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Paracoccus denitrificans Proton-Translocating ATPase: Kinetics of Oxidative Phosphorylation

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Abstract—The initial rates of ATP synthesis catalyzed by tightly coupled *Paracoccus denitrificans* plasma membrane were measured. The reaction rate was hyperbolically dependent on the substrates, ADP and inorganic phosphate (P_i). Apparent K_m values for ADP and P_i were 7-11 and 60-120 μM, respectively, at saturating concentration of the second substrate (pH 8.0, saturating Mg^{2+}). These values were dependent on coupling efficiency. The substrate binding in the ATP synthesis reaction proceeds randomly: K_m value for a given substrate was independent of the concentration of the other one. A decrease of electrochemical proton gradient by the addition of malonate (when succinate served as the respiratory substrate) or by a decrease of steady-state level of NADH (when NADH served as the respiratory substrate) resulted in a proportional decrease of the maximal rates and apparent K_m values for ADP and P_i (double substitution, ping-pong mechanism). The kinetic scheme for ATP synthesis was compared with that described previously for the proton-translocating ATP hydrolysis catalyzed by the same enzyme preparation (T. V. Zharova and A. D. Vinogradov (2006) *Biochemistry*, **45**, 14552-14558).

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$F_o{\cdot}F_1$-H^+-ATPase (synthase) of mitochondria, chloroplasts, and bacteria is a complex membrane-bound enzyme capable of $\Delta\tilde{\mu}_{H^+}$-supported ATP synthesis or $\Delta\tilde{\mu}_{H^+}$-generating ATP hydrolysis. The structural organization of the complex is similar in all species studied; it contains two oligomeric components, hydrophobic F_o and hydrophilic F_1.

F_1 is composed of five different subunits, α, β, γ, δ, and ε (nomenclature used for prokaryotic enzyme) with the stoichiometry of 3 : 3 : 1 : 1 : 1 [1-3]. Sequentially alternating α- and β-subunits are arranged in a hexamer surrounding the central γ-subunit [4]. The hexamer bears six nucleotide-binding sites: three, so-called, "catalytic" formed (largely) by β-subunit amino acid residues and three "non-catalytic" formed (largely) by α-subunits [4]. δ-Subunit is located far from the membrane plane forming a "cap" on α,β-hexamer, and ε-subunit is bound to γ-subunit connected with F_o.

F_o is an integral membrane component composed of three different subunits: *a*, b_2, and $c_{(9\text{-}14)}$ (the stoichiometry of *c*-subunits depends on particular species). F_o catalyzes transport of protons across the membrane coupled with ATP synthesis/hydrolysis [5]. It is generally accepted that the operation of an entire $F_o{\cdot}F_1$-complex occurs as a "binding change mechanism" [6], that is each catalytic site sequentially and synchronously with two others proceeds through states that are different in their relative substrate/product affinities. Any particular state of one site strictly determines the state of the other sites depending on the position of the γ-subunit within the internal cavity of a α-β-trimer. The alternating position of the γ-subunit is driven by rotation of *c*-subunits ring. The later is coupled with translocation of protons across the coupling membrane.

It was generally assumed for a long time that the synthesis of ATP is a simple reversal of its hydrolysis. However, many experimental data suggest that the mechanisms of ATP synthesis and hydrolysis are not identical ([7] and references cited therein). A complete model (kinetic scheme) of the reaction (for example coupled

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical gradient of protons; F_1 and F_o, hydrophilic and hydrophobic parts of ATP synthase, respectively; G6PDH, dehydrogenase of glucose-6-phosphate; HK, hexokinase; S-13, 2′,5′-dichloro-3-*tret*-butyl-4′-nitrosalicylanilide; SBP, inside-out plasma membrane vesicles.

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and uncoupled ATP hydrolysis) catalyzed by an enzyme that has three active sites is expected to be extremely complex: at least ten intermediates with different occupation of three binding sites by ATP and ADP should exist during the steady-state hydrolysis at any given ATP concentration, even for a simplified model where P_i binding is not accounted for. The actual kinetics is further complicated by so-called "hysteretic" behavior seen as either lag-phase or burst in the ATP hydrolytic activity depending on the substrate concentration and "history" of a particular preparation [8, 9]. Nevertheless, the kinetics of uncoupled or coupled steady-state ATP hydrolysis by $F_o{\cdot}F_1$-ATPase is satisfactorily described by relatively simple schemes [8, 9]. If $\Delta\tilde{\mu}_{H^+}$-dependent synthesis of ATP is the reversal of $\Delta\tilde{\mu}_{H^+}$-producing ATP hydrolysis, the kinetic scheme should be the same for ATP synthesis. Only a few kinetic studies of oxidative or photophosphorylation are available as compared with hundreds if not thousands of publications on ATP hydrolytic activity of $F_o{\cdot}F_1$-ATPases. The major difficulty in studies of ATP synthesis lies in the requirement of tightly coupled enzyme preparations. Traditionally explored submitochondrial particles need to be artificially coupled by treatment with oligomycin or dicyclohexylcarbodiimide, inhibitors of $F_o{\cdot}F_1$-ATPase, that makes direct comparison of the hydrolysis and synthesis ambiguous. To the best of our knowledge the only detailed kinetic studies of oxidative phosphorylation were done by Ferguson's group using coupled inside-out plasma membrane preparation (subbacterial particles, SBP) of the soil denitrifying bacterium *Paracoccus denitrificans* [11, 12]. Remarkable features of these vesicles prepared by the original procedure of John and Whatley [13] are: i) their oxidative capacity with NADH or succinate as the respiratory substrates are strongly stimulated by uncouplers or by ADP + P_i (the respiratory control phenomenon); ii) the rate of ATP hydrolysis is about only 1-2% of that measured for ATP synthesis, in other words this preparation seems to operate "unidirectionally" [14]. We have shown that the latter property is apparent, and latent ATPase can be strongly stimulated by energization of the coupling membrane [15]. A kinetic model for the $\Delta\tilde{\mu}_{H^+}$-activated, $\Delta\tilde{\mu}_{H^+}$-generating ATP hydrolysis catalyzed by *P. denitrificans* membranes has been formulated [10].

