ATP synthesis catalyzed by the mitochondrial F_1–F_0 ATP synthase is not a reversal of its ATPase activity

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Abstract The ADP(Mg^{2+})-deactivated oligomycin-sensitive F_1–F_0 ATPase of coupled submitochondrial particles treated with the substoichiometric amount of oligomycin was studied to test whether ATP synthesis and hydrolysis proceed in either direction through the same intermediates. The initial rates of ATP hydrolysis, oxidative phosphorylation, ATP-dependent, succinate-supported NAD^+ reduction, and ATP-induced $\Delta\bar{\mu}_{H+}$ generation were measured using deactivated ATPase trapped by azide [Biochem. J. (1982) 202, 15–23]. Three ATP consuming reactions were strongly inhibited when azide was present in the assay mixtures, whereas ATP synthesis was not altered by azide. The unidirectional effect of azide is not consistent with three alternating binding sites mechanism operating in ATP synthesis and support our hypothesis on the existence of nucleotide(Mg^{2+})-controlled 'synthase' and 'hydrolase' states of the mitochondrial F_1–F_0 ATPase.

Key words: F_1–F_0 ATP synthase; Oxidative phosphorylation; Nucleotide binding site

1. Introduction

The F_1–F_0 ATPase/synthase catalyzes $\Delta\bar{\mu}_{H+}$-driven ATP synthesis or $\Delta\bar{\mu}_{H+}$-generating ATP hydrolysis in the coupling membranes of mitochondria, bacteria and chloroplasts. A peripheral part (F_1) is composed of five different polipeptides in the stoichiometry $\alpha_3\cdot\beta_3\cdot\gamma\cdot\delta\cdot\varepsilon$ [1] and capable of rapid uncoupled ATP hydrolysis when detached from the membrane embedded F_0 [2]. Three out of six nucleotides bound at α-β subunits of F_1 are rapidly exchangeable with medium ATP or ADP [3,4] indicating that at least three sites may be involved in the catalytic turnovers during ATP hydrolysis/synthesis. F_0 component contains a proton conducting pathway [2] and operating together F_1–F_0 provides $\Delta\bar{\mu}_{H+}$-consuming ATP synthesis or $\Delta\bar{\mu}_{H+}$-generating ATP hydrolysis depending on physiological (in vivo) or experimental (in vitro) conditions. Several hypotheses on the molecular mechanism of the reaction with participation of three equipotential cooperative nucleotide-binding sites [5,6], two cooperative sites [7] and a single catalytic site [8] have been advanced and extensively discussed [5,6,9].

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid; Oxonol VI, *bis*-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol.

The wast majority of the studies on F_1 or F_1–F_0 ATPases reported in the literature during more than fourty years was based on explicit or implicit assumption that ATP hydrolysis is a reverse sequence of the events taking place during ATP synthesis. However, several observations on the mitochondrial F_1–F_0 ATPase are hardly in line with such a simple first approximation assumption. Many years ago we have described an unusual inhibition of F_1–F_0 and F_1 ATPase induced by ADP in the presence of Mg^{2+} [10,11]. The essential features of the phenomenon which have been detailed in a series of our papers [10–15,20–22,24] are briefly summarized below. F_1 binds ADP tightly at the enzyme specific site and its ATPase activity is ceased if medium Mg^{2+} is present [11–13]. The ATP hydrolytic activity can be very slowly (in a minutes scale) reactivated by the removal of either ADP [13] or Mg^{2+} [14]. The reactivation is greatly accelerated by ATP and prevented by azide [13,15]. It has been shown that inhibitory effect of azide on ATPase is, in fact, due to stabilization of the ADP(Mg^{2+})-deactivated form of the enzyme [15]. Such mechanism of azide inhibition was confirmed and reinforced in a number of recently published reports [16–19]. The intriguing property of the slowly reversible ADP(Mg^{2+})-induced inhibition is that deactivated F_1–F_0 ATPase is capable of oxidative phosphorylation when $\Delta\bar{\mu}_{H+}$ is created under appropriate conditions [20]. The latter finding led us to propose that two slowly interconvertible 'hydrolase' and 'synthase' states of F_1 exist and an equilibrium between them depends on ATP/ADP ratio [20] and a number of other ligands [14,21,22,24]. The ability of azide to trap ADP(Mg^{2+})-deactivated F_1 seemed to be a useful tool for more adequate assessment of whether inhibited ATPase is capable of ATP synthase activity. In this report we will show that ADP(Mg^{2+})-treated F_1–F_0 in coupled submitochondrial particles trapped by azide, being uncapable of ATP hydrolysis, ATP-dependent succinate-supported NAD^+ reduction and ATP-dependent $\Delta\bar{\mu}_{H+}$ generation, is fully active as ATP synthase. The central conclusion following from these observations is that $\Delta\bar{\mu}_{H+}$-dependent ATP synthesis catalyzed by the mitochondrial F_1–F_0 is not a simple reversal of $\Delta\bar{\mu}_{H+}$-generating ATP hydrolysis.

