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Proton-Translocating ATP-Synthase of *Paracoccus denitrificans*: ATP-Hydrolytic Activity

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Abstract—Tightly coupled membranes of *Paracoccus denitrificans* catalyze oxidative phosphorylation but are incapable of ATP hydrolysis. The conditions for observation and registration of the venturicidin-sensitive ATPase activity of subbacterial particles derived from this organism are described. The ATP hydrolytic activity does not appear after prolonged incubation in the presence of pyruvate kinase and phosphoenol pyruvate (to remove ADP), EDTA (to remove Mg^{2+}) and/or inorganic phosphate, whereas the activity dramatically increases after energization of the membranes. ATP hydrolysis by $\Delta\bar{\mu}_{H^+}$-activated ATPase is coupled with electric potential formation. Inorganic phosphate prevents and azide promotes a decline of the enzyme activity during ATP hydrolysis. The addition of uncouplers results in rapid and complete inactivation of ATPase. The $\Delta\bar{\mu}_{H^+}$-dependent ATPase activity increases upon dilution of the membranes. The results are discussed as evidence for the presence of distinct ATP-synthase and ATP-hydrolase states of F_oF_1 complex in the coupling membranes (Vinogradov, A. D. (1999) *Biochemistry* (Moscow), **64**, 1219-1229). The proposal is made that part of the free energy released from oxidoreduction in the respiratory chain is used to maintain active conformation of the energy-transducing proteins.

Key words: $F_o{\cdot}F_1$-ATP-synthase, ATP hydrolysis, submitochondrial particles, subbacterial particles, *Paracoccus denitrificans*

$F_o{\cdot}F_1$-ATPases/synthases in mitochondria, chloroplasts, and bacteria catalyze the reactions:

$$ATP + H_2O \rightarrow ADP + P_i + \Delta\bar{\mu}_{H^+} \quad (1)$$

or

$$ADP + P_i + \Delta\bar{\mu}_{H^+} \rightarrow ATP. \quad (2)$$

The enzymes catalyze either reaction (1), which provides the formation of vitally important $\Delta\bar{\mu}_{H^+}$ across the coupling membranes, or reaction (2) (synthesis of ATP during oxidative or photophosphorylation) which is the major source of ATP, depending on particular physiological or artificial conditions. At present most scholars in the field agree that the enzyme is a reversible proton-pump composed of several building blocks operating as follows.[1]

[1] Space does not permit citing numerous original papers that the generally accepted model is based on. For a comprehensive account of the problem the reader is addressed to several reviews on $F_o{\cdot}F_1$- and related ATPases [1-3].

Abbreviations: SMP) submitochondrial particles; SBP) inside-out membranous vesicles derived from *P. denitrificans*; $\Delta\bar{\mu}_{H^+}$) difference between the electrochemical potential of hydrogen ion at different sides of coupling membrane.

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$F_o{\cdot}F_1$-ATPase is an intrinsic protein of coupling membranes. Its peripheral part (F_1) is composed of three pairs of α-β subunits (3α, 3β) arranged in an almost spherical globule with a diameter of about 90 Å. F_1 bears 6 nucleotide-binding sites, and three of these are catalytically competent. The active sites are located a long distance (~100 Å) from the plane of the coupling membrane and they catalyze hydrolysis or synthesis of ATP by the so-called "alternating binding change mechanism". The essential feature of this mechanism is that three α-β dimers sequentially and cooperatively change their conformation depending on what particular specie (substrate, transition state, and product) is bound to each pair. This results in sequential alteration in binding of the "head" of a single γ-subunit situated inside the trimer. A small two-domain ε-subunit (called δ for bovine heart enzyme) is permanently bound to the "tail" of the long γ and to the membrane-bound F_o component, arranged as a ring composed of 9-12 very hydrophobic *c*-subunits. The *c*-ring is in close contact with membrane-bound $a{\cdot}b_2$ component of F_o. Each *c*-subunit contains a carboxylic group, a potential proton carrier, located within the hydrophobic bilayer of the membrane. Each time when ATP is hydrolyzed at F_1 component γ-subunit turns on, thus bringing about rotation of the *c*-ring, and one or more of the *c*-subunits come into close contact with ab_2.

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A valve/channel for protons is formed at the *c*-ring–ab_2 contact site and protons move from matrix (cytoplasm) to cytosol (outside of the cell). Thus $F_o \cdot F_1$ is a chemo-mechano-electrical machine. It is generally accepted that this machine works reversibly: during oxidative or photophosphorylation backward proton current trough the valve-channel drives rotation of the *c*-ring. This rotation brings about rotation of γ-subunit within α-β trimer of F_1 and the "head" of γ-subunit pull ATP out of the catalytic sites where it is formed isoenergetically.[1]

Several important questions regarding the mechanism of ATP-synthetase (hydrolase) remain to be answered. First, the chemistry involved in hydrolysis/synthesis at catalytic sites is not known and none of a number of speculative models [4-6] provides a clear step-by-step description of the events that take place during synchronized operation of three sites. Second, the molecular basis for a mechanically driven proton-conducting valve/channel is unknown. Last, some of the experimental data on coupled submitochondrial particles, chloroplasts, and bacterial preparations are difficult, if not impossible, to explain assuming that $F_o \cdot F_1$-ATPase is a reversible operating machine [7]. The problem of reversibility seems to be of general interest for enzymology and, particularly, for understanding of possible regulatory mechanisms of ATP synthesis and hydrolysis. For example, it is expected that strong change of energy supply, such as light–dark or aerobic–anaerobic transitions must be accompanied by decay of ATP hydrolytic activity, thus preventing futile energy dissipation. A number of possible mechanisms for such regulation have been discussed for chloroplasts [8, 9], mitochondria [7], and bacteria [10].

