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An ATP dependence of mitochondrial F_1-ATPase inactivation by the natural inhibitor protein agrees with the alternating-site binding-change mechanism

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The rate of inactivation of F_1-ATPase, isolated from beef heart mitochondria, by the active acidic form of the natural inhibitor protein depends on the ATP concentration. An increase in concentration of ATP to ~20 μM leads to a decrease in that of the inhibitor protein inducing 50% inhibition of the F_1-ATPase during 5 s preincubation (C_{50}); further increase in ATP concentration to 1 mM causes little, if any, change in C_{50}. However, the C_{50} values show a rise at ATP concentrations higher than 1 mM. This ATP dependence of the inhibitor action may be in agreement with a version of the alternating-site binding-change mechanism, which assumes that the two-site catalytic cycle intermediates possessing (i) the products (ADP + P_i) bound in the low-affinity state at one of the active sites and (ii) an ATP molecule at the other active site are the targets for the acidic form of the inhibitor protein.

ATPase, F_1-; Catalytic intermediate; Inhibitor protein

1. INTRODUCTION

The mechanism of mitochondrial F_1 regulation by the natural inhibitor protein (IP) [1] is one of the most intriguing problems in bioenergetics (review [2]). IP suppresses the activity of F_1 after binding to one of the three catalytic β-subunits [3–5] and impairs both single-site and multi-site modes of catalysis [6]. In the case of membrane-bound enzyme, energization affects the interaction of IP and F_1 and finally abolishes the effect of IP on steady-state ATP synthesis (different interpretations of this phenomenon are summarized in [2]). The fact that ATP or any other hydrolyzable substrate is required for inhibition of F_1 by IP is also well known, although the role of ATP in this process remains unclear. Investigations of the latter problem are complicated by the recent discovery of two states of IP – the active form and the inactive (or low-activity) species, interconverting as a result of protonation/deprotonation of some amino acid residue in IP molecules [7,8].

Here, the ATP dependence of the action of the acidic (active) form of IP on isolated soluble F_1 from beef heart mitochondria has been investigated. The results obtained are consistent with the alternating-site binding-change mechanism [9–11] but argue against the existence of a long-lived catalytically active complex of F_1 and IP.

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Abbreviations: F_1, mitochondrial F_1-ATPase; IP, natural inhibitor protein of mitochondrial F_1-ATPase

2. MATERIALS AND METHODS

The sources of chemicals were as described [6], except for ATP (disodium salt) which was from Sigma.

F_1 was isolated from beef heart mitochondria according to Knowles and Penefsky [12]. For experiments, an ammonium sulphate suspension of F_1 was centrifuged and F_1 was dissolved

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in a buffer containing 50 mM sucrose, 20 mM Mops-Tris (pH 8.0) and 2 mM EDTA and desalted using the column-centrifugation method [13] on Sephadex G-50 (fine), pre-equilibrated with the same buffer. An equal volume of glycerol was added to the eluate, and F_1 (40–50 μM) was stored at −15°C.

IP was purified according to a slightly modified [6] method of Frangione et al. [14]. The specific activity of IP, assayed as in [14], was 25×10^3 U/mg when the protein was determined according to Lowry et al. [15] using bovine serum albumin as standard. In titration experiments involving IP-depleted sub-mitochondrial particles, prepared as in [16] with the modifications indicated in [17], the stoichiometry of 1 mol IP per mol F_1 corresponded to the complete inhibition of F_1-ATPase activity, assuming the F_1 content in the particles to be 10% of the protein. The homogeneity of IP was also confirmed by SDS-PAGE. A molecular mass of 360 kDa for F_1 or 10.5 kDa for IP was used for calculations.

To obtain IP in the acidic form, 100–150 μM IP in 10 mM Mops-KOH (pH 6.9) was supplemented with 1/10 vol. of a solution containing 250 mM CH_3COOH-KOH (pH 4.4). The resulting solution was incubated for 5 min at room temperature, cooled to 4°C and used within 1 day. When necessary, the IP solution thus obtained was diluted with a buffer containing 23 mM CH_3COOH-KOH (pH 4.4).

