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Oxidative Phosphorylation and Respiratory Control Phenomenon in *Paracoccus denitrificans* Plasma Membrane

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Abstract—Changes in respiratory activity, transmembrane electric potential, and ATP synthesis as induced by additions of limited amounts of ADP and P_i to tightly coupled inverted (inside-out) *Paracoccus denitrificans* plasma membrane vesicles were traced. The pattern of the changes was qualitatively the same as those observed for coupled mitochondria during the classical State 4—State 3—State 4 transition. Bacterial vesicles devoid of energy-dependent permeability barriers for the substrates of oxidation and phosphorylation were used as a simple experimental model to investigate two possible mechanisms of respiratory control: (i) in State 4 phosphoryl transfer potential (ATP/ADP × P_i) is equilibrated with proton-motive force by reversibly operating $F_1 \cdot F_o$-ATPase (thermodynamic control); (ii) in State 4 apparent "equilibrium" is reached by unidirectional operation of proton motive force-activated $F_1 \cdot F_o$-ATP synthase. The data support the kinetic mechanism of the respiratory control phenomenon.

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Key words: F_oF_1-H^+-ATP synthase, oxidative phosphorylation, bacterial plasma membrane, *Paracoccus denitrificans*

An adult human being consumes about 10 mmol of oxygen per minute in the resting state (basal metabolic rate), and this value increases about 10-fold upon moderate physical exercise [1]. Since most of the oxygen consumption is due to the respiratory activity of mitochondria, a mechanism must exist which increases or decreases their respiration depending on the energy demand. Such a mechanism, called respiratory control, was originally observed in 1939 by Belitzer [2, 3] on minced muscle tissues. Thirteen years later, Lardy and Wellman, using Warburg's manometric technique, described 5-15-fold stimulation of rat liver mitochondrial oxygen consumption by ADP in the presence of inorganic phosphate and a hexokinase trap [4]. The concept of respiratory control was further developed qualitatively and quantitatively in classical papers by Chance and Williams, where reversible stimulation of respiration (measured by oxygen-sensitive electrode) [5] and redox-state transitions of the respiratory chain components [6] in response to the addition of limited amounts of ADP (and P_i) to mitochondria were analyzed. Since then measurement of respiratory control (ratio between the rates of respiration in the presence (State 3) and absence (State 4) of ADP) have become routine characteristics of isolated mitochondria. The addition of a limited amount of ADP (apparent "K_m" is in μmolar range) to tightly coupled mitochondria incubated aerobically in the presence of an excess of the oxidizable substrate and P_i (State 4) results in 2-20-fold (depending on particular preparation of mitochondria), transitory increase of oxygen consumption rate, which declines upon the conversion of ADP to ATP. This phenomenon provides a simple explanation for the strong dependence of physiological respiration on energy demand [1] and suggests that ADP is a key regulatory factor of oxidative metabolism.

In light of current spectacular achievements in bioenergetics [7], the respiratory control phenomenon is now conventionally described in well-defined terms. Free energy of substrate oxidation is accumulated as proton motive force ($\boldsymbol{p} = \Delta\psi - \Delta pH$) across the inner mitochondrial membrane, built up by operation of three proton-

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical gradient of protons; F_1 and F_o, hydrophilic and hydrophobic parts of ATP synthase, respectively; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\boldsymbol{p}$, proton motive force ($\boldsymbol{p} = \Delta\psi - \Delta pH$) where $\Delta\psi$ is a transmembrane electric potential and ΔpH is pH difference between inner (matrix) and outer space of mitochondria.

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pumping respiratory chain complexes: ***p*** is used by $F_1 \cdot F_o$-ATPase/synthase to accumulate free energy in the phosphoryl group transfer potential of ATP. When no ADP and P_i are available, $F_1 \cdot F_o$ does not turn over, and the oxidative capacity of the respiratory chain is limited by the leakage of protons across the coupling membrane. In the presence of ADP and P_i, the proton-translocating activity of $F_1 \cdot F_o$ results in increase in proton flow, thus allowing an increase in the proton-pumping activity of the respiratory chain.