Evidence for the existence of distinct ATP-hydrolase and ATP-synthase states of the membrane-bound bovine heart $F_o \cdot F_1$ has been presented in our previous papers (reviewed in [7, 11, 12]). The major difficulty to prove (or disprove) our hypothesis is that the ATPase activity of submitochondrial particles, even if they are tightly coupled, is considerably grater than their ATP synthase capacity. There exists a natural system, the soil denitrifying bacterium *Paracoccus denitrificans*, which is very similar to the mitochondrion in terms of its coupling membranes [13]. Simple procedures for preparation of tightly coupled subbacterial particles derived from this organism are available [14]. These particles thus prepared are mostly inside-out and their F_1 is exposed to the surrounding medium thus being directly accessible for the nucleotides, phosphate, Mg^{2+}, and other ligands. Remarkably, *P. denitrificans* vesicles show high catalytic activity in oxidative phosphorylation, whereas their ATPase activity is only about 1-2% of ATP synthase capacity [15]. Thus, $F_o \cdot F_1$-ATP-synthase of *P. denitrificans* is a natural example of "irreversibly" operating enzyme [16].

[1]This means that equilibrium constant for the reaction $F_1 \cdot ADP \cdot P_i \leftrightarrow F_1 \cdot ATP$ is close to 1.

A number of papers on *P. denitrificans* ATPase (Pd $F_o \cdot F_1$) were published in the late sixties and the seventies. At present, the vast majority of research in the field is focused on $F_o \cdot F_1$-ATPases of bovine heart, yeast, prokaryotes (*E. coli*, *B. thermophilus*), and studies on the unique Pd $F_o \cdot F_1$ has somehow declined. We believe that studies on the mechanisms that are responsible for unidirectional operation of Pd $F_o \cdot F_1$ would be helpful for answering the question: do energy-transducing enzymes work as reversible molecular machines? This was the reason why we initiated studies on ATP hydrolysis and synthesis by the vesicles derived from *P. denitrificans*.

This paper describes general characteristics of Pd $F_o \cdot F_1$ activities catalyzed by tightly coupled subbacterial particles, particularly the conditions required to reveal their ATPase capacity.

MATERIALS AND METHODS

Preparation of vesicles. *P. denitrificans* cells (strain Pd 1222) were grown anaerobically in the presence of succinate and nitrate [14]. Particles (SBP) were prepared essentially as described [14] with modifications [17]. The final preparation was suspended in 0.25 M sucrose, 10 mM Tris-acetate (pH 7.3), 1 mM Mg-acetate, and 0.1 mM malonate and stored in liquid nitrogen. Before the experiments the samples were thawed and diluted to 10 mg of protein per ml in the mixture comprising of 0.25 M sucrose, 0.1 M KCl, 2.5 mM Hepes, 0.1 mM EDTA (potassium salt, pH 8.0), and 5.5 mM $MgCl_2$. The respiratory control measured as the ratio of NADH oxidase in the presence and absence of uncoupler (gramicidin (0.15 μg/ml) and ammonium acetate (15 mM)) for different preparations varied from 3.0 to 4.4.

Bovine heart submitochondrial particles (SMP) essentially free of protein ATPase inhibitor were prepared as described [18] and stored (35 mg/ml in 0.25 M sucrose) in liquid nitrogen. Before the experiments the samples were thawed, diluted to 2 mg/ml in the mixture comprising of 0.25 M sucrose, 10 mM Hepes, 0.1 mM EDTA, and 5 mM phosphate (potassium salts, pH 8.0) and incubated for 1 h at room temperature (for full activation of ATPase [19]).

ATPase activity. The initial rates of ATP hydrolysis was determined as pH change [20] measured with Phenol Red (30 μM) [21] in the standard mixture (1 ml) comprising of 0.25 M sucrose, 0.1 M KCl, 2.5 mM Hepes, 0.1 mM EDTA, 5.5 mM $MgCl_2$, 2 mM phosphate, 2.5 mM succinate, 2 mM ATP (potassium salts, pH 8.0).

In some experiments ATPase activity was followed as NADH oxidation (250 μM, ε_{366} 3300) in the standard reaction mixture containing ATP regenerating system (1.5 mM PEP, pyruvate kinase and lactate dehydrogenase (20 units/ml each)). NADH oxidase activity of particles was inactivated by preincubation with piericidin

(1.5 nmol/mg protein). $\Delta\bar{\mu}_{H^+}$ generation was initiated by the addition of 2.5 mM succinate. When NADH was used as the substrate for $\Delta\bar{\mu}_{H^+}$ generation, preincubation with piericidin and succinate were omitted and ATP hydrolysis was measured after complete oxygen consumption (under anaerobic conditions).

Oxidative phosphorylation. The rates of ATP synthesis were measured at 25°C as H^+-consumption (30 μM Phenol Red as indicator) [21] in the standard reaction mixture (1 ml) comprised of 0.25 M sucrose, 0.1 M KCl, 2.5 mM Hepes, 0.1 mM EDTA, 5.5 mM $MgCl_2$, 5 mM phosphate, 5 mM succinate, and 1 mM ADP (potassium salts, pH 8.0).

Transmembrane potential. Membrane energization was followed as Oxonol VI (1.5 μM) response at 624-602 nm [22].

Protein content was determined with the biuret reagent.

All fine chemicals were from Sigma (USA). Venturicidin was a kind gift of Dr. C. Hagerhall (University of Lund, Sweden). Other chemicals were of highest purity commercially available.

RESULTS

ATP hydrolase and ATP synthase activities of the submitochondrial and subbacterial particles. Table 1 shows the relative rates of oxidative phosphorylation and ATP hydrolysis for SMP (artificially coupled by oligomycin) and coupled vesicles of *P. denitrificans* SBP as measured under identical conditions. The two preparations catalyzed oxidative phosphorylation at similar rate. However, they differ dramatically in their capacity in catalysis of the reverse reaction. The hydrolytic activity of SMP was much higher than that of oxidative phosphorylation, whereas the rate of ATP hydrolysis by the preparation of SBP was about only 5% of their ATP synthase activity.

