ATP synthesis by the F_1F_o ATP synthase of *Escherichia coli* is obligatorily dependent on the electric potential

Georg Kaim, Peter Dimroth*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelzbergstr. 7, CH-8092 Zürich, Switzerland

Received 20 July 1998

Abstract The H^+-translocating F_1F_o ATP synthase of *Escherichia coli* was purified and reconstituted into proteoliposomes. This system catalyzed ATP synthesis when energized by an acid/base transition ($pH_{in} = 5.0$; $pH_{out} = 8.3$) with succinate, malonate or maleinate but not with MES as the acidic buffer. Under these experimental conditions an electric potential of 125–130 mV is generated by the diffusion of succinate, probably the monoanionic species, whereas with MES buffer the measured potential was at background level (~5 mV). ATP was also synthesized at pH 7.2 in the absence of a ΔpH by applying a K^+/valinomycin diffusion potential. The rate of ATP synthesis increased with the potential in an exponential manner with an inflection point at about 70 mV. We conclude from these results that ΔpH and ΔΨ are kinetically unequivalent driving forces for ATP synthesis by the *E. coli* ATP synthase and that ΔΨ is a mandatory force for this synthesis. The significance of these findings for the mechanism of ATP synthesis in general is discussed.

Key words: ATP synthesis; Driving force; Electric potential; Succinate diffusion potential

1. Introduction

The F_1F_o ATP synthases catalyze the formation of ATP from ADP and phosphate utilizing the energy of a transmembrane electrochemical gradient of protons (proton motive force, $\Delta\mu H^+$) or in some cases Na^+ ions (sodium motive force, $\Delta\mu Na^+$). Closely related ATP synthases are found in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts or the cytoplasmic membrane of bacteria. The multisubunit complex consists of two structurally and functionally distinct entities. The extramembranous F_1 moiety ($\alpha_3\beta_3\gamma\delta\varepsilon$) carries the catalytic centers with the nucleotide binding sites and the membrane-embedded F_o (ab_2c_{12}) complex catalyzes the translocation of the coupling ions across the membrane [1–3].

According to Mitchell's chemiosmotic theory [4], $\Delta\mu H^+$ developed during respiration or photosynthesis consists of two distinct interconvertible parameters, an electrical potential ($\Delta\Psi$) and a chemical gradient of hydrogen ions (ΔpH) that are related by $\Delta\mu H^+ = \Delta\Psi - (2.3\ RT/F)\ \Delta pH$, where 2.3 RT/F is equal to 59 mV at room temperature. Energetic equivalence of ΔpH and the electrical potential at equilibrium is therefore an essential feature of the chemiosmotic theory. Far from equilibrium, however, these forces may contribute unequally to the kinetics of ATP formation.

In a classical study with the ATP synthase of chloroplasts Jagendorf and Uribe [5] reported that ATP was synthesized following an acid/base transition with succinate, pH 4 as the acidic and Tris, pH 8 as the basic buffer. Alternatively, ATP formation could be energized by a potassium diffusion potential [6] and its rate depended apparently equally on ΔpH and ΔΨ [7]. Kinetic equivalence of ΔpH and ΔΨ for driving ATP synthesis was also reported for other ATP synthases [8–10]. For the Na^+, Li^+ or H^+ translocating F_1F_o ATP synthase of *Propionigenium modestum* [11], ATP synthesis occurred in the presence of ΔΨ and ΔpNa^+ [12,13]. A slow rate of ATP formation by applying ΔpNa^+ only [12], however, could not be confirmed by our recent studies on the ATP synthesis mechanism (Kaim and Dimroth, unpublished). These results rather indicated that ΔΨ is a mandatory driving force for ATP formation by the *P. modestum* enzyme and that no ATP synthesis occurred in its absence with ΔpNa^+ up to 200 mV.

Except for the extended coupling ion specificity, the *P. modestum* ATP synthase is clearly related by structure and function to other members of F_1F_o ATP synthase family [3]. This is most impressively demonstrated by the formation of a functional hybrid consisting of the *Escherichia coli* subunits α, β, γ, ε and the *P. modestum* subunits a, b, c and δ [13].