The aim of these studies was to build up a kinetic scheme describing the sequence of the reactions that occur during ATP synthesis and its comparison with that previously described for ATP hydrolysis. Such a comparison seemed to shed light on the problem of reversible operation of energy-transducing enzymes.

MATERIALS AND METHODS

Chemicals. ADP, NADH, $NADP^+$ (sodium salts), Hepes, Tris, Phenol Red, malonate, succinate, $MgCl_2$, EDTA, sucrose, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), gramicidin, alcohol dehydrogenase, glucose 6-phosphate dehydrogenase (G6PDH), alamethicin, and piericidin were from Sigma (USA), hexokinase (HK) was from Ferak (Germany), venturicidin was from A. G. Scientific Inc. (USA), and other chemicals were highest purity commercially available.

Preparation of vesicles. *Paracoccus denitrificans* cells (strain Pd 1222) were grown anaerobically in the presence of succinate and nitrate [13]. Tightly coupled plasma membrane vesicles were prepared as described by John and Whatley with modifications [13]. 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 (protein concentration ~20 mg/ml) and stored in liquid nitrogen. Protein content was determined by the biuret procedure. 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 SBP preparations varied from 4.5 to 6.0. The content of inside-out vesicles determined as the ratio of NADH oxidase in the presence and absence of alamethicin (20 μg/ml) [16] was of 80-90%.

Synthesis of ATP. The initial rates of ATP synthesis were measured using two independent assays.

Continuous registration of hydrogen ion concentration. Synthesis or hydrolysis of ATP at pH > 7 is accompanied by consumption or release of scalar protons:

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

When succinate is used as the respiratory substrate its oxidation by oxygen does not result in any pH change because pK_a values of succinate and fumarate carboxylic group are almost the same:

$$2\ \text{succinate}^{2-} + O_2 \rightarrow 2\ \text{fumarate}^{2-} + 2\ H_2O, \quad (2)$$

and the observed pH change is due to the reaction (1) only.

When NADH is the respiratory substrate the NADH oxidase reaction is accompanied by consumption of protons:

$$2\ NADH + 2\ H^+ + O_2 \rightarrow 2\ NAD^+ + 2\ H_2O, \quad (3)$$

and the rate of the observed pH change during oxidative phosphorylation cannot be accounted for the reaction (1) only. The NADH-supported ATP synthesis was measured using an NADH-regenerating system (ethanol and alcohol dehydrogenase), so that the net oxidase reaction was not accompanied by pH change:

$$2\ \text{ethanol} + O_2 \rightarrow 2\ \text{acetaldehyde} + 2\ H_2O. \quad (4)$$

That the rates of alkalization were due exclusively to reaction (1) was proved by using specific inhibitors: venturicidin (specific inhibitor of prokaryotic of $F_o{\cdot}F_1$'s), pie-

ricidin (specific inhibitor of complex I), malonate (succinate dehydrogenase inhibitor), and uncoupler (CCCP). These inhibitors completely prevented alkalization of the reaction medium during coupled reactions (1)-(2) and (1)-(4). At pH 8.0 the stoichiometry H^+/ATP in reaction (1) is close to 1 [17]. The initial rates of ATP synthesis were expressed as micromoles of ATP formed per minute per milligram of protein. All measurements were performed at 30°C in 2 ml cuvettes (Hitachi 557 spectrophotometer (Japan) operating in dual wavelength mode at 557/618 nm). The standard reaction mixture was composed of 0.25 M sucrose, 2.5 mM Hepes-KOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM potassium succinate or NADH, and NADH-regenerating system (0.1 M ethanol, 30 units/ml of alcohol dehydrogenase, 60 μM NADH, 10 mM semicarbazide). Phenol Red (25 μM) was present in the reaction mixture as a pH indicator (absorption maximum of deprotonated form at 557 nm). The hydrogen ion consumption was titrated by the addition of standard HCl aliquots. The optical density responses were linearly dependent on the amount of HCl added. Respiration was started by the addition of vesicles (50 μg/ml), and ATP synthesis was initiated 2 min later by the addition of ADP.

