Energy-dependent Transformation of F₀F₁-ATPase in Paracoccus denitrificans Plasma Membranes*

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F₀F₁-ATP synthase in tightly coupled inside-out vesicles derived from Paracoccus denitrificans catalyzes rapid respiration-supported ATP synthesis, whereas their ATPase activity is very low. In the present study, the conditions required to reveal the ΔμH⁺,generating ATP hydrolase activity of the bacterial enzyme have been elucidated. Energization of the membranes by respiration results in strong activation of the venturicidin-sensitive ATP hydrolysis, which is coupled with generation of ΔμH⁺. Partial uncoupling stimulates the proton-translocating ATP hydrolysis, whereas complete uncoupling results in inhibition of the ATPase activity. The presence of inorganic phosphate is indispensable for the steady-state turnover of the ΔμH⁺-activated ATPase. The collapse of ΔμH⁺ brings about rapid deactivation of the enzyme, which has been subjected to pre-energization. The rate and extent of the deactivation depend on protein concentration, i.e. the more vesicles are present in the assay mixture, the higher the rate and extent of the deactivation is seen. Sulfite and the ADP-trapping system protect ATPase against the collapse-induced deactivation, whereas phosphate delays the rate of deactivation. A low concentration of ADP (<1 μM) increases the rate of deactivation. Taken together, the results suggest that latent proton-translocating ATPase in P. denitrificans is kinetically equivalent to the previously characterized ADP(Mg²⁺)-inhibited, azide-trapped bovine heart mitochondrial F₀F₁-ATPase (Galkin, M. A., and Vinogradov, A. D. (1999) FEBS Lett. 448, 123–126). A ΔμH⁺-sensitive mechanism operates in P. denitrificans that prevents physiologically wasteful consumption of ATP by F₀F₁-ATPase (synthase) complex when the latter is unable to maintain certain value of ΔμH⁺.

F₀F₁-ATPases (ATP synthases) are the oligomeric molecular machines that couple ATP hydrolysis (synthesis) with proton translocation across the energy-transducing membranes in mitochondria, chloroplasts, and bacteria. The structural arrangement of the subunits within F₀F₁ complexes of various organisms is assumed to be very similar (1–3). The hydrophilic F₁ is composed of a trimer of tightly packed αβ-subunit pairs and one copy each of the δ-, ε-, and γ-subunits (Escherichia coli nomenclature for the subunits is used). Long rod-like γ-subunit is asymmetrically positioned in the central cavity of the almost spherical globular αβ trimer. F₀ component is the hydrophobic complex composed of 10–14 transmembraneously positioned α- and two b-subunits. The hydrophilic parts of b-subunits are bound to F₁ (to one pair of αβ- and one δ-subunit), thus forming the peripheral stalk. The other central stalk is formed by γε complex, which interacts with c-subunit(s) arranged in a ring. F₁ bears three “catalytic” nucleotide-binding sites located on β-subunits, and F₀ serves as a proton-conducting path. It is generally believed that the coupling between the ATP hydrolysis (synthesis) and flow of protons across the membrane results from the consequence of the long distance conformational change: αβ-pair-associated chemical catalysis → γε → ab, leading to rotation of the rotor (γε bound to c-ring) within the stator (αβ trimer fixed by two b- and one δ-subunits) (4).

The kinetics of ATP hydrolysis catalyzed by the soluble F₁ or membrane-bound F₀F₁ preparations of the enzyme (coupled or uncoupled) are very complex (5). It has been documented that the key factor for such a complexity is a formation of so-called ADP(Mg²⁺)-inhibited form of the enzyme originally described (6) and kinetically characterized in a number of reports published by our (7–11) and other groups (12–16). Several phenomena such as hysteresis in onset of the catalytic activity (17), slow inhibition of ATPase by Mg²⁺ (18), activation of ATP hydrolysis by sulfite and other anions (9, 19, 20), and the inhibitory effect of azide (9) can be consistently explained by the kinetic scheme in which the slowly reversible interconversion between catalytically competent enzyme-ADP intermediate and its inactive “isomer” plays the central role (5). Perhaps the most intriguing property of the ADP(Mg²⁺)-inhibited ATPase is that being inactive in ATP hydrolysis, it is fully competent in the ATP synthase activity (21, 22).

