Biochimica et Biophysica Acta 1837 (2014) 1322–1329

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

# ATPase/synthase activity of *Paracoccus denitrificans* $F_o \cdot F_1$ as related to the respiratory control phenomenon

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ARTICLE INFO

*Article history:*
Received 3 February 2014
Received in revised form 21 March 2014
Accepted 1 April 2014
Available online 13 April 2014

*Keywords:*
Oxidative phosphorylation
$F_o \cdot F_1$-ATP synthase
Respiratory control
*Paracoccus denitrificans*

ABSTRACT

The time course of ATP synthesis, oxygen consumption, and change in the membrane potential in *Paracoccus denitrificans* inside-out plasma membrane vesicles was traced. ATP synthesis initiated by the addition of a limited amount of either ADP or inorganic phosphate proceeded up to very low residual concentrations of the limiting substrate. Accumulated ATP did not decrease the rate of its synthesis initiated by the addition of ADP. The amount of residual ADP determined at State 4 respiration was independent of ten-fold variation of $P_i$ or the presence of ATP. The pH-dependence of $K_m$ for $P_i$ could not be fitted to a simple phosphoric acid dissociation curve. Partial inhibition of respiration resulted in a decrease in the rate of ATP synthesis without affecting the ATP/ADP reached at State 4. At pH 8.0, hydrolysis of ATP accumulated at State 4 was induced by a low concentration of an uncoupler, whereas complete uncoupling results in rapid inactivation of ATPase. At pH 7.0, no reversal of the ATP synthase reaction by the uncoupler was seen. The data show that ATP/ADP × $P_i$ ratio maintained at State 4 is not in equilibrium with respiratory-generated driving force. Possible mechanisms of kinetic control and unidirectional operation of the $F_o \cdot F_1$-ATP synthase are discussed.



## 1. Introduction

Strict coordination of ATP production and demand for energy in the cell can be accomplished, at least partially, by a so-called respiratory control, the phenomenon originally discovered by Belitzer more than seventy years ago [1] and greatly elaborated by Lardy and Wellman [2] and Chance and Williams [3] in their classical studies. The essence of the respiratory control is that ADP, the product of various ATP consuming reactions, accelerates respiration, the major ATP producing metabolic pathway. Properly prepared well-coupled mitochondria incubated in the presence of oxidizable substrate and inorganic phosphate respire slowly (State 4 in Chance's nomenclature [4]) unless ADP is added. The latter increases the respiration (two- to ten- or more folds depending on the source of mitochondria and particular experimental conditions). Active State 3 (ADP stimulated) respiration decreases to the original State 4 level when ADP is converted to ATP. Numerical values of State 3 and State 4 respiration and especially their ratio are routinely used as the criteria of intactness of mitochondria and coupling efficiency of their respiration.

It should be pointed out that ATP synthesis during State 3 respiration as it proceeds in intact mitochondria or cells is a series of steps, besides the substrate oxidation by the respiratory chain, catalyzed by several energy-dependent membrane-bound enzymes, i.e. translocases of the respiratory substrates, inorganic phosphate carrier, adenine nucleotide translocase, and $F_o \cdot F_1$-ATP synthase. In this series, proton ($Na^+$ in some bacteria)-translocating $F_o \cdot F_1$-ATPase/synthase serves as an immediate operative device that connects (regulates) energy-generating respiration with ATP synthesis. Tremendous progress in the understanding of $F_o \cdot F_1$-type ATPase structure and mechanism has been achieved during recent decades. It is conventionally believed that the enzyme is the proton motive force (*pmf*)-consuming (producing) *reversibly* operating nanomotor that catalyzes synthesis (hydrolysis) of ATP by the rotary nucleotide binding change mechanism (see Refs. [5–10] for comprehensive reviews of the issue as seen by different research groups). The vast majority of information on the enzyme mechanism have been accumulated from studies on ATP hydrolytic activities and so-called partial reactions catalyzed either by its soluble nucleotide binding site containing part ($F_1$) or by the whole oligomeric complex ($F_o \cdot F_1$) purified or reconstituted into membranes. Although in most organisms ATP synthesis, not ATP hydrolysis, catalyzed by $F_o \cdot F_1$ is the physiologically relevant direction of the catalysis, much less experimental data on its ATP synthase activity are available. This is apparently due to a lack of simplified model systems where $F_o \cdot F_1$ bound to the respiratory active membranes directly interacts with the substrates of oxidative phosphorylation and show the reversible respiratory control phenomenon. To the best of our knowledge, the only available model

*Abbreviations:* FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; *pmf*, proton motive force; SBP, subbacterial particles; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference of $H^+$ electrochemical potentials; $\Delta G_p$, free energy change of phosphoryl group transfer potential, $\Delta G_p = \Delta G_p^o + RT \ln ATP/ADP \times P_i$; DCCD, *N*,*N*′-dicyclohexylcarbodiimide

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http://dx.doi.org/10.1016/j.bbabio.2014.04.002
0005-2728/

system that satisfies this requirement is inside-out vesicles of *Paracoccus denitrificans* plasma membrane (*Pd* SBP) [11,12].

Tightly coupled *Pd* SBP were shown to catalyze respiration-supported ATP synthesis, whereas, surprisingly, their ATPase and ATP-$P^{32}$ exchange activities were negligible [13,14]. This led Ferguson at al. to conclude that $F_o \cdot F_1$ of *P. denitrificans* is an irreversible component in electron transfer-linked ATP synthesis [15]. This conclusion, however important it is for mechanistic and physiological aspects of the cellular bioenergetics, has not been further elaborated in vast further publications on $F_o \cdot F_1$ ATPases. We have shown that high proton-translocating ATPase activity of *Pd* SBP is induced by pre-energization of their coupling membrane [16], similarly to the phenomena described for chloroplast [17], mammalian [18], and other bacterial $F_o \cdot F_1$s [19–21]. The *pmf*-induced ATPase activity rapidly declines upon de-energization [16], apparently due to transformation of the enzyme into so-called ADP($Mg^{2+}$)-inhibited state [22]. The presence of inorganic phosphate bound to $F_o \cdot F_1$ in an energy-dependent fashion was shown to be required for continuous *pmf*-generating ATP hydrolysis by *Pd* SBP [23] *Escherichia coli* (E $F_o \cdot F_1$), and thermophilic *Bacillus PS3* (T $F_o \cdot F_1$) [24]. Thus, the steady-state operation of $F_o \cdot F_1$ ATP synthases should be considered as a *pmf*-generating, *pmf*-requiring process, and not as a simple enzymatically catalyzed reversible reaction obeying the Haldane relationship.