Tightly coupled mitochondria, which show high respiratory control, provide naturally the preparation of choice for studies on regulation of respiration by ADP and/or P_i. It should be emphasized, however, that several enzymatic activities in addition to those of the respiratory chain components and $F_1 \cdot F_o$, are involved in the overall process of State 3 or State 4 respiration of intact mitochondria. These are: (i) translocation of the respiratory substrate into the mitochondrial matrix; (ii) operation of matrix-located dehydrogenases; (iii) P_i/OH^- exchange [8]; and (iv) adenine nucleotide translocation (ADP/ATP exchanger) [9]. A number of studies aimed toward defining the important parameters in the net ATP synthesis as related to the respiratory activity have been carried out, and two models have been extensively discussed. According to the first, the rate of respiration is a function of ADP availability [5, 10] or external ATP/ADP ratio [11] (kinetic control of respiration realized by the adenine nucleotide translocase). The other model postulates that extramitochondrial ATP/ADP × P_i ratio is the parameter that controls the rate of oxygen consumption ("thermodynamic" control of respiration) [12, 13] (see [14] for comprehensive discussion). Because the intramitochondrially (matrix) located $F_1 \cdot F_o$-ATPase/synthase is an immediate device directly interacting with ***p***, it seems of obvious importance to know how this extremely complex molecular machine operates at different ATP, ADP, and P_i concentrations and variable ***p*** in terms of the kinetic and thermodynamic parameters of ATP synthesis or hydrolysis.

Inside-out submitochondrial particles devoid of permeability barriers, a system where enzymes (i)-(iv) do not operate, although being capable of ATP synthesis, do not show ADP-induced reversible State 4–State 3–State 4 transition. Inverted (inside out) plasma membrane vesicles derived from *Paracoccus denitrificans* grown on succinate and nitrate are capable of ATP synthesis [15] and show the classical respiratory control phenomenon [16]. Some properties of *P. denitrificans* ATPase and ATP synthase activities have been described in previous reports from this laboratory [17-20]. In this paper we describe and analyze the phenomenon of respiratory control in this simple system where the respiratory chain and $F_1 \cdot F_o$-ATP synthase machinery are the only players in the overall oxidative phosphorylation reaction. The results show complex *kinetic* control of ATP synthesis catalyzed by $F_1 \cdot F_o$.

MATERIALS AND METHODS

Chemicals. ADP, Hepes, malonate, succinate, $MgCl_2$, EDTA, sucrose, FCCP, gramicidin, and pyruvate kinase were from Sigma-Aldrich (USA), venturicidin was from A.G. Scientific Inc. (USA), and other chemicals were of the highest purity commercially available.

Rat heart mitochondria were prepared essentially as described [21].

Preparation of bacterial vesicles. *Paracoccus denitrificans* cells (strain Pd 1222) were grown anaerobically in the presence of succinate and nitrate. Tightly coupled plasma membrane vesicles were prepared essentially as described by John and Whatley with some modifications [16]. The final preparations were suspended in 0.25 M sucrose, 10 mM Tris-acetate (pH 7.3), 1 mM $MgCl_2$, and 0.1 mM malonate (protein concentration ~20 mg/ml) and stored in liquid nitrogen. Protein content was determined by the biuret procedure. The respiratory control ratio, measured as the ratio of NADH oxidase activity in the presence and absence of uncoupler (gramicidin (0.05 μg/ml) and ammonium acetate (15 mM)) for different preparations, varied from 4.5 to 6.0. The content of inside-out vesicles in the preparations determined as the ratio of NADH oxidase activity in the presence and absence of alamethicin [22] was 80-90%.

Synthesis of ATP. ATP synthesis was measured by continuous registration of hydrogen ion concentration by a glass electrode according to the equation:

$$ADP^{3-}(Mg^{2+}) + P_i^{2-} + H^+ \leftrightarrow ATP^{4-}(Mg^{2+}) + H_2O. \quad (1)$$

When succinate is used as the respiratory substrate, its oxidation by oxygen does not result in any pH change because the pK_a values of succinate and fumarate carboxylic group are almost the same, and thus the observed pH change is due to reaction (1) only.

The stoichiometry $[H^+]/[ATP]$ at pH 8.0 in the presence of Mg^{2+} (25-fold excess over nucleotides) of 1.0 [23] was checked by measurement of ADP with phosphoenol pyruvate and pyruvate kinase. The photometric registration of pH change using Phenol Red as indicator was found unsatisfactory in the assay system employed (data will be reported elsewhere). ATP synthesis measured as H^+ consumption was completely sensitive to the uncouplers, malonate (excess), and venturicidin.

Respiratory activity. Respiratory rates were measured amperometrically with a covered platinum electrode.

Membrane potential was followed by safranine response [24] (intact mitochondria) or Oxonol VI response [25] (inside-out vesicles). The standard reaction mixture was composed of 0.25 M sucrose, 20 mM potassium chloride, 1 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5 mM succinate. Other additions are indicated in the legends to the figures and table. All experiments were performed at 30°C.

