

# Interaction between the mitochondrial ATP synthetase and ATPase inhibitor protein

## Active/inactive slow pH-dependent transitions of the inhibitor protein

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Received 15 March 1985

The rate of mitochondrial ATPase inactivation by the naturally occurring inhibitor protein in the presence of saturating ATP and  $Mg^{2+}$  at pH 8.0 depends hyperbolically on the amount of inhibitor added; the upper limit of an apparent first-order constant for the inactivation process is  $1.0^{-1}$  at 25°C. A dramatic difference in the inactivation rate is observed when the protein inhibitor is added to the same assay system from either acidic (pH 4.8) or alkaline (pH 8.2) solutions. The slow reversible transition of the inhibitor from its rapidly reacting 'acidic' form to the slow reacting 'alkaline' form occurs when the solution of the protein inhibitor is subjected to a pH-jump from 4.8 to 8.2 ( $t_{1/2} \sim 30$  s at 25°C). The pH-profile of the inhibitor active/inactive equilibrium suggests that a group with  $pK_a \sim 6.5$  is involved in the transition. Treatment of the inhibitor protein with a histidine-specific reagent (e.g. diethyl pyrocarbonate) abolishes its inactivating effect on the ATPase activity. It is concluded that the protonation/deprotonation of the inhibitor protein followed by its slow conformational changes is the rate-limiting step in the inhibitor-ATP synthetase interaction.

*ATP synthetase    Inhibitor protein    Acid-base transition    Mitochondria*

### 1. INTRODUCTION

The ATP hydrolytic activity of the mitochondrial ATP synthetase can be specifically abolished by the natural ATPase inhibitor (IF<sub>1</sub>) discovered by Pullman and Monroy [1] in preparations of beef heart mitochondrial coupling factor F<sub>1</sub>. Subsequent studies (review [2]) have shown that IF<sub>1</sub> or similar low- $M_r$  peptides are present in a number of membrane-bound ATP synthetase com-

plexes [3–6]. An intriguing feature of IF<sub>1</sub> which is not clearly understood is that it inhibits the ATP hydrolytic activity of the soluble [1,7,8] or membrane-bound F<sub>1</sub> [1,7–12] and the ATP-driven processes in the coupling membranes [8,9,11,13] whereas the net oxidative phosphorylation is insensitive to the inhibitor [1,13–15]. The hypothesis has been originally proposed [16] and further developed [17,18] on the  $\Delta\mu H^+$ -dependent dissociation/association of the IF<sub>1</sub>·F<sub>1</sub> complex, which explains the apparently unidirectional inhibition of the reversible  $H^+$ -ATPase by IF<sub>1</sub>. The kinetics and thermodynamics of ATPase-IF<sub>1</sub> interaction have been extensively studied ([19,20] and references cited therein), and a number of schemes which account for several complex features of the ATPase inhibition/reactivation have been proposed [10,17–21].

Here we present data demonstrating an unusual property of the mitochondrial IF<sub>1</sub>, namely the slow

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**Abbreviations:** F<sub>1</sub>, beef heart mitochondrial coupling factor; IF<sub>1</sub>, beef heart ATPase protein inhibitor; AS-particles, submitochondrial ultrasonic particles depleted of IF<sub>1</sub> by slow filtration through Sephadex; Mops, 3-(N-morpholino)propanesulphonate; Mes, 4-morpholineethanesulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine

hysteretic pH-dependent interconversion between rapidly and slowly inhibiting forms of the protein. An immediate output of this finding is that the protonation/deprotonation of  $IF_1$  followed by its slow conformation change is the rate-limiting step in the inhibitor-ATP synthetase interaction.

## 2. MATERIALS AND METHODS

Bovine heart submitochondrial particles free of the protein ATPase inhibitor (AS-particles) were prepared essentially as described [22]. The particles were activated at 23°C by phosphate [23] for 30 min in a mixture containing (final concentrations): 10 mM Mops, 10 mM KCl, 2 mM  $MgCl_2$ , 50  $\mu$ M EDTA, 0.1 M sucrose and 5 mM potassium phosphate (pH 7.0).  $IF_1$  was prepared by the method of Kanner et al. [24]. The ATPase activity was measured at 340 nm at 25°C in a reaction mixture containing (final concentrations): 10 mM Mops, 10 mM KCl, 2 mM  $MgCl_2$ , 50  $\mu$ M EDTA, 0.1 M sucrose, 2.5 mM  $KHSO_3$ , 2 mM KCN,  $10^{-6}$  M FCCP, 1 mM potassium phosphoenolpyruvate, 1 mM ATP/ $Mg^{2+}$ , 0.16 mM NAD $\cdot$ H, pyruvate kinase (3.3 units per ml) and lactate dehydrogenase (3.3 units per ml); pH is indicated in the figure legends. Sulphite was added to the assay mixture to avoid the time-dependent, ADP-specific inhibition of ATPase [25,26]. The