The kinetic parameters of *Pd* $F_o \cdot F_1$-ATP synthase [14,25,26] and ATP hydrolase [16,27] activities have been evaluated in classical initial rate and progress curve studies. The purpose of the studies reported in this paper was to inquire how complex interplay between the enzyme active and inactive states corresponds to the respiratory control phenomenon.

## 2. Material and methods

*P. denitrificans* (strain 1222) plasma membranes vesicles were prepared from a culture grown in the presence of succinate and nitrate [11] with modifications [12]. ATP hydrolysis and ATP synthesis were assayed as small pH changes [28,29] detected by a glass electrode. All ATP- or ADP-responses were completely inhibited by venturicidin. Although the stoichiometry of scalar $H^+$/ATP ratio as a function of pH has been documented [29], these values at pH 7.0 and 8.0 were determined for the particular conditions used as follows. The standard reaction mixture (2.5 ml) was comprised of 0.25 M sucrose, 1.0 mM HEPES (pH 7.0 or 8.0), 0.1 mM EDTA, 5.5 mM $MgCl_2$, potassium phosphate (variable concentrations), 5 mM potassium succinate or 5 mM semicarbazide, 50 mM ethanol, 450 units of alcohol dehydrogenase, and 60 μM NADH (30 °C), and the oligomycin-sensitive ATPase reaction catalyzed by bovine heart submitochondrial particles (SMP) was followed as $H^+$ release. SMP (17 μg protein) were added to 2.5 ml standard reaction mixture supplemented by ATP (1 or 0.5 mM), phospho*enol*pyruvate (1.5 mM), and FCCP (1 μM). Acidification was traced for 1 min, and the amount of $H^+$ released was determined after the reaction was stopped by oligomycin (6 μg/mg protein). Potassium cyanide (1 mM) was then added. Two milliliters of the mixture was transferred to a spectrophotometric cell, and potassium chloride (20 mM), NADH (150 μM) (final concentrations), and lactate dehydrogenase (12 units) were added. A decrease in NADH as induced by the addition of pyruvate kinase (5 units) was determined ($\varepsilon_{mM}^{340} = 6.22$). The stoichiometry of scalar $H^+$ release/ADP formed was 0.7 and 1.0 at pH 7.0 and 8.0, respectively. Calibrations of $H^+$ released (or consumed) during ATP hydrolysis (or synthesis) were done for all assays by the addition of a proper amount of HCl to the samples after the reactions were completed. The sensitivity of small pH-change registration depends on $P_i$ concentration and pH of a medium because of different stoichiometries and different buffer capacities. Thus, to make presentation of the data easier, the actual tracings were scanned and adjusted to the same sensitivity scale. Averaged noise/signal ratio in conditions recording ATP synthesis was 0.03. Transmembrane electric potential was followed as Oxonol (1.5 μM) response at 624–602 nm. Oxygen consumption was assessed by a membrane-covered platinum electrode. Protein content was determined by the biuret procedure with bovine serum albumin as a standard. Venturicidin B was from A.G. Scientific, Inc. (San Diego, CA). Alcohol dehydrogenase (*Saccharomyces cerevisiae*) was from Sigma, No A3263. All fine chemicals were from Sigma, and other reagents were of the highest purity available from local suppliers.

## 3. Results

Although some characteristics of *Pd* SBP have been documented [11–15,30], it seemed worthwhile to summarize the specific activities of the preparations used in this and our previous studies on *Pd* $F_o \cdot F_1$ [16,23,27]. The respiratory activities with succinate or NADH as the substrates are given in Table 1. The values of fully uncoupled oxygen consumption with either substrate as determined at pH 8.0 and 7.0 were similar and close to those reported by Ferguson et al. for NADH oxidase in the presence of ADP (0.72 μatom of $O_2 \cdot min^{-1} \cdot mg^{-1}$ at 30 °C, pH 7.3 [15]); they were, however, substantially higher than those reported by John and Hamilton for particles prepared with inclusion of ATP in osmotic lysis solution [30], the procedure employed in this and our previous studies. We are unable to explain this apparent discrepancy. Only slight stimulation of NADH oxidase by permeabilization of the membranes by alamethicin suggests that 70–80% of the vesicles were oriented inside-out. Also, in accordance with data reported in Ref. [30], uncoupled respiration with either substrate was higher than that in the presence of saturating ADP and $P_i$, thus suggesting that under the conditions employed $F_o \cdot F_1$ activity was the rate limiting step in ATP synthesis at State 3. The specific activities catalyzed by *Pd* $F_o \cdot F_1$ are summarized in Table 2. At pH 8.0, the ATP synthase and hydrolyase activities were comparable (~0.5 versus ~0.2, respectively); at pH 7.0, ATP hydrolase activity was dramatically decreased, whereas the rate of ATP synthesis and P/O ratios were essentially the same as those determined at pH 8.0. As *Pd* $F_o \cdot F_1$-related activities were essentially the same with NADH or succinate used as respiratory substrates (Table 2), further experiments were performed with either substrate and analyzed as interchangeable.

