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The Activity of the ATP Synthase from *Escherichia coli* Is Regulated by the Transmembrane Proton Motive Force*

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The ATP synthase from *Escherichia coli* was reconstituted into liposomes from phosphatidylcholine/phosphatidic acid. The proteoliposomes were energized by an acid-base transition and a K^+/valinomycin diffusion potential, and one second after energization, the electrochemical proton gradient was dissipated by uncouplers, and the ATP hydrolysis measurement was started. In the presence of ADP and P_i, the initial rate of ATP hydrolysis was up to 9-fold higher with pre-energized proteoliposomes than with proteoliposomes that had not seen an electrochemical proton gradient. After dissipating the electrochemical proton gradient, the high rate of ATP hydrolysis decayed to the rate without pre-energization within about 15 s. During this decay the enzyme carried out approximately 100 turnovers. In the absence of ADP and P_i, the rate of ATP hydrolysis was already high and could not be significantly increased by pre-energization. It is concluded that ATP hydrolysis is inhibited when ADP and P_i are bound to the enzyme and that a high $\Delta\tilde{\mu}_{H^+}$ is required to release ADP and P_i and to convert the enzyme into a high activity state. This high activity state is metastable and decays slowly when $\Delta\tilde{\mu}_{H^+}$ is abolished. Thus, the proton motive force does not only supply energy for ATP synthesis but also regulates the fraction of active enzymes.

Membrane-bound F_0F_1-ATPases catalyze ATP synthesis in bacteria, chloroplasts, and mitochondria. The enzymes consist of two major parts: the membrane embedded hydrophobic F_0 part involved in proton translocation across the membrane and the hydrophilic F_1 part containing the nucleotide- and phosphate-binding sites (for review see Refs. 1–3). In the recent years, major progress has been made toward an understanding of the enzymatic mechanism; the structure of the hydrophilic F_1 part has been solved to 2.8 Å resolution (4), and an ATP induced rotation of the γ subunit within the $\alpha_3\beta_3$ barrel has been deduced (5, 6) and visualized (7). Most recently, the ATP-induced rotation of the c-ring in the F_0 part has also been shown (Refs. 8 and 9, but see also Ref. 10).

The electron transport during respiration or photosynthesis generates a transmembrane electrochemical potential difference of protons, $\Delta\tilde{\mu}_{H^+}$ (energization of the membrane), which F_0F_1-ATPases use as driving force for ATP synthesis (11). In the reverse direction, the enzymes hydrolyze ATP and generate a transmembrane $\Delta\tilde{\mu}_{H^+}$. Whereas in chloroplasts and mitochondria this direction is inhibited by special regulations (for review see Refs. 12 and 13), ATP hydrolysis has an important physiological function in bacteria.

The proton motive force, Δp, is given by the following equation.

$$\Delta p = \frac{\Delta\mu_{H^+}}{F} = -\frac{2.3\,RT}{F}\Delta\mathrm{pH} + \Delta\varphi \qquad \text{(Eq. 1)}$$

(ΔpH being the transmembrane pH difference and $\Delta\varphi$ the transmembrane electric potential difference). According to the chemiosmotic theory (11), the two components of Δp are thermodynamically equivalent, *i.e.* a change of ΔpH by one unit will result in the same change of the ATP/ADP equilibrium as a shift of $\Delta\varphi$ by 59 mV (at 23 °C). For thylakoid membranes, chromatophores, and some bacterial F_0F_1-ATPases, it appears that ΔpH and $\Delta\varphi$ are also kinetically equivalent, *i.e.* the rate of ATP synthesis is changed by the same factor when either $\Delta\varphi$ is changed by 59 mV or ΔpH is changed by one unit (14–17).

In chloroplasts, photosynthetic bacteria, and mitochondria, it has been shown that $\Delta\tilde{\mu}_{H^+}$ is not only the free energy input for ATP synthesis but also strongly regulates the activity of the F_0F_1-ATPases, *i.e.* $\Delta\tilde{\mu}_{H^+}$ induces a metastable high activity state of the enzyme (18–23). This phenomenon is called $\Delta\tilde{\mu}_{H^+}$ activation, or only activation and occurs also under physiological conditions (24). This physiological regulation of the activity has to be distinguished from treatments (with lauryl dimethylamine oxide (LDAO), trypsin, or heat, for example) that increase the rate of ATP hydrolysis by partial denaturation of the enzyme and are sometimes also called activation. The fraction of active enzymes increases with increasing $\Delta\tilde{\mu}_{H^+}$ and the two components, ΔpH and $\Delta\varphi$, appear to be equivalent in this respect in chloroplasts (14). In contrast, in the photosynthetic bacterium *Rhodobacter capsulatus*, $\Delta\varphi$ is more effective than ΔpH (23), and there are indications that in mitochondria this might also be the case (21, 25). The stability of the metastable active state is influenced by a variety of parameters, like ADP, Mg^{2+}, and P_i concentration, and in chloroplasts F_0F_1-ATP synthase also by the redox state of the enzyme (14, 23, 26–28).