Note should be made on the statistics. The procedure employed for *P. denitrificans* coupled vesicles is well reproducible, although the absolute values for the respiratory activities and, particularly, for the respiratory control and rates of oxidative phosphorylation were slightly variable among different batches. The data shown in the figures should be considered as representative ones, and they were qualitatively reproducible when repeated using preparations derived from several culture batches. We feel that statistical treatment of the data is not required for the conclusion made.

RESULTS

Before the kinetics of oxidative phosphorylation in the system where $F_1 \cdot F_o$ is directly accessible for the substrates will be discussed, it seems worthwhile to compare actual tracing of respiration, phosphorylation, and membrane potential as they appear in the experiments carried out using coupled rat heart mitochondria and inside-out *P. denitrificans* plasma membrane vesicles. Figures 1a and 1b demonstrate synchronous recording of these parameters for mitochondria (classical well-known pattern) and for *P. denitrificans* inside-out system, respectively. Mitochondria (Fig. 1a) oxidized succinate (in the presence of rotenone) at a slow rate, which was increased 3-4-fold when 200 μM ADP was added (curve *1*). The stimulating effect of ADP was accompanied by a drop in the membrane potential (curve *3*) and immediate (within the time resolution scale) initiation of ATP synthesis, as was evident from alkalization of the medium (curve *2*). These cyclic responses to the addition of ADP could be repeated after the respiration decreased and the membrane potential returned to the original level when a limited amount of ADP was converted to ATP (State 4). The same pattern was seen for inside-out particles, although the stimulatory effect of ADP on respiration (State 4–State 3–State 4 transition) was not as prominent as in mitochondria (Fig. 1b). It was, however, adequate for analysis of the rate of ATP synthesis and, more importantly, the steady-state level of ATP/ADP during State 4 respiration.

The latter (ATP/ADP) ratio was estimated from the ratio H^+ (scalar) consumed per ADP added during the State 3–State 4 transition. The values found for the experiments shown in Fig. 1b (curve *2*) at different ADP concentrations were close to 15. Moreover, the same ratio was found for repeated cycles of the State 3–State 4 transition, i.e. under conditions where phosphorylation was initiated by limited ADP when ATP was formed by the previous cycle. The proton consumption assay was then verified by direct determination of remaining ADP in State 4 with the phosphoenolpyruvate/pyruvate kinase trap. A comparison of the data is shown in table. The complete (or almost complete) phosphorylation of added ADP argued by itself against thermodynamic equilibration between $\boldsymbol{p}$ and the external phosphoryl potential. The concentrations of ADP and P_i are equal terms in the equation describing the actual free energy accumulated in ATP ($\Delta G_p = \Delta G_p^0 + 2.3RT \cdot \log(\mathrm{ATP}/\mathrm{ADP} \times P_i)$, where ΔG_p^0 is the standard free energy of ATP hydrolysis). If $\boldsymbol{p}$ in State 4 is equal to ΔG_p, the ratio ATP/ADP is expected to vary as a function of P_i concentration. The results presented in the table show that this was not the case. Equal amounts of ATP were formed at 10-fold different P_i concentrations.

Remarkably similar patterns of ATP synthesis and change in the membrane potential were seen when phosphorylation was initiated by limited amount of either ADP or P_i if the counterpart substrate was present in excess (Fig. 2). The only difference was that when the reaction was initiated by 100 μM P_i, the phosphorylation proceeded in a close to first-order process. This is expected, because the apparent K_m for P_i is in the range of 60-120 μM [20].

It was of interest to look more closely at the kinetics of ATP synthesis and change in the membrane potential (Oxonol VI response) as induced by ADP addition. Figure 3 shows that ATP synthesis proceeds as a zero-order reaction during ADP (100 μM) phosphorylation up to about 20% of the remaining ADP. A more complex and unexpected pattern of the membrane potential was seen: the initial drop induced by the ADP addition started to be restored during phosphorylation. In other words,

Oxidative phosphorylation catalyzed by *P. denitrificans* vesicles (pH 8.0, 30°C)*

P_i, mM	ADP added, μM	H^+ consumed, μM	ADP remaining in State 4**, μM	ATP/ADP in State 4
5.0	50	47	3	15.7
	100	95	5	19
0.5	50	47	3	15.7
	100	91	7	13

* For assay mixture, see "Material and Methods" and legend to Fig. 1b.
** Determined by phosphoenolpyruvate/pyruvate kinase trap.