ATP synthesis catalyzed by the reconstituted *E. coli* ATP synthase has so far only been shown after energization by a potassium diffusion potential combined with a pH gradient [14]. Considering the relationship to the *P. modestum* enzyme we expected that for the *E. coli* ATP synthase, too, the membrane potential is an indispensable driving force. We show here that ATP synthesis by this enzyme was dependent on the electric potential and could not be energized by a pH gradient only. A K^+/valinomycin diffusion potential in the absence of ΔpH was on the other hand sufficient to drive ATP synthesis. We also report that the acid/base transition with succinate as the acidic buffer [6] that has been the method of choice to generate ΔpH in ATP synthesis experiments in addition creates an electric potential of 128 mV, probably by diffusion of the monoanionic form of succinate. Hence, the conclusions drawn from these ATP synthesis experiments have to be revised.

2. Materials and methods

2.1. Purification and reconstitution of the ATP synthase

Liposomes were formed by sonicating a suspension of 44 mg phosphatidylcholine (Sigma; type II-S) in 1 ml 5 mM sodium phosphate buffer, pH 7.2, for 2×1 min in a water bath sonicator. *E. coli* strain DH5α (Bethesda Research Laboratories) was grown in M13 minimal medium with 10 mM glucose, supplemented with thiamine-HCl (0.1 mg/l) and the ATP synthase was purified as described [13]. To obtain a protein/phospholipid ratio of 1:80, 0.55 mg purified F_1F_o (1 nmol) in sodium phosphate buffer, pH 7.2, containing 0.1 mM

*Corresponding author: Fax: (41) (1) 632 13 78.
E-mail: dimroth@micro.biol.ethz.ch

PII: S0014-5793(98)00969-7

diisopropylfluorophosphate and 0.05% Triton X-100 was added to the liposomes and the suspension was frozen in liquid nitrogen for 15 min. After thawing in an ice water bath the proteoliposomes were sonicated for 2×5 s, collected by centrifugation (200 000×*g*; 45 min) and resuspended in 0.5 ml 5 mM sodium phosphate buffer, pH 7.2.

2.2. Measurement of ATP synthesis

ATP synthesis was determined by the luciferin-luciferase assay as described [13]. The membranes of the proteoliposomes were energized by two different methods. (A) *Valinomycin-induced potassium ion diffusion*. The ATP synthase of *E. coli* was reconstituted into liposomes as described above in the additional presence of 1 mM KCl. Proteoliposomes were diluted 1:10 at 25°C into reaction buffer I (5 mM sodium phosphate, pH 7.2, 5 mM $MgCl_2$ and 2.5 mM ADP) containing KCl in the concentrations indicated in the legend of Fig. 2. The electric potential was induced by adding 20 μM valinomycin to the reaction mixture. Samples of 50 μl were taken after different incubation periods and the reaction was terminated with 5 μl trichloroacetic acid. The formed ATP was determined with 11 μl of the centrifuged samples (corresponding to an original amount of 2 pmol F_1F_o). (B) *Acid/base transition*. A 50 μl aliquot of the proteoliposomes (0.1 nmol of *E. coli* F_1F_o; 4.4 mg phospholipids) prepared as described above was incubated for 1 min with 200 μl of 100 mM of succinate, malonate or maleinate buffer, pH 5.0, respectively. In order to preserve the ΔpH during the ATP synthesis reaction, 3 μl 5 M NaOH was added to this solution prior to 1:1 dilution at 25°C into reaction buffer II (100 mM glycylglycine, pH 8.5, 5 mM sodium phosphate, 5 mM $MgCl_2$ and 2.5 mM ADP). ATP was determined as described above and the final pH was measured yielding values between 8.2 and 8.4. In control experiments, proteoliposomes were formed in the presence of 100 mM MES buffer, pH 5.0, and collected by centrifugation (200 000×*g*, 45 min). The proteoliposomes were resuspended in 500 μl reaction buffer II and ATP formed was immediately determined. A pH of 8.4 was measured after the ATP synthesis reaction.

2.3. Determination of the membrane potential (ΔΨ)

The membrane potential was determined from the distribution of the lipophilic anion [^{14}C]thiocyanate between the external and internal compartment of (proteo)liposomes subjected to succinate diffusion as described under acid/base transition. The calculation was done from the Nernst equation [15].