It is well known that the mitochondrial ATPase looses its activity in an ADP(Mg^{2+})-dependent way and the activity is slowly recovered by the removal of either Mg^{2+} (incubation in the presence of EDTA) [19] or ADP (incubation in the presence of phosphoenol pyruvate plus pyruvate kinase) or in the presence of inorganic phosphate [23, 24]. The complete ADP(Mg^{2+})-dependent deactivation occurs either as the result of preincubation with very low (almost stoichiometric) concentrations of ADP [25] or during ATP hydrolysis even in the presence of an ATP-regenerating system [26]. According to the protocol, the preparations of SBP are incubated with ATP and Mg^{2+} during the isolation procedure. Thus, it seemed conceivable that the reason for low hydrolytic activity of SBP (Table 1) was the deactivation of their ATPase during the preparation. To check this possibility we attempted to restore the latent ATPase of SBP by their preincubation with inorganic phosphate and EDTA or with phosphoenol pyruvate as it takes place for the mitochondrial enzyme. As depicted in Table 2, our attempts failed: neither treatment affected the ATPase activity of

Table 1. Comparison of ATPase/synthase activities in submitochondrial particles (SMP) and vesicles derived from *P. denitrificans* (SBP)

Parameter[a] (pH 8.0, 25°C)	SMP[b]	SBP
ATP synthesis[c], μg-ion H^+/min per mg protein	0.2-0.4	0.40-0.60
ATP hydrolysis[d], μg-ion H^+/min per mg protein	1.4-2.0	0.02-0.03
Respiratory control ratio[e]	4.0-6.0	3.0-4.5

[a] Average figures variable as indicated obtained for different preparations in many experiments are shown.
[b] SMP artificially coupled by preincubation with oligomycin (0.18 nmol/mg protein).
[c] 5 mM succinate as the substrate for respiration; 5 mM phosphate and 1 mM ADP.
[d] 2 mM ATP in the presence of 5 μM FCCP. No uncoupler was added for SBP preparations (see section "The effect of energization on ATPase of SBP" in "Results").
[e] The ratio between NADH (100 μM) oxidase activity determined after and before the addition of uncoupler (gramicidin, 0.15 μg/ml or gramicidin, 0.15 μg/ml, and 15 mM ammonium acetate for SMP and SBP, respectively).

Table 2. ATPase activity of SBP and ADP(Mg^{2+})-deactivated SMP

	SMP[a]	SBP[b]
	activity, μg-ion H^+/min per mg protein	
Control	0.10	0.03
P_i + EDTA[c]	5.10	0.03
PK + PEP[c]	5.30	0.04

[a] SMP (2 mg/ml) were preincubated for 30 min at 20°C in 0.25 M sucrose, 0.1 M KCl, 2.5 mM Hepes/KOH (pH 8.0), 2 μM ADP, and 0.5 mM $MgCl_2$.
[b] SBP (10 mg/ml) were preincubated as described for SMP except that ADP and $MgCl_2$ were omitted.
[c] 5 mM potassium phosphate and 5 mM EDTA or 2 mM phosphoenol pyruvate (PEP) and pyruvate kinase (PK, 40 units/ml) were added where indicated and the suspension was incubated for 1 h.

SBP, whereas either treatment dramatically increased the deactivated ATPase of SMP.

The effect of energization on ATPase of SBP. It has been established that azide stabilizes the ADP(Mg^{2+})-deactivated mitochondrial ATPase and no activation by ADP removal occurs in the presence of azide [27]. However, significant activation of the ADP-inhibited azide-stabilized ATPase was seen after energization of the membrane by succinate oxidation [28]. In other words, energization of coupling membrane is an important factor affecting either the enzyme affinity to ADP or the dissociation rate of the inhibiting nucleotide and/or Mg^{2+}.

It seemed of interest to find out whether the ATPase activity of SBP could be affected by energization. Figure 1 shows the simultaneous registration of ATP hydrolysis as measured by Phenol Red response (Fig. 1b, on the left) and the membrane potential change as followed by Oxonol VI response (Fig. 1a, on the left). The presence of succinate resulted in generation of the potential, which dissipated after the suspension became anaerobic (Fig. 1a, on the left, curve *3*). When particles are added to the reaction mixture containing ATP in the absence of succinate only very slow hydrolysis of ATP was observed (25 μg-ion H^+/min per mg protein, Fig. 1b, on the left, curve *1*). The ATP hydrolysis was accompanied by small transitory $\Delta\Psi$ generation (Fig. 1a, on the left, curve *1*). If particles were added to the mixture containing succinate and ATP, rapid ATP hydrolysis proceeded after the suspension became anaerobic (120 ng-ion H^+/min per mg protein, Fig. 1b, on the left, curve *2*). Small alkalinization seen after the addition of SBP was evidently due to phosphorylation of contaminating ADP. Identical patterns of ATPase activity were observed when two independent registration methods were used to follow ATP hydrolysis: H^+-release and NADH consumption in the presence of the ATP-regenerating system. In either assay system ATP hydrolytic activity was low in de-energized vesicles and the succinate-induced energization resulted in dramatic increase in their ATPase activity.