Fig. 1 demonstrates the time course of the membrane potential, oxygen consumption, and ATP synthesis induced by the addition of a limited amount of ADP to NADH-oxidizing particles (State 3–State 4 transition). The patterns observed were remarkably similar to those seen in intact mitochondria except for opposite polarity of the membrane potential, as expected. Note should be made concerning the terminology used throughout the paper. We refer to State 4 respiration as that recorded *after* a limited amount of added ADP is phosphorylated. In intact mitochondria or cells, the respirations before and (or) after

**Table 1**
Respiratory activities of *P. denitrificans* plasma membrane vesicles.[a]

| | − ADP − Uncoupler | + ADP (100 μM) | + Uncoupler[b] | Respiratory control ratio | |
|---|---|---|---|---|---|
| | μg-atom of $O_2 \cdot min^{-1} \cdot mg^{-1}$ | | | | |
| | (1) | (2) | (3) | (2)/(1) | (3)/(1) |
| Succinate oxidase[c] | | | | | |
| pH 8.0 | 0.39 ± 0.04 | 0.73 ± 0.13 | 1.05 ± 0.25 | 1.9 | 2.6 |
| pH 7.0 | 0.27 ± 0.04 | 0.50 ± 0.20 | 0.75 ± 0.05 | 2.3 | 3.0 |
| NADH oxidase[d] | | | | | |
| pH 8.0 | 0.21 ± 0.03 | 0.42 ± 0.13 | 1.00 ± 0.10 | 2.1 | 4.8 |
| pH 7.0 | 0.14 ± 0.02 | 0.29 ± 0.02 | 0.9 ± 0.05 | 2.4 | 6.4 |

[a] Averaged numbers for 5–7 different batches of SBP assayed as described in Material and methods section.
[b] 0.1 μg/ml gramicidin + 15 mM ammonium acetate. Uncoupled respiration was increased by 20–30% if the SBP were permeabilized by alamethicin [31] thus showing their 70–80% inside-out orientation.
[c] 5 mM potassium succinate.
[d] NADH-regenerating system, 60 μM NADH.

**Table 2**
$F_o \cdot F_1$-related activities of *P. denitrificans* plasma membrane vesicles.

| | P/O[a] ratio | ATP hydrolysis[b] | ATP synthesis[c] |
|---|---|---|---|
| | | μmol·min$^{-1}$·mg$^{-1}$ | |
| Succinate oxidation | | | |
| pH 8.0 | 0.7 | 0.12–0.20 | 0.53 ± 0.1 |
| pH 7.0 | 0.8 | 0.04–0.08 | 0.40 ± 0.1 |
| NADH oxidation | | | |
| pH 8.0 | 1.2 | 0.20 | 0.50 ± 0.1 |
| pH 7.0 | 1.3 | 0.04 | 0.38 ± 0.1 |

[a] Calculated as the ratio of phosphorylation rate to respiratory activity.
[b] *pmf*-activated, *pmf*-producing ATPase activity in the presence of 1 mM ATP; significant variations of the specific activity are evidently due to variable *pmf* back pressure. Apparent $K_m^{ATP}$ values (40–100 μM) were dependent on coupling efficiency of ATP hydrolysis. $K_i^{ADP}$ determined as for simple competitive inhibition was 210 ± 10 μM.
[c] Initial rates in the presence of 100 μM ADP and 5 mM $P_i$. Apparent $K_m^{ADP}$ values were dependent on *pmf* [26]. $K_i^{ATP}$ determined as simple competitive inhibition was 95 ± 25 μM for NADH-supported ATP synthesis at pH 7.0.

ADP phosphorylation are frequently referred to as State 4 interchangeably. This is justified because in either case the respiration is limited by low level of phosphate acceptor, ADP (as originally defined [4]), whereas total concentration of intramitochondrial or intracellular ATP plus ADP is in the millimolar range. The situation is quite different when inside-out particles are used: no added nucleotides are present, so

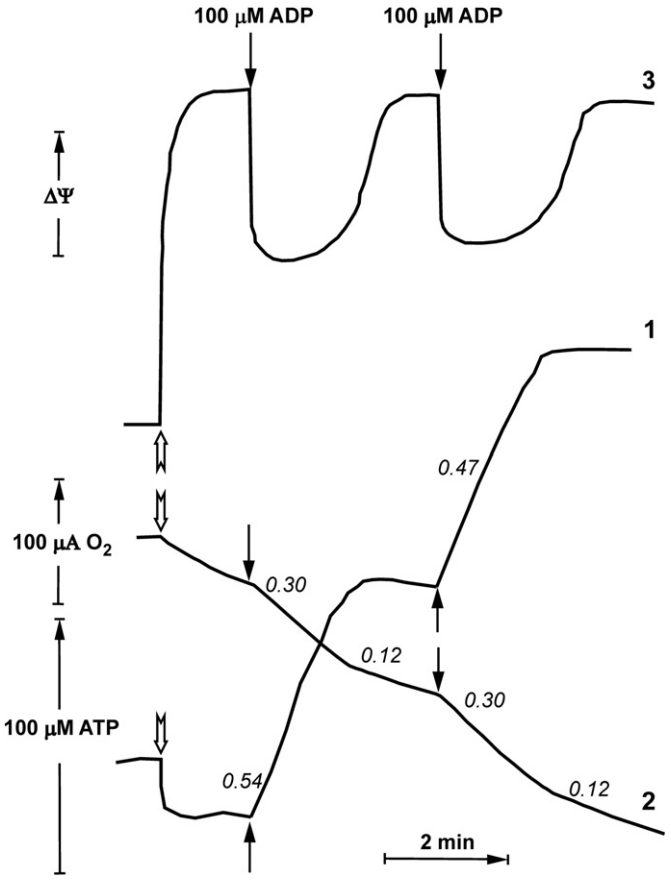



**Fig. 1.** Time course of ATP synthesis (curve 1), oxygen consumption (curve 2), and changes in membrane potential (curve 3) during aerobic oxidative phosphorylation catalyzed by *P. denitrificans* plasma membrane vesicles (SBP). The reaction was started by the addition of SBP (0.1 mg/ml, indicated by open arrows) to the standard reaction mixture supplemented with 5 mM potassium phosphate (pH 7.0), 60 μM NADH, 50 mM ethanol, 5 mM semicarbazide, and 0.3 mg/ml alcohol dehydrogenase. Oxonol (1.5 μM) was added for tracing of the membrane potential (curve 3). Figures on the curves in italic denote the specific activities, μmol and μatom of ATP and $O_2$ per min per mg of protein, respectively.