$$\Delta\Psi = \frac{2.3\ RT}{F} \cdot \log \frac{[SCN^-]_{in}}{[SCN^-]_{out}}$$

where 2.3 RT/F has a value of 54 mV at 0°C. The size of the reconstituted proteoliposomes was determined by directly measuring the diameter after freeze-fraction electron microscopy. From these data the mean inner volume of 15 μl/mg phospholipid was derived. In representative experiments, (proteo)liposomes (25 μl inner volume corresponding to 1.65 mg phospholipids) were incubated with 100 mM succinate, pH 5.0 (acid/base transition), the pH was neutralized with 5 M NaOH and the (proteo)liposomes were diluted 1:1 at 0°C into reaction buffer II (see above) containing in addition 2 μM [^{14}C]NaSCN (0.5 μCi). Alternatively, liposomes are formed in the presence of 100 mM MES, pH 5.5, collected and resuspended as described above and then diluted 1:1 at 0°C into reaction buffer II containing 2 μM [^{14}C]NaSCN (0.5 μCi). After 20 s the incubation mixture was subjected to rapid filtration through 0.22 μm GSTF filters (diameter 25 mm, Millipore). The filters were washed with 1 ml of 100 mM glycylglycine buffer, pH 8.5, and placed into scintillation plates. After addition of 1 ml scintillation fluid (Packard, Microscint 20) the entrapped [^{14}C]SCN^- was determined by liquid scintillation counting.

3. Results

ATP synthase was isolated from *E. coli* and reconstituted into proteoliposomes. These proteoliposomes converted ADP and phosphate into ATP when energized by an acid/base transition according to the procedure described by Jagendorf and Uribe [5]. To generate a ΔpH, the proteoliposomes were incubated for 1 min with 100 mM succinate, pH 5.0 which acidifies the proteoliposome interior. The mixture was subsequently diluted into an equal volume of 100 mM glycylglycine buffer, to yield an external pH of 8.3. The dilution buffer contained appropriate additions of substrates for ATP formation, and this was followed with samples taken after various incubation times with the luciferin luciferase assay system. The results of Fig. 1A show a rapid increase of the amount of ATP formed reaching a final ATP concentration of 65 mol/mol ATP synthase after 20 s. The kinetics of ATP formation followed very similar curves if the acidification of the proteoliposomes were performed with malonate or maleinate instead of succinate. No ATP synthesis was found, however, if the internal volume of the proteoliposomes was adjusted to pH

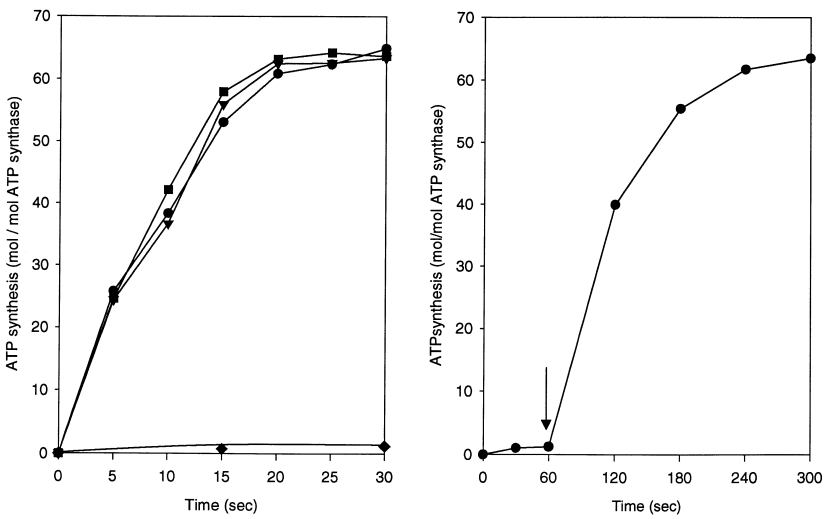

Fig. 1. ATP synthesis by proteoliposomes containing the *E. coli* ATP synthase energized by an acid/base transition. A: Proteoliposomes were incubated at 25°C for 1 min with 100 mM succinate (●), malonate (▼) or maleinate (■) buffers, pH 5.0 and diluted 1:1 into reaction buffer II. Proteoliposomes prepared with 100 mM MES buffer, pH 5.0, collected and resuspended in reaction buffer II (♦). ATP was determined by the luciferin-luciferase assay with samples taken after different time periods. B: Proteoliposomes formed in the presence of MES buffer, pH 5.0, collected and resuspended in reaction buffer II containing in addition 200 mM KCl. After 60 s ΔΨ-dependent ATP formation was induced by the addition of 20 μM valinomycin (↓). ATP was determined as described above.

