Biochimica et Biophysica Acta, 1144 (1993) 391–395

BBABIO 43890

Definition of a duration threshold in the first step for energy transduction by *Escherichia coli* by use of a microsecond electric field pulse

Nathalie Eynard and Justin Teissié

Departement des Glycoconjugués et Biomembranes, Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, Toulouse (France)

(Received 1 December 1992)
(Revised manuscript received 16 March 1993)

Key words: Energy transduction; Microsecond electric field pulse; Membrane potential; Fast energy transduction

The total ATP content increased in *Escherichia coli* when the bacteria were submitted to square wave electric pulses with field intensities of about 1 kV/cm and duration larger than 20 μs. Nothing was observed when duration was shorter. The transduction of energy from its electrical form (membrane potential) to another, stored in the membrane which was converted later in the terminal P bond of ATP, required a key-step which had to last for more than this threshold of 20 μs. Square wave electric-field pulses appeared as a new non-invasive approach for the determination of the kinetics of fast energy-transduction events.

Introduction

The basic mechanism of energy transduction during respiration is postulated to be the conversion of the electrochemical gradient evoked by the respiratory chain into the terminal phosphate bond of ATP. Creation of an artificial membrane potential by chemical means (loading by K^+ and treatment by valynomycin) gave an experimental test of this hypothesis [1] in the case of *E. coli* where the use of mutants showed that ATPase was involved in the conversion. A major criticism of these studies was the alteration of the bacterial envelope induced by the use of chemicals. A less perturbing procedure for modulation of the potential has been recently proposed [2,3]. When cell suspension is submitted to an external field, the membrane potential is altered relative to the external stimulus. ATP synthesis was induced by pulsing chloroplasts [4,5], bacterial ATPsynthase containing liposomes [6], bacteria [7] and submitochondrial particles [8,9].

Besides the advantage of being a non-invasive method, the electric pulse technique allows the modulation of membrane potentials down to a microsecond time scale. Investigation of very fast processes involved in energy transduction is therefore possible [7–9]. In most cases, long-lasting repetitive AC fields are used in order to accumulate successive stimulations [10,11]. From the dependence of the process to the pulse frequency, it was possible to get access to the kinetic parameters of enzymes [12–14]. In this study, we used a limited number of DC pulses. Our previous study lead us to postulate that the energy conversion in the bacterial membrane was a very fast process (on the microsecond range) which was induced when the membrane potential was larger than a threshold [7]. In the present communication, by use of more sophisticated technology, we gave direct evidence that the conversion of the energy from its electrical form to another, stored in the membrane, occurs when the field is applied for more than 20 μs. This process which is localized on the cell surface can occur only if the membrane potential is larger than a threshold as previously described [7]. The membrane stored form of energy is later converted in the final bond of ATP giving newly synthesized ATP.

Correspondence to: J. Teissié, Departement des Glycoconjugués et Biomembranes, Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, 118 route de Narbonne, F-31062 Toulouse, France.

Materials and Methods

Bacteria

E. coli (strain CB0129) was provided by Pr. Louarn (of this institute). The *E. coli* strain CB0129, derived from K12 (W1485 F^- $thia^-$, leu^-, thy^-, sup E42, deo B or C) was grown in the mineral medium M9, where glucose is the carbon source, supplemented with thymine (20 μg/ml) and leucine (20 μg/ml). Bacteria were grown in batches of 30 ml at 37°C with continuous air bubbling. Cells were kept for 2 h in the station-

nary phase before harvesting. The bacteria were then washed 3-times in Tris 100 mM.

Depletion of cytoplasmic ATP

The method was described in Ref. 7. Briefly, cells in the stationnary phase were washed in 100 mM Tris (pH 8) and incubated during 2 h at 37°C in 20 ml of 250 mM sucrose, 15 mM NaCN and 10 mM Tris (pH 8). The bacteria were then diluted to a suitable concentration in the same buffer. The electron-transport chain was thus blocked by cyanide.