"State 4" before and that after phosphorylation are different states. To the best of our knowledge, true State 4 respiration has never been observed in any inside-out particles except for inverted *P. denitrificans* plasma membranes. The time course of ATP synthesis during State 3 respiration is shown in Fig. 2. No lag-phase in the ATP synthesis reaction was seen within the time resolution of the experiment. The reaction could equally be initiated by either ADP ($P_i$ in excess (A)) or $P_i$ (ADP in excess (B)) (Fig. 2). If $P_i$ was present in excess (0.5 mM), ATP synthesis proceeded as a zero-order reaction up to very low residual concentrations of ADP. When excess of ADP over $P_i$ was present, the time course of the reaction was different, so that it started to deviate from zero-order kinetics at significantly higher residual $P_i$ concentration, as expected from data on apparent $K_m^{Pi}$. Simple hyperbolic dependence of the rate on $P_i$ concentration was seen for a given pH and oxidizable substrate (NADH or succinate) (Fig. 3). In an attempt to identify the ionic form of $P_i$ that binds to $F_o \cdot F_1$ as the substrate, the $K_m^{Pi}$ values were determined at various pH values in the region of $pK_a^2$ for phosphoric acid (7.2 [32]) (Table 3). No straightforward conclusion about specific ionic species could be made: only slight dependence of $K_m^{Pi}$ and $V_m$ on pH was seen, as expected if ionization of both the substrate and enzyme happen within the pH range employed (see Appendix A).

ATP accumulated during State 3 phosphorylation of a limited amount of ADP did not inhibit the rate of newly added ADP, as might be expected for a reversibly operating ATPase/synthase. Fig. 1 shows that phosphorylation of the second portion of ADP added to the vesicles respiring at State 4 showed the same pattern of time course and extent of reaction. Moreover, all the parameters depicted in Fig. 1 were the same when the concentration of $P_i$ was decreased to 0.5 mM (not shown). It was of obvious interest to determine whether ATP accumulated at State 4 could be hydrolyzed if *pmf*-generating respiration were prevented. It is worth noting that State 4 corresponds to the conditions required for ATPase activity of *Pd* $F_o \cdot F_1$, i.e. the enzyme has been pre-activated by *pmf*, and ATP (as the substrate) and $P_i$ required for ATPase activity [23] were present. Tracing of ATPase activity (Fig. 4, curves C and D) along with changes in the membrane potential (Fig. 4, curves A and B) under the State 4 conditions where generation of *pmf* was prevented by either uncoupler or cyanide is shown in Fig. 4. At pH 8.0, FCCP (0.5 μM) added to relieve back pressure of ATP hydrolysis-dependent generation of *pmf* resulted in activation of ATPase (left part of panel C) and decrease in membrane potential (left part of panel A). A certain transitory level of the membrane potential was seen until the ATP accumulated at State 4 was hydrolyzed. Lower ATPase activity and higher level of the transitory membrane potential were induced by cyanide (right parts of panels A and C, respectively). Complete discharge of the membrane potential by the addition of 10 μM FCCP (right part of panel A) resulted in immediate inhibition of ATP hydrolysis (right part of panel C). Thus, at pH 8.0 the ATP accumulated at State 4 was hydrolyzed by *pmf*-activated, *pmf*-generating $F_o \cdot F_1$. Different patterns of ATP hydrolysis and the membrane potential as induced by uncoupling and/or cyanide were seen at pH 7.0 (right panel). A much larger decrease in the membrane potential (almost complete) was induced by low FCCP (B), and no ATP hydrolysis was seen (D). Similar changes were observed when respiration-generated *pmf* was abolished by cyanide (right parts of panels B and D, respectively).

It was of obvious interest to see whether ATP phosphoryl group transfer potential ($\Delta G_p$) was equilibrated with *pmf* at State 4. The residual concentrations of ADP reached at State 4 at two ten-fold different concentrations of $P_i$ are shown in Table 4. Although the values shown in Table 4 were somewhat variable for different samples of the vesicles, no effects of $P_i$ on the residual ADP concentrations were evident. This puzzling finding (if simple equilibration of *pmf* and $\Delta G_p$ at State 4 was to be assumed) could be explained if *pmf* generated by NADH oxidation was significantly larger than the established $\Delta G_p$. Thus, the dependence of the residual ADP concentrations and the initial rates of ATP synthesis were determined at different rates of *pmf* generation during NADH oxidation (Fig. 5). Remarkably, the residual ADP concentrations

