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STEADY-STATE AND PRE-STEADY-STATE KINETICS OF THE MITOCHONDRIAL F_1F_o ATPase: IS ATP SYNTHASE A REVERSIBLE MOLECULAR MACHINE?

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Summary

H^+-ATP synthase (F_1F_o ATPase) catalyzes the synthesis and/or hydrolysis of ATP, and the reactions are strongly affected by all the substrates (products) in a way clearly distinct from that expected of a simple reversibly operating enzyme. Recent studies have revealed the structure of F_1, which is ideally suited for the alternating binding change mechanism, with a rotating γ-subunit as the energy-driven coupling device. According to this mechanism ATP, ADP, inorganic phosphate (P_i) and Mg^{2+} participate in the forward and reverse overall reactions exclusively as the substrates and products. However, both F_1 and F_1F_o demonstrate non-trivial steady-state and pre-steady-state kinetics as a function of variable substrate (product) concentrations. Several effectors cause unidirectional inhibition or activation of the enzyme. When considered separately, the unidirectional effects of ADP, P_i, Mg^{2+} and energy supply on ATP synthesis or hydrolysis may possibly be explained by very complex kinetic schemes; taken together, the results suggest that different conformational states of the enzyme operate in the ATP hydrolase and ATP synthase reactions. A possible mechanism for an energy-dependent switch between the two states of F_1F_o ATPase is proposed.

Key words: F_1F_o ATPase, microreversibility, energy transduction, mitochondrial membrane, oxidative phosphorylation.

Introduction

Mitochondrial H^+-ATP synthase (F_oF_1-H^+ ATPase) catalyzes the reaction:

$$ADP + P_i + nH^+_{out} + mH^+ \xrightarrow{Mg^{2+}} ATP + H_2O + nH^+_{in}, \quad (1)$$

where n is the quotient for stoichiometric vectorial proton translocation from the cytoplasm (H^+_{out}) to the matrix space (H^+_{in}) and m is the stoichiometric quotient for scalar proton consumption, which is determined by the difference in total acidity of the substrates (ADP and inorganic phosphate) and product (ATP). The value of n (2–4) is not definitely established; m is close to 1 at pH>7.5 and depends predominantly on pH and Mg^{2+} concentration, forming more (ATP) or less (ADP and P_i) stable complexes with the reaction product and substrates. The enzyme is composed of two distinct parts. The F_1 sector ($\alpha_3\beta_3\gamma\delta\varepsilon$ minimal subunit structure) bears catalytic sites where nucleotide-substrate/product and presumably P_i bind. The membrane-spanning F_o sector forms a proton-conducting path. It is generally accepted that work done by the flow of H^+ through F_o is transformed into the chemical work needed to form ATP as described by equation 1. Energy-dependent ATP synthesis is phenomenologically reversible: ATP hydrolysis in tightly coupled membranous preparations results in a formation of the difference between the electrochemical potentials of hydrogen ions on different sides of the coupling inner mitochondrial membrane ($\Delta\bar{\mu}_{H^+}$). Whether the ATPase activity of these preparations is a simple reversal of ATP synthesis is not known because the precise mechanisms of ATP hydrolysis and its synthesis are not known. Spectacular achievements have been made in elucidating the ternary structure of F_1 at an atomic resolution of 2.8×10^{-10} m (Abrahams et al., 1994; Bianchet et al., 1998). F_1 appears as a trimer of tightly packed αβ-subunit pairs and a long rod-like γ-subunit, which is positioned in the central cavity of the spherical globular protein. The ternary structure of smaller δ- and ε-subunits and their positions relative to the $\alpha_3\beta_3\gamma$ heptamer have not been revealed by crystallography, although structural features of the *Escherichia coli* ε-subunit and possible sites of its interaction with the γ-subunit of F_1 and F_o have been determined by nuclear magnetic resonance spectroscopy (Wilkens et al., 1995). The F_o sector has the structure ab_2c_{12} (the minimal composition); only the structure of the *c* subunit, which seems to be a key component of the proton-conducting path, is well established (Fillingame, 1997). The catalytic sites residing on F_1 are located far away from the plane of the coupling membrane. This spatial separation suggests that $\Delta\bar{\mu}_{H^+}$-dependent ATP synthesis must be a complex electro-mechano-chemical process. The rotational alternating binding change mechanism, originally based on quantitative measurements of isotope exchange reactions (Gresser et al., 1982) and supported by numerous studies including elegant demonstrations of strong negative

cooperativity in ATP binding and positive cooperativity in ATP hydrolysis (Grubmeyer and Penefsky, 1981; Grubmeyer et al., 1982), is now widely accepted (Boyer, 1998). Essential features of this mechanism are as follows: (i) the free energy produced by H^+ flow is used to release tightly bound ATP spontaneously (isoenergetically) formed at the very high affinity ATP-binding site of the enzyme; (ii) each catalytic site sequentially (in the turnover time scale) appears as: empty → tight → loose in terms of occupation and tightness of nucleotide binding; and (iii) the occupation and mode of nucleotide binding at one catalytic site within the trimer strictly determine the properties of two other sites. The most plausible way to realize such a mechanism is to rotate one element of the F_1 structure relative to the trimerically arranged catalytic sites (Boyer, 1993). The spatial structure of F_1 seems to fit ideally the proposed mechanism. Perhaps the most impressive achievement of bioenergetic enzymology during the last decade was the original prediction and further indirect demonstration (Duncan et al., 1995; Junge et al., 1996; Sabbert et al., 1996) and final direct visualization (Noji et al., 1997) of ATP-dependent rotation of the γ-subunit (the rotor) within three αβ-subunit pairs (the stator).