2. Materials and methods

Bovine heart submitochondrial particles (SMP) essentially free of protein ATPase inhibitor were prepared as described [23] and stored in liquid nitrogen as a suspension of 40–50 mg of protein (determined by the biuret procedure) per ml of 0.25 M sucrose. The suspension was thawed and diluted (4 mg/ml) at room temperature (~20°C) in the mixture (pH 7.4) containing: 0.25 M sucrose, 0.1 M KCl, 10 mM HEPES, 0.1 mM EDTA, 1 mM malonate (to activate succinate dehydrogenase), bovine serum albumin (1 mg/ml) and oligomycin (0.25 nmol/mg of protein). 2 μM ADP, 2.4 mM $MgCl_2$ and 100 mM sodium azide were added to one sample of the suspension ('deactivated' SMP) and incubation was continued for 10 min before the assays. 5 mM potassium phosphate (pH 7.4) was added to another sample ('activated' SMP [24])

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and incubation was continued for 1.5 h. The specific activities of 'activated' SMP assayed at 30°C as described below were (initial rates expressed in mmoles of product formed or substrate consumed per min per mg of protein): ATPase, 1.8–2.0; ATP synthesis, 0.3–0.5; ATP-dependent, succinate-supported NAD^+ reduction, 0.1–0.2; succinate oxidase, 0.25–0.32.

ATPase reaction was followed as H^+ release [25] measured with Phenol Red as a decrease of absorption at 557–620 nm in the reaction mixture (2 ml) containing: 0.25 M sucrose, 0.1 M KCl, 10 mM potassium phosphate (pH 7.4), 5.5 mM $MgCl_2$, 16 mM potassium succinate, 0.1 mM EDTA, 30 μM Phenol red, bovine serum albumin (1 mg/ml) and 1 mM ATP. The $[H^+]$ released/[ADP] formed ratio of 0.75 under the experimental conditions employed was determined in the separate experiments where the amount of ADP was determined in the coupled enzymatic assay (lactate dehydrogenase, pyruvate kinase, phospho*enol*-pyruvate and NADH in the presence of 4 μM rotenone) after the reaction was stopped by the addition of oligomycin (12 nM).

ATP synthesis was followed as H^+ consumption measured by the same method (Phenol red) in the same reaction mixture except for ATP which was substituted for 1 mM ADP. The same $[H^+]$ consumed/[ATP] formed ratio (0.75) was determined after the reaction was stopped and the amount of ATP was determined using enzymatic coupled assay (hexokinase, glucose-6-phosphate dehydrogenase, glucose, $NADP^+$). ATP-dependent, succinate-supported NAD^+ reduction [26] was followed as an increase of absorption at 340 nm in the reaction mixture used for ATPase assay (Phenol Red and rotenone were absent and 0.5 mM NAD^+ and 1.5 mM antimycin A were added).