Voltage-pulse experiments

200 μl of the bacterial suspension were put in a pulsing chamber (two parallel flat stainless steel electrodes at a distance of 5 mm). The high voltage was provided by a high power generator (Cober 605). In some experiments, another generator (CNRS Cell Electropulser) was used when longer pulses were needed. The common characteristic of the pulses delivered by these generators was that they were square, i.e., the field intensity E remained constant during the pulse duration t.

As the electrodes are parallel and flat, the field is uniform in any position of the pulsed volume.

The associated temperature increase was computed by assuming that all the electrical energy generated during the pulse was converted into Joule heating of the sample. At 2 kV/cm with a duration of 0.1 ms, the temperature increase was less than 1°C.

Orientation of bacteria observed by changes in transmitted light

The change of transmitted light by the bacterial suspension during the electric pulse was followed on line on a very fast spectrophotometer designed by our group and described elsewhere [15].

ATP content increase determination

A differential method was used. The content of the unpulsed sample was first measured. It was then compared to the content of the pulsed sample. ATP was measured by bioluminescence as in Ref. 7. Calibration was obtained by counting samples of a known ATP concentration which received the same treatment as the bacterial suspension. 20 pmol of nucleotides gave a signal of 10^5 cpm and unpulsed samples 15000 cpm (average value). The increase due to the electric pulse was of this order of magnitude (15000 cpm).

Results

Influence of the pulse duration and electric field strength on ATP synthesis

Our previous work shown that cytoplasmic ATP content in intact *E. coli* increased with a capacitor discharge generator [7]. Evidence that F_1F_0-ATPase was implicated in this process is given by the observation that synthesis was blocked by uncouplers, F_1F_0-ATPase inhibitor and ionophores (valinomycin) and not induced in unc mutants [7]. Results presented here were obtained with square wave electric pulses, which are more controlled than exponential decay fields. This technology allow us to analyse effet of the pulse duration without modification of the others experimental parameters (ionic composition or field amplitude).

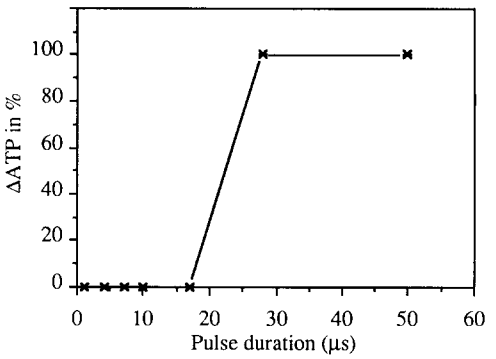

Fig. 1. Relative ATP change as a function of the pulse duration. After depletion of cytoplasmic ATP, bacteria were pulsed with two pulses (field intensity 1.4 kV/cm). The ATP content increase determination was made by bioluminescence assay (see Materials and Methods). The 100% change was about 1000 pmol ATP per mg of bacteria.

Using a field with a 1.4 kV/cm magnitude, effect of the pulse duration was studied (Fig. 1). A pulse duration threshold was present, no ATP synthesis was observed with pulses shorter than 16 μs. For longer pulsations, a strong increase of cytoplasmic ATP was present followed by a plateau when the pulse lasted more than 28 μs. The plateau was present with pulse durations as large as 180 μs (data not shown).

With a 50 μs pulse, ATP synthesis was triggered only with a field intensity larger than a threshold of about 0.1–0.2 kV/cm (Fig. 2). Yield of synthesis did not increase in a significant way if the field strength was increased up to 0.75 kV/cm and then slightly decreased for larger field intensities.