Recently, we reconstituted the ATP synthase from *Escherichia coli*, EF_0F_1, into liposomes and measured ATP synthesis after energization by an acid-base transition. (29, 30). Surprisingly, the data gave hints that the *E. coli* enzyme too might be activated by $\Delta\tilde{\mu}_{H^+}$. Therefore, we decided to investigate this phenomenon in more detail. Activation by $\Delta\tilde{\mu}_{H^+}$ is detected most easily by measuring the increase of the uncoupled rate of ATP hydrolysis after the enzyme has been exposed to a $\Delta\tilde{\mu}_{H^+}$

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(31), and with this reconstituted system we were able to carry out these experiments.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of EF_0F_1 into Liposomes—E. coli strain DK8 (Δ*unc*) transformed with plasmid pBWU13 (32) was a gift from Prof. Futai (Osaka, Japan). The bacteria were grown in a minimal medium and EF_0F_1 was isolated as described (29, 32). The enzyme was obtained in a solution containing 10 mM Mes[1] and 10 mM Tricine/NaOH, pH 7.0, 500 μM $MgCl_2$, 5 mM thioglycerol, 10 g/liter octylglucoside, and 300 g/liter sucrose with a protein concentration between 1 and 2 g/liter. EF_0F_1 was rapidly frozen and stored in liquid nitrogen. The protein concentration was determined with Amido Black (33), and a molecular mass of 530 kDa was used.

Liposomes from phosphatidylcholine and phosphatidic acid were prepared by dialysis (30), and EF_0F_1 was reconstituted into these preformed liposomes using Triton X-100 (29, 34). The proteoliposomes were obtained in a buffer at pH 8.0 containing 13 mM Tricine, 8 mM succinate, 9 mM NaOH, 0.5 mM KCl, and 4 mM $MgCl_2$ with a lipid concentration of approximately 8 g/liter and a EF_0F_1 concentration of 30 nM. The proteoliposomes were concentrated by centrifugation at 265,000 × *g* for 2 h at 18 °C. The pellet was resuspended in a minimal volume of buffer LP (20 mM succinate, 20 mM Tricine, titrated with NaOH to pH 8.0) to get a final concentration of approximately 1 μM EF_0F_1. KCl was added to a final concentration of 0.5 mM. These concentrated proteoliposomes were kept at room temperature (23 °C) for up to 3 days. For energization at lower Δφ or in the absence of Δφ, KCl was added to final concentrations of 8 and 100 mM, respectively, and the proteoliposomes were incubated at room temperature for 4 h in the presence of 50 μM valinomycin prior to measurements.

ATP Hydrolysis in an ATP Regenerating System—The continuous measurement of ATP hydrolysis was carried out in a spectrophotometer at 23 °C by detecting the decrease of NADH in an enzymatic coupled assay (35). The reaction medium contained 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 4 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 18 units/ml pyruvate kinase, 16 units/ml lactate dehydrogenase, and 0.2 mM NADH. For measuring the uncoupled rate of ATP hydrolysis, the reaction medium contained in addition 1 μM valinomycin and 1 μM nigericin.

ATP Hydrolysis after Energization—Proteoliposomes were energized by an acid-base transition and an additional K^+/valinomycin diffusion potential in the presence of 100 μM ATP and different concentrations of P_i and ADP at room temperature (23 °C). Proteoliposomes (10 μl) were mixed with 20 μl of acidic medium (±Δφ) and incubated for 2 min. Thereafter, $\Delta\tilde{\mu}_{H^+}$ was generated by adding 50 μl basic medium (±Δφ) (energization). After 1 s, $\Delta\tilde{\mu}_{H^+}$ was dissipated by adding 10 μl of uncoupling medium (±Δφ) containing nigericin to a final concentration of 1 μM (uncoupling). The time of uncoupling and addition of [γ-^{32}P]ATP is the start of ^{32}P release, and this is $t = 0$ for all of the following experiments. The uncoupling medium (±Δφ) contained [γ-^{32}P]ATP (Amersham Pharmacia Biotech) to a specific activity of 40 kBq/ml. The reaction was stopped at different times by adding 40 μl of perchloric acid to a final concentration of 0.9 M. The points at $t = 0$ were obtained by adding perchloric acid to proteoliposomes after the acid base jump and then adding, after about 5 s, uncoupling medium (±Δφ). The released $^{32}P_i$ was separated from [γ-^{32}P]ATP by organic solvent extraction of a molybdate-P_i complex, and the radioactivity was measured by liquid scintillation counting in a Tri-Carb 2100TR (Canberra Packard). The $^{32}P_i$ found at $t = 0$ amounted to about 4% of the total and was subtracted from all data.

Compositions of the buffers were as follows: acidic medium (+Δφ) contained 20 mM succinate, 0.5 mM KOH, 2.5 mM $MgCl_2$, either 0 or 15 mM NaH_2PO_4, 0 ± 150 μM ADP, 80 μM valinomycin (freshly added from a 10 mM ethanolic solution) titrated to the desired pH values with NaOH (from 4.0 to 8.3); basic medium (+Δφ) contained 200 mM Tricine, 100 mM KOH, 2.5 mM $MgCl_2$, either 0 or 10 mM NaH_2PO_4, 150 μM ATP, and ADP concentrations between 0–100 μM titrated to the desired pH values with NaOH (from 8.0 to 8.6); and uncoupling medium (+Δφ) was like basic medium (+Δφ), except that the ATP concentration was 100 μM, and it contained 9 μM nigericin (freshly added from a 1 mM ethanolic solution) and [γ-^{32}P]ATP (360 kBq/ml). For energization with a lower diffusion potential, composition of buffers was as above except that the basic medium and uncoupling medium contained 8 mM KOH instead of 100 mM. For energization in the absence of diffusion potentials, the following media were used: acidic medium (−Δφ) was like acidic medium (+Δφ), except that 100 mM KOH/KCl were present instead of 0.5 mM KOH; basic medium (−Δφ) was like basic medium (+Δφ); and uncoupling medium (−Δφ) was like uncoupling medium (+Δφ).

