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 \(\Delta\mu_H^+\) 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 \(\Delta\mu_H^+\) 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\(_0\)F\(_1\)-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 molecule 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 \(\Delta\mu_H^+\) is abolished. Thus, the proton motive force does not only supply energy for ATP synthesis but also regulates the fraction of active enzymes.

\[
\Delta p = \Delta \mu_H^+ = \frac{2.3RT}{F}\Delta pH + \Delta \varphi
\]

(\(\Delta \varphi\) being the transmembrane pH difference and \(\Delta \varphi\) the transmembrane electric potential difference). According to the chemiosmotic theory (11), the two components of \(\Delta p\) are thermodynamically equivalent, i.e. a change of \(\Delta p\) 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\(_0\)F\(_1\)-ATPases, it appears that \(\Delta \varphi\) and \(\Delta \varphi\) are also kinetically equivalent, i.e. the rate of ATP synthesis is changed by the same factor where either \(\Delta \varphi\) is changed by 59 mV or \(\Delta \varphi\) is changed by one unit (14–17).

In chloroplasts, photosynthetic bacteria, and mitochondria, it has been shown that \(\Delta \mu_H^+\) is not only the free energy input for ATP synthesis but also strongly regulates the activity of the F\(_0\)F\(_1\)-ATPases. In F\(_0\)F\(_1\)-ATPases, \(\Delta \mu_H^+\) induces a metastable high activity state of the enzyme (18–23). This phenomenon is called \(\Delta \mu_H^+\)-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 dimethyl-amine 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 \(\Delta \mu_H^+\) and the two components, \(\Delta \mu_H\) 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 \(\Delta \mu_H\) (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\(_0\)F\(_1\)-ATP synthase also by the redox state of the enzyme (14, 23, 26–28).

Recently, we reconstituted the ATP synthase from *Escherichia coli*, EF\(_0\)F\(_1\), 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 \(\Delta \mu_H^+\). Therefore, we decided to investigate this phenomenon in more detail. Activation by \(\Delta \mu_H^+\) is detected most easily by measuring the increase of the uncoupled rate of ATP hydrolysis after the enzyme has been exposed to a \(\Delta \mu_H^+\).
EF$_{0}$F$_{1}$ Activation

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

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of EF$_{0}$F$_{1}$ into Liposomes—E. coli strain DK5 (Δmnc) transformed with plasmid pBWU13 (32) was a gift from Prof. Futai (Osaka, Japan). The bacteria were grown in a minimal medium and EF$_{0}$F$_{1}$ was isolated as described (29, 32). The enzyme was obtained in a solution containing 10 mM Mes and 10 mM Tricine/NaOH, pH 7.0, 500 mM MgCl$_{2}$, 5 mM thiglycolic, 10 g/liter octylglucoside, and 300 g/liter sucrose with a protein concentration between 1 and 2 g/liter. EF$_{0}$F$_{1}$ 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$_{0}$F$_{1}$ 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$_{0}$F$_{1}$ concentration of 30 nM. The proteoliposomes were concentrated by centrifugation at 265,000 g at 4°C for 2 h at 18°C. The pellet was resuspended in a minimal volume of buffer LP (20 mM succinate, 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 mM valinomycin and 1 μM nigericin.

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 NaOH, 2.5 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 mM valinomycin and 1 μM nigericin.

ATP Hydrolysis after Energization—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$_{2}$PO$_{4}$, 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$_{2}$PO$_{4}$, 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$_{0}$F$_{1}$ was reconstituted into liposomes at a concentration of 30 nM. The proteoliposomes were concentrated by centrifugation to about 1 μM EF$_{0}$F$_{1}$, 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 ΔpH 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 it was also increased 5-fold after uncoupling.

Activation of ATP Hydrolysis by Δμ$^\text{H}^+$—To investigate whether reconstituted EF$_{0}$F$_{1}$ can be activated by Δμ$^\text{H}^+$, the experiments were carried out as follows. EF$_{0}$F$_{1}$ proteoliposomes were incubated for 2 min in acidic medium, and Δμ$^\text{H}^+$ was generated by addition of basic medium (Δμ$^\text{H}$ΔpH jump).

One second after the Δμ$^\text{H}$ΔpH jump, the Δμ$^\text{H}$ was collapsed by addition of an uncoupler (nigericin), and simultaneously, ATP hydrolysis was started by adding [$\gamma$$^{32}$P]ATP. ATP hydrolysis was stopped after various times between 1 and 20 s by addition of perchloric acid and the released Pi was measured. In the following, the rate of ATP hydrolysis measured in this way is called measured ATP hydrolyzed ATP.