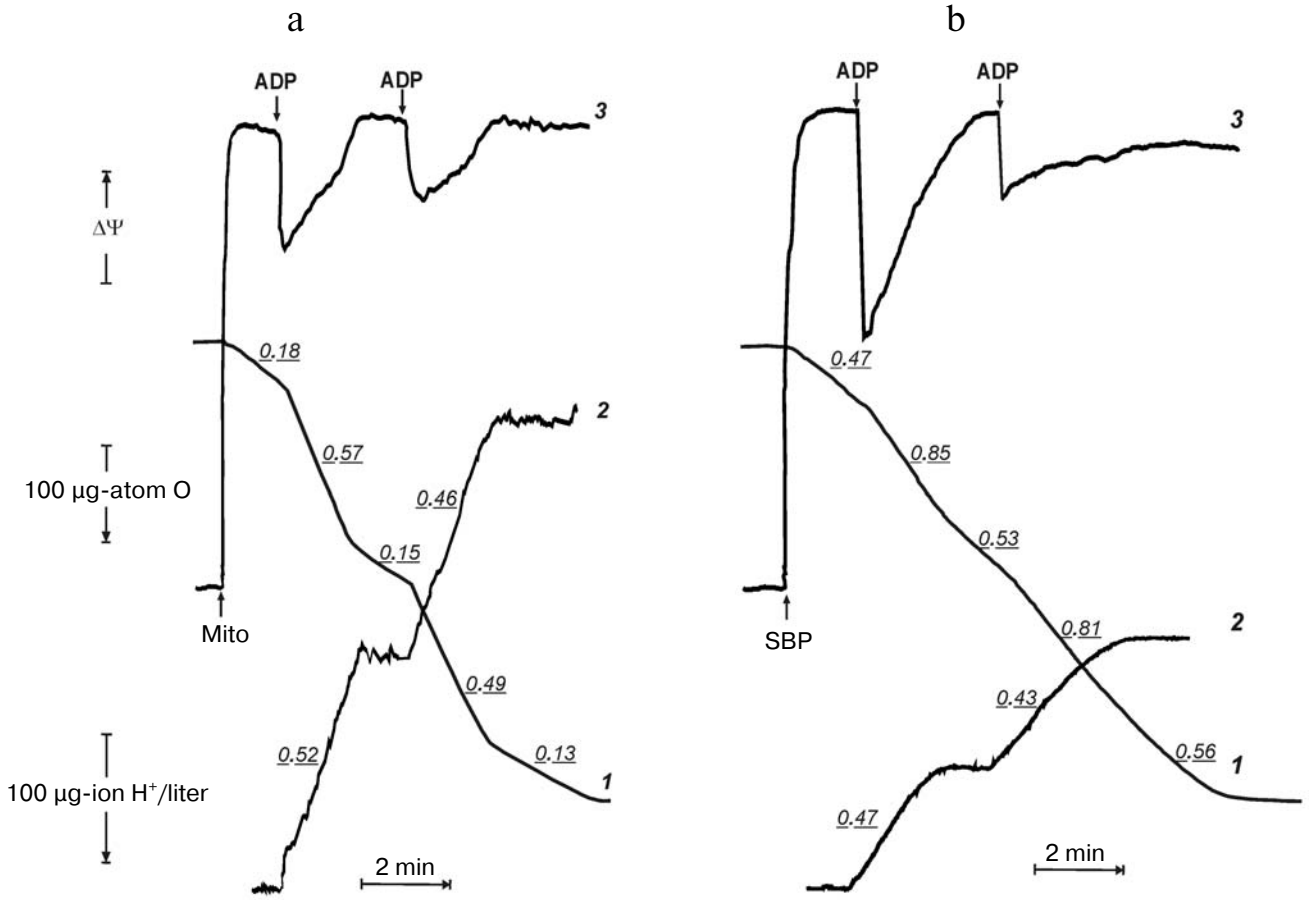

Fig. 1. Oxidative phosphorylation catalyzed by rat heart mitochondria (a) and *P. denitrificans* plasma membranes (b). a) Rat heart mitochondria (Mito, 0.2 mg/ml) were added to the reaction mixture comprised of 0.25 M sucrose, 10 mM KCl, 5 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 2.5 mM potassium succinate, 1 μM rotenone, and 10 μM safranine. ADP (200 μM) was added where indicated. Curve *1-3* show oxygen consumption, hydrogen ion consumption, and safranine response, respectively. Underlined figures on curves *1* and *2* indicate the rates of oxygen consumption and ATP synthesis, respectively (μmoles/min per mg protein). b) *P. denitrificans* plasma membrane vesicles (SBP, 250 μg/ml) were added to the reaction mixture comprised of 0.25 M sucrose, 20 mM KCl, 5 mM potassium phosphate, 1 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM potassium succinate, and 1.5 μM Oxonol VI. ADP (100 μM) was added where indicated. Curve *3* is the Oxonol VI response, other curves as in (a).