The ADP(Mg²⁺)/ATP- and possibly ΔμH⁺-induced rearrangement within F₀F₁ complex that triggers its ATP hydrolysis and ATP synthase activities remains unclear. The ε-subunit, an endogenous inhibitor of the ATP hydrolyase activity of F₁ and F₀F₁ (23, 24), seems to be the most likely candidate for the triggering function. It has been shown that two distinct domains of the ε-subunit can exist in different conformations interacting differently with γ-subunit (25–27). Most recently, it has been reported that the isolated ε-subunit of F₁ from thermophilic Bacillus PS3 specifically binds free ATP, thus suggesting its possible role as a sensor for the cellular ATP level (28). Several schemes describing different conformations of ATP synthase and ATP hydrolysis states of F₀F₁ complex have been published (3, 5, 27).

Tightly coupled vesicles from Paracoccus denitrificans have been shown to catalyze only very low rates of ATP hydrolysis (29, 30) while being capable of high rates of oxidative phosphorylation (31, 32). The reason(s) why ATP synthase in P. denitrificans carries out apparently unidirectional catalysis is not known. An explanation of this phenomenon seems to be of...
importance for uncovering physiologically relevant mechanisms involved in in situ operation of the energy-transducing enzymes. Clearly, this problem cannot be solved using highly purified soluble F₁ preparations, which catalyze only uncoupled ATP hydrolysis. The membrane-bound FₐF₁ in tightly coupled P. denitrificans vesicles is ideally suited for studies on the reversibility of the ΔψM-supported ATP synthesis. In some respects, FₐF₁ synthase of P. denitrificans seemed to be remarkably similar to the mitochondrial ADP(Mg²⁺)-inhibited enzyme form stabilized by azide (22) or to the thermophilic Bacillus PS3 mutant FₐF₁, which is incapable of ATP binding to the “non-catalytic” sites on α-subunits (33). This similarity is corroborated by recent reports demonstrating that the ATPase activity of coupled vesicles derived from P. denitrificans is susceptible to activation by sulfite and/or by energization (34, 35). Analogous phenomena have been reported for chloroplasts (36), photosynthetic purple bacteria (37), cyanobacterium Synechococcus 6716 (38, 39), E. coli (40), and the mitochondrial enzyme (41). In this work, we report the results of studies aimed to delineate the conditions required for activation of the latent ATP hydrolyase activity of P. denitrificans FₐF₁. We conclude that latent proton-translocating ATPase is, indeed, kinetically equivalent to the mitochondrial ADP(Mg²⁺)-axidetrapped FₐF₁.

EXPERIMENTAL PROCEDURES
Preparation of Vesicles—P. denitrificans cells (strain Pd 1222) were grown anaerobically in the presence of succinate and nitrate (42). Particles were prepared essentially as described (42) with modifications (45). The final preparation was suspended in 0.25 M sucrose, 0.1 M KCl, 2.5 mM Hepes, 1 mM ADP (potassium salts, pH 8.0), and 5.5 mM MgCl₂.

Fig. 1. Activation of the latent ATPase activity by energization of the coupling membrane. A. upper curves, inside-out plasma membrane vesicles of P. denitrificans (P, 140 μg/ml) were added to the standard reaction mixture, and the ATPase hydrolysis registered as H⁺ release was initiated by the addition of malonate (20 mM) where indicated. The lower curves show the synchronous change of the membrane potential of NADH oxidase in the presence and absence of uncoupler (gramicidin S-13) was 2 μM. B, inorganic phosphate was omitted where indicated. The rate of activated ATP hydrolysis was 0.12 μmol/min/mg of protein, and the reaction was abolished if venturicidin (1 μg/mg) was added to the assay mixture.

Membrane Energization—Membrane energization was followed as Oxonol VI (1.5 μM) response at 624 and 602 nm (45).

Oxygen Consumption—Oxygen consumption was measured amperometrically with oxygen-sensitive platinum electrode.

All fine chemicals were from Sigma. Venturicidin was a kind gift of Dr. C. Hägerhall (University of Lund, Lund, Sweden). A number of protonophoric uncouplers were tested in the preliminary experiments. Most of them inhibited the NADH oxidase activity of the membranes. S-13 (46) was used as uncoupler in all the experiments. It was a kind gift of Dr. V. P. Skulachev (A. N. Belozersky Institute of Physical-Chemical Biology, Moscow State University, Moscow, Russian Federation). Other chemicals were of the highest purity commercially available.