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 Δμ$^\text{H}$ was generated. After one second, [$\gamma$$^{32}$P]ATP was added, and ATP was generated. The abbreviations used are: Mes, 2-[N-morpholino]ethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; EF, soluble portion of the E. coli F$_{0}$F$_{1}$ ATP synthase.
hydrolisis 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 an activation factor of about 9 was obtained. The high rate was observed only transiently and decayed to a level close to the basal activity decays back to the basal activity within 10 to 20 s. (ii) In the absence of ADP and Pi, 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 mM ATP in the reaction medium. The same basal rates and pre-energized rates were obtained compared with the measurements shown in Fig. 1A. This indicates that the presence of ATP during pre-energization is not required for activation.

**ADP and Pi Inhibition of the Basal ATP Hydrolysis Rate**—In further experiments, we measured the effects of increasing concentrations of ADP and Pi on the basal rate of ATP. Fig. 2 shows the results obtained in the presence of either 10 mM Pi or 100 mM 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
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 Pi was not caused by preincubation in the acidic medium.

In Fig. 3A the basal rate of ATP hydrolysis is shown as a function of ADP concentration in the presence of 10 mM Pi. 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 Pi concentration in the presence of 100 μM ADP. These data are shown in Fig. 3B, indicating a half-maximal inhibition at 470 μM Pi. We conclude that the basal rate observed in Fig. 1A could not be significantly decreased by a further increase in ADP or Pi concentration.

ATP Hydrolysis after Exposure to Various Δμ\textsubscript{H} Values—In additional experiments we varied the Δμ\textsubscript{H} values generated in the pre-energization step. First we checked whether one of the two components of Δμ\textsubscript{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. 1B, i.e., in the presence of 50 μM ADP and 10 mM Pi. For ΔpH = 0, pH\textsubscript{in} and pH\textsubscript{out} were both 8.3, and the [K\textsuperscript{+}]\textsubscript{out}/[K\textsuperscript{+}]\textsubscript{in} ratio was 100 mM/0.5 mM, equivalent to a Nernst potential of 134 mV. For Δφ = 0, [K\textsuperscript{+}]\textsubscript{out} and [K\textsuperscript{+}]\textsubscript{in} were both 100 mM with pH\textsubscript{in} = 4.9, pH\textsubscript{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 Δμ\textsubscript{H} (ΔpH ≈ 240 mV). Fig. 4 shows the effect of pre-energization on ATP hydrolysis when, at constant Δμ\textsubscript{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 Δμ\textsubscript{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\textsubscript{1} part (for review see Ref. 1). In this work, we have investigated ATP hydrolysis catalyzed by EF\textsubscript{F\textsubscript{1}} reconstituted into liposomes. First, we have shown that the steady state rate of ATP hydrolysis was limited by the back pressure of Δμ\textsubscript{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. 3A and B) was observed. Because all measurements reported in this work were carried out in the presence of 2.5 mM Mg\textsuperscript{2+}, ADP was present almost completely as MgADP, which
suggests that it is the binding of this complex to EF\textsubscript{F}1 that inhibits ATP hydrolysis. For EF\textsubscript{F}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\textsubscript{1}F\textsubscript{0} ATP synthase (38–40). These authors have proposed that this inhibitory complex could be responsible for EF\textsubscript{F}1 occurring as an heterogeneous mixture of active and inactive enzyme, as initially suggested by kinetic studies (41).

Binding of P\textsubscript{i} to the E. coli enzyme has not been seen in competition studies with ATP using mutagenized tryptophans as reporters of nucleotide binding to catalytic and non catalytic sites (42, 43). On the other hand, relatively low concentrations of P\textsubscript{i} (2 mM) were shown to inhibit uni- and multi-site ATP hydrolysis in EF\textsubscript{F}1 and P\textsubscript{i} binding to EF\textsubscript{F}1 (K\textsubscript{D} = 50 \mu M) has been shown to change the rate of trypsin cleavage of the € subunit (44). Possibly, the binding of P\textsubscript{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\textsubscript{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 € subunit could be considered to play a main role in the \( \Delta \mu_{ATP} \)-induced inhibited-to-active transition. It is also interesting that, within the complexity of P\textsubscript{i} effects on the activity of various ATP synthases to be found in the literature, there are reports of an ADP-requiring P\textsubscript{i} inhibition of the mitochondrial F\textsubscript{1} (45) and of the chloroplasts F\textsubscript{0}F\textsubscript{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. 1A). Remarkably, this activation effect was only evident in the presence of inhibitory ADP and P\textsubscript{i}. In their absence, the enzyme was able to catalyze high rates of ATP hydrolysis that could not be increased significantly by a \( \Delta \mu_{ATP} \) (Fig. 1D).

