Comparison of ΔpH- and Δφ-driven ATP synthesis catalyzed by the H^+-ATPases from *Escherichia coli* or chloroplasts reconstituted into liposomes

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Abstract The H^+-ATPases from *Escherichia coli*, EF_0F_1, and from chloroplasts, CF_0F_1, were reconstituted in liposomes from phosphatidylcholine/phosphatidic acid. The proteoliposomes were energized by an acid-base transition and a K^+/valinomycin diffusion potential and the initial rate of ATP synthesis was measured as a function of the transmembrane pH difference, ΔpH, and the electric potential difference, Δφ. With EF_0F_1, a rate of 80 s^{-1} is observed at ΔpH = 4.1 and Δφ ≈ 140 mV. The rate decreases sigmoidally with Δφ and at Δφ ≈ 0 mV, the rate is about 1 s^{-1} although ΔpH is still 4.1. Under the same conditions with CF_0F_1, a rate of 280 s^{-1} is observed which decreases to 190 s^{-1} when Δφ is abolished, i.e. ATP synthesis catalyzed by EF_0F_1 and CF_0F_1 depends in a different way on ΔpH and Δφ. EF_0F_1-catalyzed ATP synthesis was measured as a function of ΔpH at a constant Δφ. The rate depends sigmoidally on ΔpH reaching a maximal rate which cannot be further increased by increasing ΔpH. However, this maximal rate depends on Δφ, i.e. ΔpH and Δφ are not kinetically equivalent in driving ATP synthesis. We assume that EF_0F_1 must be converted into a metastable, active state before it catalyzes proton transport-coupled ATP synthesis. For EF_0F_1, this activation step depends only on Δφ, whereas for CF_0F_1, the activation depends on ΔpH and Δφ.

Key words: ATP synthase; EF_0F_1; Diffusion potential; ATP synthesis; Proteoliposome; *Escherichia coli*

1. Introduction

Membrane-bound H^+-ATPases catalyze ATP synthesis in bacteria, chloroplasts and mitochondria. In these organelles, the electron transport leads to the generation of a transmembrane electrochemical potential difference of protons, $\Delta\tilde{\mu}_{H+}$, and the proton flux driven by $\Delta\tilde{\mu}_{H+}$ provides the Gibb's free energy for ATP synthesis [1,2]. In chloroplasts, ATP synthesis can be driven by both components of $\Delta\tilde{\mu}_{H+}$, i.e. by the transmembrane pH difference, ΔpH, and the transmembrane electric potential difference, Δφ. Most convincingly, this was shown by ATP synthesis driven by an artificially generated ΔpH [3] (acid-base transition) and by an artificially generated Δφ [4] (external electric field pulse). According to the chemiosmotic theory, ΔpH and Δφ are energetically equivalent [2] and it has been shown with thylakoid membranes, chromatophores and bacterial F_0F_1 that both parameters are also kinetically equivalent in driving ATP synthesis [5–8], i.e. the rate of ATP synthesis is changed by the same factor when either Δφ is changed by 58 mV or ΔpH is changed by one unit.

Recently, we reconstituted the H^+-ATPase from *Escherichia coli*, EF_0F_1, into liposomes and energized the liposomes with an acid-base transition in combination with a K^+/valinomycin diffusion potential. We observed ATP synthesis catalyzed by reconstituted EF_0F_1 and measured the K_M values for ADP and phosphate [9]. During these investigations, we noted that the presence of Δφ seems to be necessary for high rates of ATP synthesis, whereas in our earlier investigations with reconstituted H^+-ATPase from chloroplasts, CF_0F_1, high rates of ATP synthesis were observed at a high ΔpH, also in the absence of Δφ [10]. Therefore, we decided to carry out a comparative study of ΔpH- and Δφ-driven ATP synthesis catalyzed by reconstituted CF_0F_1 and EF_0F_1 under the same conditions.

2. Materials and methods

2.1. Purification and reconstitution of EF_0F_1 and CF_0F_1 into liposomes

E. coli strain DK8 (Δ*unc*) transformed with plasmid pBWU13 [11] was a gift from Prof. Futai, Osaka, Japan. The bacteria were grown in a minimal medium and EF_0F_1 was isolated as described [9,11]. The enzyme was obtained in a solution containing 10 mM Mes and 10 mM tricine/NaOH, pH 7.0, 500 μM $MgCl_2$, 5 mM thioglycerol, 10 g/l octylglucoside and 300 g/l sucrose with a protein concentration between 1–2 g/l. CF_0F_1 was purified from spinach as described [12] and obtained in 30 mM NaH_2PO_4/NaOH, pH 7.2, 2 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 4 mM dodecylmaltoside and 420 g/l sucrose with a protein concentration between 2–4 g/l. EF_0F_1 and CF_0F_1 were rapidly frozen and stored in liquid nitrogen. The protein concentration was determined with amido black [13]. Molecular masses of 530 kDa for EF_0F_1 and of 550 kDa for CF_0F_1 were used.