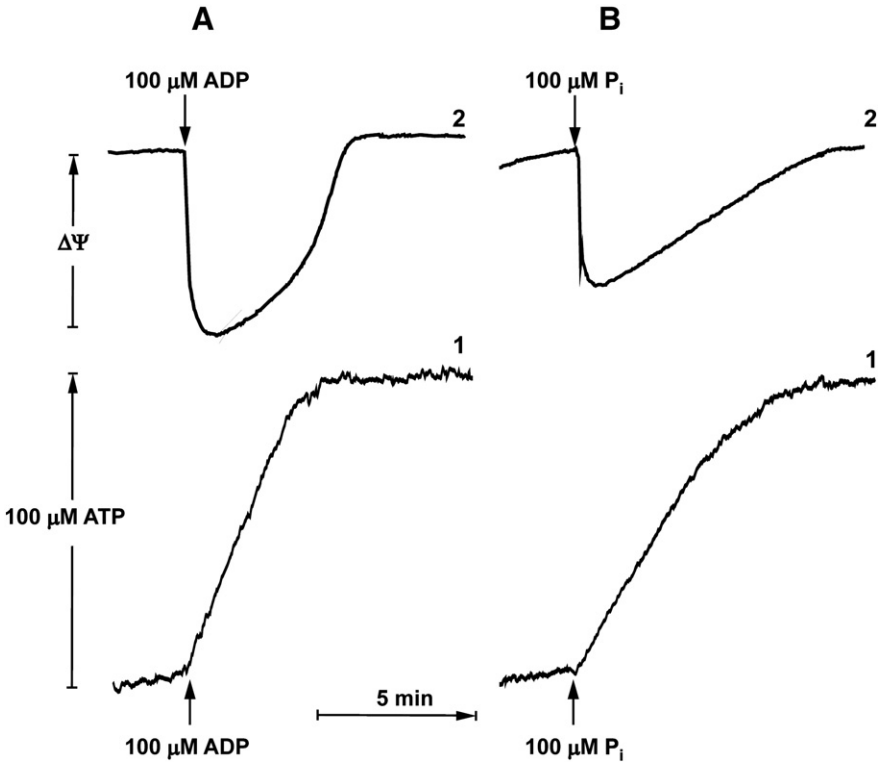



**Fig. 2.** ATP synthesis (curves 1) and membrane potential (curves 2) as initiated by the addition of ADP (panel A) or $P_i$ (panel B). Standard reaction mixture (pH 7.0) was supplemented with 0.5 mM potassium phosphate (panel A) or 0.5 mM ADP (panel B). SBP (0.15 mg/ml) and NADH-regenerating system (as in Fig. 1) was added before initiation of the reaction.

determined after the phosphorylation was completed were not changed even if the rate of the synthesis was significantly reduced.

## 4. Discussion

This paper confirms and extends previous original studies on *P. denitrificans* plasma membranes [11–16,26,27]. The data in Tables 1 and 2 particularly those depicted in Fig. 1 show that tightly coupled vesicles of *P. denitrificans* are exceptionally well suited for studies aimed to elucidate the respiratory control phenomenon. Two alternative mechanisms of respiratory control have been under debate in the literature. One is so-called "thermodynamic" control [33,34] based on a proposal that equilibrium (or close to equilibrium) between the driving force and intracellular or intramitochondrial $\Delta G_p$ is reached at State 4 respiration. In this model, an increase in the respiratory activity by ADP is considered as a change in ATP/ADP ratio that results in activation of the redox reactions to restore disturbed equilibrium. An alternative view is that respiration is kinetically controlled by ADP via either the adenine nucleotide translocase (in mitochondria) or some intrinsic mechanisms of $F_o \cdot F_1$ ATPase/synthase [35,36]. Only slight stimulation of the respiratory activities by ADP without restoration of the original level has been reported in studies where $F_o \cdot F_1$ was directly accessible for the substrates, such as submitochondrial particles [37] and other than *P. denitrificans* bacterial plasma membranes [38] because of either poor coupling or high ATPase activity of uncoupled vesicles present in notoriously heterogeneous preparations. Tremendous efforts were undertaken to relate $\Delta G_p$ to *pmf* on coupling membranes of mitochondria, chloroplasts, and bacteria at the time when Mitchell's chemiosmotic

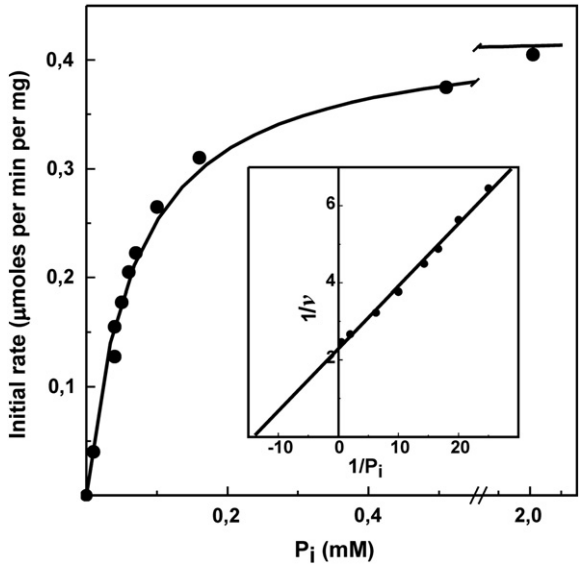



**Fig. 3.** Rate of ATP synthesis as a function of $P_i$ concentration. The standard reaction mixture (pH 8.0) was supplemented with 5 mM potassium succinate and vesicles (0.1 mg/ml). The reaction was initiated by the addition of 200 μM ADP. Inset, double reciprocal plot.

**Table 3**
Apparent affinity of $P_i$ at variable pH.[a]

| pH | $K_m^{Pi}$ (μM) | $V_{max}$[b] (μmol ATP·min$^{-1}$·mg$^{-1}$) |
|---|---|---|
| Succinate oxidase | | |
| 7.0 | 70 | 0.34 |
| 7.5 | 68 | 0.48 |
| 8.0 | 75 | 0.36 |
| 8.5 | 115 | 0.32 |
| NADH oxidase | | |
| 7.0 | 90 | 0.45 |
| 8.0 | 60–120 | 0.45 |

[a] Succinate- and NADH-supported ATP syntheses assayed as indicated in Tables 1 and 2.
[b] Initial rates in the presence of 100 μM ADP.