After 2 min of incubation of the proteoliposomes in acidic medium (±Δφ), the internal pH (pH_{in}) was assumed to be equal to the pH measured after mixing two parts of the acidic medium with one part of the proteoliposomes. This time was enough to reach the maximal ATP synthesis rate (30). The external pH (pH_{out}) was the pH value that resulted after mixing three parts of the acidified proteoliposomes with five parts of the basic medium (±Δφ).

Measurement of ATP Synthesis—The rate of ATP synthesis catalyzed by EF_0F_1 was measured at 23 °C as described earlier (30). Proteoliposomes were energized by an acid-base transition and an additional K^+/valinomycin diffusion potential. Proteoliposomes (15 μl) were mixed with acidic medium S (60 μl), and after 2 min of incubation 50 μl of the acidified suspension were injected into 950 μl of basic medium S. The ATP concentration was monitored continuously with luciferin/luciferase (Merlin) in a luminometer (LKB 1250). The following media were used in the acid-base transition: acidic medium S contained 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; and basic medium S contained 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. After mixing, pH_{in} was 4.7, and pH_{out} was 8.8.

RESULTS

EF_0F_1 was reconstituted into liposomes at a concentration of 30 nM. The proteoliposomes were concentrated by centrifugation to about 1 μM EF_0F_1 and stored at room temperature for up to 3 days. The rate of ATP synthesis was measured before and after centrifugation and on every following day. Centrifugation caused about 45% loss of ATP synthesis activity, *i.e.* from 65 to 35 s^{-1}. For comparison with our previous ATP synthesis data, all the following enzyme concentrations were corrected by this factor. A correction factor was also applied to compensate for the 10% loss of ATP synthesis activity observed after 3 days of storage.

ATP hydrolysis catalyzed by proteoliposomes after centrifugation was measured with an ATP regenerating system in the absence and presence of uncouplers (nigericin and valinomycin). At 4 mM ATP, the steady state rate in the absence of uncouplers was 40 s^{-1}; it increased after addition of valinomycin to 80 s^{-1} and after addition of nigericin to 210 s^{-1}. The 5-fold increase of the rate indicates that in the absence of uncouplers, proton transport-coupled ATP hydrolysis generates $\Delta\tilde{\mu}_{H^+}$ across the membrane, and its back pressure limits the rate. When the proton permeability of the membrane was increased by the uncouplers nigericin and valinomycin, this back pressure was released, and the rate was increased. The stimulation by a factor 5 indicates a good coupling between ATP hydrolysis and proton transport, *i.e.* a good efficiency of reconstitution of the enzyme into the liposomes. The same experiment was carried out with 100 μM ATP. In this case, the coupled rate was 15 s^{-1} and was also increased 5-fold after uncoupling.

Activation of ATP Hydrolysis by $\Delta\tilde{\mu}_{H^+}$—To investigate whether reconstituted EF_0F_1 can be activated by $\Delta\tilde{\mu}_{H^+}$, the experiments were carried out as follows. EF_0F_1 proteoliposomes were incubated for 2 min in acidic medium, and $\Delta\tilde{\mu}_{H^+}$ was generated by addition of the basic medium (Δφ/ΔpH jump). One second after the Δφ/ΔpH jump, the $\Delta\tilde{\mu}_{H^+}$ was collapsed by addition of an uncoupler (nigericin), and, simultaneously, ATP hydrolysis was started by adding [γ-^{32}P]ATP. ATP hydrolysis was stopped after various times between 1 and 20 s by addition of perchloric acid and the released P_i was measured. In the following, the rate of ATP hydrolysis measured in this way is called pre-energized ATP hydrolysis.

For comparison ATP hydrolysis was measured under identical conditions but without pre-energization. In this case, after incubation in the acidic medium, the uncoupler was added simultaneously with the basic medium, so that no $\Delta\tilde{\mu}_{H^+}$ was generated. After one second, [γ-^{32}P]ATP was added, and ATP

[1] The abbreviations used are: Mes, 2-[*N*-morpholino]ethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; EF_1, soluble portion of the *E. coli* F_1F_0 ATP synthase.