Succinate oxidase was measured with platinum oxygen-sensitive electrode. Other details and additions to the reaction mixtures are indicated in the legends to the figures.

All the activities (except for succinate oxidase) were more than 95% oligomycin-sensitive.

3. Results

The time-course of ATP hydrolysis catalyzed by F_1 or F_1–F_0 ATPase in the presence or abscence of ATP regenerating system shows either lag or burst depending on the enzyme preconditioning [13]. When submitochondrial particles are preincubated with phospho*enol*-pyruvate and pyruvate kinase [13], or P_i [24] or EDTA [14] their initial ATPase activity appears as azide-insensitive burst followed by the slower, azide-sensitive rate [15]. In contrast, when the enzyme is preincubated with stoichiometric amount of ADP in the presence of Mg^{2+} the ATP hydrolysis is completely blocked and the reaction is slowly accelerated in the ATP concentration-dependent fashion. This reactivation does not proceed if azide is present in the assay medium and ATPase remains inhibited for quite a long time [15]. It seemed to be of great interest to find how these active/inactive transitions of F_1–F_0 ATPase correlate with the ability of the enzyme to catalyze other ATP-dependent reactions and, most importantly, oxidative phosphorylation. Fig. 1 shows the time course of ATP hydrolysis and synthesis by P_i-activated and ADP(Mg^{2+})-deactivated azide-trapped SMP under the identical conditions where the only difference was nucleotide used as the substrate. The reaction pattern seen in the ATPase assay was expected: the enzyme activity was almost completely inhibited when azide was present in the reaction mixture and ATP hydrolysis was initiated by addition of ADP(Mg^{2+})-deactivated particles (Fig. 1B). Rapid initial rate was seen when the reaction was started by the P_i-activated particles (Fig. 1A). Both small residual activity of deactivated SMP observed in the presence of azide, and rapid hydrolysis catalyzed by activated SMP were enhanced by uncoupler. The same pattern of ATP hydrolysis was seen when the reaction was followed by NADH decrease in the coupled enzyme assay system (not shown). No significant difference in the time course of ATP synthesis was seen when oxidative phosphorylation was initiated by the addition of either 'activated' or 'deactivated' SMP. An excess of oligomycin or the presence of an uncoupler completely prevented ATP synthesis. The abscence of inhibitory effect of azide on ATP synthesis is also evident from the Table 1, where the rates of oxygen consumption, ATP synthesis and calculated P/O ratio for the oligomycin-coupled SMP are presented.

It might be proposed that only a small part of net ATP hydrolysis measured as shown in Fig. 1 was 'coupled' and azide-insensitive ATP synthesis was catalyzed by that 'coupled' ATPase. To check this possibility the time course of 'coupled' ATP-dependent reactions were measured. Fig. 2 depicts simultaneous registration of ATP-dependent reverse electron transfer and ATP hydrolysis as catalyzed by 'activated' and 'deactivated' SMP. The presence of azide almost completely prevented the initial rates of net ATP hydrolysis and also completely prevented ATP-dependent reverse electron transfer when the