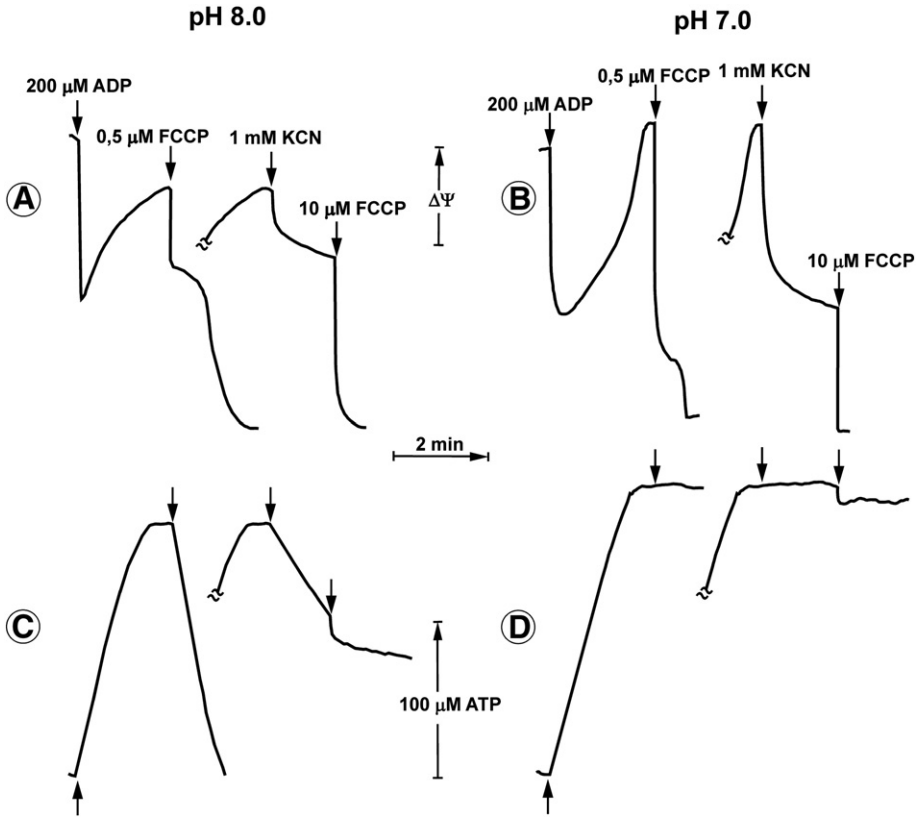



**Fig. 4.** The membrane potential changes (panels A and B) and ATP synthesis/hydrolysis activities (panels C and D) at different pH. Succinate (5 mM), 1 mM potassium phosphate and vesicles (0.27 mg/ml) were added to the standard reaction mixture and ATP synthesis was initiated by the addition of ADP. Further additions were made after ATP synthesis has been completed. See text for further explanation.

theory was under debate (see Refs. [39,40] for comprehensive reviews). Particularly, comparison of *pmf* magnitude with $\Delta G_p$ generated by NADH and succinate oxidation in Pd SBP has been thoroughly investigated [41]. Interestingly $\Delta G_p$ was not altered in the presence of potassium nitrate whereas no NADH oxidase was detected. Also, no ATP-induced *pmf* generation was detected; the observation was interpreted as the irreversibility of $F_o \cdot F_1$-ATPase [41] in accord with the previously reported data [15]. The conclusions derived from those studies, particularly those concerning required $\bar{H}^+$/ATP ratio, were

**Table 4**
Residual ADP concentrations at State 4 at different nucleotides and $P_i$ concentrations.[a]

| Nucleotide added (μM) | Residual ADP at State 4 (μM) | |
|---|---|---|
| | $P_i$ (mM) | |
| | 0.5 | 5.0 |
| Exp. 1[b] | | |
| ADP (50) | 2.8 | 2.5 |
| ADP (100) | 4.6 | 3.5 |
| ADP (50) | 6.3 | 4.6 |
| ATP (500) | | |
| ADP (50) | 8.6 | 5.3 |
| ATP (1000) | | |
| Exp. 2[b] | | |
| ADP (50) | 2.3 | 1.6 |
| ADP (100) | 3.5 | 2.7 |
| ADP (50) | 5.1 | 4.3 |
| ATP (500) | | |

[a] NADH-supported ATP synthesis, pH 7.0. The reactions were initiated by addition of ADP to the standard reaction mixture supplemented with $P_i$ and ATP (where indicated). The residual concentrations of ADP at State 4 were determined as follows. NADH (0.2 mM) oxidation in the standard reaction mixture supplemented with phospho*enol*pyruvate (1.5 mM) was followed at 340 nm. ADP was added to stimulate respiration and after transition to State 4 (see Fig. 1) venturicidin (1 μg/mg of protein) and potassium cyanide (1 mM) (to prevent further reactions), and potassium chloride (20 mM) (required for pyruvate kinase activity) were added. The sensitivity of the instrument was changed (increased), lactate dehydrogenase (20 units) was added and the amount of ADP was determined as a decrease of NADH induced by the addition of pyruvate kinase (10 units). The pyruvate kinase induced response was further titrated by the addition of 5–10 μM ADP.
[b] Two different vesicle preparations were assayed.

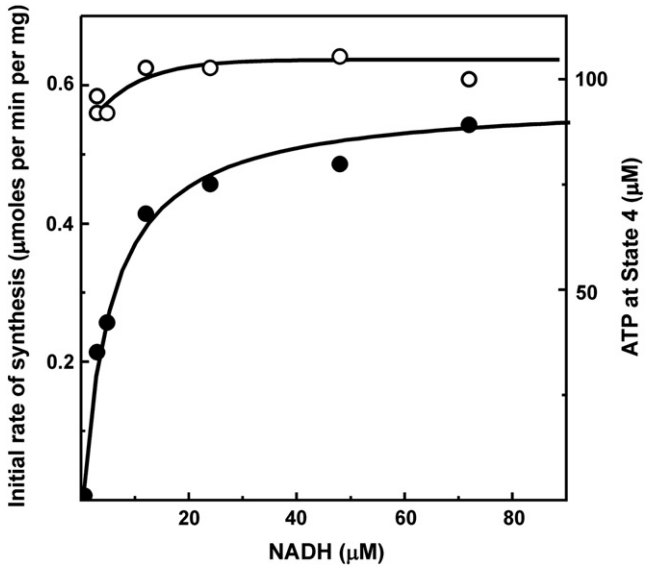



**Fig. 5.** Rate of ATP synthesis (closed circles, left ordinate) and ATP concentration at State 4 (open circles, right ordinate) as dependent on the respiratory activity. The reaction was initiated by the addition of 105 μM ADP to the standard reaction mixture (pH 7.0) supplemented with 0.5 mM potassium phosphate, NADH-regenerating system, and various concentrations of NADH (indicated on abscissa).