Liposomes from phosphatidylcholine and phosphatidic acid were prepared by dialysis. 18 g/l phosphatidylcholine and phosphatidic acid in a mass ratio 19:1 were suspended in a solution containing 7.2 g/l cholic acid, 3.6 g/l sodium desoxycholate, 500 μM dithiothreitol, 100 μM ethylenediaminetetraacetic acid and 10 mM tricine/NaOH (pH 8). The lipid detergent solution was sonicated in an ice bath for 3×30 s (Branson sonifier at 20 kHz and 150 W) and dialyzed against the 4000-fold volume of buffer (2.5 mM $MgCl_2$, 250 μM dithiothreitol, 200 μM ethylenediaminetetraacetic acid and 10 mM tricine/NaOH, pH 8) at 30°C for 5 h using a Diachema membrane type 10.14 MWC 5000. The lipid concentration of the liposomes after dialysis was approximately 16 g/l.

EF_0F_1 and CF_0F_1 were reconstituted into the pre-formed liposomes as described earlier [9]. In short, the liposome membrane was slightly destabilized by Triton X-100, the enzyme detergent micelles were added and liposome-micelle aggregates were formed. Removal of the detergent by BioBeads leads to insertion of the enzyme into the membrane and the lipid bilayer is stabilized again [14]. Finally, the lipid concentration of the proteoliposomes was approximately 8 g/l with a F_0F_1 concentration of 80 nM.

For measurement of ATP synthesis with Δφ, either EF_0F_1 or CF_0F_1 was reconstituted using buffer A (20 mM succinate, 20 mM tricine, 80 mM NaCl, 0.6 mM KOH titrated with NaOH to pH 8.0). The

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resulting proteoliposomes contained 0.2 mM K^+, they are called proteoliposomes A in the following. For measurement of ATP synthesis without $\Delta\phi$, EF_0F_1 or CF_0F_1 was reconstituted using buffer B (20 mM succinate, 20 mM tricine, 276 mM KCl, 47 mM KOH, pH 8.0). The resulting proteoliposomes contained 130 mM K^+, they are called proteoliposomes B in the following.

2.2. Measurement of ATP synthesis

The rate of ATP synthesis catalyzed by F_0F_1 was measured at 23°C as described earlier [9]. Proteoliposomes were energized by an acid-base transition and an additional K^+/valinomycin diffusion potential. Experiments in the absence of $\Delta\phi$ were carried out with a high internal and external K^+ concentration in the presence of valinomycin. This abolishes almost completely $\Delta\phi$ generated by the acid-base transition. The ATP concentration was monitored continuously with luciferin/luciferase (Merlin) in a luminometer (LKB 1250). In order to generate $\Delta\phi$, the following media were used in the acid-base transition. Conditions A: acidic medium A: 20 mM succinate, 0.6 mM KOH, 2.5 mM $MgCl_2$, 10 mM NaH_2PO_4, 0.1 mM ADP, 20 μM valinomycin (freshly added) titrated to pH 4.5 with NaOH. Basic medium A: 200 mM tricine, 130 mM KOH, 2.5 mM $MgCl_2$, 10 mM NaH_2PO_4, 0.1 mM ADP, titrated to pH 8.8 with NaOH. In order to eliminate $\Delta\phi$, the following media were used. Conditions B: acidic medium B: 20 mM succinate, 130 mM KCl, 2.5 mM $MgCl_2$, 10 mM NaH_2PO_4, 0.1 mM ADP, 20 μM valinomycin (freshly added) titrated to pH 4.5 with NaOH. For the basic stage, basic medium A was used.