5.0 with MES buffer instead of a dicarboxylic acid and the proteoliposomes were subsequently collected by centrifugation and resuspended in glycylglycine buffer, pH 8.5 containing the substrates for ATP synthesis. However, ATP synthesis was observed if the suspension of the proteoliposomes was performed in the additional presence of 200 mM KCl and 20 μM valinomycin was added to induce a K^+ diffusion potential (Fig. 1B). Hence, the synthesis of ATP was not impaired by preparing the proteoliposomes with MES buffer, pH 5.0. These results are not consistent with the idea that the pH gradient established between the internal and external vesicular compartments can be used as the exclusive driving force for ATP synthesis as was described for the chloroplast enzyme [5,7].

Therefore, the possibility was considered that by the acid/base transition an electric potential may be created by diffusion of the single protonated species of the dicarboxylic acids. For instance, the pK_a values of maleinate are 1.8 and 6.0. Therefore, at pH 5.0, the undissociated maleinate species hardly exists, whereas that with one carboxylate protonated and one unprotonated predominates. If this species traverses the membrane, the proton dissociates in the external medium of high pH, resulting in a large gradient of the singly protonated species. As the transport is electrogenic, a diffusion potential of around 150 mV may be generated. In contrast, performing the acid/base transition with proteoliposomes loaded with the membrane-impermeable MES buffer should not generate a diffusion potential.

If this was a valid explanation the membrane-permeable anion [^{14}C]thiocyanate should be accumulated within proteoliposomes subjected to the acid/base transition with succinate but not with MES as the acidic buffer. The results of Table 1 show that this was the case. The diffusion potential calculated from the distribution of [^{14}C]thiocyanate between internal and external volumes of liposomes was 128 or 5 mV, if the acidic internal pH was adjusted with succinate or MES buffer, respectively. Proteoliposomes with the *E. coli* ATP synthase established a diffusion potential of 126 mV after incubation with succinate, pH 5.0 and dilution into glycylglycine pH 8.5. Hence, the 'acid bath procedure' with succinate as acidic buffer that has been used routinely in ATP synthesis experiments [5,7–10,14], establishes not only a ΔpH but also a significant ΔΨ, probably by diffusion of the singly protonated species of the dicarboxylic acid.

Table 1
Formation of an electric potential by succinate^{-1} diffusion over the membrane of (proteo)liposomes

Internal buffer	Calculated membrane potential (mV)	
	Liposomes	Proteoliposomes containing the ATP synthase of *E. coli*
Succinate (method A)	128 ± 7	126 ± 8
MES (method B)	5 ± 1	n.d.

The liposomes or proteoliposomes containing the ATP synthase of *E. coli* were incubated with 100 mM succinate, pH 5.0 and diluted 1:1 into glycylglycine buffer, pH 8.5, containing 2 μM Na-[^{14}C]SCN (0.5 μCi) (acid/base transition: method A). Alternatively, the proteoliposomes were formed in the presence of 100 nM MES buffer, pH 5.5 followed by the transition into glycylglycine buffer, pH 8.5, containing 2 μM Na-[^{14}C]SCN (0.5 μCi) (method B). After 20 s internal [^{14}C]SCN^- was determined after rapid filtration by scintillation counting. The mean results from four different experiments are depicted. n.d. = not determined.

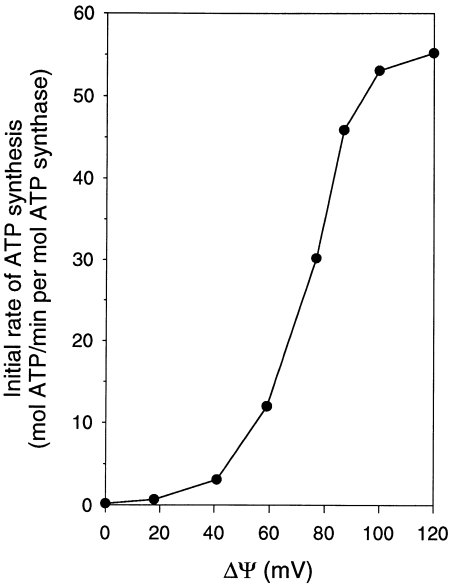

Fig. 2. Dependence of ATP synthesis rate on the size of a valinomycin-induced K^+ diffusion potential (ΔΨ). The electrical potential was induced by adding 20 μM valinomycin to proteoliposomes (0.1 nmol F_1F_o; 4.4 mg phospholipids) containing 5 mM sodium phosphate buffer, pH 7.2, on both sides of the membrane, 1 mM KCl on the inside and KCl concentrations of 1 mM, 2 mM, 5 mM, 10 mM, 20 mM, 30 mM, 50 mM and 110 mM on the outside to yield ΔΨ of 0 mV, 18 mV, 41 mV, 59 mV, 77 mV, 87 mV, 100 mV and 120 mV, respectively. The outside compartment also contained 2.5 mM ADP and 5 mM $MgCl_2$. The ATP synthesized was determined as described in the legend to Fig. 1.