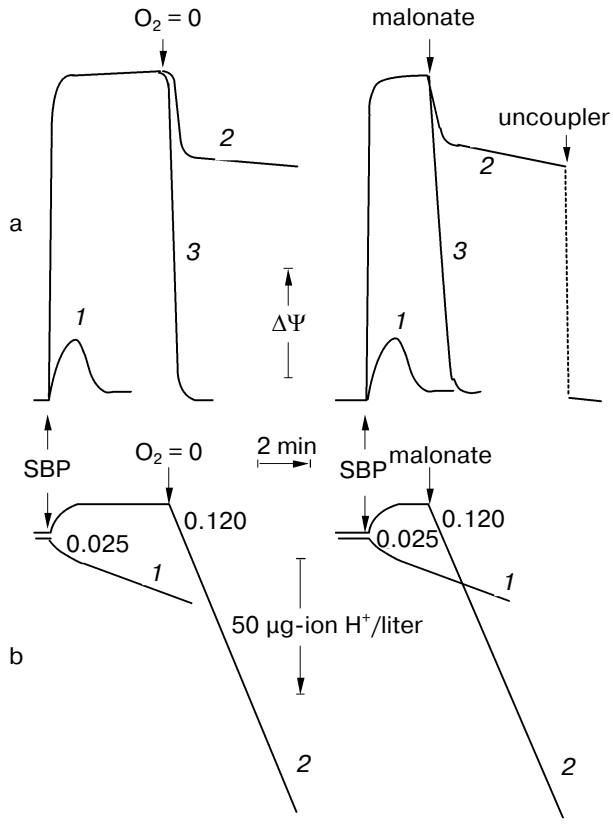

Fig. 1. Effect of energization on ATP-dependent generation of $\Delta\Psi$ (a) and ATP hydrolysis (b). SBP (0.14 mg/ml) were added to the standard reaction mixture containing: *1*) 2 mM ATP and no succinate; *2*) 2 mM ATP and 2.5 mM succinate; *3*) no ATP and 2.5 mM succinate. Further additions are indicated by the arrows (malonate, 20 mM; gramicidin, 0.15 μg/ml plus 15 mM ammonium acetate). Figures on the curves in part (b) are the rates of ATP hydrolysis in μg-ion H^+/min per mg protein.

It is thus evident that ATPase of SBP is strongly activated by succinate oxidation. After the suspension became anaerobic the energy-dependent ATPase activity maintained significant $\Delta\bar{\mu}_{H^+}$ (Fig. 1a, on the left, curve *2*) indicating that the reaction is electrochemically coupled. The energy-dependent activation of ATPase was relatively slow. Complete activation was reached after 2-3 min incubation in the presence of succinate.

Malonate, the inhibitor of succinate oxidation, affected the ATPase activity exactly as anaerobiosis did (Fig. 1, on the right). ATP was not required for the activation. If SBP were preincubated with succinate and the reaction was than started by the addition of ATP and malonate, the rate of ATP hydrolysis was the same as seen after preincubation with succinate and ATP (the results are not shown). $\Delta\bar{\mu}_{H^+}$-activated hydrolysis was 90%-sensitive to venturicidin, a specific inhibitor of the bacterial ATPases [29]. Thus, we conclude that the proton-translocating venturicidin-sensitive ATPase activity of *P. denitrificans* is energy-dependent.

In contrast to the inhibitors of respiration, uncouplers prevented the $\Delta\bar{\mu}_{H^+}$-activated ATP hydrolysis (Fig. 2). The addition of an uncoupler to SBP pre-energized by succinate does not induce ATP hydrolysis (curve *3*). Moreover, the addition of an uncoupler to the $\Delta\bar{\mu}_{H^+}$-activated samples (after malonate) resulted in instant and complete inhibition of ATP hydrolysis (curve *2*). The inhibitory effect of the uncouplers can not be due to their direct interaction with the enzyme: the inhibition was equally efficient when uncoupling was induced by gramicidin in the presence of ammonium acetate, or by S-13, and/or by alamethicin. This suggests that $\Delta\bar{\mu}_{H^+}$ is required not only for activation of ATPase but also for maintaining of the enzyme in the catalytically active state.

The effect of temperature, pH, and inorganic phosphate on $\Delta\bar{\mu}_{H^+}$-activated ATPase. $\Delta\bar{\mu}_{H^+}$-activated ATPase

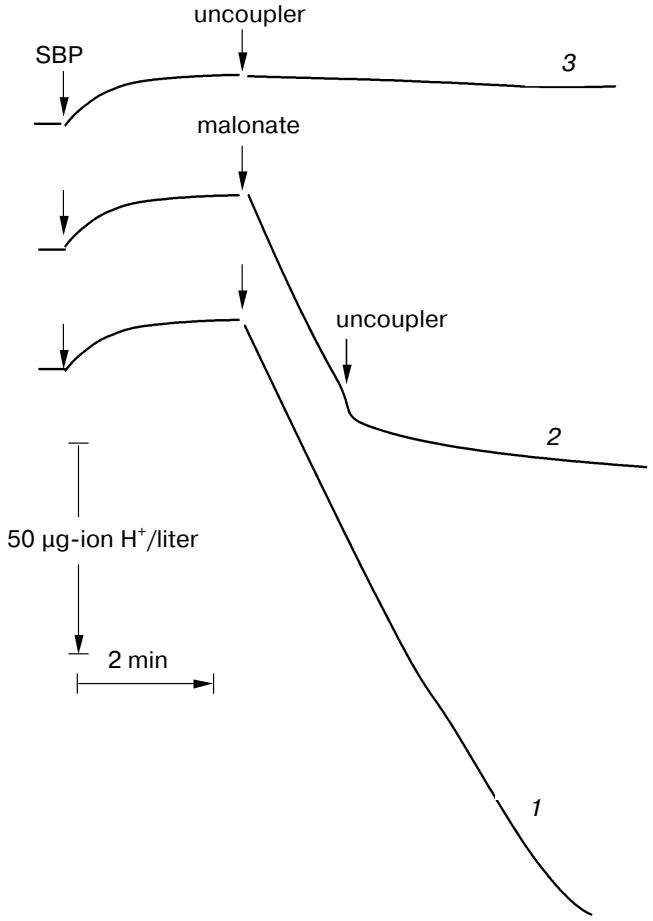

Fig. 2. Effect of uncoupling on $\Delta\bar{\mu}_{H^+}$-activated ATP hydrolysis. SBP (0.14 mg/ml) were added to the standard reaction mixture containing ATP and succinate. The ATP hydrolysis was initiated by the addition of 20 mM malonate (indicated by the arrows on curves *1* and *2*) or uncoupler (*3*). Uncoupling was induced by the addition of either gramicidin (0.15 μg/ml) plus 15 mM ammonium acetate, or by S-13 (2 μM), or by alamethicin (40 μg/ml), where indicated.

activity was optimal at pH 8.0 and it was strongly temperature-dependent. The specific activity at 20 and 40°C was 65 and 240 nmol of ATP hydrolyzed per 1 min per 1 mg protein, respectively, at pH 8.0.