ATP synthesis and detection of ATP with the luciferin/luciferase assay were carried out simultaneously as follows: 930 μl of the basic medium was mixed with 20 μl luciferin/luciferase reagent, placed in the luminometer and the base line was recorded. Proteoliposomes (20 μl, F_0F_1 concentration 80 nM) were mixed with 80 μl acidic medium and incubated at 23°C for 1–6 min. ATP synthesis was initiated by injection of 50 μl of this solution with a Hamilton syringe directly into the basic medium. The increase of the ATP concentration was followed by the increased luminescence. When the signal reached a constant level, it was calibrated by addition of a ATP standard solution. The rate of ATP synthesis was calculated from the initial slope. The internal and external pH were measured with a glass electrode. The internal pH after equilibration was assumed to be equal to the pH measured after mixing four parts of the acidic medium with one part of the proteoliposomes. Under standard conditions, the result was $pH_{in} = 4.7$. The pH of the strongly buffered basic medium does not change after addition of the acidified liposomes, i.e. under standard conditions, the result was $pH_{out} = 8.8$.

The Nernst potential for K^+ was calculated from

$$\Delta\phi = \frac{RT}{F} \ln \frac{[K^+]_{out}}{[K^+]_{in}}$$

It resulted for conditions A (presence of $\Delta\phi$, $[K^+]_{in} = 0.5$ mM, $[K^+]_{out} = 120$ mM) in $\Delta\phi \approx 140$ mV, for conditions B (absence of $\Delta\phi$, $[K^+]_{in} = [K^+]_{out} = 130$ mM) in $\Delta\phi \approx 0$ mV.

3. Results

CF_0F_1 was reconstituted in phosphatidylcholine/phosphatidic acid liposomes, an acid-base transition was carried out in the presence as well as in the absence of $\Delta\phi$ and ATP synthesis was measured as described in Section 2. Fig. 1 shows some original traces of these experiments. Fig. 1a shows the result in the presence of $\Delta\phi$. The arrow indicates the addition of the acidified proteoliposomes resulting in an increase of luminescence. After calibration of the luminescence with an ATP standard, the rate of ATP synthesis is calculated from the slope directly after injection of the proteoliposomes ($t = 0$) as indicated by the dashed line. In this case, it resulted in a rate of 200 nM/s ATP. Since the CF_0F_1 concentration was 0.8 nM, the rate per enzyme (turnover) is 250 s^{-1}. In the absence of $\Delta\phi$ (Fig. 1e), the rate is 20% lower than in its presence. When the transmembrane ΔpH is abolished by nigericin, no ATP synthesis is observed in any case (Fig. 1b and f). In a second set of experiments, EF_0F_1 was reconstituted into phosphatidylcholine/phosphatidic acid liposomes and acid-base transitions were carried out under exactly the same conditions as used for CF_0F_1. The results are also depicted in Fig. 1. In the presence of $\Delta\phi$, a rate of 62 nM/s is observed (Fig. 1c). The turnover is in this case 78 s^{-1} ($EF_0F_1 = 0.8$ nM). In the absence of $\Delta\phi$ (Fig. 1g), almost no ATP synthesis is detected. However, when a longer reaction time is used (Fig. 1i), a very low rate of ATP synthesis (1 nM/s) can be seen. When the transmembrane ΔpH is abolished by nigericin, no ATP synthesis is observed (data not shown). As a further control, we have measured the effect of addition of acidified liposomes without F_0F_1 on luminescence. The data in Fig. 1d, h and k show that the addition of liposomes does not change the luminescence, i.e. the activity of the luciferin/luciferase system is not changed.

Obviously, at $\Delta pH = 4.1$ and without $\Delta\phi$, a high rate of ATP synthesis is observed with CF_0F_1, but almost no ATP synthesis is found with EF_0F_1 under the same conditions. However, the fact that no ATP synthesis is found in a reconstituted system might have different reasons. For example, it is possible that the reconstitution into the liposomal membranes was not successful at the high K^+ concentrations used for conditions B. Therefore, we have carried out several control experiments and the results are listed in Table 1.

First, the rate of ATP synthesis was measured with EF_0F_1 at standard conditions with proteoliposomes A, resulting in a high rate of ATP synthesis. These proteoliposomes A were then incubated with 130 mM KCl at 23°C for 18 h and an acid-base transition was carried out with acidic medium B and basic medium A, i.e. without a K^+ diffusion potential. The observed rate was 1 s^{-1}. When the acid-base transition was carried out with proteoliposomes B, the same low rate was observed. Obviously, after incubation of highly active proteoliposomes A with a high KCl concentration, the same rate is observed as with proteoliposomes B (which are reconstituted already with a high KCl concentration). When the same procedures were carried out with CF_0F_1, high rates of ATP synthesis (190 s^{-1}) were found when $\Delta\phi$ was abolished either by using proteoliposomes B or proteoliposomes A after incubation with a high KCl concentration. These results show that the same rates are obtained irrespective of whether the high internal K^+ concentration is obtained by entrapping it during reconstitution or by incubation of low K^+ containing proteoliposomes with a high KCl concentration. Obviously, the reconstitution efficiency under reconstitution conditions A (low K^+ concentration) is almost the same as under conditions B (high K^+ concentration).