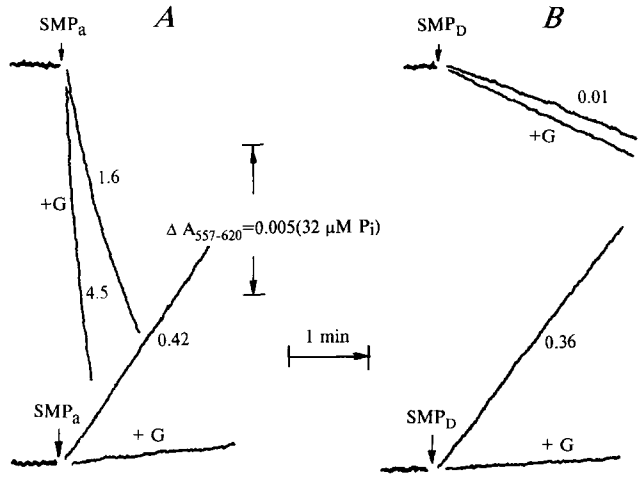

Fig. 1. Time-course of ATP hydrolysis (upper curves) and synthesis (lower curves) catalyzed by the coupled active (A) and ADP(Mg^{2+})-deactivated, azide-trapped (B) F_1–F_0 ATPase. The reactions were initiated by the addition of submitochondrial particles (SMP; 0.1 mg) to 2 ml of the standard reaction mixture containing either 1 mM ATP (ATP hydrolysis) or 1 mM ADP (ATP synthesis). Other components of the reaction mixture and pretreatment of active (SMP_a) and deactivated (SMP_D) are described in section 2. 100 μM sodium azide was present in the assay mixtures when the reactions catalyzed by deactivated SMP were measured (B). Gramicidin D (G; 0.2 μg) was added where indicated. Figures on the curves indicate the rates expressed in mmoles of ATP hydrolyzed or formed per min per mg of protein.

Table 1
The efficiency of oxidative phosphorylation catalyzed by submitochondrial particles with 'activated' and 'deactivated' F_1–F_0 ATPase[a] (pH 7.4, 30°C)

Sample	Oxygen consumed μg-atom/2 min	ATP formed μmol/2 min	P/O ratio
Activated particles, no azide	0.07	0.09	1.29
Deactivated particles, 100 μM azide	0.06	0.08	1.33

[a]16 mM succinate, 1 mM ADP and 10 mM phosphate were added as the substrates. Preparation of activated and deactivated particles and other details are described in section 2.

reactions were initiated by the 'deactivated' SMP. The ATP-induced $\Delta\bar{\mu}_{H+}$-generaton as seen from the response of the membrane potential-sensitive probe [27] was also inhibited by azide when particles with 'deactivated' F_1–F_0 ATPase were used (Fig. 3).

4. Discussion

Most of the present-day ideas concerning the mechanism of oxidative phosphorylation are based on the structural and kinetic studies of purified F_1's or preparations of F_1–F_0 which are uncapable of net ATP synthesis. Numerous reports on the kinetics of ATP hydrolysis by F_1 or F_1–F_0 preparations different degree of resolution have appeared in the literature but advanced studies on the kinetics of ATP synthesis have been relatively rare [28–30] although it has been claimed that oxidative phosphorylation is much less sensitive to azide than ATPase [31,32]. In fact, we were not aware of any reports where the time-course of ATP hydrolysis and synthesis were studied under the same experimental conditions. Many years ago we have reported that ADP(Mg^{2+})-deactivated F_1–F_0 ATPase is capable of ATP synthesis [20] and concluded that the inhibitory ADP binds to a site which is not the same as that where ADP leaves F_1 during the steady-state ATP hydrolysis [12,20]. Much effort have been put forward over past 15 years to attribute ADP-specific inhibition to catalytic or non-catalytic ATPase sites but clear-cut consensus does not appear to be reached ([33–36] and references cited therein). The results presented in this report strongly support and reinforce our original observation on the catalytic competence of ADP(Mg^{2+}) inhibited F_1–F_0 ATPase in ATP synthesis. If inhibitory ADP resides at ATPase catalytic site, as it have been concluded from the location of 2-azido-ADP trapped by azide on the catalytic site peptide of β-subunit [19,37], then our data unumbiguesly show that three equipotential nucleotide-binding site mechanism [5,6] is not