Coupled enzyme system. The alternative assay with coupled enzyme system for continuous tracing of oxidative phosphorylation was used when succinate was the respiratory substrate:

$$\mathrm{ADP} + \mathrm{P_i} \xrightarrow{\mathrm{Fo \cdot F1}} \mathrm{ATP} \qquad (5)$$

$$\mathrm{ATP} + \mathrm{glucose} \xrightarrow{\mathrm{HK}} \mathrm{glucose\ 6\text{-}phosphate} + \mathrm{ADP} \qquad (6)$$

$$\mathrm{glucose\ 6\text{-}phosphate} + \mathrm{NADP^+} \xrightarrow{\mathrm{G6PDH}} \mathrm{6\text{-}phosphoglucolactone} + \mathrm{NADPH} \qquad (7)$$

and an increase of absorption at 340 nm was followed. The standard reaction mixture (2 ml) was supplemented by ADP-regenerating system (1 mM glucose, 0.6 mM $NADP^+$, 20 units/ml of HK (lyophilized bacterial preparation), 10 units/ml of G6PDH (lyophilized bacterial preparation)) and 5 mM succinate. Note should be made that yeast G6PDH could not be used for correct assays because of contamination with adenylate kinase. It has been shown that G6PDH catalyzes the glucose dehydrogenase reaction stimulated by P_i (the substrate of oxidative phosphorylation) [18, 19]. To avoid this complication the concentration of glucose in the assay system was decreased (1 mM) to make the glucose dehydrogenase reaction negligible, whereas G6PDH activity did not limit the rate of oxidative phosphorylation. An increase of absorption at 340 nm during assays was abolished in the presence of malonate or uncoupler. In the coupled assay system the respiration was started by the addition of vesicles, and ATP synthesis was initiated by the addition of either ADP (in the presence of P_i) or P_i (in the presence of ADP). The addition of particles to the assay system containing ADP in the absence of P_i resulted in uncoupler- and malonate-sensitive NADPH formation. The rate of this reaction was dependent on the amount of particles added (contamination by P_i) and was taken into account when K_m values for P_i were determined.

Respiratory activity of SBP. Respiratory rates were measured amperometrically with a covered platinum electrode. The coupling efficiency was determined as the ratio of the initial rates of ATP synthesis to the rate of respiration, the parameter similar to classic P/O ratio. Average values for the NADH- and succinate-supported oxidative phosphorylation were 2.1 and 1.7, respectively.

All the data are presented as representative results for a single experiment with a particular preparation of the vesicles. As will be shown, the absolute values of V_{max} and K_m depend on the coupling efficiency ($\Delta\tilde{\mu}_{H^+}$ magnitude), which was various in different preparations. The statistical treatment of data would require multiple repeats of all experiments to be done for variable particular preparations, a condition which was practically impossible. This does not mean that the data presented are results from a single experiment. All the dependencies of the reaction rates on variable parameters were determined several times (not less than three).

RESULTS

The vesicles prepared as described catalyzed succinate- and NADH-supported ATP synthesis (at saturating concentration of all substrates) at the rates of 0.2-0.3 and 0.3-0.5 μmole/min per mg of protein, respectively.

To create a kinetic model for any enzymatic reaction the dependencies of the reaction rate on concentration of all substrates, activators (inhibitors), and other ligands are required. When the energy transducing enzymes are under investigation an obvious difficulty arises, that is the participation of $\Delta\tilde{\mu}_{H^+}$ as an obligatory "substrate" of the reaction. The magnitude of $\Delta\tilde{\mu}_{H^+}$ built up by the enzyme under investigation or by another generator depends on multiple difficultly controlled factors and in most cases only qualitative estimation of its effect on the reaction rate is possible. Consequently, other standard parameters such as K_m and V_{max} can also be determined only qualitatively. Thus, the aim of this study was to answer only two questions: are the affinities for ADP and P_i to $F_o \cdot F_1$ mutually dependent, and what is the effect of $\Delta\tilde{\mu}_{H^+}$ (if any) on these affinities?

Figure 1 shows a dependence of the initial rate of the NADH-supported ATP synthesis on P_i concentration at saturating ADP. The dependence was satisfactory fitted to simple hyperbolae with corresponding to V_{max} and $K_m^{P_i}$

values of 0.45 μmole/min per mg of protein and 115 μM, respectively.

Attempts to evaluate the same dependence on ADP concentration using the same (pH-metric) assays were not successful. The pilot experiments showed that apparent affinity to ADP was too high to be measured by pH change due to the sensitivity limitation, and the rates of ATP synthesis at low ADP concentrations were measured as the succinate-supported reaction in the presence of ADP-regenerating system (see "Materials and Methods" section). Figure 2 shows that at saturating P_i the dependence was also simple hyperbolic with the parameters V_{max} and K_m^{ADP} of 0.24 μmole/min per mg of protein and 8 μM, respectively.

Next, the dependence of the initial rates of ATP synthesis on P_i at different ADP and vice versa was determined (Fig. 3).