ATP synthesis proceeded at constant rate at variable membrane potential. It should be noted that only the electrical component of ***p*** ($\Delta\psi$) was traced, and we do not know what ΔpH change (if any) occurred during the transition.

The experiments shown in Figs. 1 and 2a were carried out in the presence of "kinetic" and "thermodynamic" excess of P_i. A peculiar effect of P_i on proton-translocating activity of *P. denitrificans* $F_1 \cdot F_o$ was described in our previous publications [17, 19]. It is well known that P_i does not inhibit ATPase activity catalyzed by either F_1 or $F_1 \cdot F_o$ at concentrations, which are orders of magnitude higher than its apparent K_m for oxidative phosphorylation (see [26], however, for the opposite). Moreover, the energy-dependent binding of P_i is required for the proton-translocating ATPase activity of $F_1 \cdot F_o$ [19, 27]. To gain insight into these puzzling phenomena, the effect of P_i on ATP synthesis was further investigated. The rate of ATP synthesis as expected depended on P_i concentration as shown in Fig. 4a, with apparent K_m of about 70 μM, in accord with our previously reported data [20]. More interesting, P_i was quantitatively consumed during oxidative phosphorylation: the amount of ATP formed was equal to that of P_i added if ADP was present in excess (Fig. 4b). Considered together, the data shown in the table and Fig. 4 suggest that under the conditions employed, $F_1 \cdot F_o$ catalyzes ***p***-dependent irreversible synthesis of ATP from ADP and P_i. The possibility exists that the magnitude of ***p*** under the experimental conditions employed (aerobic succinate oxidation by coupled vesicles) was always significantly higher than that required to poise a detectable equilibrium between the substrate/product of reversibly operating ATPase/synthase. This possibility was scrutinized by the experiment where the respiration-supported ***p*** was gradually decreased by malonate, a competitive inhibitor of succi-

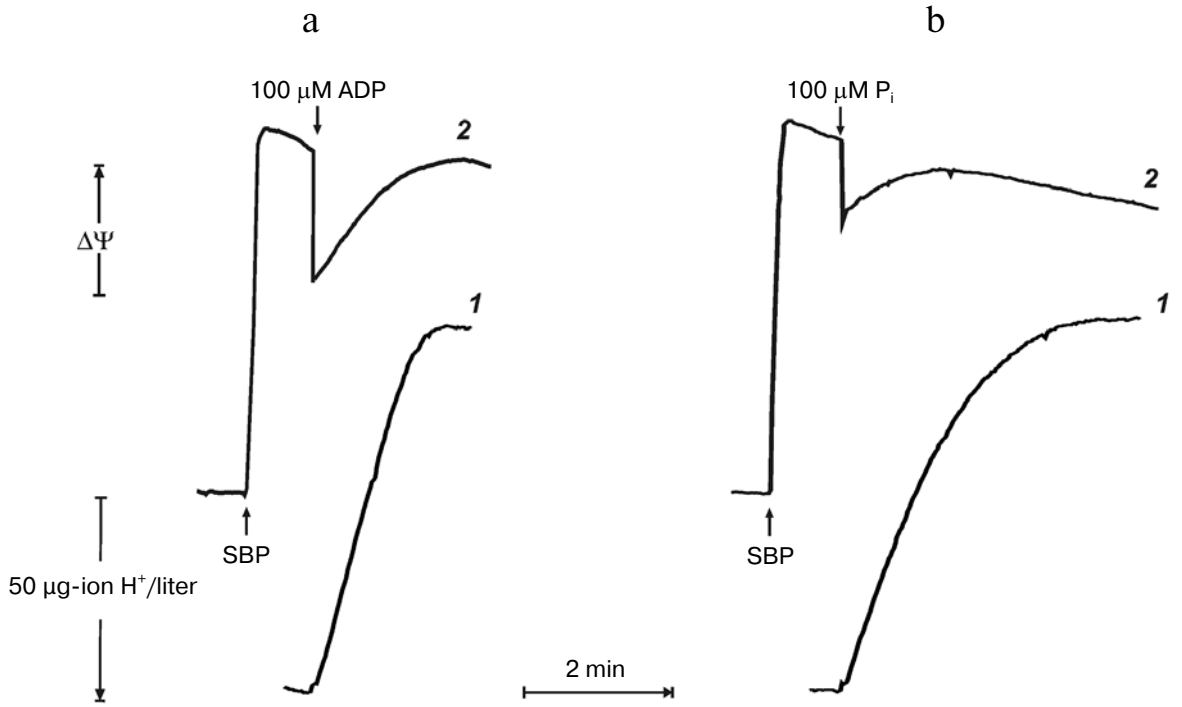

Fig. 2. ATP synthesis by *P. denitrificans* vesicles (140 μg/ml) and membrane potential change during ATP synthesis initiated by ADP (a) or inorganic phosphate (b). Potassium phosphate (0.5 mM) (a) and 0.5 mM ADP (b) were present in the reaction mixture (other components were as in Fig. 1b). Curves *1* and *2* are hydrogen ion consumption and Oxonol VI response, respectively.