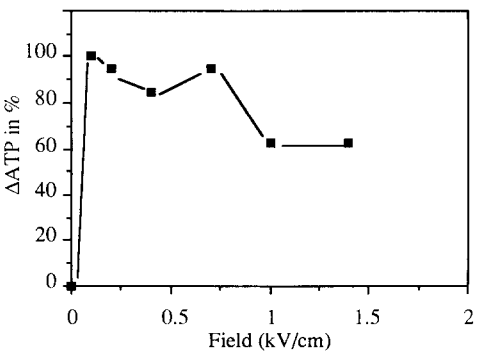

Fig. 2. Relative ATP change as a function of the field intensity. Bacteria were treated as in Fig. 1, the pulse duration was 50 μs and the number of pulses set at 2.

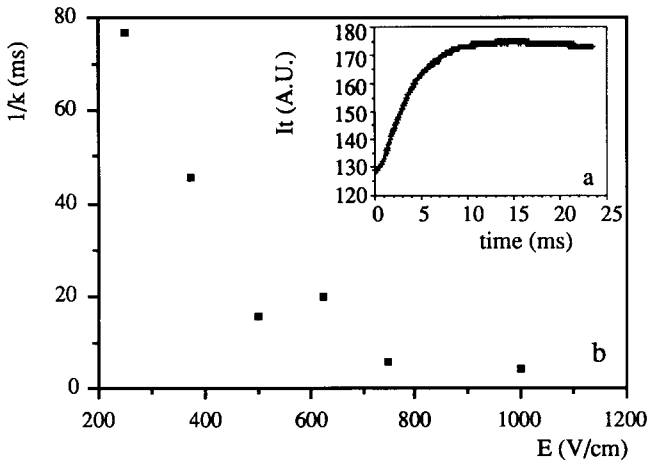

Fig. 3. Orientation of bacteria during a pulse. The measurements were made on a home-made apparatus, increase of transmited light was correlated with the aligment of the long axis of the bacteria along field lines. (a) Kinetics of transmitted light variation during a pulse of 24 ms at 750 V/cm. Orientation can be fitted by an exponential law $(1-\exp(-kt))$. (b) Evolution of the orientation rise time $(1/k)$ as a function of the field intensity.

Induced potential is correlated with orientation changes of the bacteria during the pulse

In this study the use of NaCN, for the depletion of ATP, blocked the electron transfer chain and thus abolished the two components of protonmotive force ($\Delta\Phi$ and ΔpH). The only transmembrane potential difference then available for the cell was the electrical-induced one.

From Bernhardt and Pauly's calculations [17], the field-induced membrane-potential difference depends on the relative position of the main axis of the rod relative to the field lines and on the shape factor F, i.e., the ratio between the main and the minor axis. For non spherical cells, the orientation of the long axis of the cell during the electric pulse must be taken into account for these calculations.

Orientation of rod-shaped cells like *E. coli* was observed by the associated turbidity change previously described [15]. During the pulse, the orientation process occurs according an exponential law (Fig. 3a). At the end of this process (equilibrium) the final degree of orientation imposed on the suspension, depends on the field strength. The kinetics of the orientation process could then be measured (Fig. 3b).

Under field conditions used in this study (smaller than 1 kV/cm) the orientation process occurred with a rise time longer than 1 ms (i.e., much longer than the pulse duration), then, the field is applied to a bacterial population in which the rods have a random distribution.

A range of induced potential differences prone to stimulate ATP synthesis were then computed and listed in Table I. As the length of a bacteria was very small, the loading time of the membrane was very short (less than 1 μs [17]) and under our experimental conditions the steady-state value was reached.