Inorganic phosphate failed to activate ATPase activity of de-energized SBP (Table 1). Phosphate only slightly affected the initial rate of ATP hydrolysis by $\Delta\bar{\mu}_{H^+}$-energized particles: at pH 7.4 a slight decrease (~20%) and at pH 8.0 a slight increase (~30%) was observed in the presence of 2-5 mM phosphate. On the other hand, the presence of phosphate prevents the time-dependent decrease in the hydrolytic activity accompanied by $\Delta\bar{\mu}_{H^+}$ generation (Fig. 3).

Effect of azide on ATP hydrolysis. The rate of ATP hydrolysis by SMP decays slowly (Fig. 4a). Deactivation of ATPase during the reaction was previously shown to be due to accumulation of the inactive ADP-Mg^{2+}–enzyme complex [27]. In the presence of azide the apparent rate constant for the deactivation was increased (Fig. 4a). Qualitatively the same kinetic pattern was seen for the effect of azide on $\Delta\bar{\mu}_{H^+}$-activated ATP hydrolysis by SBP (Fig. 4b).

The deactivation rate of ATP hydrolysis was decreased in the presence of phosphate and increased in the presence of azide. These findings suggest that the decay of the ATPase activity during the reaction catalyzed by SBP is, at least partially, due to the formation of ADP-Mg^{2+}–enzyme complex as it has been documented for SMP [27].

Protein content and activation/deactivation of ATPase. The results described above show that ATP hydrolase activity of *P. denitrificans* of $F_o{\cdot}F_1$-ATPase is

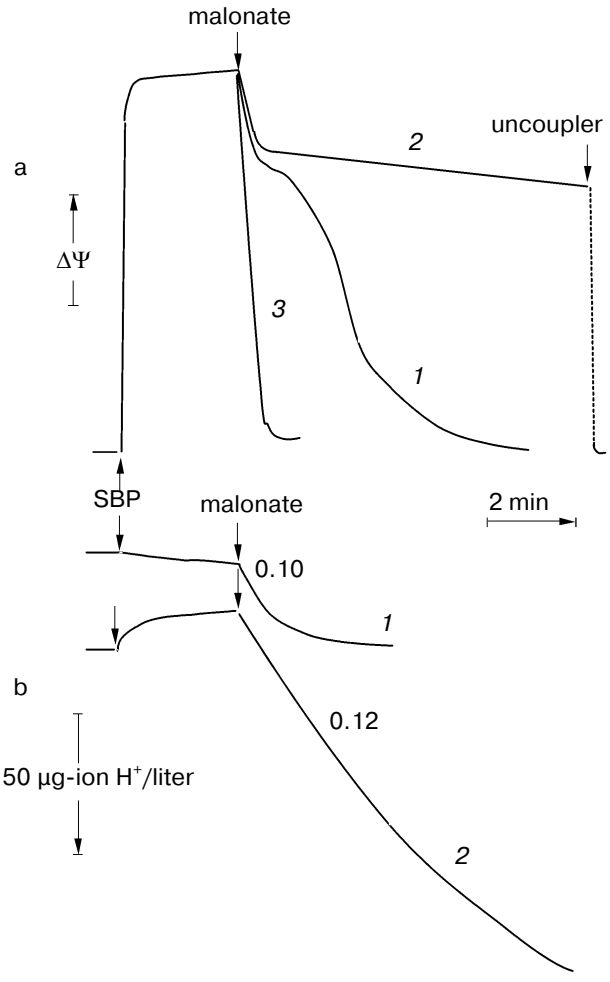

Fig. 3. Effect of inorganic phosphate on ATP-dependent generation of $\Delta\bar{\mu}_{H^+}$ (a) and ATP hydrolysis (b). SBP (0.14 mg/ml) were incubated for 2.5 min in the standard reaction mixture containing 2.5 mM succinate and 2 mM ATP (*1*, *2*); *3*) no ATP was present; *1*, *2*) 2 mM potassium phosphate was absent or present, respectively.