Inhibition of F_1 by IP was studied in a medium (final volume 10 or 20 μl) containing 50 mM Mops-KOH (pH 6.8), 2 mM Na_2SO_3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mg/ml pyruvate kinase, phospho*enol*pyruvate and MgATP. The concentration of phospho*enol*pyruvate was 1.5 or 3 mM at MgATP concentrations $\leqslant$1 mM or $\geqslant$0.1 mM, respectively (in the latter case, 2 mM $MgCl_2$ was additionally present). Throughout the range 0.1–1.0 mM MgATP, the results obtained did not depend on the concentration of phospho*enol*-pyruvate. The reaction was initiated by addition of F_1. After 5 s, the acidic form of IP was added for another 5 s and the reaction mixture was diluted 10- or 40-fold using a solution of 1 μM [γ-^{32}P]ATP ($2–5 \times 10^5$ cpm/nmol) in a medium containing 40 mM Tris-HCl (pH 8.5), 2.5 mM $MgCl_2$, 2 mM Na_2SO_3 and 0.2 mM EDTA. After incubation of the diluted mixture for 20 s, [γ-^{32}P]ATP hydrolysis was stopped by adding 2 M $HClO_4$ to a final concentration of 0.5 M. The unhydrolyzed [γ-^{32}P]ATP was removed by charcoal precipitation and the radioactivity of $^{32}P_i$ formed was measured as Cherenkov radiation.

3. RESULTS

As shown in fig.1, the acidic form of IP induced rapid inactivation of F_1 hydrolysing ATP at pH 6.8. To investigate the degree of efficiency of the action of IP over a wide range of ATP concentrations, F_1 was preincubated for 5 s in the presence of IP at each ATP concentration, the residual activity of the enzyme being then determined using [γ-^{32}P]ATP. The action of IP during the [γ-^{32}P]ATP hydrolysis step was diminished by the following factors: (i) short duration of [γ-^{32}P]ATP hydrolysis (20 s); (ii) dilution of IP in the [γ-^{32}P]ATP hydrolysis step; (iii) high pH (8.5) of the dilution medium (at this pH, the acidic form of IP lost its inhibitory activity with $\tau_{1/2} \sim 5$ s). These factors ensured that the IP-induced decrease in F_1 activity during [γ-^{32}P]ATP hydrolysis was less than 10% (verified in experiments without ATP at the pre-incubation step).

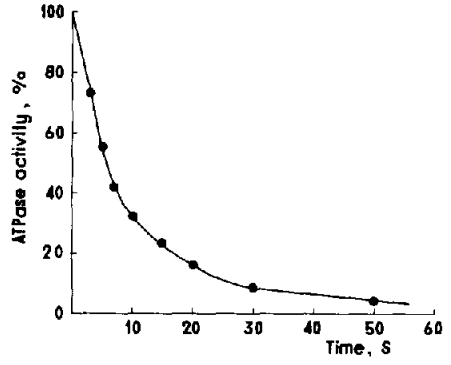

Fig.1. Kinetics of F_1-ATPase inactivation induced by 0.4 μM IP in the presence of 0.1 mM ATP.

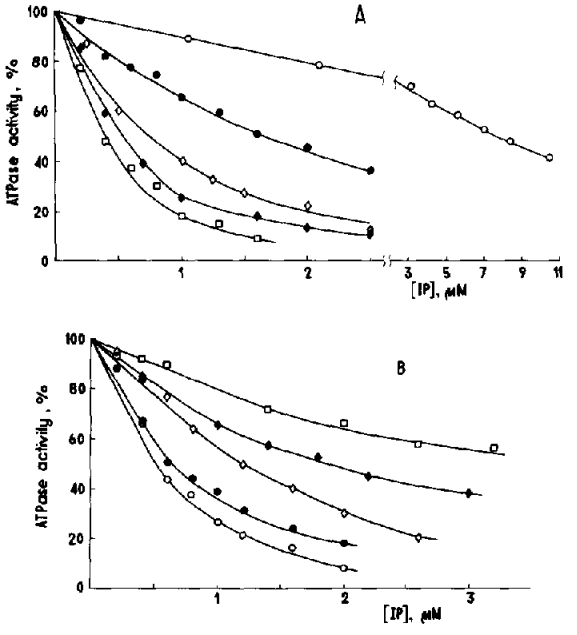

Fig.2. Inactivation of F_1-ATPase during 5 s preincubation in the presence of IP and ATP. ATP concentration: (A) 0.5 (○), 2 (●), 4 (◇), 10 (◆), 20 μM (□); (B) 0.1 (○), 1 (●), 3 (◇), 7 (◆) or 10 mM (□).