A simple way to explain these observations is that membrane-bound EF\textsubscript{F}1 exists in at least two different states: an inhibited state, E\textsubscript{i}, with bound ADP and P\textsubscript{i}, and an active state, E\textsubscript{a}. Energization of the proteoliposomes by \( \Delta \mu_{ATP} \) induces the transition from E\textsubscript{i} to E\textsubscript{a}. The high rates of ATP hydrolysis are detected only after dissipation of \( \Delta \mu_{ATP} \) by uncoupling, because the back pressure of \( \Delta \mu_{ATP} \) inhibits the ATP hydrolysis reaction. The active state is metastable and decays after dissipation of \( \Delta \mu_{ATP} \) 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.

\[
\Delta \mu_{ATP} \rightarrow E_i \cdot ADP \cdot P_i \rightarrow E_a \cdot ADP \cdot P_i + \Delta \mu_{ATP} + ADP + P_i
\]

**Scheme 1**

According to this scheme, the E\textsubscript{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\textsubscript{i} and whether the conformational change results in an increase of the dissociation constants of bound ADP and P\textsubscript{i} or a \( \Delta \mu_{ATP} \)-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 \mu M ATP, 100 \mu M ADP and 10 mM P\textsubscript{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 \( \Delta \mu_{ATP} \) applied in this work (\( \Delta \mu_{H} = 3.4 \), \( \Delta \varphi \approx 134 \) 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\(^{-1} \) (\( \Delta \mu_{ATP} \), absence of ADP and P\textsubscript{i}), whereas the hydrolysis rate observed after \( \Delta \mu_{ATP} \) activation in the presence of the highest ADP/P\textsubscript{i} concentration was 26 s\(^{-1} \), i.e. only 40%. This lower rate might be due to two different effects: (i) the applied \( \Delta \mu_{ATP} \) activates all enzymes; in this case the lower activity can be attributed to competitive product inhibition of the ATP hydrolysis; and (ii) the applied \( \Delta \mu_{ATP} \) activates only 40% of the enzymes, and, correspondingly, the rate is lower.

It is striking that the activation of EF\textsubscript{F}1 requires \( \Delta \mu_{H} \) values that are higher than the thermodynamic threshold for ATP synthesis under these conditions. Taking \( \Delta G^0 = 35 \) kJ mol\(^{-1} \) (49) and [ATP] = 100 \mu M, [ADP] = 50 \mu M, [P\textsubscript{i}] = 10 mM, the thermodynamic threshold of \( \Delta \mu_{H} \) for ATP synthesis is 12 kJ mol\(^{-1} \) (\( \Delta \rho \approx 125 \) mV) for \( \Delta \mu_{ATP} \approx 4 \) or 16 kJ mol\(^{-1} \) (\( \Delta \rho \approx 166 \) mV) for \( n_{ATP} = 3 \). Fig. 4 shows that no activation took place up to \( \Delta \rho \approx 4.1 \). On the other hand, an increase of ATP hydrolysis by a factor of 2 was seen at the same \( \Delta \mu_{ATP} \), but in the presence of a \( \Delta \varphi \) (\( \Delta \rho \approx 2.9 \), \( \Delta \varphi \approx 70 \) 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 \( \Delta \rho = 4.1 \) but that...
about 20% of the maximal rate could be observed at $\Delta \phi = 2.9$, $\Delta \phi \sim 70$ 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 $\Delta \phi$ than to $\Delta \phi$. 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 $\Delta \phi$ relative to $\Delta \phi$ in driving the activation process (23). It is even possible that in *E. coli* the presence of a $\Delta \phi$ 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$_{1}$F$_{1}$, by the $\Delta \mu_{1}^{-}$ (24, 31, 52, 53). Similar activation phenomena have been reported for the photosynthetic bacteria *R. rubrum* (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 24, 31, 52, 53). Similar activation phenomena have been reported for the photosynthetic bacteria *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 24, 31, 52, 53).

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