The rotational alternating binding change mechanism apparently leaves no alternative but for ADP, P_i, ATP and $\Delta\tilde{\mu}_{H^+}$ to participate in the overall reaction in a way different from that required for the substrate/product binding/release and driving force as depicted in equation 1. However, the kinetic properties of either soluble F_1 or membrane-bound F_1F_o are far from being trivial. The purpose of this review is twofold. First, I will try to summarise the standard (steady-state) and non-standard (slow pre-steady-state) kinetic properties of the enzyme. They will be described mainly for bovine heart mitochondrial ATPase, and data for other ATPases of the F_1 family (*Escherichia coli*, chloroplasts, thermophilic bacteria) will be referred to only when absolutely necessary. Countless papers relevant to the subject have been published during last 20–30 years. A newcomer to the field is often faced by a number of contradictory statements circulating in various reviews. This paper is by no means a comprehensive review, but rather a summary of some studies performed by my own group.

The second problem to be discussed briefly concerns the reversibility of F_1F_o ATP synthase. Analysis and further development of the rotational mechanism of F_1 ATPase, which is now often considered to be the smallest electro-mechanical machine, has resulted in the introduction of new terminology to describe the enzyme: a number of terms previously unknown to enzymology, such as rotor, stator, torque, shaft, spring, etc., are now widely used in the current literature. If, as according to several language dictionaries, a general definition of a machine is an 'assemblage of parts that transit forces, motions and energy from one to another in a predetermined manner' is to be accepted, the question arises: do reversible molecular machines exist? In my opinion, the molecular machines created by Nature and those created by mankind are made exclusively for unidirectional energy transduction. Thus, a special mechanism that can be switched on or off by the applied driving force, thus permitting unidirectional energy transmission, must be a component of any machine.

General remarks on the kinetics of mitochondrial ATPase

When soluble F_1, oligomycin-sensitive purified F_1F_o or submitochondrial particles are added to a reaction mixture containing ATP and Mg^{2+}, rapid hydrolysis of ATP is observed. The enzyme activity under 'optimal' conditions has a turnover number of several hundreds per second. It might be expected that the standard kinetic parameters of this remarkably stable and highly active enzyme could easily be determined. However, this is not the case. Meaningful interpretation of enzyme kinetics is only possible when initial steady-state rates are measured. When the hydrolytic activity of F_1-type ATPase is considered, the initial rate, as such, is not an unambiguous term. There are several reasons for this. First, many preparations contain a small natural inhibitory peptide, an ATPase inhibitor protein (Pullman and Monroy, 1963), which is an intrinsic part of the mitochondrial F_1F_o ATP synthase. The inhibitory effect of this protein is time-, pH-, ionic-strength- and ATP-dependent (Harris and Das, 1991; Panchenko and Vinogradov, 1985, 1989) and may also be dependent on $\Delta\tilde{\mu}_{H^+}$ and on the presence of divalent cations (Vasilyeva et al., 1989; Yamada and Huzel, 1988). It appears that uncontrolled contamination of the protein inhibitor may be a source of serious errors in measurements of the actual initial velocities of ATPase in many early studies, including our own (Akimenko et al., 1972). Second, the product of ATP hydrolysis, ADP, is an inhibitor of all ATPase preparations. One type of inhibition (rapidly equilibrating competitive inhibition) can be easily prevented if ATP-regenerating systems, such as phosphoenolpyruvate and pyruvate kinase or phosphocreatine and creatine kinase, are used in the assays. However, another unusual time-dependent inhibition by ADP (Fitin et al., 1979) is a potential source of serious errors in the ATPase assays even in the presence of ATP-regenerating systems. When ATP hydrolysis is followed using continuous methods, the rate of product release is almost always biphasic or triphasic, depending on the 'history' of a particular preparation. It shows a 'burst' or lag, and the time-dependence of the active → inactive or inactive → active transition is a very complex function of the concentrations of nucleotides and $[Mg^{2+}]$ (Bulygin and Vinogradov, 1988; Vasilyeva et al., 1980, 1982a; Vinogradov, 1984). Thus, the possibility that erroneous conclusions may be reached from the measurements of apparent 'initial' rates is considerable. Although the details of the unusual hysteresis behaviour of ATPase have been discussed (Vinogradov, 1984), only a few research groups have paid attention to this phenomenon, and the results of ATPase kinetics studies should be interpreted with caution.