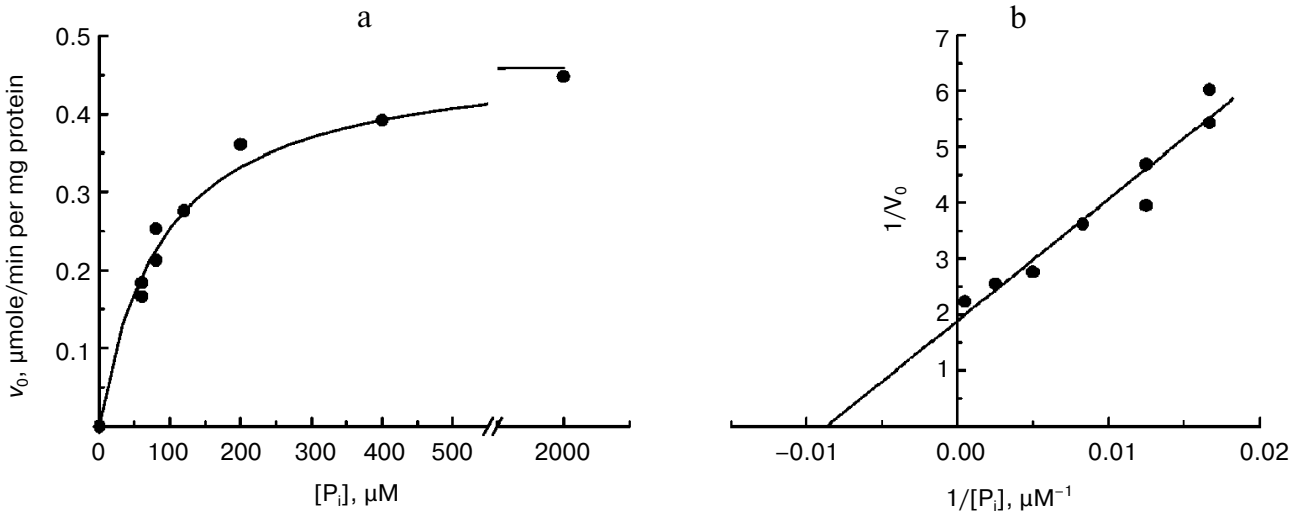

Fig. 1. Initial rates of ATP synthesis as a function of phosphate concentration in direct (a) and reciprocal (b) coordinates. The rates were measured as pH change in standard mixture containing NADH-regenerating system, 60 μM NADH, 25 μM Phenol Red, and potassium phosphate (concentrations are indicated on the abscissa). The reaction was started by the addition of 100 μM ADP after the mixture was preincubated for 2 min. The kinetic parameters determined from the double-reciprocal plot were: $K_m^{P_i}$ = 115 μM, V_{max} = 0.45 μmole/min per mg of protein.

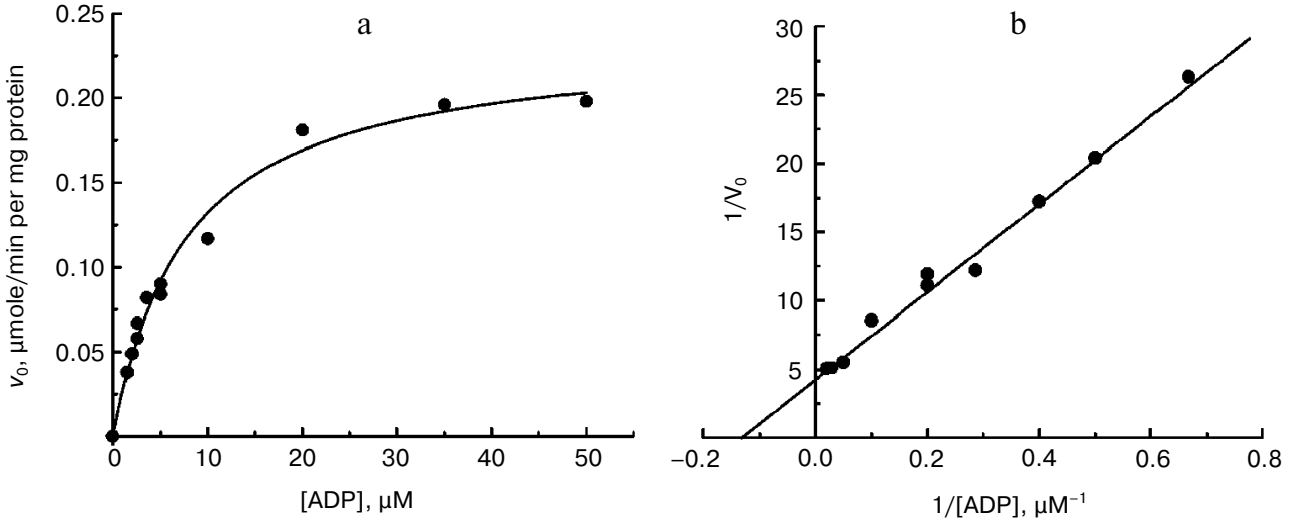

Fig. 2. Initial rates of ATP synthesis as a function of ADP concentration in direct (a) and reciprocal (b) coordinates. The rates were measured as absorption increase at 340 nm in the standard mixture containing 5 mM succinate, 1 mM potassium phosphate, ADP-regenerating system, and membrane preparation (25 μg protein per 1 ml). The reaction was initiated by the addition of ADP (concentrations are indicated on the abscissa) after the mixture was preincubated for 2 min. The kinetic parameters determined from the double-reciprocal plot were: K_m^{ADP} = 8 μM, V_{max} = 0.24 μmole/min per mg of protein.