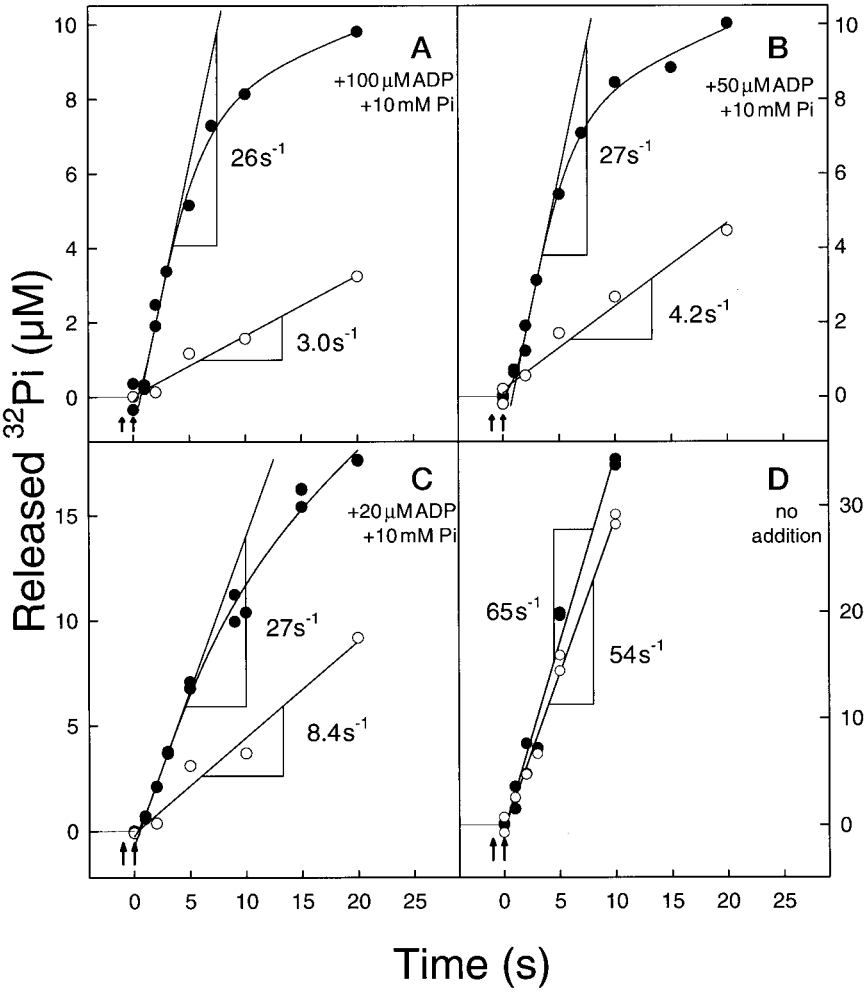

FIG. 1. **ATP hydrolysis with and without pre-energization.** EF_0F_1 proteoliposomes were energized by an acid-base transition. A ΔpH of 3.4 units (pH_{in} = 4.9, pH_{out} = 8.3) and a Δφ of about 134 mV ($[K^+]_{out}$ = 100 mM, $[K^+]_{in}$ = 0.5 mM in the presence of valinomycin) were generated. After 1 s, nigericin and [γ-^{32}P]ATP were added to dissipate $\Delta\tilde{\mu}_{H^+}$ and to start the release of $^{32}P_i$. ●, pre-energized rate. In each panel, the *left arrow* indicates the acid-base transition (addition of basic medium), and the *right arrow* represents the addition of nigericin and [γ-^{32}P]ATP (addition of uncoupling medium). EF_0F_1 and ATP concentrations were 54 nM and 100 μM, respectively. The reaction was stopped at various times by adding perchloric acid, and samples were analyzed for $^{32}P_i$ content. As controls, the same experiments were carried out except that nigericin was present during the acid-base transition, *i.e.* no $\Delta\tilde{\mu}_{H^+}$ was generated (basal rate, ○). The *solid lines* represent least square fits of the data. The numbers at the slopes give the rates of ATP hydrolysis in mol ATP/mol EF_0F_1/s, *i.e.* in s^{-1}. In all cases, the maximum slope is indicated. ADP and P_i concentrations were as follows: *A*, 100 μM ADP, 10 mM P_i; *B*, 50 μM ADP, 10 mM P_i; *C*, 20 μM ADP, 10 mM P_i; *D*, no added ADP and P_i.

hydrolysis was assayed as described above. In the following, the rate of ATP hydrolysis measured under these conditions, *i.e.* without pre-energization, is called the basal rate. Both the basal and the pre-energized rates of ATP hydrolysis are assayed under the same experimental conditions (composition of medium, substrates, uncouplers, inhibitors etc.) except for the enzyme that has been exposed to $\Delta\tilde{\mu}_{H^+}$ or left untreated. Notice that both the pre-energized and the basal rates of ATP hydrolysis were measured under uncoupled conditions.

In a first set of experiments the effect of pre-energization on ATP hydrolysis was measured in the presence of 10 mM P_i for ADP concentrations between 20 and 100 μM (Fig. 1, *A–C*) and in the absence of ADP and P_i (Fig. 1*D*). Throughout these experiments, $\Delta\tilde{\mu}_{H^+}$ was the same during pre-energization, with pH_{in} = 4.9, pH_{out} = 8.3 (ΔpH = 3.4), and a $[K^+]_{out}/[K^+]_{in}$ ratio of 100 mM/0.5 mM, equivalent to a Nernst potential of 134 mV. The data for the pre-energized and basal rates are shown in *filled* and *open circles* respectively, and the rates of ATP hydrolysis are given by the *sloped lines*.