Simulation of the field-induced activity

As described above, external fields induced a transmembrane potential difference (ΔV_E).

$$\Delta V_E = fgrE \cos\theta \tag{1}$$

Assuming that (i) ATP synthesis occurs only when ΔV_E was larger than a critical value ΔV_S; (ii) ΔV_E was locally induced on a cell surface defined by the angle θ. Then, ATPase activity would have appeared only inside a cap of angle θ_S such as:

$$fgrE \cos\theta_S = \Delta V_S \tag{2}$$

The critical field value E_S (corresponding to the minimal surface activated, $\cos\theta = 1$) is defined by:

$$E_S = \Delta V_S / fgr = E \cos\theta_S \tag{3}$$

If we assume that the density of ATPsynthases is uniform on the cell surface, the number of synthesized ATP molecules is a function of the surface defined by θ_S, and then of the field strength.

$$\text{ATP} = K(1-\cos\theta_S) = K(1-E_S/E) \tag{4}$$

where K is a constant

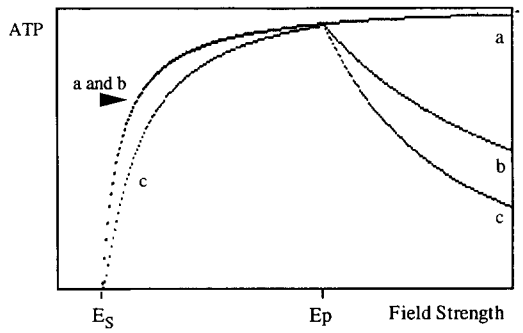

Fig. 4. Simulation of the field-induced activity. Simulated profile of ATP synthesis by the bacteria when submitted to an electric field. (a) This profile is obtained from Eqn. 4. The number of synthesized ATP molecules is a linear function of the surface inside the cap defined by the electric field. (b) Simulation of the field-induced activity when permeabilization occurs. This profile is obtained from Eqn. 6. Taking in account the inhibition of ATP synthesis in permeabilized areas, ATP is synthesized only in the part of the cell surface where the field (E) induced potential difference is large enough to induce the effect (E_S) but lower than the permeabilizing threshold (E_p). In this field range, the ATPase activity does not depend on the magnitude of the transmembrane potential ('all or nothing' law). Profiles a and b have the same rising phase. (c) Simulation of field-induced ATP synthesis, when ATPase activity is dependent on the potential difference. The profile is obtained from Eqn. 8. In this hypothesis, the synthesis is possible only in non permeabilized areas, but the rate of synthesis is proportional to the difference between the local potential difference and the critical value needed to induce ATP synthesis.

The simulation is plotted in Fig. 4a and could not explain the decrease in synthesized ATP observed with high field pulses. As we observed that synthesis was present even when cells where permeabilized, we had to use a localized description of the energy transducing event.

Taking into account the correlation between the occurrence of this decrease and the induction of the permeabilized state of the bacterial membrane [15], another condition had to be added to our simulation. A local inhibition of synthesis could occur when the membrane had been permeabilized (i.e., where the potential difference had been brought to a critical value [18,19]). If E_p was the field inducing this critical value for ($\theta = 0$), then for a field E, the area which was permeabilized is:

$$A_p = A_t(1 - E_p/E) \tag{5}$$

The number of synthesized ATP molecules would then be:

$$(\mathrm{ATP}) = K((1 - E_S/E) - (1 - E_p/E)) = K((E_p - E_S)/E) \tag{6}$$

The simulated plot from Eqn. 6 is given in Fig. 4b.

However, this model used an 'all or nothing' dependence of the synthesis rate on the potential difference. Results by other groups suggested that at constant ΔpH, the rate would be dependent on the potential difference [20–22]. This observation could be included in our simulation with the restriction that the rate was proportional to the difference between the local potential difference and the critical value needed to induce the ATP synthesis:

$$(ATP)(\theta) = K_1(\Delta V(\theta) - \Delta V_S) = K_1(fgrE\cos\theta - fgrE_S) \tag{7}$$

By use of the previous model, i.e., synthesis activated inside a cap and prevented where electropermeabilization was triggered, we obtained:

$$(\mathrm{ATP})(\mathrm{cell}) = K_2\left((1 - E_S/E)^2 - (1 - E_p/E)^2\right) \tag{8}$$

Results from Eqn. 8 are plotted in Fig. 4c.