Steady-state ATP hydrolysis

Some standard kinetic parameters of ATP hydrolysis by the

Table 1. *Steady-state kinetic parameters for mitochondrial F_oF_1 ATPase (submitochondrial particles)*

Substrate or product	Effect on the initial rate	Apparent affinity
ATP	Simple Michaelis–Menten kinetics at saturating [Mg^{2+}]. Inhibition at high concentrations when [Mg^{2+}] is limiting	$K_m^{ATP\cdot Mg}=10^{-4}$ mol l^{-1}
ADP	Competitive inhibition	$K_i^{ADP}=3\times10^{-4}$ mol l^{-1}
P_i	No effects	
Mg^{2+}	Simple Michaelis–Menten kinetics at constant [ATP·Mg]	$K_s=2\times10^{-5}$ to 10^{-4} mol l^{-1}, pH-dependent
pH	Very broad pH maximum at saturating [ATP·Mg] and free [Mg^{2+}]	pKa=7.4 at limiting [Mg^{2+}]

The quantitative data (right-hand column) are given for pH 7.5, 25 °C, at an ionic strength of 0.1 mol l^{-1} (KCl), and refer to the initial rates (10–30 s) of ATP hydrolysis by activated (preincubation with either 10 mmol l^{-1} potassium phosphate or phosphoenolpyruvate and pyruvate kinase) F_oF_1 ATPase.

mitochondrial ATPase are summarized in Table 1 and briefly discussed below.

Adenosine triphosphate

ATP and other nucleotides (ITP, GTP) are hydrolyzed at significant rates only in the presence of Mg^{2+} or some other divalent cations (Mn^{2+}, Co^{2+}, Fe^{2+}). At high Mg^{2+} concentrations (2–10 mmol l^{-1} at pH 7.0–8.0), the dependence of the hydrolytic rate on ATP concentration is a simple hyperbolic function within the range 2–5000 μmol l^{-1} with an apparent K_m of approximately 10^{-4} mol l^{-1}. This finding contrasts with several reports in the literature in which two or three K_m values for ATP hydrolysis have been found. There are strong reasons to believe that the non-hyperbolic dependence of ATPase activity on ATP concentration is due to an erroneous perception of the observed rates as the initial ones (see above). It is worth noting that the experimentally well-documented strong negative cooperativity in ATP binding and extremely positive cooperativity in hydrolytic performance (Grubmeyer and Penefsky, 1981; Grubmeyer et al., 1982) do not conflict with the simple Michaelis–Menten kinetics of steady-state ATP hydrolysis. So-called unisite catalysis is seen under conditions when the enzyme concentration is several times higher than that of ATP. The affinity for ATP binding to the 'first' catalytic site is extremely high (approximately 10^{-12} mol l^{-1}; Grubmeyer et al., 1982; Penefsky, 1985; Souid and Penefsky, 1995), and this concentration range is never covered in the steady-state kinetic measurements. Also, simple Michaelis–Menten kinetics do not conflict with the alternating binding change mechanism (see Introduction): if cooperativity of binding is very strong or absolute (as in the 'flip-flop' mechanism; Lazdunski, 1972), a single 'entrance' for the substrate and a single 'exit' for the products would exist. An interesting possibility is that, during the steady-state performance of F_1-type ATPase/synthase, a 'gate' and special 'path' operate to determine the penetration of ATP (or ADP) from the medium into a 'catalytic cavity' where three exchangeable sites (out of a total of six) are located. In the light of the structural arrangement of F_1, it seems unlikely that what are assigned as 'catalytic' and 'noncatalytic' sites on the β and α subunits are readily accessible to nucleotides in solution.

The K_m value for ATP depends strongly on Mg^{2+} concentration. A high ATP concentration (a significant molar excess over [Mg^{2+}]) inhibits ATPase activity. This inhibition is most likely due to a decrease in free [Mg^{2+}], which is needed (in addition to that bound as an ATP·Mg complex) for the catalysis (Syroeshkin et al., 1999).

Adenosine diphosphate

ADP, the product of the ATPase reaction, is an inhibitor of the hydrolytic activity. Quantitative and qualitative parameters for inhibition by ADP in terms of steady-state kinetics are difficult to determine because ADP slowly deactivates the enzyme (the term 'deactivation' will be used throughout this article to discriminate between a decrease of only the initial velocity (i.e. appearance of the lag phase in the enzyme performance) and the usual inhibition of the activity (see below). From the initial rate measurements, it has been concluded that ADP is a purely competitive inhibitor (with ATP) of hydrolysis (Hammes and Hilborn, 1970). We found that free ADP, not the ADP·Mg complex, competes with the ATP·Mg complex for the 'substrate-binding' site (Syroeshkin et al., 1999). This finding is of particular interest because the ADP·Mg complex has been shown to be a true substrate for photophosphorylation in chloroplasts (Zhou and Boyer, 1992) and for oxidative phosphorylation in submitochondrial particles (Galkin and Syroeshkin, 1999). Certainly, as pointed out above, one needs to define clearly what is assumed to be the 'substrate-binding site': one of these that are seen in the resolved F_1 structure, or the 'gate-specific path' step which may contribute significantly to the overall steady-state reaction.