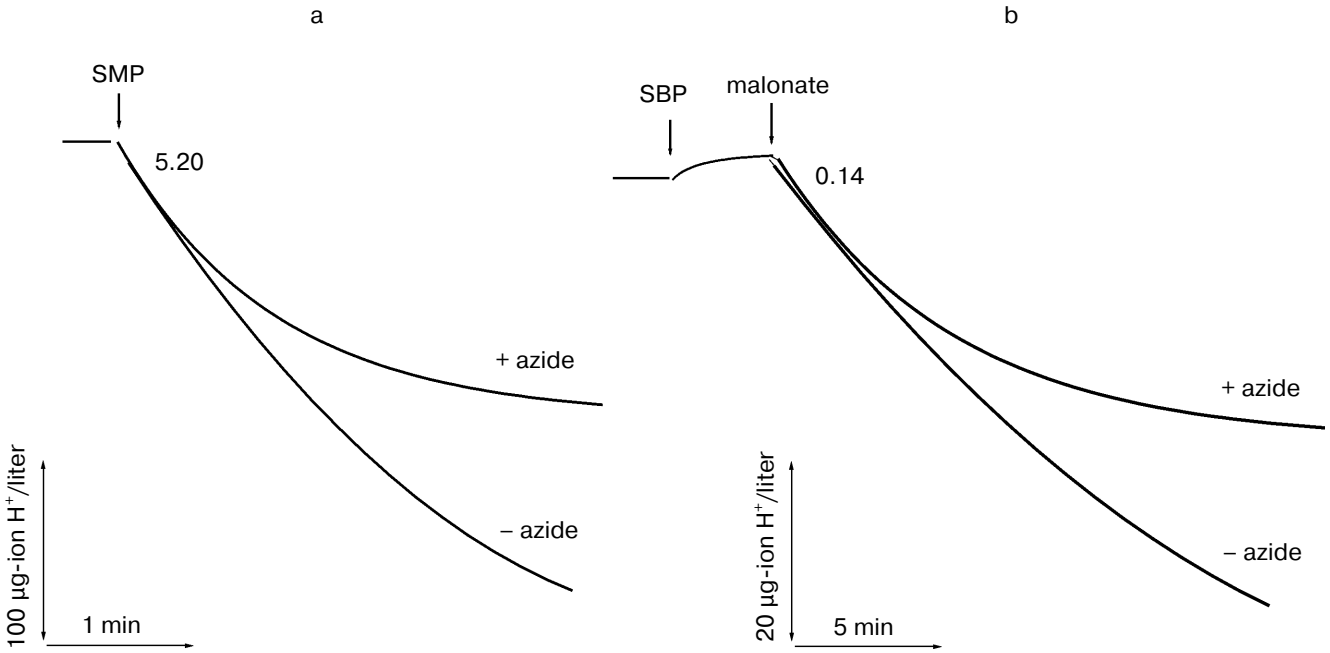

Fig. 4. Effect of azide on ATP hydrolysis catalyzed by SMP (a) or SBP (b). SMP (82 μg/ml) or SBP (160 μg/ml) were added to the reaction mixture containing 2 mM ATP (no phosphate was present) (a) and 2 mM ATP and 2.5 mM succinate (b). Sodium azide (0.4 mM) was added where indicated. The addition of 20 mM malonate to initiate ATP hydrolysis by SBP is indicated by the arrow.

strongly $\Delta\bar{\mu}_{H^+}$-dependent. At least two mechanistic possibilities for this phenomenon are worth considering. The energization may result either in intramolecular rearrangement of the enzyme or in dissociation (association) of tightly-bound inhibitor (activator). The latter is expected to be ADP, if all data on the inhibitory effect of ADP on the mitochondrial ATPase are taken into account. Other components such as one of the multiple enzyme subunits may also be involved in the activation/deactivation transition. If intramolecular rearrangements are involved the observed phenomena would not be dependent on dilution of the enzyme preparations. If the alternative mechanism (dissociation of an inhibitor) operates, the activation/deactivation process is expected to be protein content-dependent. To distinguish these alternatives the dependence of the activation/deactivation on SBP content was studied.

Figure 5 shows that the specific ATPase activity of SBP increased in parallel with dilution of preparation in the assay mixture. The $\Delta\bar{\mu}_{H^+}$-induced ATPase activity of SBP rapidly declines after $\Delta\bar{\mu}_{H^+}$ dissipation (Fig. 6). In these experiments SBP were preactivated by succinate oxidation, malonate was then added and the ATP hydrolysis was initiated by the addition of ATP. Almost complete deactivation was seen 2.5 min after generation of $\Delta\bar{\mu}_{H^+}$ was prevented by malonate (Fig. 6, curve *1*). Both the rate of the activation and its final level were decreased upon dilution (Fig. 6, curve *2*). The data depicted in Fig. 3b (curve *2*) show that ATPase activity was the same if ATP was added before or simultaneously with malonate. The comparison of the results shown in Figs. 3 and 6 suggests that the presence of ATP protects ATPase from the deactivation (Fig. 6, curve *3*).

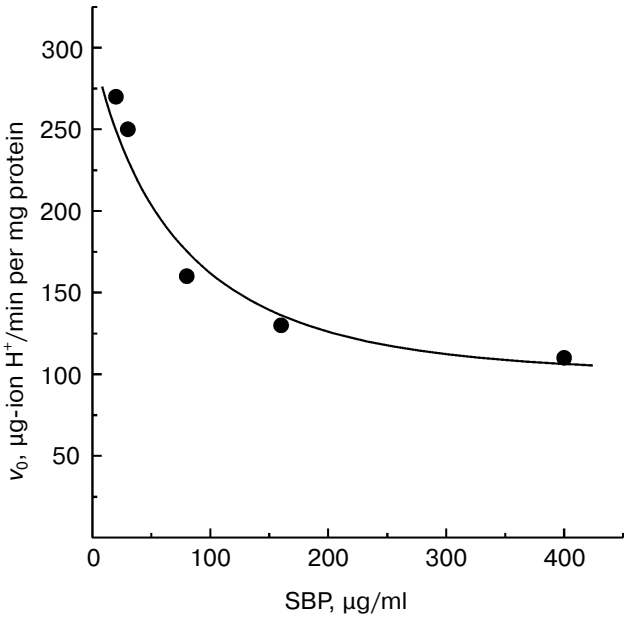

Fig. 5. Dependence of the specific ATPase activity of SBP on protein content in the assay mixture. SBP (protein content is indicated on abscissa) were added to the standard reaction mixture, and their ATPase activity measured at 35°C was initiated by the addition of 20 mM malonate.