For EF_0F_1, the observed rates without $\Delta\phi$ are almost zero and it might be that the prolonged incubation with a high KCl concentration leads to an irreversible inactivation of the enzyme. Therefore, we have carried out an experiment where the proteoliposomes B were re-activated. The K^+ concentration of the proteoliposomes B was reduced to 13 mM by incubation of one part proteoliposomes B with nine parts acidic medium A for 4 h. The acid-base transition was carried out with basic medium B ($K^+_{out} = 130$ mM), i.e. the resulting Nernst potential was about 57 mV. For comparison, proteoliposomes A were first incubated with 130 mM KCl, then, the K^+ concentration was reduced to 13 mM by appropriate incubation and, finally, an acid-base transition was carried out with basic medium B. A rate of ATP synthesis of 7–8 s^{-1} is

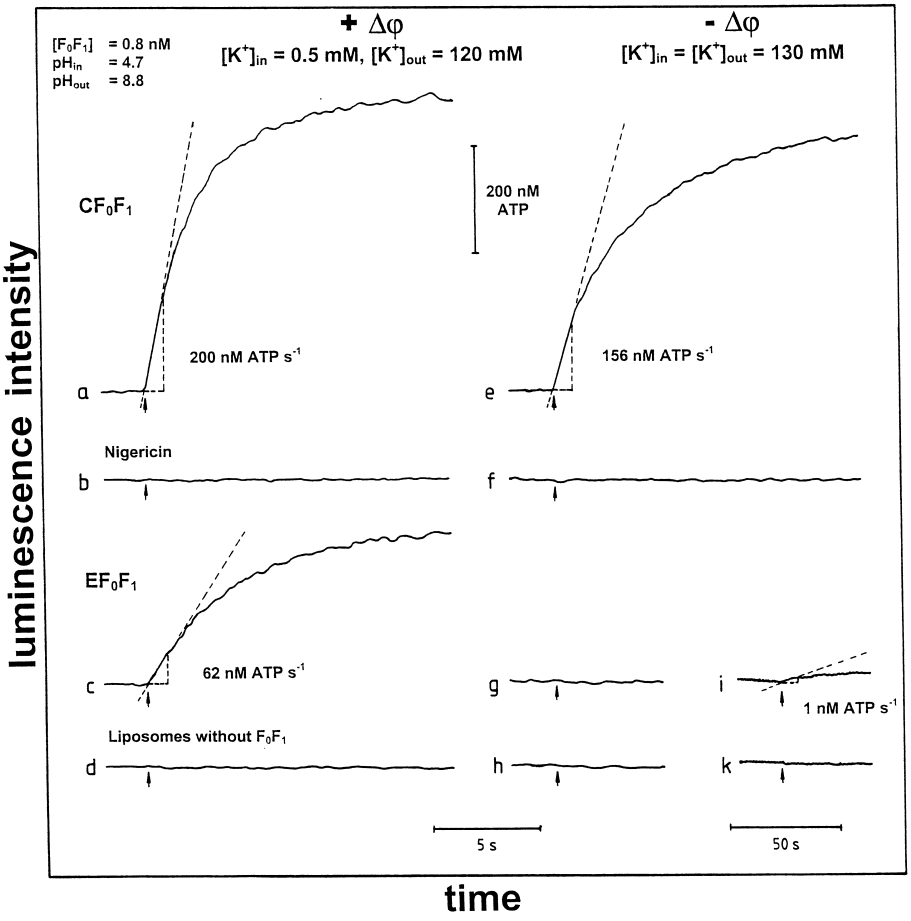

Fig. 1. ATP synthesis catalyzed by EF_0F_1 liposomes and by CF_0F_1 liposomes. The ATP concentration was measured as a function of time after a $\Delta pH/\Delta\phi$ jump with luciferin/luciferase. The arrow indicates the addition of the acidified proteoliposomes to the basic medium containing the luciferase assay. pH_{in} was 4.7, pH_{out} was 8.8. The reaction medium contains after mixing 0.8 nM F_0F_1. The slope directly after mixing ($t = 0$) is the initial rate of ATP synthesis and the numbers give the rates in nM/s ATP. At the end of the reaction (approximately after 20 s), the luminescence signal was calibrated by addition of standard ATP. Left: acid-base transition in the presence of $\Delta\phi$ ($[K^+]_{in} = 0.5$ mM, $[K^+]_{out} = 120$ mM). Right: acid-base transition in the absence of $\Delta\phi$ ($[K^+]_{in} = [K^+]_{out} = 130$ nM). Traces a and e: CF_0F_1 in the presence and in the absence of $\Delta\phi$, traces b and f: CF_0F_1 in the presence of 100 nM nigericin, traces c and g: EF_0F_1 in the presence and in the absence of $\Delta\phi$, traces d and h: liposomes without F_0F_1, traces i and k: the same as g and d with a longer time scale.