Inorganic phosphate

Although P_i is equally as important as ADP as a substrate/product of ATP synthase, only a few studies, compared with those on nucleotide binding, are available on its specific effects on the mitochondrial ATPase. Soluble F_1

has been shown to bind P_i with a relatively high affinity (Kasahara and Penefsky, 1978; Penefsky, 1977). It would be logical to expect that P_i must inhibit ATP hydrolysis with an apparent K_i similar to its K_m value for oxidative phosphorylation (in the millimolar range). However, this is not the case, and P_i either has no effect on ATP hydrolysis or it slightly stimulates ATPase activity, as do several other anions. It is extremely difficult, if not impossible, to construct any kinetic scheme that includes reversible P_i release and that does not include a P_i inhibition term in an equation for the steady-state reaction rate. This problem has somehow been overlooked in detailed discussions and experimental studies of the enzyme. The only possible explanation for the absence of an inhibitory effect is that P_i irreversibly dissociates from the active site during ATP hydrolysis: this proposal immediately creates an unavoidable problem in viewing ATP synthase as a reversible ATPase.

Mg^{2+}

It is well established that Mg^{2+} (or some other divalent cation) is needed for ATPase activity (Selwin, 1967; Akimenko et al., 1972; Fleury et al., 1980). Since the early publications on ATPase kinetics (Selwin, 1967; Fleury et al., 1980), statements on the inhibition of ATPase by excess free Mg^{2+} have been widely circulated in the literature. Recently, we have reinvestigated the problem and found no inhibition of enzyme activity by Mg^{2+} (up to 10–15 mmol l^{-1}) provided that true initial rates are measured. It appears that the previously reported inhibitory effects of Mg^{2+} were due to underestimations of another effect: Mg^{2+}-induced, ADP-dependent (or *vice versa*) slow deactivation of the enzyme (Bulygin and Vinogradov, 1991; Guererro et al., 1990). Moreover, we found that free Mg^{2+}, in addition to that bound in the true substrate ($ATP{\cdot}Mg^{+}$), is needed for ATPase activity. The kinetic analysis suggests that activating Mg^{2+} is involved in the overall reaction according to the so-called 'ping-pong' mechanism, i.e. it participates after irreversible dissociation of one of the products from the active site. When only a limiting concentration of free Mg^{2+} is available, ATPase activity becomes strongly pH-dependent, as to be expected if Mg^{2+} binds to a deprotonated group with a pKa of 7.4 (Syroeshkin et al., 1999).

Proton-motive force

The ATPase activity of intact mitochondria is greatly stimulated by uncoupling agents. Unfortunately, the ATPase activity of coupled submitochondrial particles is only slightly (not more than twofold) accelerated when $\Delta\bar{\mu}_{H^+}$ is collapsed. Our knowledge on the effects of $\Delta\bar{\mu}_{H^+}$ on steady-state ATP hydrolysis is very limited.

Summary

To summarise, the simplest kinetic scheme for the steady-state ATPase reaction is depicted in Fig. 1. The initial steady-state rate reaction mechanism (under conditions where slow deactivation of the enzyme is prevented) is shown as the sequence of steps 1–5. ATP·Mg is the species taken up as the substrate. After the hydrolytic step (step 2), P_i leaves the active site before ADP. The absence of any inhibitory effect of P_i on F_1F_o ATPase suggests that it leaves the enzyme during