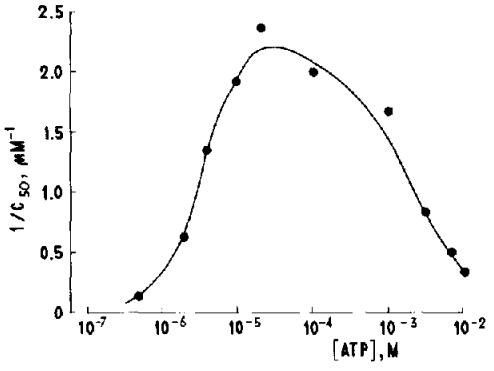

Fig.3. ATP dependence of the reciprocal of the IP concentration that induced 50% inhibition of F_1-ATPase during 5 s preincubation (C_{50}). C_{50} values were obtained from the data in fig.2.

The curves in fig.2 show the dependence of residual F_1 activity on the concentration of IP added for 5 s in the pre-incubation step. As demonstrated in fig.2A, the IP concentration which lowered the F_1-ATPase activity by 50% (C_{50}) decreased from 8 μM at 0.5 μM ATP to 0.4 μM at 20 μM ATP. The increase in ATP concentration from 20 to 100 μM did not exert any significant effect on the C_{50} value, however, further increase in ATP concentration resulted in an increase in C_{50} (fig.2B). In the presence of 10 mM ATP the C_{50} value was >3 μM.

Fig.3 summarizes the data of fig.2 and shows the dependence of the $1/C_{50}$ value on ATP concentration.

4. DISCUSSION

In accordance with the two-step scheme (reaction 1) proposed for the interaction of F_1 and IP [18]:

$$IP + F_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} IP \cdot F_1 \xrightarrow{k_2} IP \cdot F_1^* \qquad (1),$$

the initially formed $IP \cdot F_1$ complex retains catalytic activity and may undergo irreversible isomerization to yield a catalytically inactive $IP \cdot F_1^*$ complex. From the data obtained using the alkaline form of IP [8] or a mixture of the alkaline and acidic forms of IP [19], k_2 was evaluated to be ~ 0.02 s^{-1}. However, as follows from the data of Hashimoto et al. [20], the k_2 value for F_1 and IP obtained from the yeast *Saccharomyces cerevisiae* (the inhibitor form was not indicated) may be higher than 0.3 s^{-1}.

The acidic form of IP was shown to inactivate F_1-ATPase of submitochondrial particles more rapidly as compared to the alkaline species, the rate constant for inactivation, k_{app}, being linearly dependent on the concentration of acidic IP and equalling 0.25 s^{-1} at 13 μM IP [8]. As may be seen from fig.2, 2 μM IP inactivates F_1 by more than 90% during 5 s preincubation over the ATP concentration range 20–100 μM. Since attainment of such a degree of inactivation required at least three half-times of the reaction to occur, the k_{app} value should be greater than 0.5 s^{-1}. Thus, either the catalytically active $IP \cdot F_1$ complex (reaction 1), if it exists, should be short-lived ($k_2 > 0.5$ s^{-1}), or interaction of F_1 with the acidic form of IP should proceed according to the simple one-step scheme [21]:

$$IP + F_1 \xrightarrow{k'} IP \cdot F_1^* \qquad (2).$$

Although the present data cannot discriminate between these schemes (reactions 1,2), one can derive from figs 1,2 a second-order rate constant for IP and F_1 interaction (k_{+1} or k') of k_{app}/[IP] or $\ln 2/(C_{50} \times t)$ (where t is the time of preincubation of F_1 with IP, 5 s). Calculated in this way, k_{+1} is about 4×10^5 $M^{-1} \cdot s^{-1}$ over the ATP concentration range 20–100 μM.

The ATP dependence of k_{+1} (which is proportional to the $1/C_{50}$ value) exhibits a complex pattern (fig.3) and, at first glance, contradicts the assumption [21–23] that some catalytic intermediate of the enzyme is a target for the action of IP. Thus, Panchenko and Vinogradov [24] observed that the rate of IP-induced inactivation of F_1 was half-saturated at about 5 μM ATP and remained unchanged over the ATP concentration range 0.02–1.0 mM, while F_1-ATPase activity was shown to have a K_m for ATP of ~0.1 mM [25]. Proceeding from these results it was suggested [24] that the binding of ATP with $K_d \sim 5$ μM at a specific (different from catalytic) site is required for the productive interaction of the enzyme and IP.