made with implicit or explicit assumptions on thermodynamic equilibrium (or close to equilibrium) between *pmf* and $\Delta G_p$ as catalyzed by reversibly operating proton translocating ATPase/synthase. The data reported in this paper show that at least in *P. denitrificans* membranes this is not the case: ten-fold variation of $P_i$ did not change ATP/ADP ratio poised at State 4 respiration (Table 4). In fact, only a small response of ATP/ADP ratio to $P_i$ variation has been noted, not explained, in previous studies [42,43]. $P_i$ does not inhibit ATP hydrolytic activity of various $F_1$ or $F_o{\cdot}F_1$ (see Introduction section in Ref. [23] for a brief review, but Ref. [44] for the opposite), and it does not affect ATP-induced rotation of γ-subunit in immobilized *Thermus thermophilus* $F_1$ [45]. Insensitivity of ATPase to $P_i$ corroborates the data shown in Table 4 and also argues against thermodynamic equilibration of *pmf* and $\Delta G_p$ at State 4.

In contrast to the data for yeast $F_o{\cdot}F_1$ interpreted as identification of $P_i$ monoanion as the substrate of ATP synthesis [46], we were unable to reach the same conclusion from the pH-dependence of $K_m$ for $P_i$ (Table 3). Since the structural arrangement of a putative $P_i$ binding area at the catalytic site of various $F_1$ is similar [47–49], the difference between the data of Förster et al. [46] and those reported here can hardly be explained by the difference between *Paracoccus* $F_1$ and yeast $F_1$.

Presently, no unequivocal explanation for nonequilibrated ATP/ADP ratio at State 4 can be offered. The cycling of ATP caused by the presence of uncoupled vesicles seems unlikely because $F_o{\cdot}F_1$ ATPase activity of *P. denitrificans* membranes is latent unless they are activated by *pmf* [16], similarly to the energy-dependent transformation of ADP($Mg^{2+}$)-inhibited, azide stabilized heart $F_o{\cdot}F_1$ [50]. Studies of the ADP($Mg^{2+}$)-inhibited mitochondrial $F_1$, the dead-end catalytic intermediate evident from the steady-state or pre-steady-state of ATP hydrolysis discovered more than 30 years ago [51] led us to suggest that "hydrolase" and "synthase" forms of $F_o{\cdot}F_1$ exist as controlled by tight ADP binding, and that the ATP synthase reaction is not a reversal of the ATP hydrolase reaction [52,53]. This hypothesis has been elaborated [54] and supported by recent analysis of single molecular turnover experiments on T $F_1$ [55]. The absence of thermodynamic equilibrium is expected if ATP/ADP ratio at State 4 is maintained by the futile cycle catalyzed by putative "hydrolyase" and "synthase" forms of $F_o{\cdot}F_1$, and the ratio between them is controlled by a number of intrinsic (ε-subunit [56], protein inhibitor [57], recently described ξ-subunit of *Pd* $F_1$ [58]) and exogenous factors (ATP, ADP, $P_i$, and *pmf*). Among the latter ADP seems to play a central role, as the ADP($Mg^{2+}$)-inhibited states have been identified in all $F_1$-type ATPases studied so far. Interestingly, the hydrolytic activity of prokaryotic vacuolar-type *Thermus thermophilus* (T $V_o{\cdot}V_1$ $H^+$-ATPase), which physiologically functions as ATP synthase, is severely inhibited by entrapment of ADP-$Mg^{2+}$ [59], whereas eukaryotic and *Enterococcus hirae* $V_o{\cdot}V_1$, which functions as an ATP hydrolysis-driven proton pumps does not exhibit sensitivity to ADP-$Mg^{2+}$ [60]. Synthesis/hydrolysis cycling at State 4 would decrease actual efficiency of ATP production, but it would be beneficial for fine control (maintenance of constant ATP/ADP ratio in bacterial cytoplasm or in the mitochondrial matrix). The utility of the "futile" cycles for sensitive control of metabolic pathways has been proposed and discussed by Newsholme and Start [61].

Another puzzling observation that merits brief discussion is that the ATP/ADP ratio at State 4 was essentially constant at different rates of respiration, and only the rate of ATP synthesis, not the final $\Delta G_p$, was decreased upon limitation of *pmf*-generating NADH oxidase (Fig. 5). Free energy required for ATP synthesis can be provided by any *pmf* if ATP synthase would operate with proper ($n$) $\vec{H}^+$/ATP stoichiometry ($\Delta G_p = n \times pmf$). According the original proposal [62] and current models [6–10], the latter is strictly determined by the number of *c*-subunits which form a rotating proton-translocating drive gear for rotation of γ-, ε-subunits within a 3α, 3β-subunit stator fixed by *b*-subunits. The number of *c*-subunits in the $F_o$-ring of different species varies from 8 to 15, and these variations have been related to bioenergetic cost of ATP synthesis [63–65]. What factor(s) determines the assembly of a particular stoichiometry of *c*-subunits, besides variation in their primary structures, is not known. Neither $\vec{H}^+$/ATP stoichiometry nor the number of *c*-subunits in *Pd* $F_o$ is known, and the possibility that different isoforms of $F_o{\cdot}F_1$ with different numbers of *c*-subunits are present in *P. denitrificans* and other bacterial membranes should not be overlooked. Possible mechanisms that permit ATP synthesis at low *pmf*, a problem particularly important for alkalophilic bacteria, have been discussed [66].