RESULTS
Fig. 1 depicts the synchronous recording of ATP hydrolysis (upper curves) and the transmembranous electric potential, Δψ (lower curves) in the suspension of tightly coupled P. denitrificans vesicles. A very low rate of ATP hydrolysis (0.03 μg of ion
The presence of inorganic phosphate was found to be required for the continuous steady-state ATP hydrolysis induced by malonate (after pre-energization by coupled succinate oxidation), as shown in the Fig. 1B. Only a short jump of the ATPase activity accompanied by a short transitory maintenance of the potential was seen, and both the rate of ATP hydrolysis and the membrane potential rapidly declined in the absence of phosphate.

The results shown in Fig. 1 show that latent proton-translocating ATPase can be activated by the membrane energization and complete uncoupling leads to complete inhibition of ATP hydrolysis. The latter unexpected phenomenon was further investigated in the experiments shown in Fig. 2. Gradual uncoupling was induced by increasing concentrations of S-13, and the ATPase activity and Δψ were followed as described in Fig. 1 except that malonate addition was excluded. The energization-activated ATP hydrolysis was stimulated by low concentration of S-13, which was unable to dissipate the membrane potential generated by succinate oxidation (Fig. 2, left part). Under these conditions, the ATPase activity was high enough to support energization of the membrane, as was evident from the substantial Δψ seen after the suspension became anaerobic. The addition of a high concentration of S-13 resulted in almost the same steady-state rate of ATP hydrolysis, which declined rapidly, after the suspension became anaerobic concomitantly with dissipation of the potential (Fig. 2, right part). Intermediate patterns of ATP hydrolysis and the membrane potential change were seen at intermediate concentrations of the uncoupler (Fig. 2, middle part). Qualitatively the same pattern was seen with other uncouplers, i.e., gramicidin or alamethicin (the data are not shown).

The dependence of the steady-state ΔμH⁺-activated ATPase activity on uncoupler concentration measured as described in Fig. 2 is shown in Fig. 3. The bell-shaped curve suggests that the optimal value of ΔμH⁺ that is low enough to avoid its back pressure inhibitory effect on the ATP-dependent proton flow across the membrane, and high enough to maintain the enzyme in the active state, is required to reveal full ATP hydrolytic activity. This interpretation of the bell-shaped curve was supported by the experiments in which the ΔμH⁺ generation (succinate oxidation) was partially decreased by malonate. As predicted, less uncoupler was required for the maximal ATPase rate, and a smaller fraction of the enzyme was activated (as was evident from the lower level of the ATPase activity) when ΔμH⁺ generation (succinate oxidation) was limited. It should be noted that no quantitative correlation between the degree of activation and absolute value of the steady-state Δψ can be estimated from the data shown in Figs. 2 and 3 because the observed amplitude of the Oxonol VI response is only a semiquantitative indicator of Δψ.

The ΔμH⁺-dependent activation of ATPase was shown to be reversible; when ΔμH⁺ was collapsed either by inhibition of respiration (excess of malonate, anaerobiosis) or by the addition of uncoupler before ATP, the enzyme activity rapidly declined. In the preliminary trials, we have experienced poor reproducibility of the specific (expressed as units per mg of protein) ΔμH⁺-activated ATPase activity and its deactivation time course. Eventually, it was found that both the degree of...
activation and the rate of deactivation were dependent on protein content in the assay mixture. Fig. 4 shows the time course of the deactivation process as a function of protein content. A substantially higher level of the specific $\Delta \mu_{H}^{-}$-induced activity and slower rate and lower degree of the deactivation were seen in very diluted samples, whereas smaller activation and rapid and complete deactivation occurred at high protein content. This behavior is expected if the activation phenomenon is due to the $\Delta \mu_{H}^{-}$-induced dissociation of an inhibitory ligand and the deactivation results from to the backward binding of the ligand in the second-order kinetics reaction that took place after $\Delta \mu_{H}^{-}$-collapses. ADP seemed to be the most conceivable inhibitory ligand. As shown in Fig. 5A, added ADP significantly increased the rate of deactivation. The affinity of ADP to its inhibitory binding site could not be determined because the actual content of the enzyme and enzyme-bound ADP pre-existed in the vesicles prior to $\Delta \mu_{H}^{-}$-induced activation were not known. However, very low (less than 1 $\mu$M) concentrations of added ADP were sufficient for significant acceleration of the deactivation process (Fig. 5B).