The results of Fig. 2 show that the *E. coli* ATP synthase synthesizes ATP also if the proteoliposomes are energized by a K^+/valinomycin diffusion potential at $pH_{in} = pH_{out} = 7.2$. At ΔΨ of 40 mV and below, the ATP synthesis rate is very low. It increases exponentially with increasing electric potentials with an inflection point around 70 mV and approaching the maximal velocity above 100–120 mV. The exponential dependence of the reaction rate on the electric potential is indicative for a rate-limiting voltage-dependent reaction step in the synthesis of ATP [16]. Please note that maximal turnover numbers under these conditions were about 1/s, whereas ATP synthesis rates were 5/s following the acid/base transition with succinate, maleinate or malonate. In a previous study, about five times higher ATP synthesis rates were observed with proteoliposomes containing the *E. coli* ATP synthase energized by the acid/base transition with succinate and superimposed by a K^+/valinomycin diffusion potential [14]. These higher rates could be explained by higher electrical potentials or might be due to superior reconstitution conditions.

In summary, our data are consistent with the notion that both parameters of the proton motive force, ΔpH and ΔΨ contribute to the rate of ATP formation. More importantly, however, they indicate that in contrast to general opinion, ΔΨ is an indispensable driving force for ATP synthesis that cannot be compensated by a ΔpH of the same size.

4. Discussion

We show here that an electric potential is kinetically indispensable as a driving force for ATP synthesis by the *E. coli* ATP synthase. With a calculated K^+/valinomycin diffusion

potential of 100 mV and no ΔpH, ATP was synthesized at an initial rate of 1/s, whereas no ATP was formed with a calculated ΔpH of 194 mV [pH_{in} = 5.0 (MES); pH_{out} = 8.3 (glycylglycine)] and no ΔΨ. These results are in contrast to the general view that ΔpH and ΔΨ are kinetically equivalent driving forces for ATP synthesis, where one parameter of sufficient magnitude can completely compensate for the other [5–10]. In a previous study with the *E. coli* enzyme, Fischer et al. [14] were unable to demonstrate ATP synthesis in the absence of ΔpH. This may be due to the high pH used in these experiments ($pH_{in} = pH_{out} = 8.8$) as compared to $pH_{in} = pH_{out} = 7.2$ in ours. The authors further noticed inhibition of ATP synthesis in the presence of 20 mM NH_4Cl and interpreted this to be caused by the dissipation of ΔpH. However, there are other possibilities for this effect and without further controls this conclusion seems to be premature. In any case, the results reported here show clearly that ΔΨ alone is a suitable driving force for ATP synthesis by the *E. coli* enzyme.

Most studies on the kinetic equivalence of ΔpH and ΔΨ as driving forces were performed with the chloroplast ATP synthase, but ATP synthesis experiments with ATP synthases from other sources seemed to support this equivalence. Ever since the introduction of the so called acid bath procedure in 1966 by Jagendorf and Uribe [5], this has been the method of choice to generate ΔpH as driving force for ATP synthesis. In their classical experiments, these authors incubated chloroplasts shortly with succinate, pH 4–5, to acidify the interior compartment and subsequently diluted the chloroplasts into Tris buffer, pH 8.5 to generate a ΔpH. Unfortunately, it has never been considered that the diffusion of the succinate monoanion might generate an electric potential of considerable magnitude which could contribute or even be essential to drive ATP formation. We show here that this is indeed the case and that electrical potentials of about 125–130 mV are created by the diffusion of succinate from the acidic into the alkaline environment. Interestingly enough, ATP synthesis by the chloroplast ATP synthase was highly dependent on the nature of the acid used to lower the pH of the lumen of the chloroplasts. It was noticed that bringing the internal pH to 4 by HCl or by glutamate was not sufficient to drive ATP synthesis following the transition into the basic environment, but by including 10 mM succinate the basal level of ATP synthesis increased 15-fold [5]. On the basis of these and of our present results it is likely, therefore, that the original conclusion of an entirely ΔpH-driven ATP synthesis by the chloroplast ATP synthase is erroneous. Rather, the electrical potential generated by succinate diffusion seems to be an indispensable driving force also for the chloroplast ATP synthase. All subsequent investigations following the same protocol for ΔpH formation using succinate as acidic buffer probably also derived at erroneous conclusions, because formation of a succinate diffusion potential was not taken into account [7–10].