Biochemical studies provide evidence for the ATP-dependent, DCCD- and azide-sensitive movement (rotation?) of the *c*-ring relative to the *a*-subunit in *E. coli* $F_o{\cdot}F_1$ [67]. Visually detected ATP-supported rotation of the c-ring in immobilized single molecule $F_o{\cdot}F_1$ has been reported [68], although interpretation of these results was questioned [69]. If a proposal on different mechanisms (intermediates) in ATP hydrolysis and synthesis [52,53] is to be accepted, other than rotary binding change mechanisms of ATP synthesis catalyzed by $F_o{\cdot}F_1$ cannot be excluded.

Strong sensitivity of the ATPase of $F_o{\cdot}F_1$ to pH (Table 2 and Fig. 4) may have physiological significance. ATP hydrolysis at pH > 7.0, whether or not it builds up *pmf*, is inevitably accompanied by a release of scalar $H^+$ into the surrounding solution. If it proceeds inside small poorly $H^+$-permeable vesicles, it would result in acidification of the intravesicular space (bacterial cytoplasm or mitochondrial matrix), and inhibition of the ATPase activity at acidic pH may serve as physiologically important mechanism (along with several others [70]) to keep intracellular (intramitochondrial) pH constant.

## Acknowledgements

We are grateful to Dr. V.S. Kozlovsky (A.N. Belozersky Institute of Physico-Chemical Biology) for his help in programming. This study was supported by the Russian Foundation for Fundamental Research (grant 14-04-00279 to ADV).

## Appendix A

Consider the simplest model where one of the two existing ionic species forms of the substrate ($S^-$) binds to an enzyme active site:

$$\begin{array}{ccccc} E + S^- & \xleftrightarrow{K_s} & E{\cdot}S^- & \xrightarrow{k_{cat}} & E + \text{product(s)} \\ \Big\downarrow{\scriptstyle K_a^S} & & & & \\ SH + H^+ & & & & \end{array} \quad (1)$$

In the equilibrium approximation, the initial steady-state rate ($v$) is:

$$v = \frac{V \cdot S_o}{S_o + K_s(1 + H^+/K_a^S)} \quad (2)$$

where $V = k_{cat}{\cdot}E_o$, $E_o$ is the total enzyme concentration, and $S_o = (S^- + SH)$. The apparent $K_m$ for the substrate thus appears as:

$$K_m = K_s\left(1 + 10^{\left(pK_a^S - pH\right)}\right). \quad (3)$$

Note, that Eq. (2) appears exactly as that for simple competitive inhibition by $H^+$, and it may appear that the ionic form of the substrate can be identified from Eq. (3) if the dependence of $K_m$ on pH and $K_a^S$ are known.

However, for a model:

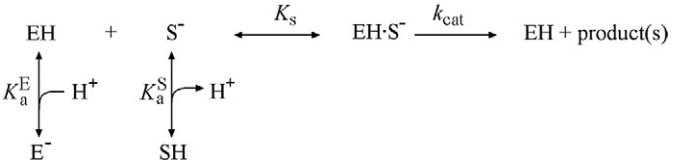
$$\begin{array}{ccccc} E^- + S & \xleftrightarrow{K_s} & E^-{\cdot}S & \xrightarrow{k_{cat}} & E^- + \text{product(s)} \\ \Big\downarrow{\scriptstyle K_a^E} & & & & \\ EH + H^+ & & & & \end{array} \quad (4)$$

where two ionic forms of an enzyme exist and only deprotonated ($E^-$) binds to the substrate, the initial rate is

$$v = \frac{V \cdot S_o}{S_o + K_s\left(1 + H^+/K_a^E\right)} \tag{5}$$

where $V = k_{cat} \cdot E_o$ and $E_o = (E^- + EH)$. Eq. (5) is exactly the same as Eq. (2), and the dependence of $K_m$ on pH corresponds to ionization of the enzyme active site. In other words, pH-dependence of $K_m$ does not distinguish between changes in ionic forms of either substrate or enzyme.

If both the substrate and enzyme ionic forms are variable within the pH interval corresponding to $K_a^S$ and $K_a^E$:




(6)

The initial steady-state rate is again pH dependent as described by Eq. (7)

$$v = \frac{V \cdot S_o}{S_o + K_s\left(1 + 10^{(pK_a^S - pH)} + 10^{(pH - pK_a^E)} + 10^{(pK_a^S - pK_a^E)}\right)}. \tag{7}$$

Note that Eqs. (2), (5), and (7) are qualitatively the same, i.e. only apparent $K_m$ values, not V, are pH dependent.

For model (6), the apparent $K_m = \alpha \cdot K_s$,

$$\alpha = 1 + 10^{(pK_a^S - pH)} + 10^{(pH - pK_a^E)} + 10^{(pK_a^S - pK_a^E)} \tag{8}$$

where $V = k_{cat} \cdot E_o$, and $E_o = E^- + EH$ and $S_o = S^- + SH$. Depending on $K_a^S$ and $K_a^E$, the pH dependence of apparent $K_m$ appears as more or less steep curves in the pH interval where dissociation of the enzyme active site group(s) and substrate takes place. For example, if $K_a^S$ equals to 7.2 (second step dissociation of orthophosphoric acid) [32], and $K_a^E$ is taken as 9.0 (a reasonable assumption for some positively charged group(s) participating in phosphate anion binding) [47], the value of $\alpha$ only slightly depends on pH (~3.0, 1.5, and 1.5 at pH 6.5, 7.5 and 8.5, respectively). Certainly an equation more complex than Eq. (7) is expected if $k_{cat}$ is also pH dependent.

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