nate oxidation (Fig. 5). No substantial decrease of the State 4 ATP level was seen when respiration was gradually decreased upon increase in malonate concentration up to about 1 mM (Fig. 5a), i.e. within the range where the rate of phosphorylation was significantly inhibited (Fig. 5b).

DISCUSSION

It was shown many years ago that membrane vesicles derived from *P. denitrificans* show very low ATPase activity compared with their capacity to synthesize ATP, and irreversibility of $F_1 \cdot F_o$ operation during NADH oxidation supported ATP synthesis has been suggested [28]. More recently, we have shown that active proton-translocating ATPase in coupled *P. denitrificans* vesicles can be induced by the energization of the membrane [17, 18]. ATP hydrolysis catalyzed by the energized ATPase rapidly declines if ***p*** is abolished by an uncoupler [18]. The requirement of ***p*** for ATPase activity was shown to be brought about by energy-linked P_i binding [19] that prevents formation of ADP(Mg^{2+})-inhibited enzyme, a dead-end intermediate and a central species accounting for very complex kinetics of ATP hydrolysis [29, 30]. We have emphasized that $F_1 \cdot F_o$ ATPase is both ***p***-generating

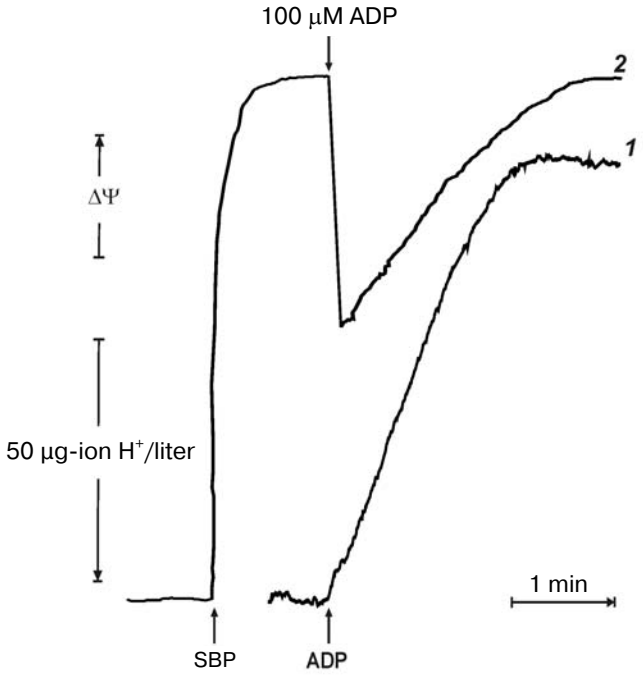

Fig. 3. Hydrogen ion consumption (curve *1*) and membrane potential change (Oxonol VI response, curve *2*) during oxidative phosphorylation catalyzed by *P. denitrificans* membranes (110 μg/ml). The reaction mixture composition was as in Fig. 1b.