In the presence of 100 μM ADP (Fig. 1*A*), the rate of ATP hydrolysis was increased from 3 to 26 s^{-1} by pre-energization, *i.e.* an activation factor of about 9 was obtained. The high rate was observed only transiently and decayed to a level close to the basal one within about 15 s. When the ADP concentration in the reaction medium was lowered to 50 μM (Fig. 1*B*) or to 20 μM (Fig. 1*C*), the pre-energized rates were not affected. In contrast, the basal rates obtained were higher for lower ADP concentrations, and this resulted in a lower activation factor (6.4 and 3.6 for 50 and 20 μM ADP, respectively). In the absence of added ADP and P_i (Fig. 1*D*), the pre-energized and basal rates were nearly the same and were about two times higher compared with the pre-energized rates in the presence of ADP and P_i. Under pre-energized conditions, the kinetics of ATP hydrolysis show a small lag (Fig. 1, *A–C*). The most likely explanation for this lag is a delay in reaching a complete dissipation of $\Delta\tilde{\mu}_{H^+}$ under these experimental conditions. In such cases the rates in Fig. 1 refer to the highest slope of the curves.

These results can be summarized as follows: (i) In the presence of ADP and phosphate, the rate of ATP hydrolysis is low but it can be increased by pre-energization. (ii) Once $\Delta\tilde{\mu}_{H^+}$ is dissipated the high activity decays back to the basal activity within 10 to 20 s. (iii) In the absence of ADP and P_i the enzyme activity is already high and cannot be further increased by pre-energization.

Throughout these experiments, ATP was always present during pre-energization, being present in the basic medium. In some experiments, ATP was omitted from the basic medium, and, correspondingly, the ATP concentration in the uncoupling medium was increased to reach the same final concentration of 100 μM ATP in the reaction medium. The same basal rates and pre-energized rates were obtained compared with the measurements shown in Fig. 1*A*. This indicates that the presence of ATP during pre-energization is not required for activation.

ADP and P_i Inhibition of the Basal ATP Hydrolysis Rate—In further experiments, we measured the effects of increasing concentrations of ADP and P_i on the basal rate of ATP. Fig. 2 shows the results obtained in the presence of either 10 mM P_i or 100 μM ADP alone and in the presence and absence of both. The two substrates alone inhibited the ATP hydrolysis rate, to 23 and 42% of the maximal rate, respectively. The inhibition was

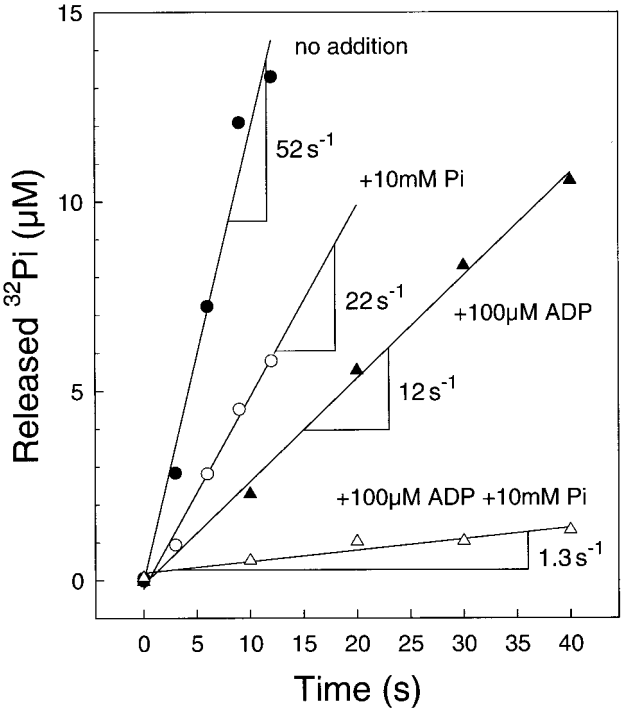

FIG. 2. **ATP hydrolysis without pre-energization at different ADP and P_i concentrations.** Proteoliposomes (10 μl) were mixed with 80 μl of reaction medium containing 20 μl of acidic medium (+Δφ), 50 μl of basic medium (+Δφ), and 10 μl of uncoupling medium (+Δφ). The final ATP concentration was 100 μM. The reaction was stopped at various times by adding perchloric acid, and samples were analyzed for $^{32}P_i$ content. The *solid lines* represent least square fits of the data. EF_0F_1 concentration was 23 nM. ●, no added ADP or P_i; ○, 10 mM P_i; ▲, 100 μM ADP; △, 100 μM ADP, 10 mM P_i. The *numbers* at the slopes give the rates of ATP hydrolysis in s^{-1}.