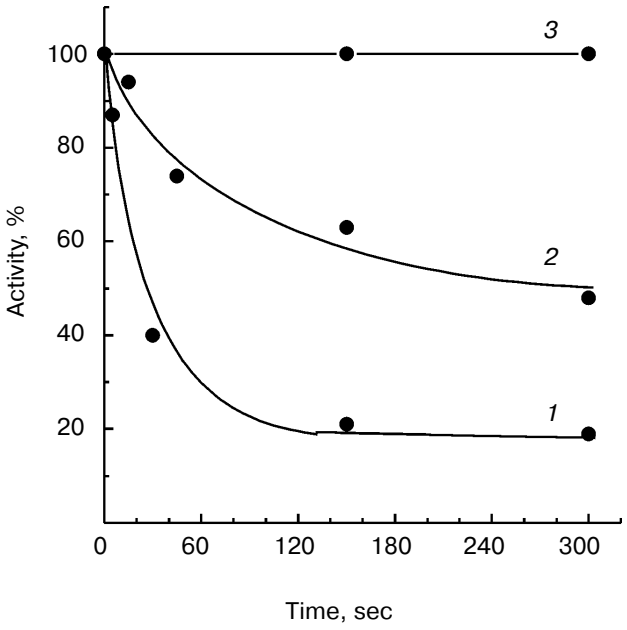

Fig. 6. De-energization-induced decay of ATPase activity. SBP (160 μg/ml) were added to the standard reaction mixture containing 2.5 mM succinate and incubated for 2.5 min. At zero time 20 mM malonate was added and 2 mM ATP was than added at the time intervals indicated on the abscissa (curve *1*). Curves: *3*) malonate and ATP were added simultaneously; *2*) the same as curves *1* and *3*, except for the protein content was 80 μg/ml. 100% activity correspond to 120 (curves *1* and *3*) and 160 (curve *2*) ng-ion H^+/min per mg protein, respectively.

DISCUSSION

The major purpose of this study was to find the experimental conditions for registration of the ATP hydrolase activity of $F_o{\cdot}F_1$ ATP synthase in tightly coupled inside-out vesicles derived from *P. denitrificans* and to describe the general properties of the reaction. The results presented in Table 1 show the advantage of using SBP over SMP in studies of $F_o{\cdot}F_1$ ATP synthase complex. SBP have high respiratory control ratio and they do not require artificial coupling by oligomycin for active oxidative phosphorylation. The artificial coupling makes straight forward interpretation of the results difficult because the mechanism of coupling effect of oligomycin is not understood. On the other hand, it seems very likely that the structural characteristics and mechanism of the reactions catalyzed by the mitochondrial and prokaryotic proton-translocating $F_o{\cdot}F_1$ complexes are very similar.

Our results agree with the data from other laboratories [15, 16, 30] on the absence of ATP hydrolase activity in phosphorylating preparations of SBP. They also show that ATP hydrolase state of the enzyme is induced and maintained by $\Delta\bar{\mu}_{H^+}$.

The activation of ATPase activity by membrane energization is a well known phenomenon demonstrated for chloroplasts [8, 9], cyanobacteria [31], *E. coli* [10], *Rhodobacter capsulatus* chromatophores [32], and SMP [28]. Recently data on activation of *P. denitrificans* ATPase by succinate oxidation and by sulfite has been published [30]. The authors have confirmed the latency of ATPase and they concluded that *different* states of $F_o{\cdot}F_1$ complex are induced by those effectors. Detailed discussion of all available data on $\Delta\bar{\mu}_{H^+}$-dependent activation of ATPase is space-limited and only some general points concerning apparent reversibility of the proton-translocating ATPase will be briefly discussed.

Many years ago we proposed that two kinetically distinct forms of $F_o{\cdot}F_1$—ATP-hydrolase and ATP-synthase—exist in mitochondria [33, 34] and the microreversibility principle does not hold for the reactions catalyzed by the enzyme. In other words, the system does not obey the Haldane relationships [35]. The experiments aimed to verify this hypothesis have led to a model describing *three* states of the complex and "hydrolase" (H-state) and "synthase" (S-state) arise as the result of $\Delta\bar{\mu}_{H^+}$- and nucleotide-dependent rearrangements of the "original" (N-state) form, which is inactive in either hydrolytic or synthetic reactions [7, 12]. It should be emphasized that according to our hypothesis $\Delta\bar{\mu}_{H^+}$ serves not only as driving force for ATP synthesis but it is also used to maintain catalytically competent strain conformation of the enzyme, thus it kinetically contributes to the overall forward or reverse reactions. Therefore, energy-required support of catalytically competent $F_o{\cdot}F_1$ conformation should be added to the list of several other energy-linked reactions in coupling membranes such as ATP-synthesis, ion-translocation, ATP/ADP exchange, pyridine nucleotide transhydrogenase, and reverse electron transfer.

The results presented in Fig. 1 show that both $\Delta\bar{\mu}_{H^+}$-activation and deactivation of $F_o{\cdot}F_1$ ATPase are *slow* compared with the enzyme turnover and in the absence of $\Delta\bar{\mu}_{H^+}$ equilibrium between H- and N-form is strongly shifted to the deactivated ATPase.

Phenomenologically $F_o{\cdot}F_1$ complex of SBP corresponds to the S-state of the mitochondrial enzyme "frozen" by azide. The latter is not capable of activation by pyruvate kinase (removal of ADP) or by removal of phosphate and Mg^{2+} (Table 2), but it is activated by $\Delta\bar{\mu}_{H^+}$ [28] and fully capable in the ATP synthesis reaction [34]. For the mitochondrial enzyme the conditions and factors involved in stabilization of S-state are well known; that is binding of stoichiometric ADP in the absence of phosphate and in the presence of Mg^{2+} [19]. The structural characteristics of this state remain to be established.

Recently the hypothesis on *two* different states of $F_o{\cdot}F_1$ complex has been reinforced by structural studies [36]. It has been shown that one of the enzyme subunits (ε in the nomenclature accepted for *E. coli*, which is

equivalent to δ in the nomenclature accepted for the mitochondrial F_1), which is part of the stalk region, exists in two different conformations [37]. It has been shown that ε-subunit inhibits the ATP hydrolysis catalyzed by F_1 [38] and by $F_o{\cdot}F_1$ complex [39]. The contact between ε- and other subunits (γ- and β-) as evident from sensitivity of ε to proteolytic digestion depends on the nucleotides binding [40]. It can be speculated that binding of "inhibitory" ADP (and Mg^{2+}) stabilizes the conformation of ε-subunit (δ- in mammals), which inhibits the ATP hydrolytic reaction, and this subunit serves as the $\Delta\bar{\mu}_{H^+}$-dependent switch (see Scheme 3 in the review [12]).