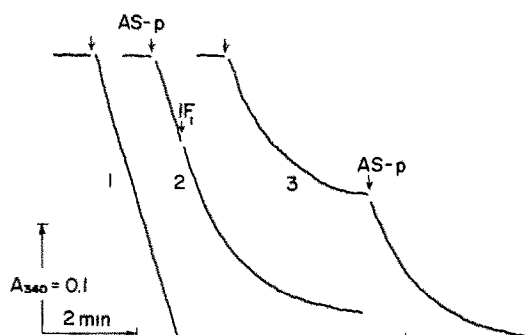


Fig. 1. Time course of ATPase inhibition by  $IF_1$ . The reaction was started by the addition of activated AS-particles (AS-p) (14  $\mu$ g) to 1.5 ml of the standard assay mixture (see section 2) at pH 8.0. 16  $\mu$ g  $IF_1$  dissolved in a mixture containing 50 mM sucrose, 50  $\mu$ M EDTA, 5 mM Tris, and 5 mM Mes (pH 8.2) were added to the assay cuvette during ATP hydrolysis (curve 2) or before the reaction was initiated (curve 3). Curve 1, no  $IF_1$  present. The specific ATPase activity without added  $IF_1$  was 2.3  $\mu$ mol ATP hydrolyzed/min per mg protein.

nucleotides (potassium salts), pyruvate kinase and lactate dehydrogenase were obtained from Reanal (Hungary). The enzymes were extensively dialyzed before use. Mops and Mes were obtained from Calbiochem; other chemicals were of the purest grade commercially available.

## 3. RESULTS AND DISCUSSION

Fig. 1 demonstrates the time course of ATP hydrolysis and inhibition of the reaction by an excess of  $IF_1$ . The patterns of inhibition are the same irrespective of whether the reaction is started by the particles and  $IF_1$  is added during the steady-state hydrolysis, or  $IF_1$  is present in the assay mixture before hydrolysis is initiated. As evident from the experiment documented by trace 3, the amount of  $IF_1$  is indeed in excess, since the same rate of inhibition is observed when the second portion of AS-particles is added to the assay mixture after the inactivation of the first portion has been completed. When the apparent first-order rate constant for the inhibition process was plotted as a function of the inhibitor amount (fig. 2, curve 1), a

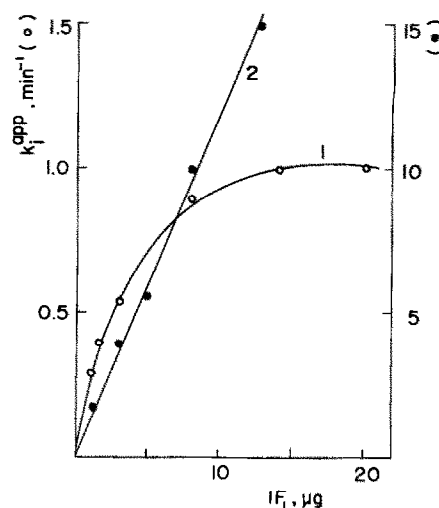


Fig. 2. Dependence of the inhibition apparent first-order rate constant on the amount of  $IF_1$  added. Experimental conditions as in fig. 1. Curve 1 (left ordinate),  $IF_1$  was added to the assay mixture (pH 8.0) from the solution adjusted to pH 8.2. Curve 2 (right ordinate),  $IF_1$  was added to the assay mixture (pH 8.0) from the solution adjusted to pH 4.8. The pH changes in the assay mixture after additions of  $IF_1$  were negligible.