found in both cases (Table 1). These results show that neither the reconstitution with a high KCl concentration nor the incubation of proteoliposomes with a high KCl concentration inactivates the enzyme irreversibly. Since there is again ATP synthesis after generating $\Delta\phi$, it can be concluded that the loss of activity is due to the absence of $\Delta\phi$. As a control, proteoliposomes A were incubated for 4 h in acidic medium A to detect acidic denaturation. The rate of ATP synthesis was 50

Table 1
Rate of ATP synthesis (turnover) catalyzed by EF_0F_1 and CF_0F_1 in the presence and in the absence of $\Delta\phi$ and different controls

Conditions	Rate of ATP synthesis/s^{-1}	
	CF_0F_1	EF_0F_1
$pH_{out} = 8.8$, $pH_{in} = 4.7$		
Proteoliposomes A ($K^+_{in} = 0.5$ mM, $K^+_{out} = 120$ mM, $\Delta\phi$ (K^+) ≈ 140 mV)	280	78
Proteoliposomes B ($K^+_{in} = K^+_{out} = 130$ mM, $\Delta\phi$ (K^+) ≈ 0 mV)	190	1
Proteoliposomes A incubated with 130 mM KCl ($K^+_{in} = K^+_{out} = 130$ mM, $\Delta\phi$ (K^+) ≈ 0 mV)	190	1
$pH_{out} = 8.8$, $pH_{in} = 5.1$		
Proteoliposomes A ($K^+_{in} = 0.5$ mM, $K^+_{out} = 120$ mM, $\Delta\phi$ (K^+) ≈ 140 mV)	230	50
Proteoliposomes B ($K^+_{in} = 13$ mM, $K^+_{out} = 120$ mM, $\Delta\phi$ (K^+) ≈ 57 mV)	190	7
Proteoliposomes A incubated with 130 mM KCl ($K^+_{in} = 13$ mM, $K^+_{out} = 120$ mM, $\Delta\phi$ (K^+) ≈ 57 mV)	220	8

The rate of ATP synthesis was measured after an acid-base transition in the absence and in the presence of a K^+/valinomycin diffusion potential. Proteoliposomes A were prepared in the presence of 0.2 mM K^+, proteoliposomes B in the presence of 130 mM K^+. In addition, proteoliposomes A were incubated with 130 mM KCl for 18 h. The acid-base transitions were carried out with basic medium A (pH 8.8, 130 mM K^+). Top: for measurements with $\Delta\phi$ (K^+) = 140 mV, acidic medium A (pH 4.5, 0.6 mM K^+) and for $\Delta\phi$ (K^+) = 0 mV, acidic medium B (pH 4.5, 130 mM K^+) was used. Bottom: proteoliposomes were incubated in acidic medium A (pH 4.9) for 4 h. For proteoliposomes B and A, incubated with 130 mM KCl, the high K^+ concentration inside was reduced to 13 mM K^+ and $\Delta\phi$ (K^+) = 57 mV was obtained.