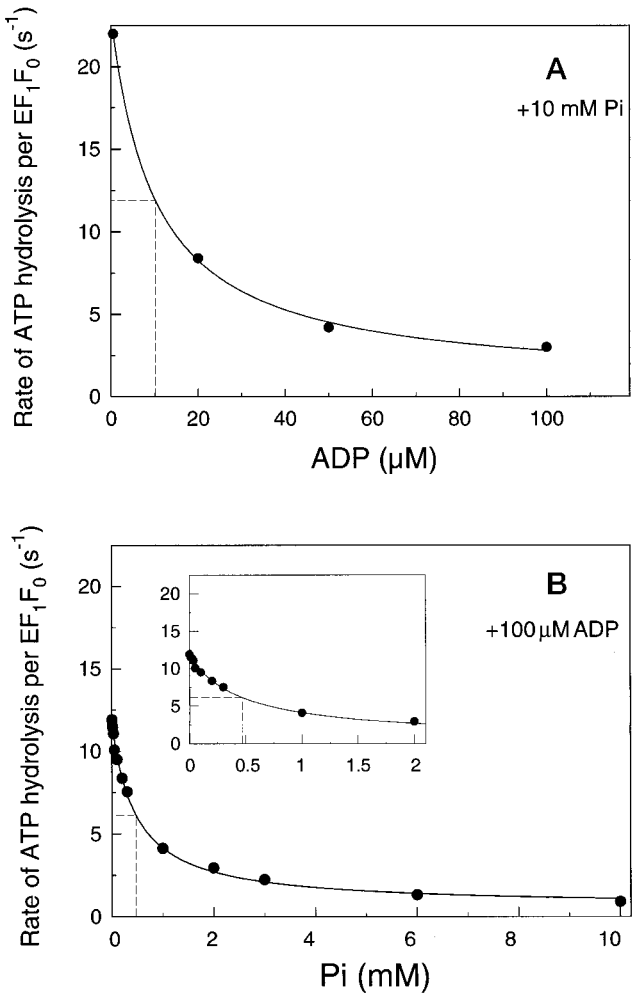

FIG. 3. **Rate of ATP hydrolysis without pre-energization as a function of ADP and P_i concentration.** Basal rates of ATP hydrolysis are from Figs. 1 and 2 and additional measurements. *A*, rates of ATP hydrolysis in the presence of 10 mM P_i were measured as a function of the ADP concentration. *B*, rates of ATP hydrolysis in the presence of 100 μM ADP as a function of P_i concentration.

much more pronounced when both substrates were present together, leaving 2.5% of the maximal rate. Note that the experimental conditions for the measurements shown in Fig. 2 are similar to those of Fig. 1 (*A* and *D*, *open circles*). The only difference is that in this case acidic medium, basic medium, and uncoupling medium were first mixed, and then the proteoliposomes were added. Because the rates are the same within experimental error, it appears that the inhibitory effect of ADP and P_i was not caused by preincubation in the acidic medium.

In Fig. 3*A* the basal rate of ATP hydrolysis is shown as a function of ADP concentration in the presence of 10 mM P_i. Because 0.5% ADP was found as a contaminant in the commercial ATP, 0.5 μM was the ADP concentration when no ADP was added to the reaction medium. The half-maximal inhibition is observed at 10 μM ADP. ATP hydrolysis was also measured as a function of P_i concentration in the presence of 100 μM ADP. These data are shown in Fig. 3*B*, indicating a half-maximal inhibition at 470 μM P_i. We conclude that the basal rate observed in Fig. 1*A* could not be significantly decreased by a further increase in ADP or P_i concentration.

ATP Hydrolysis after Exposure to Various $\Delta\tilde{\mu}_{H^+}$ Values—In additional experiments we varied the $\Delta\tilde{\mu}_{H^+}$ values generated in the pre-energization step. First we checked whether one of the two components of $\Delta\tilde{\mu}_{H^+}$, Δφ or ΔpH alone, was responsible for the activation phenomenon. ATP hydrolysis was measured after pre-energization with either Δφ or ΔpH alone. The experiments were carried out in the same way as those presented in Fig. 1*B*, *i.e.* in the presence of 50 μM ADP and 10 mM P_i. For ΔpH = 0, pH_{in} and pH_{out} were both 8.3, and the $[K^+]_{out}/[K^+]_{in}$ ratio was 100 mM/0.5 mM, equivalent to a Nernst potential of 134 mV. For Δφ = 0, $[K^+]_{out}$ and $[K^+]_{in}$ were both 100 mM with pH_{in} = 4.9, pH_{out} = 8.3 (ΔpH = 3.4). In both cases no significant activation could be seen, indicating that neither Δφ nor ΔpH alone was responsible for the activation seen in Fig. 1.

We also checked whether ΔpH and Δφ have the same effect on the activation by varying the relative contribution of Δφ and ΔpH at the same level of $\Delta\tilde{\mu}_{H^+}$(Δp ≈ 240 mV). Fig. 4 shows the effect of pre-energization on ATP hydrolysis when, at constant $\Delta\tilde{\mu}_{H^+}$, the relative contribution of ΔpH and Δφ were varied. It can be seen that no activation is observed for Δφ = 0; however, at the same $\Delta\tilde{\mu}_{H^+}$, the pre-energized rate was increased by a factor 2 when Δφ was increased to about 80 mV. Therefore, Δφ appears to be more effective than ΔpH in driving the activation step.

DISCUSSION

Most investigations on the catalytic mechanism of the ATP synthase from *E. coli* have been carried out with the EF_1 part (for review see Ref. 1). In this work, we have investigated ATP hydrolysis catalyzed by EF_0F_1 reconstituted into liposomes. First, we have shown that the steady state rate of ATP hydrolysis was limited by the back pressure of $\Delta\tilde{\mu}_{H^+}$, indicating that the proteoliposomes were well coupled. Correspondingly, these proteoliposomes catalyzed high rates of ATP synthesis.