Further evidence for the crucial role of tightly bound ADP in the active/de-active ATPase transition was obtained in the experiments with the ADP-trapping system (pyruvate kinase plus phosphoenol pyruvate). The experimental setup was different from that employed in Fig. 5 because the presence of an ATP-regenerating system in the assay mixture was expected to interfere with the pH-metric measurement of ATP hydrolysis. To avoid this, the $\Delta \mu_{H}^{-}$-induced activation and subsequent deactivation were performed in the separately preincubated samples, and the final level of ATPase activity was measured after strong dilution of the suspension in the assay during the activation and deactivation significantly delayed the latter process.

FIG. 3. The effect of uncoupling on the steady-state rates of ATP hydrolysis catalyzed by the activated ATPase. The rates of ATP hydrolysis were measured as described in the legend for Fig. 2. 1 mM malonate was present in the assay mixture where indicated.

FIG. 4. Deactivation of the $\Delta \mu_{H}^{-}$-activated ATPase activity at different protein content. Vesicles were added to the standard reaction mixture (ATP was omitted), and after 2.5 min of incubation, respiration was stopped by the addition of 20 mM malonate (zero time). ATP (2 mM) was added after malonate at the time indicated on abscissa, and the initial steady-state rate of ATP hydrolysis was measured as shown in Fig. 1. In curves 1, 2, and 3, the protein content in assay mixture was 342, 114, and 38 $\mu$g/ml, respectively.

FIG. 5. Effect of ADP on deactivation of the ATPase activity. A, the deactivation process was followed at 35°C as described in the legend for Fig. 4 at the protein concentration in the assay mixture of 50 $\mu$g/ml. Malonate (20 mM) and ADP (where indicated) were added at zero time simultaneously. B, the residual rates of ATP hydrolysis (20 s after the deactivation was started as described in A) were measured as a function of ADP concentration.

to be indispensable for the $\Delta \mu_{H}^{-}$-activated steady-state ATP hydrolysis (Fig. 1B). If phosphate was absent in the assay in which the deactivation process was followed, the steady-state ATPase rate was negligible. Please note that the true initial rate could not be determined at the time resolution of the assay. On the other hand, the presence of inorganic phosphate in the assay during the activation and deactivation significantly delayed the latter process.

DISCUSSION

The soluble $F_{1}$-, and membrane-bound $F_{0}$-$F_{1}$-ATPases from all sources studied so far undergo strong, slowly reversible inhibition of their ATP hydrolytic activity when exposed to very low concentrations of ADP in the presence of Mg$^{2+}$ (see Refs. 5, 47, and 48 for reviews). There exist several ways to reactivate the ADP(Mg$^{2+}$)-inhibited ATPase. (i) The mitochondrial enzyme is slowly reactivated ($k_{obs} \sim 0.06$ min$^{-1}$) by “irreversible” removal of ADP in pyruvate kinase reaction (8). (ii) This slow activation is accelerated by a factor of about 10 in the presence of ATP (6, 7). (iii) Inorganic phosphates have been shown to reactivate the inhibited form (49) and to permit the steady-state turnover of the bacterial $F_{1}$ mutated at the nucleotide-binding site located on the $\alpha$-subunit (50, 51). (iv) Removal of Mg$^{2+}$ by prolonged incubation with EDTA results in activation of the inhibited form, and this reactivation is considerably accelerated by $P_{i}$, free ATP, and sulfite (10). (v) In tightly coupled membranous preparation in which $F_{0}$-$F_{1}$-ATPase is originally inactive, as in chloroplasts (36) and some bacteria.
incubation with phosphate, or phosphoenol pyruvate and pyruvate kinase, or EDTA (pathways of activation (iii), (i), and (iv), as depicted above). These observations might seem to rule out the ADP(Mg$^{2+}$) inhibition as a possible reason for the absence of active ATPase. However, closer inspection, as reported here, suggests that similar to the chloroplast enzyme (36), the latent ATPase in \textit{P. denitrificans} is present as the ADP(Mg$^{2+}$)-inhibited form. The data shown in Fig. 3 suggest that \(\Delta\mu\text{H}^+\)-induced activation results from dissociation of an inhibitory ligand, and the data depicted in Figs. 4–6 show that this ligand is ADP. The failure of pyruvate kinase, \(P_i\), and EDTA to activate ATPase can be explained by much higher affinity of ADP to its inhibitory site as compared with that for the mitochondrial enzyme. This affinity is likely to be as high as is seen in the azide-trapped ADP(Mg$^{2+}$)-inhibited mitochondrial \(F_0F_1\) (9). This explanation is in accord with the previously reported data of Harris et al. (30), who have shown that \textit{P. denitrificans} coupling ATPase contains tightly bound nucleotides, which became exchangeable upon the membrane energization. Our data somehow agree with those recently reported by Pacheco-Moisés et al. (34). However, their speculative proposal on two conformationally different active states of the enzyme as induced by either succinate or sulfite can hardly be deduced from the activating effect of trypsin on the ATPase activity in such a complex system as vesicular preparation of the bacterial membranes. Moreover, as is shown in this report (Fig. 3), in coupled \textit{P. denitrificans} vesicles, great precautions should be taken if the enzyme activity is to be quantitatively characterized, and the optimal level of \(\Delta\mu\text{H}^+\) dissipation should be determined for any particular preparation under given conditions. At present, we do not know whether sulfite-protected active ATPase in \textit{P. denitrificans} vesicles is fully coupled or uncoupled, as has been demonstrated for \textit{Rhodobacter capsulatus} membranes (37). We were unable to follow \(\Delta\psi\) response in the presence of sulfite because of non-enzymatic interaction of the latter with Oxonol VI.