Comparing the experimental data (Fig. 2) and the simulations (Fig. 4) led to the following conclusions:

(1) The increase in synthesized ATP was very steep for field values just above the threshold as obtained with the 'all or nothing' hypothesis; (2) the decrease when permeabilization was induced was small (about 30% at 1.5 E_p) as obtained with the 'all or nothing' hypothesis.

The conclusion is that a fair correlation is obtained between the experimental results and the 'all or nothing' hypothesis.

Discussion

As largely discussed in other works on the electric pulsation of energy-transducing membrane containing closed vesicular systems (thylakoids [4]; mitochondria [8,9] or bacteria [6,7]), the major effect of this perturbation was not the temperature increase (less than 1°C in the present experiments) but the electric field induced membrane potential difference change [2]. This effect was known to follow very closely the application of the external field; the delay being less than 0.1 μs in the case of *E. coli* (from the mathematical work in Ref. 2). As a consequence, we were able to consider that under the present experimental conditions (time scale: tens of microseconds), the kinetics of the membrane potential difference change were almost the same as the ones of the external field (i.e., of the applied voltage). Using a square-wave electric pulse, membrane potential difference of each point at the surface of the bacteria was altered from a well-defined value during a given time.

The field requirement was explained by the induced membrane potential difference change which had to increase beyond a given threshold in order to induce the synthesis of ATP by ATPase. Using as values 1.5 for f (impermeable sphere) and 1 μm for r, this threshold in membrane potential increase was of the order of 20–30 mV. Another feature of the dependence of the ATP synthesis on the magnitude of the field was the decrease which was observed at high field values (0.75 kV/cm; Fig. 2). This effect was linked to the electropermeabilization of the membrane [2,23]. A fair analogy was present between field intensities where permeabilization was triggered [15] and ATP synthesis began to decrease.

The electroconformational coupling described the effect of the external field as mediated by a conformational change of the membraneous enzyme involved in the energy transduction [10]. A simple model considers a two-state conformational transition:

$$P_1 \underset{K_{-1}}{\overset{K_1}{\longleftrightarrow}} P_2 \tag{15}$$

where K_1 and K_{-1} are the rate constants of the conformational changes. ATP can be released from ATP synthetase only from the P_2 conformation. When no external field is present, only the P_1 state is present. The external field induced a decrease in the activation energy barrier between P_1 and P_2. The P_2 state may then be populated and ATP will be released from ATP synthetase. A net ATP synthesis is observed after the external field pulse, the enzyme relaxes to the P_1 conformation where a bound ATP molecule is formed from ADP and P_i.

But if the ATP synthesis we observed was simply due to a two-state conformational transition, we should

have observed an exponentional dependence of the increase in ATP on the pulse duration. From our results, a duration threshold was observed (sigmoidal profile), we then must conclude that the conformational change to the activated state P_2 occurs through a complex pathway. No clear-cut explanation can be proposed for the time requirement which is present in the bacterial and mitochondrial systems [7–9]. We must take into account that the energy-transducing system is a complex set of macromolecules embedded in the phospholipidic matrix.

As a general conclusion, this work provides definitive evidence that energy transduction in membranes is a fast process (microsecond range). Nevertheless, due to the technique which was used to monitor the ATP synthesis (measurements on extracts) we were not following the direct synthesis during the pulse. During the pulsation the energy was converted from an electrical form (membrane potential difference) into another which was stored in the membrane, this conversion requiring at least 20 μs. This stored energy, when it was large enough as indicated by the existence of a field threshold, was then converted in a chemical bond during the synthesis of ATP. This last process was known to be much slower (on the millisecond–second range) [31] and has to be explained.

Acknowledgements

This work was supported by the CNRS ("ATP Bioenergétique" grant to J.T.). N.E. was the recipient of a predoctoral fellowship from the MRT. Thanks are due to Mr G. Holland for rereading of the manuscript.