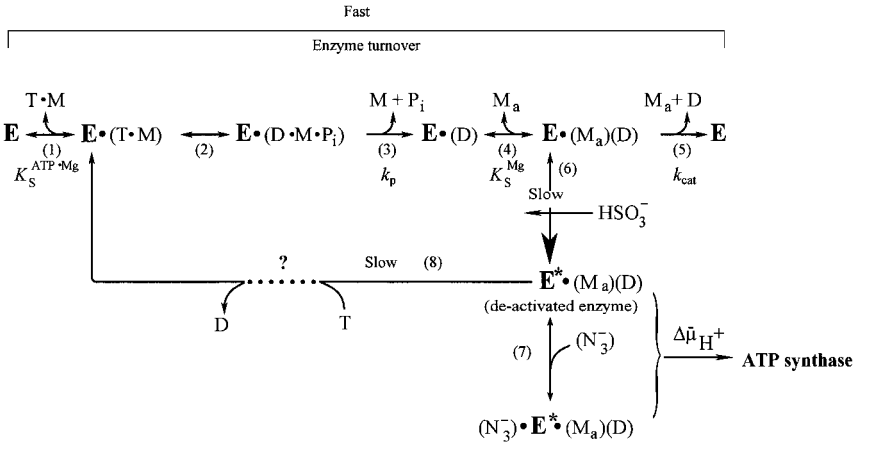

Fig. 1. Reaction pathways for ATP hydrolysis catalyzed by F_1F_o ATPase. **E**, T, D, P_i, M and M_a stand for the enzyme, ATP, ADP, inorganic phosphate, Mg^{2+} originating from the ATP·Mg complex (T·M) and free activating Mg^{2+}, respectively. The scheme does not attempt to portray the interactions between the enzyme subunits. The hydrolytic steps, steps 2–5, are almost certainly dependent on cooperative interactions within the multisubunit enzyme [the alternating binding change mechanism (Boyer, 1993), which may include γ-subunit rotation (Noji et al., 1997)]. The scheme is intended to describe the fate of the substrate and enzyme–substrate complexes during catalytic turnover steps 1–5. Steps 4 and 5 are shown as binding and release of Mg^{2+} for the sake of clarity; no differences in the kinetic behaviour are expected if activating Mg^{2+}, participating in the reaction cycle after P_i release, remains permanently bound to the enzyme during catalysis. K_s is the dissociation constant of the first enzyme–substrate complex $\mathbf{E}_{\cdot(T\cdot M)}$; the relative contributions of the first-order rate constants of the dissociation of P_i (k_p) and ADP (k_{cat}) to the apparent K_m for the ATP·Mg complex, compared with that of K_s, depends on the concentration of activating Mg^{2+}. Slow deactivation of the enzyme is described by step 6 (isomerization of the enzyme–product complex, which is prevented by some anion activators such as sulphite) or by a combination of steps 5 and 6 (deactivation by added ADP and Mg^{2+}). The irreversibility of step 5 holds only for the initial rates, when the concentration of ADP is small enough to be neglected. Step 8 describes ATP-dependent activation of the deactivated enzyme; the ionic species of ATP and the mechanism of activation (Fitin et al., 1979; Jault and Allison, 1994) remain to be established. Deactivated forms of the enzyme (**E***) (whether or not stabilized by azide; step 7) are proposed to be transformed into ATP synthase *via* an energy-dependent mechanism (see Fig. 2). What particular component of the total proton-motive force ($\Delta\bar{\mu}_{H^+}$) [electrical potential difference ($\Delta\psi$) or pH, or both] is involved in the energy-dependent transformation of the enzyme is not known.

uncoupled ATP hydrolysis from a site with very low or no affinity and is taken as the thermodynamic basis for the kinetic sequence of product release. The simple competitive inhibition of ATPase by free ADP is in accord with the reaction sequence depicted in Fig. 1, in which free ADP leaves the catalytic site *via* a Mg^{2+}-dependent mechanism. The data on tight binding of ADP and some nucleotide derivatives to F_1 in the absence of Mg^{2+} and on rearrangements of the nucleotide-binding sites after subsequent addition of Mg^{2+} (Burgard et al., 1994; Yoshida and Allison, 1983) are in accord with the proposed sequence. Since the structure of $\alpha_3\beta_3\gamma$ at 2.8×10^{-10} m resolution is now available (Abrahams et al., 1994; Bianchet et al., 1998), an obvious question arises: where is the Mg^{2+}-specific 'activating' site located? The structures of the nucleotide-binding sites in asymmetric F_1 containing Mg·nucleotide complexes bound to the α and β subunits have been visualised (Abrahams et al., 1994). Surprisingly, no Mg^{2+} other than that originating from the Mg·nucleotide complexes was seen, despite the presence of adenosine-5′-phosphoimidophosphate, ADP, Mg^{2+} and sodium azide and the absence of P_i in the crystallization medium (Lutter et al., 1993). There are several ways of explaining the absence of visualized Mg^{2+}. Significant rearrangement of F_1 subunits induced by an interaction between F_1 and F_o is one possibility (Böttcher et al., 1995). Another possibility is that the Mg^{2+}-specific site is located on the disordered 'invisible' part of the γ-subunit. This proposal may be substantiated by the finding that ATP hydrolysis catalyzed by the reconstituted $\alpha_3\beta_3\gamma$ complex of *Thermus thermophilus* F_1 is not sensitive to azide, which is known to inhibit complete $\alpha_3\beta_3$ ATPase by a Mg^{2+}-dependent mechanism (Bulygin and Vinogradov, 1991; Bulygin et al., 1993; Vasilyeva et al., 1982a). If the proposal for γ-subunit Mg^{2+}-binding is correct, the structural arrangements of the events shown as the reaction scheme in Fig. 1 would be as follows: free ADP that is left at the catalytic site after the departure of P_i·Mg is then released to the solution *via* an interaction with the Mg^{2+}-liganded arm of the γ-subunit. The key role of Mg^{2+} in energy-transducing enzymes was suggested many years ago (Jenkins et al., 1984; Racker, 1977, 1979) and merits further study.