The Na^+, Li^+ or H^+ translocating ATP synthase of *P. modestum* is another example for which ΔΨ has been shown to be a mandatory driving force for ATP synthesis (Kaim and Dimroth, unpublished). Previous reports of slow rates of ATP synthesis in the presence of ΔpNa^+ [12] only could not be confirmed. Even at ΔpNa^+ of about 200 mV, ATP synthesis was not detectable in the absence of an electric potential. These results are in accord with observations on the Na^+ ion permeability of the reconstituted F_o complex of *P. modestum* [17]. It was found that the unidirectional translocation of Na^+ ions through F_o could not be observed with ΔpNa^+ between 60 and 180 mV. The initial rate of Na^+ uptake depended strongly on the presence of a membrane potential with no significant activity below −40 mV. Interestingly, the rate of Na^+ uptake increased exponentially with the electric potential in much the same way as the exponential increase of the ATP synthesis rate with increasing electrical potentials. Hence, the two reactions probably depend on the same rate-limiting voltage-dependent reaction step. It was also observed that Na^+ ions were freely exchanged between both sides of the membrane with no significant interference by ΔpNa^+. With ΔΨ, however, the exchange activity was abolished and F_o switched to catalyze unidirectional Na^+ movements following the electrical gradient [17]. Considering the rotational mechanism this membrane potential induced switch could be the key element to convert the motor from idling into a torque-generating directed rotation [18]. This voltage-dependent switch is not only observed with the isolated F_o moiety but also with the F_1F_o complex. The ATP synthase catalyzed Na^+ shuttling between the two sides of the membrane that was not affected by ΔpNa^+ but completely abolished by ΔΨ (Kaim and Dimroth, unpublished).

In summary, we have shown here that ATP synthesis by the *E. coli* ATP synthase requires an electrical potential across the membrane. This is probably needed to overcome the activation energy of a voltage-dependent step in the ion translocation mechanism. We report elsewhere that ATP synthesis by the *P. modestum* ATP synthase is also mandatorily dependent on voltage (Kaim and Dimroth, unpublished) and from published data on the chloroplast ATPase [5] one may conclude that this enzyme is also not functional in the absence of an electric potential. These results disprove previous assumptions on the kinetic equivalence of ΔΨ and ΔpH as driving force for ATP synthesis [5–10] and shed new light on the bioenergetics of this important reaction in general.

References

[1] Deckers-Hebestreit, G. and Altendorf, K. (1996) Annu. Rev. Microbiol. 50, 791–824.
[2] Weber, J. and Senior, A.E. (1997) Biochim. Biophys. Acta 1319, 19–58.
[3] Dimroth, P. (1997) Biochim. Biophys. Acta 1318, 11–51.
[4] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[5] Jagendorf, A.T. and Uribe, E. (1966) Proc. Natl. Acad. Sci. USA 55, 170–177.
[6] Uribe, E.G. (1973) FEBS Lett. 36, 143–147.
[7] Junesch, U. and Gräber, P. (1991) FEBS Lett. 294, 275–278.
[8] Bokranz, M., Mörschel, E. and Kröger, A. (1985) Biochim. Biophys. Acta 810, 332–339.
[9] Turina, P., Melandri, B.A. and Gräber, P. (1991) Eur. J. Biochem. 196, 225–229.
[10] Slooten, L. and Vandenbranden, S. (1989) Biochim. Biophys. Acta 976, 150–160.
[11] Laubinger, W. and Dimroth, P. (1988) Biochemistry 27, 7531–7537.
[12] Dmitriev, O., Deckers-Hebestreit, G. and Altendorf, K. (1993) J. Biol. Chem. 268, 14776–14780.
[13] Kaim, G. and Dimroth, P. (1994) Eur. J. Biochem. 222, 615–623.
[14] Fischer, S., Etzold, C., Turina, P., Deckers-Hebestreit, G., Altendorf, K. and Gräber, P. (1994) Eur. J. Biochem. 225, 167–172.
[15] Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
[16] Läuger, P. (1991) Electrogenic Ion Pumps, Sinauer Associates, Sunderland, MA.
[17] Kluge, C. and Dimroth, P. (1992) Biochemistry 31, 12665–12672.
[18] Dimroth, P., Kaim, G. and Matthey, U. (1998) Biochim. Biophys. Acta 1365, 87–92.