References

1 Wilson, D.M., Alderete, J.F., Maloney, P.C. and Wilson, T.H. (1975) J. Bacteriol. 126, 327–337.
2 Kinosita, K. and Tsong, T.Y. (1977) Proc. Natl. Acad. Sci. USA 74, 1923–1927.
3 Teissié, J. and Tsong, T.Y. (1981) Biochemistry 20, 1548–1554.
4 Witt, H.T., Schlodder, E. and Graber, P. (1976) FEBS Lett. 69, 272–276.
5 Vinkler, C. and Korenstein, R. (1982) Proc. Natl. Acad. Sci. USA 79, 3183–3187.
6 Rögner, M., Ohno, K., Hamamoto, T., Sone, N. and Kagawa, Y. (1979) Biochem. Biophys. Res. Commun. 91, 362–367.
7 Teissié, J. (1986) Biochemistry 25, 368–373.
8 Teissié, J., Knox, B.E., Tsong, T.Y. and Wehrle, J. (1981) Proc. Natl. Acad. Sci. USA 78, 7473–7477.
9 Knox, B.E. and Tsong, T.Y. (1984) J. Biol. Chem. 259, 4757–4763.
10 Tsong, T.Y., Liu, D.S., Chauvin, F., Gaigalas, A. and Astumian, R.D. (1989) Bioelectrochem. Bioenerg. 21, 319–331.
11 Graziana, A., Ranjeva, R. and Teissié, J. (1990) Biochemistry 29, 8313–8316.
12 Robertson, B. and Astumian, R.D. (1992) Biochemistry 31, 138–141.
13 Astumian, R.D. and Robertson, B. (1989) J. Chem. Phys. 91, 4891–4901.
14 Robertson, B. and Astumian, R.D. (1990) Biophys. J. 58, 969–974.
15 Eynard, N., Sixou, S., Duran, N. and Teissié, J. (1992) Eur. J. Biochem. 209, 431–436.
16 Neumann, E. (1989) in Electroporation and Electrofusion in Cell Biology (Neumann, E., Sowers, A.E. and Jordan, C., eds.), pp. 61–82, Plenum, New York.
17 Bernhardt, J. and Pauly, H. (1973) Biophysik 10, 89–98.
18 Kinosita, K., Ashikawa, Y., Saita, N., Yoshimura, H., Itoh, H., Nagayama, K. and Ikegami, A. (1988) Biophys. J. 53, 1015–1018.
19 Hibino, M., Shigemori, M., Itoh, H., Nagayama, K. and Kinosita, K. (1991) Biophys. J. 59, 209–220.
20 Boork, J. and Wennerstrom, H. (1984) Biochim. Biophys. Acta 767, 314–320.
21 Boork, J., Strid, A. and Baltscheffsky, M. (1985) FEBS Lett. 180, 314–316.
22 Gräber, P. and Witt, H.T. (1976) Biochim. Biophys. Acta 423, 141–163.
23 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
24 Farkas, D.L., Malkin, S. and Korenstein, R. (1984) Biochim. Biophys. Acta 767, 507–514.
25 Westerhoff, H., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) FEBS Lett. 165,1–5.
26 Westerhoff, H.V., Kell, D.B. and Astumian, R.D. (1988) J. Electrost. 21, 257–298.
27 Tsuji, K. and Neumann, E. (1983) Biophys. Chem. 17, 153–163.
28 Teissié, J., Prats, M., Soucaille, P. and Tocanne, J.F. (1985) Proc. Natl. Acad. Sci. USA 82, 3217–3221.
29 White, S.H. and Chang, W. (1981) Biophys. J. 36, 449–453.
30 Vaz, W.L.C., Derzko, Z.I. and Jacobson, K.A. (1982) in Membrane reconstitution (Poste, G. and Nicholson, G.L., eds.), pp. 83–136, Elsevier, Amsterdam.
31 Fillingame, R.H. (1981) Curr. Top. Bioenerg. 11, 35–100.