaustion of ATP comes about faster than in the absence of the ionophore. Apparently, the cell spends its ion gradients with great parsimony to prevent the bankruptcy of the cellular energetics.

Such a control continues even when the energy level proves insufficient to support ATP formation. This conclusion is supported by our finding that in the Na^+ medium, bacterial motility can be still observed after a 9 h anaerobic dark incubation when ATP synthesis does not occur. Under such conditions, the cation gradients support those types of $\Delta\bar{\mu}H^+$-linked work that can operate at low (non-phosphorylating) levels of $\Delta\bar{\mu}H^+$. Besides bacterial motility, it may be uphill transport of metabolites.

The very fact that some membrane energization survives 9 h storage of bacteria with a Na^+/K^+-gradient as the only energy source points to the following policy persued by the halobacterial cell during day-light hours: they invest some energy in a Na^+/K^+-gradient, to regain it during the night.

Acknowledgements

The authors wish to thank Dr D. Oesterhelt for providing the *Halobacterium halobium* R_1M_1 strain; Dr L. A. Drachev for helpful suggestions; Mr M. Galperin for help in determining ATP concentration.

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