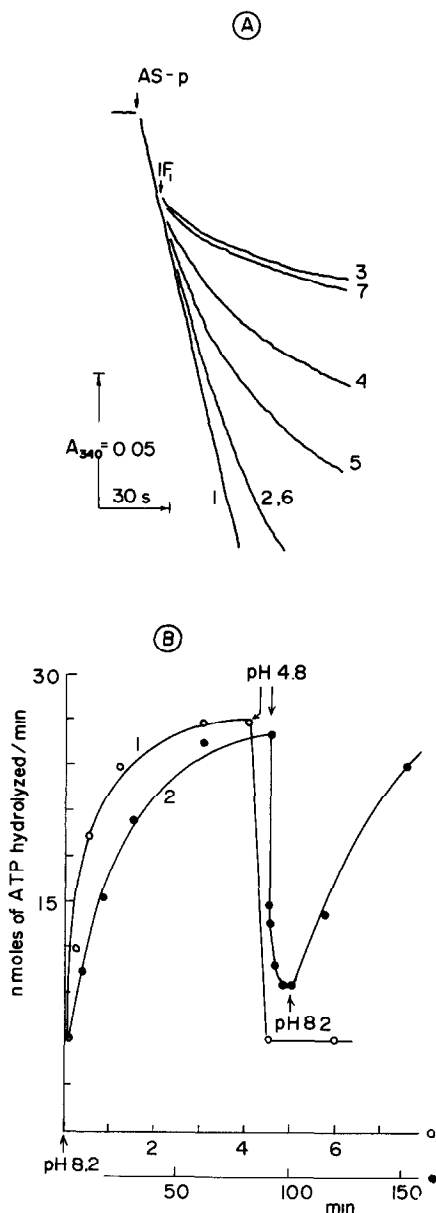


Fig.3. Time course of the pH-dependent active/inactive transitions of IF<sub>1</sub>. (A) The actual traces of the ATPase assays at pH 8.0 after addition of IF<sub>1</sub>. pH of the buffers where IF<sub>1</sub> was dissolved was adjusted to 8.2 (curve 2) or 4.8 (curve 3) and IF<sub>1</sub> was added to the assay mixture (pH 8.0) where indicated. The buffer containing IF<sub>1</sub> at pH 4.8 was then rapidly adjusted to pH 8.2, and IF<sub>1</sub> was added to the assay mixture 15, 30 and 300 s after the pH jump (curves 4, 5 and 6, respectively); pH was then readjusted to 4.8 and 60 s later IF<sub>1</sub> was added to the assay mixture (curve 7). Curve 1, no IF<sub>1</sub> added. (B) IF<sub>1</sub>

saturation curve was obtained with an upper limit of  $1.0\text{ min}^{-1}$ . In accordance with [1,7], it was observed that the maximal rate of inhibition is strongly pH-dependent. At pH 6.0, the inhibition rate increases up to the level which does not permit an accurate determination of the first-order rate constant. Under these conditions (pH 6.0), the rate of the ATPase reaction itself is only slightly ( $\sim 20\%$ ) decreased as compared with that at pH 8.0, thus suggesting that the increase in inhibition rate is most probably due to a change of IF<sub>1</sub> rather than of F<sub>1</sub>.

An unexpected finding shown in fig.3A is that when equal amounts of IF<sub>1</sub> were added to ATP hydrolyzing particles from acidic or alkaline solutions of the inhibitor the inhibition patterns were quite different, i.e., acidic IF<sub>1</sub> almost instantly inactivates ATPase, whereas alkaline inhibitor slowly inhibits the reaction. The reversible transition from the rapidly reacting to the slowly reacting form of IF<sub>1</sub> is slow enough to be registered at  $25^\circ\text{C}$  as shown in fig.3B. The transition is strongly temperature-dependent, indicating significant structural rearrangements which occur during the pH jump induced transformation ( $t_{1/2}$  for inter-conversion of 'active' to 'inactive' IF<sub>1</sub> is about 30 s and 20 min at 25 and  $0^\circ\text{C}$ , respectively). When the inhibition rate was plotted as a function of acidic IF<sub>1</sub>, a straight line was obtained (fig.2, line 2), indicating a simple second-order reaction between F<sub>1</sub> and acidic IF<sub>1</sub>, which is a first-order process with respect to the inhibitor.

