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

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ABSTRACT

The time course of ATP synthesis, oxygen consumption, and change in the membrane potential in *Paracoccus denitrificans* inside-out plasma membrane vesicles (SBP) was traced. ATP synthesis initiated by the addition of a limited amount of either ADP or inorganic phosphate (P_i) 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 (FCCP), 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 $\times$ 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.

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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 of which was 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, which is the product of various ATP consuming reactions, accelerates respiration, which is 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; ΔG_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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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 of 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^+ was released. 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 hydrolyase 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 ammonia 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.

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