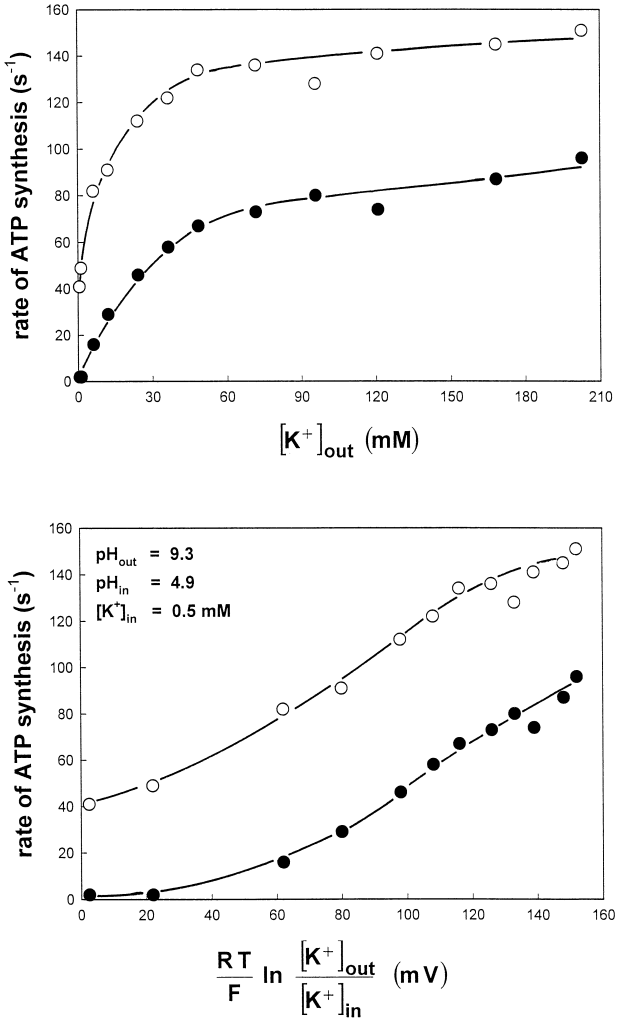

Fig. 2. Rate of ATP synthesis catalyzed by EF_0F_1 and CF_0F_1 as a function of the external K^+ concentration. The initial rate of ATP synthesis was measured with reconstituted EF_0F_1 and CF_0F_1 after energization of the membrane by an acid-base transition, $pH_{in} = 4.9$, $pH_{out} = 9.3$. In addition to the transmembrane ΔpH, a transmembrane K^+/valinomycin diffusion potential was generated with an internal K^+ concentration of 0.5 mM and a varying external K^+ concentration. Filled circles indicate data obtained with EF_0F_1, open circles indicate data with CF_0F_1. Top: rate of ATP synthesis as a function of the external K^+ concentration. Bottom: rate of ATP synthesis as a function of the Nernst potential of K^+.

s^{-1}. Since the rate of ATP synthesis at a high $\Delta\phi$ does not depend on pH_{in} between pH_{in} 4.7 and pH_{in} 5.1 (see Fig. 3), the difference in rates indicates a slight denaturation by the prolonged acidic incubation (compare line 1 with line 4 in Table 1). When the same treatments were carried out with CF_0F_1 proteoliposomes, the influence of $\Delta\phi$ on the enzyme activity was small (Table 1).

In further experiments, the rate of ATP synthesis was measured as a function of $\Delta\phi$ at ΔpH 4.4 ($pH_{in} = 4.9$, $pH_{out} = 9.3$). EF_0F_1 and CF_0F_1 were reconstituted with buffer A and then incubated in acidic medium A so that the internal K^+ concentration was 0.5 mM. The acid-base transition was carried out with basic medium B which was titrated to pH 9.3 using different ratios of KOH and NaOH in order to obtain different external K^+ concentrations. Fig. 2, top, shows the rate of ATP synthesis as a function of the external K^+ concentration. The highest rate observed with EF_0F_1 is about 90 s^{-1} and with CF_0F_1 about 150 s^{-1}, i.e. both rates are of a similar magnitude at high external K^+ concentrations. At the lowest K^+ concentration, the rate with EF_0F_1 is less than 2 s^{-1}, that with CF_0F_1 is about 40 s^{-1}. From the ratio of the internal and external K^+ concentration, the Nernst potential was calculated and the data are re-plotted as a function of this potential (Fig. 2, bottom). It can be seen that at low $\Delta\phi$, the rate catalyzed by EF_0F_1 is less than 2 s^{-1}, although the magnitude of ΔpH is energetically sufficient to drive ATP synthesis. For CF_0F_1, the rate decreases by a factor of three, whereas for EF_0F_1, the rate decreases by more than a factor 50 in the same range of $\Delta\phi$.