We were also not able to discriminate what particular component of \(\Delta\mu\text{H}^+\), \(\Delta\psi\) or \(\Delta\phi\) or both, serves as the driving force for the activation of ATPase (dissociation of ADP). Our attempts to distinguish these possibilities with standard approaches such as the use of valinomycin and nigericin to transform \(\Delta\mu\text{H}^+\) into \(\Delta\phi\) and \(\Delta\psi\), respectively, have failed, presumably because of the presence of some unidentified cation (anion) transporting system in \textit{P. denitrificans} plasma membrane.

Another feature of \textit{P. denitrificans} ADP(Mg$^{2+}$)-de-activated ATPase, in addition to higher affinity for inhibitory ADP, which differs from that of the mitochondrial enzyme, is its susceptibility to inorganic phosphate (Fig. 1B). Although \(P_i\) has been shown to decrease apparent affinity of the mitochondrial \(F_0F_1\) to ADP (49), it does not affect the bi-phase kinetics of the uncoupled ATP hydrolysis during the assay up to 10–50 mM.
concentration. The possibility that $\Delta \mu_{H^+}$--induced activation, as reported here, is due to phosphorylation of tightly bound inhibitory ADP seems to be unlikely because phosphate is not required for activation per se but only needed for the continuous steady-state turnover of the activated enzyme (Fig. 1B). Please note that the presence of tightly bound P, in the latent deactivated enzyme cannot be excluded, and its presence in bovine heart $F_0F_1$ preparations has been reported (55).

The requirement of a high level of $\Delta \mu_{H^+}$ on the coupling membrane to maintain the proton-translocating ATPase activity (Fig. 3) appears to be an important physiologically relevant regulatory feature of the bacterial $F_0F_1$ complex. The electron transfer-generated $\Delta \mu_{H^+}$ is used for a number of processes, i.e. active transport of nutrients. If the electron transfer activity becomes temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can become temporarily limited, ATP produced by anaerobic carboxydrates breaks down via the Entner-Doudorff pathway can be utilized by $F_0F_1$-ATPase to maintain $\Delta \mu_{H^+}$, thus providing a driving force for the uptake of necessary metabolites. If the ATPase-supported $\Delta \mu_{H^+}$ would drop to a certain level under such conditions, further wasteful ATP hydrolysis would be prevented via the $\Delta \mu_{H^+}$--sensitive deactivation mechanism.

A similar regulatory mechanism realized by small ATPase inhibitor peptides has been suggested for the eukariotic mitochondrial $F_0F_1$-ATPase complex (56–59). Interestingly, the ratio between the active and deactivated enzyme forms is apparently poised in equilibrium with $\Delta \mu_{H^+}$, thus suggesting that the energy provided by either the electron transfer reactions or ATP hydrolysis is permanently utilized not only as the thermodynamic driving force for ATP synthesis or ATP-supported $\Delta \mu_{H^+}$ generation but also to maintain the catalytically competent state of the enzyme.

The last point to be mentioned concerns the problem of the $F_0F_1$-ATPase (synthase) reversibility, which has been discussed elsewhere (5). The $\Delta \mu_{H^+}$--dependent ATP hydrolysis activity of $P$. denitrificans $F_0F_1$ was shown to be capable of proton pumping. Since several parameters for the ATPase activation and deactivation processes have been established, it is of obvious interest to find out whether these parameters are the same for the transformation between the active and de-active ATP synthase forms. Work in this direction is currently under way in our laboratory.

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