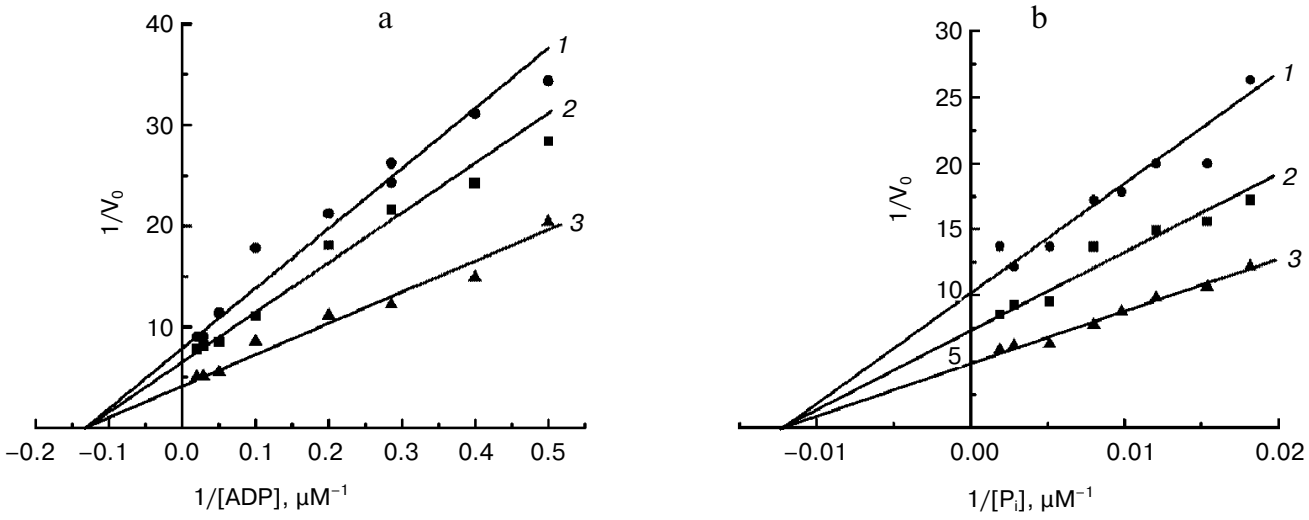

Fig. 3. Initial rates of ATP synthesis as a function of ADP (a) and P_i (b) concentration. a) Lines: *1-3*) concentrations of potassium phosphate were 50, 100, and 500 μM, respectively. b) Lines: *1-3*) concentrations of ADP were 5, 10, and 50 μM, respectively. The initial rates were measured as described in Fig. 2.

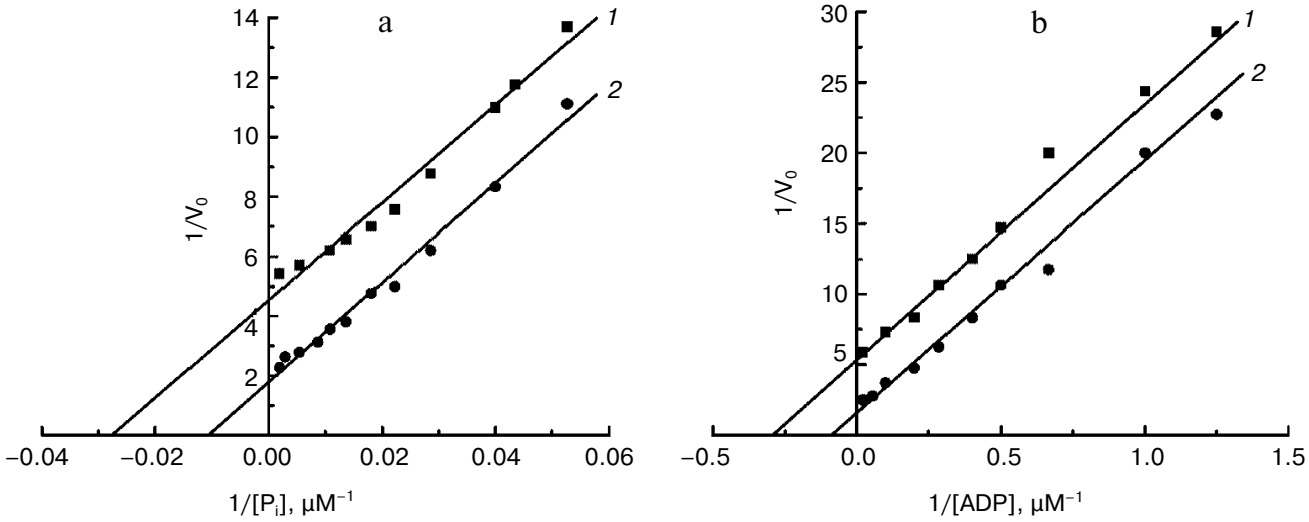

Fig. 4. Effect of malonate on the kinetic parameters of ATP synthesis: a) K_m for P_i; b) K_m for ADP. The initial rates were measured as described in Fig. 2. Lines: *1*) 0.4 mM malonate was added; *2*) control (no malonate).