In order to obtain further information on the correlation of the rate of ATP synthesis and ΔpH and $\Delta\phi$, the rate was measured as a function of the internal proton concentration with different K^+ concentration ratios. EF_0F_1 proteoliposomes A were incubated in acidic medium A containing, in this case, 5 mM NaH_2PO_4 instead of 10 mM NaH_2PO_4. The pH was varied between $pH_{in} = 4.1$ and 6.7 and the internal K^+ concentration was 0.5 mM. The acidified proteoliposomes were mixed with basic medium B containing 5 mM phosphate and either 13 mM (reaction conditions 1), 39 mM (reaction conditions 2) or 218 mM KOH (reaction conditions 3).

The time for equilibration was varied between 1 min at $pH_{in} = 4.1$ and 6 min at $pH_{in} = 6.7$. Since the time needed for equilibration during acidic incubation is higher for a higher pH_{in}, the appropriate incubation time was determined by measuring the ATP synthesis as a function of the incubation time. When the rate showed no further increase with increasing incubation time, this time was used for further experiments. Between $pH_{in} = 4.1$ and 4.6, a decrease of the rate was observed with an increasing incubation time which is

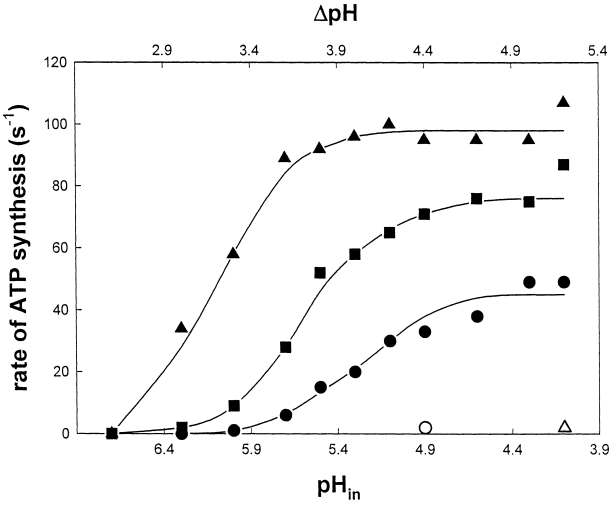

Fig. 3. Rate of ATP synthesis catalyzed by EF_0F_1 as a function of the internal proton concentration at different Nernst potentials of K^+. The proteoliposomes were energized by an acid-base transition. pH_{out} was 9.3 and pH_{in} was changed between 4.1 and 6.7. The internal K^+ concentration of the proteoliposomes was 0.5 mM, the external K^+ concentration was either 13 mM (filled circles), 32 mM (filled squares) or 218 mM (filled triangles). The Nernst potential of K^+ was 80 mV, 108 mV or 152 mV, respectively. When $\Delta\phi$ was abolished by equal internal and external K^+ concentrations, the rates were smaller than 2 s^{-1} at any ΔpH (open symbols).

presumably due to a denaturation of the enzyme in the acidic medium. This effect was corrected as described earlier [24]. First, ATP synthesis was measured at $pH_{in} = 5.1$ (rate 1) and at 4.1 (rate 2). In a second experiment, proteoliposomes were incubated for 1 min at pH = 4.1, then, the pH was changed to 5.1, incubated for another 2 min and then, the acid-base transition was carried out (rate 3). The ratio between rate 1 and 2 was then used as a correction factor for rate 2.

Fig. 3 shows the rate of ATP synthesis as a function of pH_{in}. The rates at the different $\Delta\phi$s depend non-linearly on pH_{in}. Half maximal rates were observed at $pH_{in} = 5.2$ (reaction conditions 1), $pH_{in} = 5.6$ (reaction conditions 2) and $pH_{in} = 6.1$ (reaction conditions 3). The maximal rate observed at a high ΔpH depends on the magnitude of $\Delta\phi$. This is surprising since for reaction conditions 1, the ΔpH can be changed from ΔpH = 4.5 to 5 without a significant effect on the rate, for reaction conditions 2, the ΔpH can be changed from 4 to 5 without an effect on the rate and for reaction conditions 3, ΔpH can be changed from 3.5 to 5 without an effect on the rate. Obviously, ΔpH has reached a saturation, i.e. all available groups, which must be protonated for proton translocation, are protonated. Nevertheless, an increase in $\Delta\phi$ leads to a further increase of the rate. When $\Delta\phi$ is completely abolished, the rate of ATP synthesis is less than 2 s^{-1} even when ΔpH is between 4 and 5 (open symbols in Fig. 3). We have to conclude from these results that ΔpH (i.e. protonation) and $\Delta\phi$ (i.e. charge translocation) act in two different steps of the reaction sequence of the enzyme.