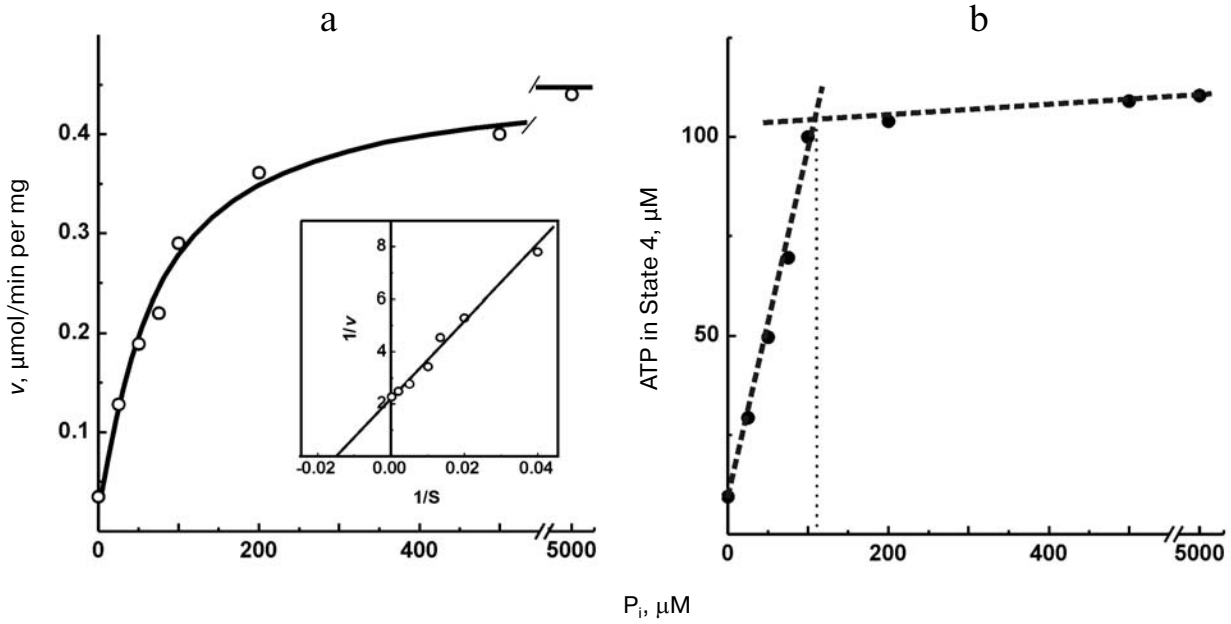

Fig. 4. Oxidative phosphorylation catalyzed by *P. denitrificans* membranes (150 μg/ml) as a function of P_i concentration. a) Initial rate of ATP synthesis (the insert is a double-reciprocal plot); b) amount of ATP formed in State 4.

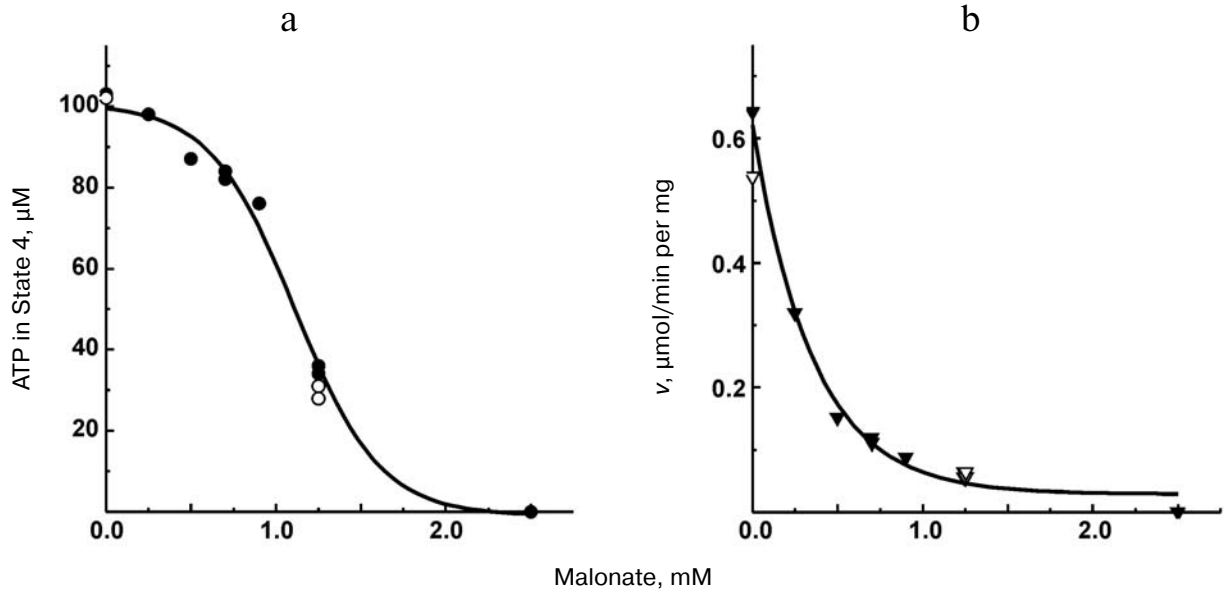

Fig. 5. State 4 ATP level (a) and rate of oxidative phosphorylation (b) during limited respiratory activity. Phosphorylation was initiated by the addition of 113 μM ADP to the reaction mixture comprised as in the Fig. 1b. Closed and open symbols indicate that 5 or 0.5 mM P_i was present, respectively.

and ***p***-consuming machinery that uses free energy of the catalyzed reaction to maintain its catalytically competent state [31]. Thus, *a priori* it seems unlikely that $F_1 \cdot F_o$ is a reversibly operating enzyme equilibrating ***p*** and phosphoryl group transfer potential of ATP as it is widely stated in numerous reviews and in some textbooks. The energy-dependent transformation of $F_1 \cdot F_o$ is a characteristic phenomenon described for chloroplast [32], bacterial [33], and mammalian [34] enzymes.