To find out what step in the overall ATP synthesis is energy-dependent, the dependence of apparent ADP and P_i affinities to $F_o \cdot F_1$ was evaluated. The magnitude of $\Delta\tilde{\mu}_{H^+}$ could be varied by either a decrease of respiratory activity or by increase of the proton permeability by protonophoric uncoupler. With succinate as the respiratory substrate, malonate the competitive inhibitor of succinate dehydrogenase [20] was used. As shown in Fig. 4, gradual inhibition of respiration resulted in a decrease of the maximal rates and parallel decrease of apparent K_m values for ADP and P_i. Our attempts to measure the same dependence when NADH was the respiratory substrate met some difficulties. Use of high-affinity specific inhibitors of NADH oxidation such as rotenone [21], piericidin, NADH-OH [22] acting at the amount comparable with Complex I content seemed unjustified because this type of inhibitors were expected to induce heterogeneity of the enzyme preparation, that is apparent decrease of Complex I content, not its activity. Capsaicin, an inhibitor with relatively low affinity to Complex I (I_{50} ~ 50 μM [23]), showed uncoupling effect (the data are not presented). The other low affinity competitive Complex I inhibitor, ADP-ribose

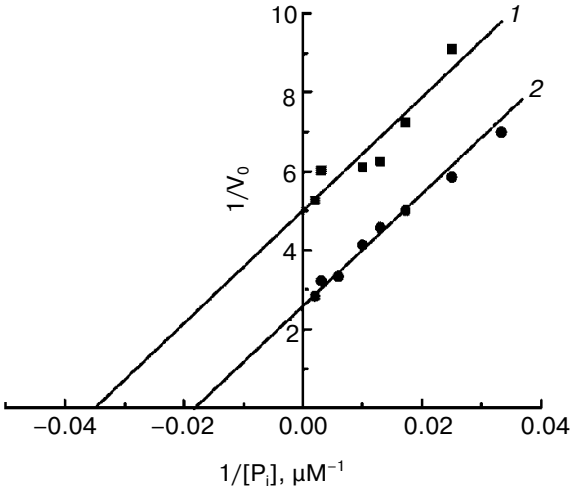

Fig. 5. Initial rates of ATP synthesis at different rates of NADH oxidation. The rates of hydrogen ions consumption were measured as Phenol Red response. The standard reaction mixture was supplemented with the NADH-regenerating system and 3 μM (*1*) and 60 μM NADH (*2*). Concentrations of potassium phosphate are indicated on the abscissa. The reaction was initiated by the addition of 100 μM ADP after 2 min preincubation.

(K_i = 80 μM [24]), could also not be used because it inhibited $F_o{\cdot}F_1$ as was evident by a decrease of the succinate-supported ATP synthesis. Our trials to use protonophoric uncouplers were also unsuccessful: high affinity uncoupler S-13 (2′,5′-dichloro-3-*tret*-butyl-4′-nitrosalicylanilide) could induce apparent heterogeneity (see above) and the other uncoupler, CCCP, was found to inhibit $F_o{\cdot}F_1$ itself. Finally, the rate of NADH oxidation was altered by the addition of various concentrations of NADH to the system containing the NADH-regenerating system (alcohol dehydrogenase plus ethanol, see "Materials and Methods" section). With this experimental setup results similar to those obtained with the succinate-supported $\Delta\tilde{\mu}_{H^+}$ generation were obtained. Figure 5 shows that a decrease of steady-state NADH level from 60 to 3 μM resulted in proportional decrease of the maximal ATP synthesis rate and apparent K_m for P_i. Obviously, the experimental setup with the NADH-regenerating system could not be used for oxidative phosphorylation assay with glucose 6-phosphate dehydrogenase, and we were unable to evaluate the effect of $\Delta\tilde{\mu}_{H^+}$ on apparent affinity of $F_o{\cdot}F_1$ to ADP.

DISCUSSION

This study was aimed to describe oxidative ATP synthesis in terms of a first approximation kinetic scheme for the same enzyme preparation and the same experimental conditions as has been done previously for ATP hydrolysis [10]. The results obtained are schematically summarized in Fig. 6a and briefly discussed below.

Hyperbolic dependence of ATP synthesis and hydrolysis rates on the substrates concentrations. The substrate-binding part of $F_o{\cdot}F_1$ (F_1) bears at least three nucleotide-binding sites rapidly equilibrating with the surrounding medium, and non-trivial dependence of the catalytic activity on substrate concentration (ATP, ADP, and P_i) is obviously expected. Indeed, kinetic cooperativity in the ATP hydrolysis reaction has been described in many reports published before the 1980s. In 1979 we showed that apparent cooperativity of ATP hydrolysis catalyzed by bovine heart $F_o{\cdot}F_1$ or F_1 is due to unusual hysteretic properties of the enzyme [8, 9], and now almost all groups agree that F_1-ATPase catalyzes ATP hydrolysis with simple Michaelis–Menten kinetics (K_m^{ATP} ~ 0.1 mM). This certainly does not mean that the catalytic sites operate independently; rather it suggests that the cooperativity is absolute (or close to absolute) and deviation from simple hyperbolic kinetics can be seen only at very low ATP concentration (<0.1 μM). A simple single-binding site model thus can be used for the $F_o{\cdot}F_1$-ATPase kinetics within (10^{-3}-1) mM substrate range. The results presented in this paper show that the simple "single site" kinetics (for both ADP and P_i) is observed for the ATP synthesis reaction (Figs. 1 and 2). To the best of our knowledge, this study is the only one where the initial rates of oxidative phosphorylation were continuously followed. The kinetics of oxidative phosphorylation catalyzed by *P. denitrificans* membranes have been previously studied [11] using $^{32}P_i$ incorporation technique (glucose–hexokinase coupled assay system). The cooperativity in ADP dependence with two different K_m (7-13 μM and ~50 nM) corresponding to 50-fold difference in V_{max} has been reported [11]. The first K_m^{ADP} value agrees with that determined in this report (Fig. 2). We believed that the second K_m and V_{max} values should be taken with great precautions because of the sensitivity and assay precision limits. The dependence