Non-steady-state enzyme behaviour

Mitchell and Moyle (1971) were the first to observe a relatively slow time-dependence of some kinetic properties of the ATPase. This phenomenon, although demonstrated for ATPases from a variety of sources (Moyle and Mitchell, 1975; Rectenwald and Hess, 1977, 1979), was somehow ignored for a long time. In 1979, it was discovered that ADP is responsible for the slow transient kinetics of ATPase (Fitin et al., 1979). Since then, a number of detailed studies have shown that an ADP(Mg^{2+})-dependent slow active/inactive transition is a universal property of F_1-type ATPases (Jault and Allison, 1994; Matsui et al., 1997; and references cited therein) and, as has been shown more recently, is also a characteristic of V-type ATPases (Yokoyama et al., 1998). Some essential features of this still enigmatic phenomenon are briefly summarized below.

When ATP at relatively low concentrations ($\leqslant K_m$) is hydrolyzed by ATPase free of protein inhibitor in the absence of an ATP-regenerating system, a rapid decline in the activity is observed which is not due to a decrease in the substrate concentration. The addition of further enzyme results in an increase in activity that would be expected if the enzyme added previously had been irreversibly inactivated (Fitin et al., 1979).

Brief preincubation of F_1F_o (submitochondrial particles) or soluble F_1 with very low, almost stoichiometric, concentrations of ADP in the presence of Mg^{2+} results in a considerable lag (on a time scale of minutes) in the onset of ATPase activity measured in the presence of an ATP-regenerating system. Qualitatively, the same phenomenon is seen for standard F_1 preparations after preincubation with Mg^{2+} alone, but is absent when nucleotide-depleted F_1 is assayed with an ATP-regenerating system after preincubation with Mg^{2+} (Minkov et al., 1979).

The initial rates of ATP hydrolysis by submitochondrial particles or soluble F_1 measured under unified conditions (with or without regenerating enzymes) are poorly reproducible. The activities are markedly increased and become quite reproducible after prolonged preincubation of the preparations in the presence of phosphoenolpyruvate plus pyruvate kinase or phosphocreatine and creatine kinase. After such activation, the ATPase activity measured with an ATP-regenerating system becomes biphasic: a rapid initial rate slowly decreases to 50–70 % of the original level, and the reaction then proceeds at a constant rate (Vasilyeva et al., 1980, 1982a).

Neither azide (a specific inhibitor of F_1 ATPase) nor sulphite (an activator of the enzyme) affects the initial rapid phase of ATP hydrolysis, whereas the delayed steady-state rate is inhibited by azide and stimulated by sulphite (Vasilyeva et al., 1982b). Hydrolysis of other nucleotides (ITP, GTP) by activated ATPase occurs at a constant rate (no lags or 'bursts') and is insensitive to azide or sulphite. Preincubation of the enzyme with IDP or GDP has no effect on its ATPase activity, whereas preincubation with ADP completely blocks the initial rates of hydrolysis of other nucleotides.

The duration of the lag phase in the ATP regeneration assay depends on the ATP concentration approximately in the same way as the steady-state ATPase activity does itself. Thus, activation of the enzyme during preincubation with pyruvate kinase plus phosphoenolpyruvate (see above) occurs much more slowly than in the assay system in the presence of added ATP.

When ATPase is first deactivated by ADP+Mg^{2+} and then passed through a Sephadex column in the presence of EDTA to remove Mg^{2+}, the enzyme becomes 'active' and can again be deactivated by added Mg^{2+} (Bulygin and Vinogradov, 1991). In other words, F_1F_o bears a specific site for Mg^{2+} binding with an apparent affinity close to that measured in the steady-state kinetic experiments. Mg^{2+} affinity, as measured using these two independent approaches, is strongly pH-

dependent. It increases at alkaline pH and decreases upon acidification (Bulygin et al., 1993; Syroeshkin et al., 1999).

P_i, which does not affect the rate of ATP hydrolysis, strongly modulates the ADP(Mg^{2+})-induced deactivation. It decreases the affinity of the ADP-specific deactivating site, so that the enzyme can be reactivated equally well by either pyruvate kinase or P_i (Yalamova et al., 1982). P_i also significantly increases the rate of EDTA-induced activation (see above) (Bulygin and Vinogradov, 1991).

The results listed above show that, in addition to the rapid ATP hydrolytic steady-state turnovers (steps 1–5, Fig. 1), an additional pathway of enzyme transformation exists (as described in Fig. 1 by steps 6, 7 and 8) which explains the pre-steady-state kinetics of ATP hydrolysis and also the inhibitory and activating effects of azide and sulphite. This scheme as presented is an extension of that suggested previously (Vasilyeva et al., 1982b).