4. Discussion

The data presented in this work show that it is possible to obtain high rates of ATP synthesis (about 100 s^{-1}) when EF_0F_1 is reconstituted into liposomes and energized by an acid-base transition and a K^+/valinomycin diffusion potential. It is shown in Fig. 1 that at ΔpH = 4.1, almost no ATP synthesis is observed, whereas with an additional $\Delta\phi$ of 80 mV, a rate of about 70 s^{-1} is observed. It might be argued that the proton motive force is not sufficient to drive ATP synthesis in the absence of $\Delta\phi$. However, for CF_0F_1 in the absence and in the presence of $\Delta\phi$, almost the same rate is observed (see Fig. 1). We have to conclude from this comparison that either the H^+/ATP ratio for CF_0F_1-catalyzed ATP synthesis is higher than for EF_0F_1 or that for EF_0F_1, ΔpH and $\Delta\phi$ are not equivalent in driving ATP synthesis. The data presented in Fig. 3 support the latter possibility, an increase of ΔpH does not change the rate whereas an increase of $\Delta\phi$ raises the rate.

Membrane-bound H^+-ATPases are strongly regulated enzymes [15–22]. It has been shown for CF_0F_1 that this enzyme is usually in an inactive state, E_i, and it can be converted into an active metastable state, E_a, by energization of the membrane [15,16,23]. Only in this active state, the enzyme can catalyze ATP synthesis and ATP hydrolysis. CF_0F_1 can be activated by $\Delta\phi$ and/or ΔpH in an energetically equivalent way [24]. In addition, it has been shown for ATP synthesis that ΔpH and $\Delta\phi$ are kinetically equivalent [23,24]. The observed rate, V_{obs}, of ATP synthesis and ATP hydrolysis can therefore be described by

$$V_{obs} = \eta(\Delta\,pH,\ \Delta\phi)\,V_{cat}(\Delta pH,\ \Delta\phi)$$

where η is the fraction of active H^+-ATPases depending on ΔpH and $\Delta\phi$ and V_{cat} is the rate of ATP synthesis per active enzyme, which depends also on ΔpH and $\Delta\phi$.

A similar analysis has been carried out with the H^+-ATPase from *Rhodobacter capsulatus* and it was found that both activation and the catalytic reaction depend on ΔpH and $\Delta\phi$. However, both parameters do not seem to be equivalent. The fraction of active H^+-ATPases was measured as a function of ΔpH and $\Delta\phi$. Its magnitude depends on both ΔpH and $\Delta\phi$. However, at a high ΔpH, it cannot be increased further by increasing ΔpH, whereas an increase of $\Delta\phi$ increases this fraction [21,22]. The data reported in Fig. 3 are similar to those found in *R. capsulatus*. The main difference seems to be that activation of the H^+-ATPase from *E. coli* is regulated by $\Delta\phi$ only. Therefore, we conclude that all H^+-ATPases might be regulated by an activation process. The regulation might be different in different H^+-ATPases, however, the mechanism of catalysis is the same. With this assumption, our data on the non-equivalence of ΔpH and $\Delta\phi$ in driving ATP synthesis for EF_0F_1 can be described by the following scheme:

$$E_i \overset{\Delta\phi}{\leftrightarrows} E_a + ADP + P_i \overset{\Delta\tilde{\mu}_{H^+}}{\longrightarrow} E_a + ATP$$

where E_i is the inactive and E_a the active enzyme form.

The non-equivalence of ΔpH and $\Delta\phi$ in driving ATP synthesis has been reported recently [25]. Whereas our data also indicate a non-equivalence of both parameters for EF_0F_1, our conclusion is different. It should also be pointed out that the highest rates reported in that work are a factor 20–100 lower than those reported here, i.e. they have the magnitude of the zero controls of our measurements. In addition, the authors observed ATP synthesis driven by diffusion potentials with $\Delta\phi \geq 40$ mV (without ΔpH) [25]. In our hands, significant rates of ATP synthesis required the presence of $\Delta\phi$ and ΔpH, i.e. we were not able to detect ATP synthesis driven by a diffusion potential only. This is not surprising since the minimal proton motive force thermodynamically required for ATP synthesis under our experimental conditions is higher than that provided by the diffusion potential. However, it was shown earlier for CF_0F_1 that it is possible to drive ATP synthesis only by $\Delta\phi$ when the field is high enough [4,26–28].

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