A strong inhibition of the rate of ATP hydrolysis by ADP (Fig. 3*A*) and P_i (Fig. 3*B*) was observed. Because all measurements reported in this work were carried out in the presence of 2.5 mM Mg^{2+}, ADP was present almost completely as MgADP, which

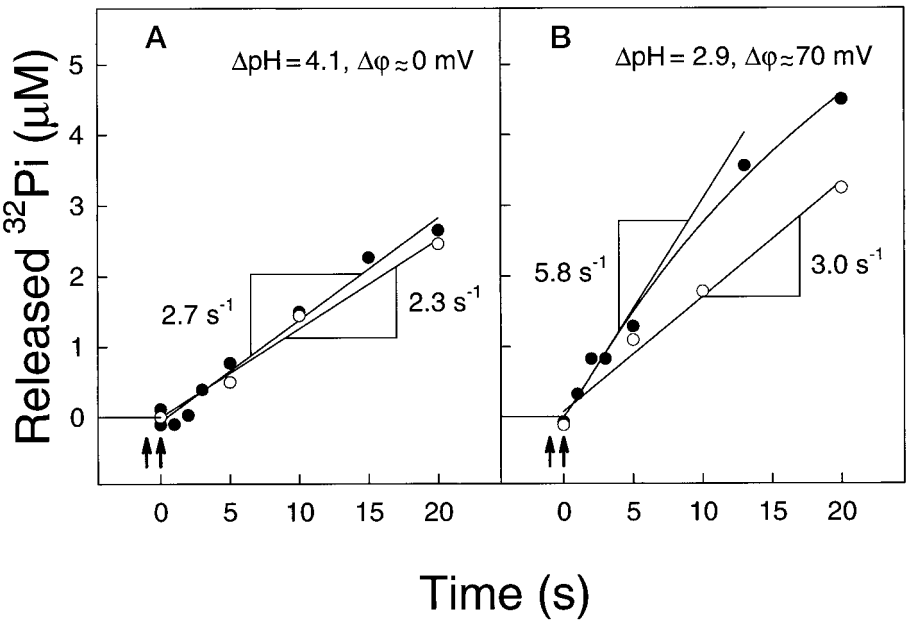

FIG. 4. **ATP hydrolysis with and without pre-energization under different $\Delta\tilde{\mu}_{H^+}$ conditions.** EF_0F_1 proteoliposomes were energized by an acid-base transition. After 1 s, nigericin and [γ-^{32}P]ATP were added to dissipate $\Delta\tilde{\mu}_{H+}$ and to start the release of $^{32}P_i$. The *left arrow* indicates the acid-base transition (addition of basic medium), and the *right arrow* represents the addition of nigericin and [γ-^{32}P]ATP (addition of uncoupling medium). EF_0F_1 and ATP concentrations were 54 nM and 100 μM, respectively. The reaction was stopped at various times by adding perchloric acid, and samples were analyzed for $^{32}P_i$ content. *Solid circles* indicate data after pre-energization, and *open circles* indicate data without pre-energization. The *solid lines* represent least square fits of the data, and the *numbers* at the slope give the rate of ATP hydrolysis in s^{-1}. *A*, pH_{in} = 4.7, pH_{out} = 8.8, $\Delta\varphi \approx 0$, *i.e.* $\Delta p \approx 240$ mV. *B*, pH_{in} = 5.9, pH_{out} = 8.8, $[K^+_{in}]/[K^+_{out}]$ = 0.5 mM/8 mM, *i.e.* $\Delta p \approx 240$ mV.

suggests that it is the binding of this complex to EF_0F_1 that inhibits ATP hydrolysis. For EF_1, the occurrence of an inhibitory MgADP catalytic site complex has been reported by Hyndman *et al.* (36), similar to what was found for soluble portions of the chloroplast (37) and mitochondrial F_1F_0 ATP synthase (38–40). These authors have proposed that this inhibitory complex could be responsible for EF_1 occurring as an heterogeneous mixture of active and inactive enzyme, as initially suggested by kinetic studies (41).

Binding of P_i to the *E. coli* enzyme has not been seen in competition studies with ATP using mutagenized tryptophanes as reporters of nucleotide binding to catalytic and non catalytic sites (42, 43). On the other hand, relatively low concentrations of P_i (2 mM) were shown to inhibit uni- and multi-site ATP hydrolysis in EF_1 (41) and P_i binding to EF_1 (K_D = 50 μM) has been shown to change the rate of trypsin cleavage of the ϵ subunit (44). Possibly, the binding of P_i is sensitive to the particular pattern of nucleotide occupancy of the enzyme. Because a small amount of ADP, as a contaminant of ATP, was present in our experiments, it is also possible that inhibition by P_i occurs only when ADP is present. The data of Mendel-Hartvig and Capaldi (44), considered in the light of the present work, do suggest that the ϵ subunit could be considered to play a main role in the $\Delta\tilde{\mu}_{H^+}$-induced inhibited-to-active transition. It is also interesting that, within the complexity of P_i effects on the activity of various ATP synthases to be found in the literature, there are reports of an ADP-requiring P_i inhibition of the mitochondrial F_1 (45) and of the chloroplasts F_0F_1 (27, 46).

We then investigated the effect of pre-energization on ATP hydrolysis. The rate of ATP hydrolysis was significantly increased compared with the rate without energization (Fig. 1*A*). Remarkably, this activation effect was only evident in the presence of inhibitory ADP and P_i. In their absence, the enzyme was able to catalyze high rates of ATP hydrolysis that could not be increased significantly by a $\Delta\tilde{\mu}_{H^+}$ (Fig. 1*D*).