Unidirectional transformations of ATPase/synthase

ADP dissociates from its ATPase inhibiting site very slowly (the first-order rate constant varies from 0.1 to 1 min^{-1}). This site cannot therefore be considered as participating in the ATPase reaction. Many years ago, we showed that ADP(Mg^{2+})-deactivated ATPase is able to catalyze oxidative phosphorylation without any lag in ATP formation (Minkov et al., 1980). This observation led us to conclude that, when F_1 catalyzes ATP hydrolysis or $\Delta\bar{\mu}_{H^+}$-dependent ATP synthesis, it is in an alternative, slowly interconvertible, conformation. In other words, the microreversibility principle, which is usually quantitatively expressed in enzymology as Haldane relationships, does not hold for F_1 ATPase. The main difficulty in obtaining experimental evidence for or against such a proposal is that the conditions for measurement of the reaction in the two directions are quite different. Some recent findings relevant to this problem are briefly discussed below.

The ADP(Mg^{2+})-deactivated enzyme can be trapped by azide (see Fig. 1). Surprisingly, tightly coupled submitochondrial particles in which ADP-deactivated F_1 is trapped by azide catalyze succinate-supported ATP synthesis at the same rate as control samples, whereas both uncoupled ATPase activity and ATP-dependent $\Delta\bar{\mu}_{H^+}$ generation cease (Syroeshkin et al., 1995). Further studies of this unidirectional inhibition have revealed that $\Delta\bar{\mu}_{H^+}$ (a 'substrate' or 'product' of the reactions) is a strong effector of the F_1F_o complex (Galkin and Vinogradov, 1999). If ATP synthesis catalyzed by the azide-trapped deactivated enzyme is stopped because of the collapse of $\Delta\bar{\mu}_{H^+}$, no further hydrolysis of added ATP occurs. However, if ATP is added first, rapid hydrolysis occurs after subsequent addition of an uncoupler to the submitochondrial particles, and the reaction rate decreases slowly over time because of inhibition by azide. It has been shown that this striking effect of $\Delta\bar{\mu}_{H^+}$ is not due to back pressure (inhibition by a 'product'), and the only ligand that prevents $\Delta\bar{\mu}_{H^+}$-dependent enzyme transformation is free ADP. A similar (if not identical) phenomenon of $\Delta\bar{\mu}_{H^+}$-dependent transformation of ATPase in chloroplasts is well documented (Du and Boyer, 1989; Groth and Junge, 1995; Junge, 1970; Zhou and Boyer, 1993; and references cited therein) and has been interpreted using a quite complex kinetic scheme (Gräber, 1994). More recently, the unidirectional inhibition of ATPase activity by azide has been confirmed in elegant studies on a reconstituted system composed of *Thermus thermophilus* F_1F_o ATP synthase and bacterial rhodopsin (Bald et al., 1998).

How the different reaction pathways in ATP synthesis and hydrolysis are catalyzed by F_1F_o has been discussed in our previous publications (Vinogradov, 1984; Vinogradov et al., 1985) using a language for enzyme kinetics that is not agreeably accepted by most scholars in the field. The remarkable progress in structural studies of the enzyme enable us to present our working hypothesis as the scheme shown in Fig. 2. The key point in this scheme is that the 'clutch' between a rotating γ-subunit and the $\alpha_3\beta_3$ complex is changed when the enzyme operates in different directions. The simplest driving mechanism to shift the 'clutch' from one mode of operation to the other could be the vertical (perpendicular to the plane of the membrane) $\Delta\bar{\mu}_{H^+}$-dependent movement of one or several subunits of F_o that are firmly attached to the F_1 sector. The minimal requirement for such a movement would be the positioning of a charged (or potentially charged) group within the membrane-embedded area to function as a $\Delta\bar{\mu}_{H^+}$-sensitive sensor. This model is different from those in which an elastic spring (γ-subunit) is proposed for the transmission of a torque from F_o to the catalytic sites of F_1 (Cherepanov et al., 1999; Yasuda et al., 1998). I believe that a rigid ratchet mechanism operates in the electro-mechanical energy transduction. If Nature has created an electro-mechanical motor, it has also provided it with a special mechanism to allow it to operate effectively as a mechano-electrical generator, just like similar devices created by mankind are differently constructed and operated although based on the same physical principles. It would be of great interest to apply several modern techniques, such as cryoelectron microscopy (Gogol et al., 1990) and scanning force microscopy (Singh et al., 1996), to visualise the structure of F_1F_o under the conditions of $\Delta\bar{\mu}_{H^+}$ generation.

What are the biological advantages of an enzyme operating differently in the forward and reverse directions? At least two possible answers can be offered. First, this arrangement may increase the efficiency of energy transmission. Second, perhaps more importantly, it makes independent metabolic control of the enzyme activities possible, since different conformations of the protein are likely to be susceptible in different ways to the same (or different) ligands. An analogy is anabolic and catabolic pathways, which are widely exploited for metabolic regulation in cells. Different enzymes, e.g. phosphofructokinase and fructoso-*bis*-phosphatase, operate in glycolysis and gluconeogenesis; again, isoenzymes, such as different lactate dehydrogenases, participate in the metabolism of the same substrate in different organs or cell compartments. The rapidly expanding concept that considers an enzyme as a molecular machine leads me to believe that 'the one-way