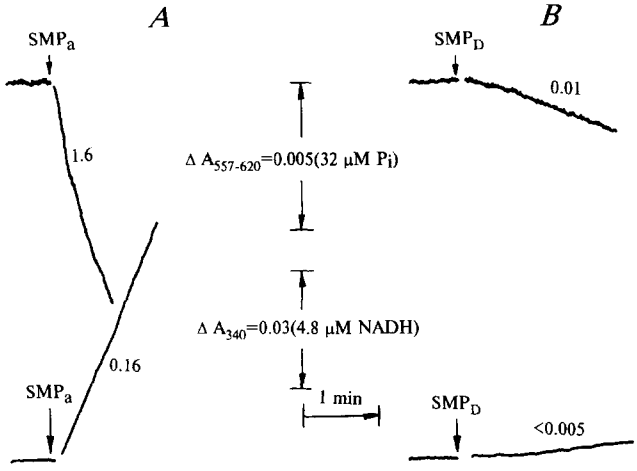

Fig. 2. Time-course of ATP hydrolysis (upper curves) and ATP-dependent, succinate-supported NAD^+ reduction catalyzed by the coupled active (A) and deactivated (B) F_1–F_0 ATPase. The standard reaction mixture contained 1 mM ATP and 0.5 mM NAD^+ (other components are indicated in section 2). 100 mM sodium azide was present in (B). Both ATP hydrolysis and NAD^+ reduction were more than 95% sensitive to oligomycin (12 nM); the reverse electron transfer was completely prevented by uncoupler (not shown). Figures on the curves indicate the rates expressed in mmoles of ATP hydrolyzed or NAD^+ reduced per min per mg of protein.

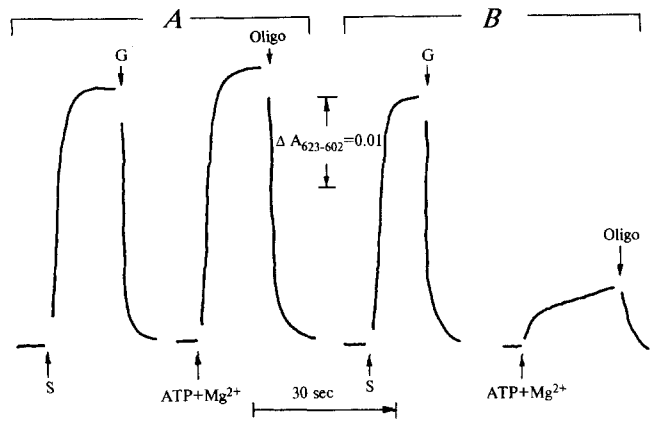

Fig. 3. ATP-dependent Oxonol-VI response in coupled submitochondrial particles. SMP (0.1 mg) were placed in 2 ml of the standard reaction mixture containing no bovine serum albumin nucleotides and succinate. 1.5 mM oxonol VI added before the spectral responce induced by further additions was monitored as indicated. (A) Activated particles no azide was present. (B) ADP(Mg^{2+})-deactivated particles, 100 mM azide was present. The final concentrations were: succinate (S) 16 mM; ATP 1 mM; $MgCl_2$ 5 mM; gramicidin D (G) 0.2 mg; oligomycin (Oligo) 12 nM.

operating during ATP synthesis. Several reports in the literature contradict equipotentiality of the nucleotide-binding sites on β-subunits (e.g. [38] and references cited therein). Mention should be made that the nucleotide-binding site(s) which is 'catalytic' during the steady-state ATP hydrolysis may function as 'regulatory' site(s) during the steady-state ATP synthesis. The central issue of this report concerns the questionable identity of the molecular events during ATP synthesis and hydrolysis by F_1–F_0 ATPase/synthase. It worth noting that F_1–ATPase protein inhibitor (IF_1) which is an intrinsic part of the mammalian ATP synthase complex is also known to be a 'unidirectional' inhibitor of ATP hydrolysis [39]. Although $\Delta\bar{\mu}_{H+}$ dependence of F_1–F_0–IF_1 interaction have been proposed to explain the lack of inhibitory effect of IF_1 on oxidative phosphorylation [40], such explanation is hard to reconcile with our more recently reported data [41]. In summary, we would like to emphasize that the difference in the catalytic step-by-step mechanism of ATP synthesis and hydrolysis may be a general phenomenon for F_1–F_0 type ATP synthases.

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