A simple way to explain these observations is that membrane-bound EF_0F_1 exists in at least two different states: an inhibited state, E_i, with bound ADP and P_i, and an active state, E_a. Energization of the proteoliposomes by $\Delta\tilde{\mu}_{H^+}$ induces the transition from E_i to E_a. The high rates of ATP hydrolysis are detected only after dissipation of $\Delta\tilde{\mu}_{H^+}$ by uncoupling, because the back pressure of $\Delta\tilde{\mu}_{H^+}$ inhibits the ATP hydrolysis reaction. The active state is metastable and decays after dissipation of $\Delta\tilde{\mu}_{H^+}$ to the inhibited state within about 15 s. During this time, the enzyme carries out about 100 turnovers. This can be summarized by the following scheme.

$$E_i \cdot ADP \cdot P_i \xrightarrow{\Delta\mu_{H^+}} E_i \cdot ADP \cdot P_i \rightarrow E_a + ADP + P_i$$

SCHEME 1

According to this scheme, the E_a form is able to catalyze high rates of both ATP hydrolysis and synthesis, the direction being determined by the phosphate potential and membrane energization. It is not yet clear what causes the release of ADP and P_i and whether the conformational change results in an increase of the dissociation constants of bound ADP and P_i or a $\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis. In chloroplasts it has been shown that, after energization, ADP is released from the enzyme without previous phosphorylation (19, 47, 48).

At 100 μM ATP, 100 μM ADP and 10 mM P_i were enough to inhibit the enzyme almost to completion (about 2% of the maximal activity was left; Figs. 1 and 2). One might ask whether the maximum $\Delta\tilde{\mu}_{H^+}$ applied in this work (ΔpH = 3.4; $\Delta\varphi \approx 134$ mV; Fig. 1) was enough to activate all enzymes. Comparison of *D* and *A* of Fig. 1 shows that the highest hydrolysis rate was 64 s^{-1} (+$\Delta\tilde{\mu}_{H^+}$, absence of ADP and P_i), whereas the hydrolysis rate observed after $\Delta\tilde{\mu}_{H+}$ activation in the presence of the highest ADP/P_i concentration was 26 s^{-1}, *i.e.* only 40%. This lower rate might be due to two different effects: (i) the applied $\Delta\tilde{\mu}_{H^+}$ activates all enzymes; in this case the lower activity can be attributed to competitive product inhibition of the ATP hydrolysis; and (ii) the applied $\Delta\tilde{\mu}_{H^+}$ activates only 40% of the enzymes, and, correspondingly, the rate is lower.

It is striking that the activation of EF_0F_1 requires $\Delta\tilde{\mu}_{H^+}$ values that are higher than the thermodynamic threshold for ATP synthesis under these conditions. Taking $\Delta G^{0'}_P$ = 35 kJ mol^{-1} (49) and [ATP] = 100 μM, [ADP] = 50 μM, [P_i] = 10 mM, the thermodynamic threshold of $\Delta\tilde{\mu}_{H^+}$ for ATP synthesis is 12 kJ mol^{-1} (Δp = 125 mV) for n_{H^+} = 4 or 16 kJ mol^{-1} (Δp = 166 mV) for n_{H+} = 3. Fig. 4 shows that no activation took place up to ΔpH = 4.1. On the other hand, an increase of ATP hydrolysis by a factor of 2 was seen at the same $\Delta\tilde{\mu}_{H^+}$ but in the presence of a $\Delta\varphi$ (ΔpH = 2.9, $\Delta\varphi \approx 70$ mV). It is interesting to compare these data with those of Fischer and Gräber (30), who found that no ATP synthesis could be detected at ΔpH = 4.1 but that

about 20% of the maximal rate could be observed at $\Delta pH = 2.9$, $\Delta\varphi \approx 70$ mV. Both sets of data could fit together if it is assumed that the ATP synthesis reaction was limited by the activation process and if the activation process is more sensitive to $\Delta\varphi$ than to ΔpH. This last point awaits the support of more experimental data. For the moment, though, we consider it as very likely also in the light of data previously obtained in the photosynthetic bacterium *R. capsulatus*, indicating a much higher efficiency of $\Delta\varphi$ relative to ΔpH in driving the activation process (23). It is even possible that in *E. coli* the presence of a $\Delta\varphi$ is obligatory for activation.

Activation phenomena have first been found in the chloroplast ATP synthase (18, 19, 50, 51). ATP synthesis/hydrolysis in the light/dark cycles of plants has been shown to be regulated, in addition to a redox reaction of a disulfide bridge in CF_0F_1, by the $\Delta\tilde{\mu}_{H^+}$ (24, 31, 52, 53). Similar activation phenomena have been reported for the photosynthetic bacteria *R. rubrum* (22) and *R. capsulatus* (23). Activation of the mitochondrial ATP synthase has been shown to involve an inhibitor protein that dissociates from the enzyme in an energy-dependent manner leading to an activated enzyme (Refs. 21, 54, and 55; for a recent review see Ref. 12). More recently, activation by $\Delta\tilde{\mu}_{H^+}$ has been observed also in the absence of the inhibitor protein (56). In this work we have provided evidence that, similar to F_0F_1-ATPases from chloroplasts, mitochondria, and photosynthetic bacteria, EF_0F_1 is strongly regulated by $\Delta\tilde{\mu}_{H^+}$. Possibly, this type of regulation is a common feature of all F_0F_1-ATPases.

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The Activity of the ATP Synthase from *Escherichia coli* Is Regulated by the Transmembrane Proton Motive Force

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