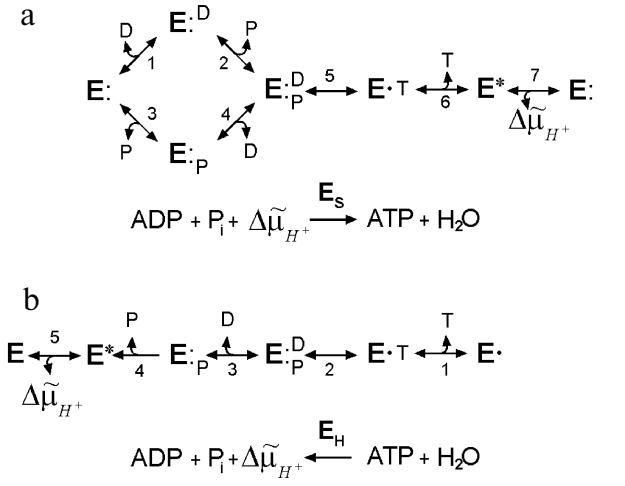

Fig. 6. Schemes describing kinetics of ATP synthesis (a) and hydrolysis (b) catalyzed by $F_o{\cdot}F_1$. See text for details and discussion.

of the reaction rates on P_i concentration is also hyperbolic with K_m of 60-120 μM (Fig. 1), a value that agrees with that reported by other groups [11, 25-28]. It worth noting that at least three P_i-binding sites should be present in the F_1 molecule according to the conventional models of its operation. The presence of three nucleotide (ATP, ADP, and their analogs)-binding sites has been directly demonstrated by their binding, and F_1 was found to bind only one or two $^{32}P_i$ molecule [29, 30].

Formation of the productive enzyme–substrate complex. The results shown in Fig. 3 suggest random formation of the enzyme ADP·P_i complex. The same conclusion has been reached in studies where the inhibition of the initial rates of ATP synthesis by ATP and kinetics of the reaction progress curves were analyzed [11]. Both approaches seem to be difficult to use for ATP synthesis kinetics. The inhibitory effect of a product on the initial rates as well as the reaction progress curves for a bi-substrate one-product reaction is informative only if the reaction is irreversible. Indeed, *P. denitrificans* $F_o{\cdot}F_1$ does not catalyzed ATP hydrolysis under "usual" experimental conditions [31]. Apparently this feature was an assumption for the analysis that was done in reference [11]. We have shown, however, that high proton-translocating ATP hydrolase activity of *P. denitrificans* vesicles does exist if the coupling membrane is energizes, i.e. under the condition required for ATP synthesis [15]. In other words, both ATP synthesis and hydrolysis proceed at any ADP and ATP concentration (in the presence of P_i) under energized conditions, and the net ATP synthesis reaction is not irreversible.