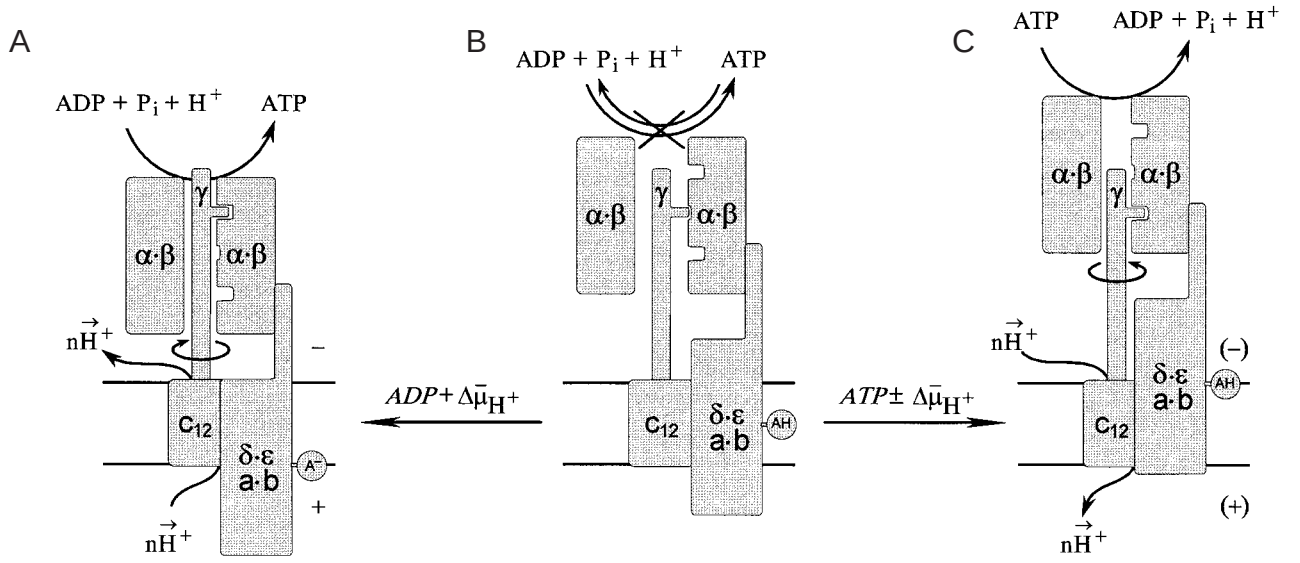

Fig. 2. Schematic representation of the unidirectional operation of the mitochondrial ATPase/ATP synthase. Only one (out of three functioning αβ-subunit pair) is shown as that which has the γ–αβ catch. The relative positions and molecular masses of the $ab_2\delta\varepsilon$ complex are not specified. Readers should consult the series of excellent mini-reviews describing this aspect and the dynamics of the different subunits of F_1F_o (Pedersen, 1996). The key proposal is that there is an energy-driven switch between three types of transmission: (B) no enzyme turnover, no leak for H^+ through the F_o component; (A) the ATP synthase state; and (C) the ATP hydrolase state. For the sake of simplicity, the proton-motive force ($\Delta\bar{\mu}_{H^+}$) 'sensor' is shown as an acidic group AH, although the same type of mechanism can easily be constructed by placing a positive charge in the appropriate position. It is proposed that the 'switch' mechanism is operated by both $\Delta\bar{\mu}_{H^+}$ and nucleotides (ATP or ADP in italic type), which are bound at non-catalytic sites. The model predicts at least two different $\Delta\bar{\mu}_{H^+}$-dependent conformations for the enzyme. It is worth noting that different forms of F_1 knob in submitochondrial particles (collapsed and extended) were observed many years ago (Hatase et al., 1972).

traffic' principal is also realized at the level of a single enzyme. Several recent findings indicate that such a principal applies to another mitochondrial molecular machine, i.e. complex I in the mitochondrial respiratory chain (Vinogradov, 1998).

Conclusions and perspectives

Tremendous experimental effort has been made for more than half a century to determine the molecular mechanism of ATP synthesis during oxidative phosphorylation and photophosphorylation. One recent review on the subject contains 284 references, citing almost exclusively papers published since 1990 (Weber and Senior, 1997)! In spite of spectacular progress in the field, it cannot be denied that this problem remains to be solved. The great majority of mechanistic studies in the past have focused on the ATP hydrolysis reaction catalyzed by F_1 which, by definition, is a hydrolase. These studies have been performed with the explicit or implicit assumption that the enzyme operates reversibly within the F_1F_o $\Delta\bar{\mu}_{H^+}$-dependent ATP synthase complex. I believe that, in the future, more emphasis will be focused on F_1F_o operating in the 'physiological direction' (at least for the mammalian enzyme) and that many current dogmas concerning the mechanism of the enzyme will be re-evaluated. Another direction for future studies, which at present is at a very primitive stage, is the physiological regulation of ATP synthesis. It is hard to believe that 'The ATP synthase – a splendid molecular machine' (Boyer, 1997) is not the subject of fine metabolic control.

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