Our results for ATP concentrations <1 mM (fig.3) are in accord with the data of Panchenko

and Vinogradov [24]. However, taking into account the alternative-site binding-change mechanism of F_1 functioning [10,11], the results obtained allow us to propose catalytic cycle intermediates which are the target for the action of IP. The two-site scheme of the F_1 catalytic cycle is represented in fig.4 where the substrate and products bound in the high-affinity state are denoted by an asterisk and the intermediates, the targets for IP action, are underlined. One of these intermediates ($ATP \cdot F_1 \cdot ADP \cdot P_i$), in which the nucleotides and P_i are bound to both catalytic sites in the low-affinity state, interacts with IP more rapidly than does the second one ($ATP^* \cdot F_1 \cdot ADP \cdot P_i$) having an ATP bound in the high-affinity state. A characteristic feature of both these intermediates is the existence of ADP and P_i bound to one of the catalytic sites in the low-affinity state.

At each ATP concentration, according to fig.4, the k_{+1} value for binding of IP to F_1 should be determined by the sum of the steady-state concentrations of the intermediates $ATP \cdot F_1 \cdot ADP \cdot P_i$ and $ATP^* \cdot F_1 \cdot ADP \cdot P_i$. After saturation of single-site catalysis in F_1 with K_m for ATP of 5–20 nM [26,27], the concentration of the $F_1 \cdot ADP \cdot P_i^*$ complex is practically independent of the ATP concentration and, consequently, the k_{+1} value in the submicromolar ATP range should be proportional to the ATP concentration. In the micromolar ATP range, the steady-state concentration of the $F_1 \cdot ADP \cdot P_i^*$ complex decreases due to competitive formation of an $ATP \cdot F_1 \cdot ATP^*$ intermediate (as indicated by Cross et al. [28], this situation most likely explains the decrease in the intermediate oxygen water/P_i-exchange during ATP hydrolysis) and, as a result, the steady-state concentration of the $ATP \cdot F_1 \cdot ADP \cdot P_i$ intermediate (and, consequently, k_{+1}) ceases to depend on ATP concentration. At higher ATP concentrations, the steady-state concentration of the $ATP \cdot F_1 \cdot ADP \cdot P_i$ complex decreases due to the fall in concentration of the $F_1 \cdot ATP^*$ complex. However, this is compensated by an increase in steady-state concentration of the $ATP^* \cdot F_1 \cdot ADP \cdot P_i$ intermediate which is also the target for IP.

The reason for the decrease in k_{+1} at ATP concentrations >1 mM is unclear. It should be noted that this decrease is not determined by possible lowering of pyruvate kinase activity as a result of an increase in concentration of Na^+ introduced

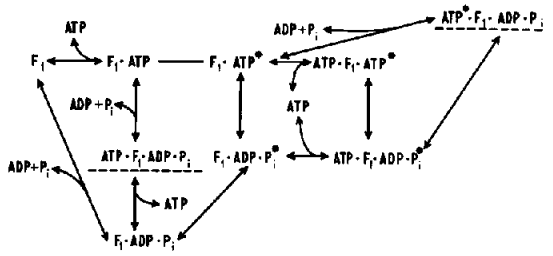

Fig.4. Kinetic scheme of the F_1-ATPase. Asterisks denote substrate and products bound in the high-affinity state. Underlined species designate intermediates that are targets for the action of IP.

with ATP and by Cd^{2+} and Zn^{2+}, possibly contaminating the ATP preparations (Cd^{2+} and Zn^{2+} have been reported to lower the rate of F_1 inactivation by IP [19]), since neither 20 mM Na^+ (the concentration that was added with 10 mM ATP) nor 1 mM Zn^{2+} was found to affect the inactivation of F_1 by IP in the presence of 0.1 mM ATP. The latter result is in contradiction with the data of Chernyak et al. [19] and means that, at least in the case of the active acidic form of IP, Zn^{2+} (and, possibly, Cd^{2+}) does not affect the interaction of F_1 and IP. The rate of association of IP and F_1 was reported to decrease with increasing ionic strength [21]. Since the preincubation medium is of high ionic strength (see section 2), an increase in ionic strength due to a rise in MgATP concentration appears unlikely to be the cause of the decrease in k_{+1} observed at high ATP concentrations. It may be speculated that the fall in k_{+1} at high ATP concentrations results from a decrease in steady-state concentration of the $ATP^* \cdot F_1 \cdot ADP \cdot P_i$ intermediate (fig.4) owing to the possible transition of F_1-ATPase to three-site catalysis.

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