Effect of $\Delta\tilde{\mu}_{H^+}$ on the kinetics of oxidative phosphorylation. We showed that limitation of respiratory activity that is a parameter affecting the steady-state $\Delta\tilde{\mu}_{H^+}$ magnitude results in proportional decrease of the maximal rate and apparent K_m values for the substrates (parallel lines in Figs. 4 and 5, double-substitution kinetics, ping-pong mechanism). Oxidative phosphorylation (if respiration is to be excluded from consideration) is a three-substrate reaction in which proton (or hydroxyl anion) moving across the coupling membrane serves as a substrate along with ADP and P_i. Since the productive enzyme·ADP·P_i complex is formed randomly, the ATP synthesis can be analyzes as a bi-substrate reaction where ADP + P_i is one substrate and $\Delta\tilde{\mu}_{H^+}$ is the other one. The ping-pong mechanism (double-substitution) is the process (by definition) where irreversible step between the enzyme complexes with the first and the second substrate exists. This certainly does not mean that the enzymes operating by this mechanism catalyze irreversible processes only: "irreversibility" arises because of the initial rates measurement, i.e. under the conditions where concentration of at least one product is negligible. If this reasoning is to be applied for the reaction sequence depicted in Fig. 6a, the irreversible steps are either 5 (ATP formation from ADP and P_i bound at the active site) or 7 ($\Delta\tilde{\mu}_{H^+}$-dependent transformation of "relaxed" enzyme into its catalytically competent form). The first possibility seems unlikely: the equilibrium constant for the reaction E·(ADP + P_i) ↔ E·(ATP) at the F_1 active site was found to be close to 1 [32]. Mechanistically $\Delta\tilde{\mu}_{H^+}$ involvement in step 7 (Fig. 6a) means that only "empty" α·β-subunit pair is capable of energy-dependent transformation into the state that binds the substrates (ADP and P_i). One-step participation of $\Delta\tilde{\mu}_{H^+}$ in the overall catalysis is considered in the scheme for sake of simplicity. It appears that synthesis of one ATP molecule is coupled with more than one proton translocation (the exact number is not known), so participation of $\Delta\tilde{\mu}_{H^+}$ may be distributed among several elementary steps. Numerous reports on the effects of $\Delta\tilde{\mu}_{H^+}$ on the kinetics of ATP synthesis or hydrolysis have been published [12, 33-40]. A decrease of apparent affinity to the substrates (ADP and P_i) upon de-energization was observed in the majority of those reports. Great precaution should be made when high affinity protonophores and ionophores (valinomycin, nigericin) or high affinity respiratory inhibitors are used to decrease $\Delta\tilde{\mu}_{H^+}$. First, high affinity modifiers are expected to induce enzyme heterogeneity: the observed half-maximal uncoupling may equally result from either complete uncoupling of half of the vesicles or from real half-maximal uncoupling of all vesicles. It is hard, if not impossible, to discriminate these possibilities. The contribution of induced heterogeneity to the observed effects depends on the relative rates of several processes, mostly on the rate of the modifier distribution between the vesicles. Second, lipophilic compounds (uncouplers, ionophores, respiratory inhibitors) may modify the properties of phospholipid bilayer, thus affecting $F_o{\cdot}F_1$. We believe that use of hydrophilic competitive (rapidly equilibrating) inhibitor, malonate, and a decrease of the steady-state level of NADH is better suited for gradual decrease of $\Delta\tilde{\mu}_{H^+}$ than highly lipophilic modifiers. Our results (Fig. 4) showed proportional decrease of the maximal rates and apparent K_m values, thus suggesting that the substrate concentration required for the half-maximal ATP synthesis rate is not a measure of this affinity to the enzyme active site but should be interpreted as pure kinetic parameters.

A comparison of the kinetics of ATP synthesis and hydrolysis. The steady-state proton-translocating ATPase activity is depicted in Fig. 6b [10]. To make a comparison of the synthetic and hydrolytic reactions easier, the sequence of the latter is shown from right to left. What particular step in the hydrolytic reaction is coupled with proton translocation is not known. To answer this question the data on the effects $\Delta\tilde{\mu}_{H^+}$ magnitude on the kinetic parameter of ATP hydrolysis are evidently needed. One obvious difficulty in such studies is expected to be a proper choice of the instrumental way of varying $\Delta\tilde{\mu}_{H^+}$ (see above). The other problem is that, as we have pointed out previously [7], the proton-translocating $F_o{\cdot}F_1$-ATPase is not a catalyst in the strict sense because part (unknown) of the free energy released during ATP hydrolysis is used to

maintain the enzyme in its active state. This seems to be important for physiology: an enzyme which spends cellular phosphoryl potential to keep the membrane energized, the condition required for metabolite uptake, must "be informed" that its activity is not futile. The important well-documented although often ignored fact is that inorganic phosphate, the product of ATP hydrolysis and the substrate for ATP synthesis, does not inhibit coupled or uncoupled $F_o \cdot F_1$ or F_1 ATPase activity even at concentrations much higher than its apparent K_m in the ATP synthase reaction (see reference [10] for a brief review of this problem). This suggests that dissociation of P_i from the enzyme active site during ATPase activity proceeds irreversibly. We propose that irreversible P_i release is coupled with the energization of the enzyme that contains no product at the active site (empty $\alpha \cdot \beta$-subunits pair), and its relaxation to the structure capable of ATP-binding (step 5 in Fig. 6b) is the $\Delta\tilde{\mu}_{H^+}$-generating step. The prominent difference in the sequences depicted in Figs. 6a ($\Delta\tilde{\mu}_{H^+}$-dependent synthesis) and 6b ($\Delta\tilde{\mu}_{H^+}$-generating hydrolysis) is the existence of the intermediate containing ADP only at the active site in sequence of Fig. 6a and its absence in sequence of Fig. 6b.

As we have emphasized previously [7, 15], the ATP synthesis and ATP hydrolysis reactions are catalyzed by different forms of $F_o \cdot F_1$, synthase and hydrolase, respectively. The proportion between these forms depends on $\Delta\tilde{\mu}_{H^+}$ and ATP/ADP ratio. The ε-subunit of $F_o \cdot F_1$, which is shown to change its conformation upon ATP binding [41], may serve as the ATP concentration sensor. The presence of $F_o \cdot F_1$ ATPase/ATP synthase "isoenzymes" should result in futile ATP hydrolysis cycle at any given ATP/ADP ratio and $\Delta\tilde{\mu}_{H^+}$ magnitude. The possible physiological function of such a cycle may consist in high sensitivity of the overall oxidative phosphorylation rate to the cellular energetic status.

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