The data obtained in his study confirm and extend the original report by Ferguson et al. on ATPase as an irreversible component of electron transfer supported ATP synthesis [28]. A limited amount of ADP was almost completely (more than 90%) transformed into ATP when

P_i was present in excess (Fig. 2a) and, a limited amount of P_i was also quantitatively incorporated into ATP when ADP was in excess (Fig. 2b). Moreover, 10-fold decrease in P_i concentration did not change the amount of ATP formed from limited ADP (the table). These results do not agree with a paradigm describing State 4 as a state where ***p*** is equilibrated with ATP phosphoryl potential ($\boldsymbol{p} = \Delta G_p^0 + RT\ln(ATP/ADP \times P_i)$). In agreement with our previously reported data [20], the rate, not the amount of ATP formed, was decreased (in a limited range) when ***p***-generating succinate oxidation was gradually inhibited by malonate (Fig. 5). It worth noting that when the rate of ATP synthesis was slow, the true State 4 value for ADP consumed could not be accurately measured because the vesicles become spontaneously uncoupled if incubated for a long time under ***p***-generating conditions (unpublished observation from this laboratory).

Transitory change of $\Delta\psi$ induced by initiation of ATP synthesis is unexpected and merits brief discussion. As shown in Fig. 2, the addition of ADP causes an immediate drop in the membrane potential followed by its rapid restoration, whereas ATP synthesis started and proceeded at a constant rate up to the level of the remaining ADP close to or less than its K_m. This unexpected pattern is hard to explain in terms of a simple model where ATP-synthesizing $F_1 \cdot F_o$ operates just as a proton-conducting load for the respiration-driven ***p*** generator.

If possible artifacts in $\Delta\psi$ quantitation by Oxonol VI response are to be excluded, several explanations can be offered. A drop in $\Delta\psi$ induced by an increased load may be followed by an immediate compensation by a putative ATP-dependent (activated) K^+/H^+-exchanger, which would increase $\Delta\psi$ along with formation of ATP. Another more likely possibility is that there is a ***p***- and ATP/ADP ratio-dependent interplay between ATP synthase and proton-translocating ATPase conformations of $F_1 \cdot F_o$ during the time course of oxidative phosphorylation. Accumulating evidence suggests that two interconvertible conformational states of $F_1 \cdot F_o$, ATP synthase and ATP hydrolase exist, and that ATP synthesis is not the reversal of ATP hydrolysis [30, 35-37]. A number of effectors modulating the hydrolytic activity are known, these are: tightly bound inhibitory ADP(Mg^{2+}) [29, 30], P_i [19, 27], inhibitory ATP-binding ε-subunit [38], protein inhibitor [39-41], activating ***p***, and recently discovered in *P. denitrificans* ξ-subunit [42] (see [43] for review). Their interplay in the control of ATP synthase/ATP hydrolase activities of $F_1 \cdot F_o$ is complex and poorly understood, and further studies in this direction are needed.

Perhaps the most important conclusion from the original [28] and this study is that when ***p*** and the substrates of oxidative phosphorylation are available, *P. denitrificans* $F_1 \cdot F_o$ catalyzes synthesis of ATP irreversibly. If the binding-change rotary mechanism of $F_1 \cdot F_o$ is operative (note should be made that no evidence for rotation in the ***p***-dependent ATP synthase reaction are available), the rotation proceeds unidirectionally as a ratchet-and-pawl mechanism, a device widely used in many man-made machines. It remains to be established what particular part(s) of the enzyme serves as a ratchet (specific hydrogen bond arrangements of γ and α-β subunit interaction [44]) and as a pawl (*P. denitrificans* ξ-subunit [42], protein inhibitor [39-41], inhibitory ADP(Mg^{2+}) [29, 30]). Certainly such a device is not able to violate constrains of thermodynamics as it has been elegantly discussed by R. Feynman [45]. It is conceivable that other than bulk ***p*** force(s) is(are) involved in ATP synthesis, as has been discussed many years ago [46]. Also, variation of H^+ translocated/ATP synthesized stoichiometry upon variation of ***p*** cannot be excluded. Whatever the mechanical arrangement that furnishes the one-way operation of the enzyme are, a simple model of $F_o \cdot F_1$ as an enzyme reversibly equilibrating free energy of ATP hydrolysis and ***p*** does not hold at least for the *P. denitrificans* system. Whether this is true for $F_o \cdot F_1$ from other sources remains to be experimentally tested.

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