The results shown in Figs. 3, 5, and 6 suggest that, at least partially, energization results in dissociation of an unidentified ligand, most likely ADP. Further work is needed to prove or disprove this proposal.

Another important question remaining to be answered is whether $\Delta\bar{\mu}_{H^+}$-induced ATPase activity is a simple reversal of the ATP synthase reaction. To answers this question further studies of the reactions coupled with ATP hydrolysis, e.g., ATP-dependent succinate-supported NAD^+ reduction, and direct comparison of their kinetics with the initial rates of oxidative phosphorylation are needed.

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REFERENCES

1. Walker, J. (ed.) (2000) *Biochim. Biophys. Acta*, **1458**, No. 2-3.
2. Pedersen, P. L. (ed.) (2000) *J. Bioenerg. Biomembr.*, **32**, No. 4-5.
3. Harvey, W. R., Boutilier, R. G., and Nelson, N. (eds.) (2000) *J. Exp. Biol.*, **203**, No. 1.
4. Ko, Y.-H., Bianchet, M., Amzel, L. M., and Pedersen, P. L. (1997) *J. Biol. Chem.*, **272**, 18875-18881.
5. Senior, A. E., Nadanaciva, S., and Weber, J. (2000) *J. Exp. Biol.*, **203**, 35-40.
6. Ren, H., and Allison, S. (2000) *Biochim. Biophys. Acta*, **1458**, 221-233.
7. Vinogradov, A. D. (2000) *J. Exp. Biol.*, **203**, 41-49.
8. Junge, W. (1970) *Eur. J. Biochem.*, **14**, 582-592.
9. Graber, P. (1994) *Biochim. Biophys. Acta*, **1187**, 171-176.
10. Fisher, S., Graber, P., and Turina, P. (2000) *J. Biol. Chem.*, **275**, 30157-30162.
11. Vinogradov, A. D. (1984) *Biokhimiya*, **49**, 1220-1238.
12. Vinogradov, A. D. (1999) *Biochemistry (Moscow)*, **64**, 1219-1229.
13. John, P., and Whatley, F. R. (1975) *Nature*, **254**, 495-498.
14. John, P., and Whatley, F. R. (1970) *Biochim. Biophys. Acta*, **216**, 342-352.
15. Perez, J. A., and Ferguson, S. J. (1990) *Biochemistry*, **29**, 10503-10518.
16. Ferguson, S. J., John, P., Lloyd, W. J., Radda, G. K., and Whatley, F. R. (1976) *FEBS Lett.*, **62**, 272-275.
17. John, P., and Hamilton, W. A. (1970) *FEBS Lett.*, **10**, 246-248.
18. Kotlyar, A. B., and Vinogradov, A. D. (1990) *Biochim. Biophys. Acta*, **1019**, 151-158.
19. Bulygin, V. V., and Vinogradov, A. D. (1991) *Biochem. J.*, **276**, 149-156.
20. Chance, B., and Nishimura, M. (1967) *Meth. Enzymol.*, **10**, 641-650.
21. Galkin, A. S., Grivennikova, V. G., and Vinogradov, A. D. (2001) *Biochemistry (Moscow)*, **66**, 435-443.
22. Waggoner, A. S. (1979) *Meth. Enzymol.*, **55**, 689-695.
23. Vasilyeva, E. A., Fitin, A. F., Minkov, I. B., and Vinogradov, A. D. (1980) *Biochem. J.*, **188**, 807-815.
24. Yalamova, M. V., Vasilyeva, E. A., and Vinogradov, A. D. (1982) *Biochem. Int.*, **4**, 337-344.
25. Fitin, A. F., Vasilyeva, E. A., and Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun.*, **86**, 434-439.
26. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 9-14.
27. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 15-23.
28. Galkin, M. A., and Vinogradov, A. D. (1999) *FEBS Lett.*, **448**, 123-126.
29. Ferguson, S. J., and John, P. (1977) *Biochem. Soc. Trans.*, **5**, 1525-1527.
30. Pacheco-Moises, F., Garcia, J. J., Rodriquez-Zavala, J. S., and Moreno-Sanchez, R. (2000) *Eur. J. Biochem.*, **267**, 993-1000.
31. Bakels, R. H. A., van Walraxen, H. S., Scholts, M. J. C., Krab, K., and Kraayenhof, R. (1991) *Biochim. Biophys. Acta*, **1058**, 225-234.
32. Cappellini, P., Turina, P., Fregni, V., and Melandri, A. B. (1997) *Eur. J. Biochem.*, **248**, 496-506.
33. Minkov, I. B., Vasilyeva, E. A., Fitin, A. F., and Vinogradov, A. D. (1980) *Biochem. Int.*, **1**, 478-485.
34. Syroeshkin, A. V., Vasilyeva, E. A., and Vinogradov, A. D. (1995) *FEBS Lett.*, **366**, 29-32.
35. Haldane, J. B. S. (1930) *Enzymes*, Longmans, Green and Co., London-N. Y.-Toronto, pp. 80-83.
36. Bald, D., Amano, T., Muneyuki, E., Pitard, B., Rigaud, J.-L., Kruip, J., Hisabori, T., Yoshida, M., and Shibata, M. (1998) *J. Biol. Chem.*, **273**, 865-870.
37. Tsunoda, S. P., Rodgers, A. J. W., Aggeler, R., Wilce, M. C. J., Yoshida, M., and Capaldi, R. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 6560-6564.
38. Kato, Y., Matsui, T., Tanaka, N., Muneyuki, E., Hisabori, T., and Yoshida, M. (1997) *J. Biol. Chem.*, **272**, 24906-24912.
39. Schulenberg, B., and Capaldi, R. (1999) *J. Biol. Chem.*, **274**, 28351-28355.
40. Capaldi, R., and Schulenberg, B. (2000) *Biochim. Biophys. Acta*, **1458**, 263-269.