The pH profile of the active/inactive inter-conversion of IF<sub>1</sub> (not shown) suggested the participation of a single group with an apparent  $pK_a$  of 6.5. Although a straightforward interpretation

was dissolved in the buffer (see legend to fig.1) at pH 4.8 and its inhibitory effect was estimated as the amount of ATP hydrolyzed by AS-particles during 1 min after the addition of IF<sub>1</sub>. At zero time, the pH of the IF<sub>1</sub> solution was adjusted to 8.2, and the inhibitory efficiency was measured as a function of time after the pH jump. Curves 1 and 2, the solutions where the proper amounts of IF<sub>1</sub> for the assays were taken from were incubated at 25 and  $0^\circ\text{C}$ , respectively. The amounts of AS-particles and IF<sub>1</sub> added to 1.5 ml of the assay mixture were 14 and  $16\text{ }\mu\text{g}$ , respectively.

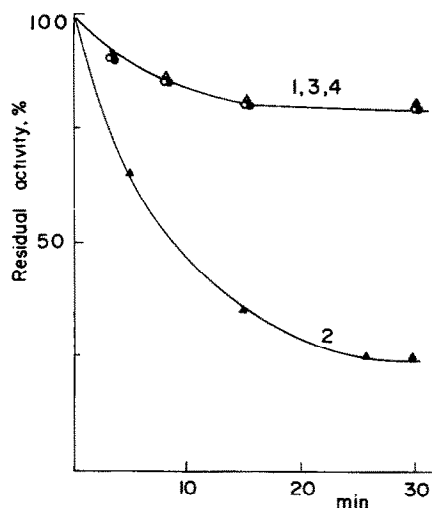


Fig.4. Inactivation of IF<sub>1</sub> by a histidine-specific reagent. 14  $\mu$ g AS-particles were preincubated at 25°C for the time indicated on abscissa in 200  $\mu$ l of the buffer used for activation (see section 2) which contained 4  $\mu$ M ATP, 5 mM phosphoenolpyruvate, 10 units of pyruvate kinase and 0.045  $\mu$ g IF<sub>1</sub> pretreated as described below. The ATPase activity was then assayed in the standard mixture made by the adjustment of the volume to 1.5 ml with the buffer containing all the necessary components. 4.5  $\mu$ g IF<sub>1</sub> were preincubated at 25°C for 10 min in 100  $\mu$ l of a mixture containing 10 mM Mops, 10 mM KCl, 50 mM sucrose and 1 mM diethyl pyrocarbonate (pH 6.0). Aliquots of the mixture were added to the suspension of AS-particles, and the incubation was started as described above. Curve 1, no additions to AS-particles; curve 2, IF<sub>1</sub> preincubated without diethyl pyrocarbonate was added to AS-particles; curve 3, IF<sub>1</sub> treated with diethyl pyrocarbonate was added to AS-particles; curve 4, an aliquot containing diethyl pyrocarbonate without IF<sub>1</sub> was added to AS-particles. The IF<sub>1</sub> to F<sub>1</sub> molar ratio in the incubation mixture was 0.8. 100% corresponds to a specific activity of 2.3.

of the pH-dependent equilibrium between the active and inactive IF<sub>1</sub> can hardly be made, an apparent  $pK_a$  of 6.5 might indicate a histidine residue as a feasible participant of the process. Indeed, when IF<sub>1</sub> was treated with a histidine-specific reagent (diethyl pyrocarbonate), its inhibitory potency was lost (fig.4).

The simplest model for the IF<sub>1</sub>-F<sub>1</sub> interaction which accounts for the findings reported here as well as for other data reported in the literature may be formulated as follows. Apparently, a specific site exists in one of the intermediate F<sub>1</sub>-substrate

(product) complexes (presumably on the  $\beta$ -subunit [12,27]), where IF<sub>1</sub> binds, thus inhibiting the ATP hydrolytic activity. The IF<sub>1</sub> as prepared by conventional procedures [1,6,7,24,28,29] should be regarded as a denatured protein capable of slow spontaneous renaturation at acidic pH. It also renaturates at any given pH in the presence of ATP hydrolyzing F<sub>1</sub> as a result of the inhibitor natural conformation stabilization within the ATP synthetase complex (e.g., at pH 8.0 the rate constant for renaturation is 1 min<sup>-1</sup>; fig.2, curve 1). A very high sensitivity of IF<sub>1</sub> to tryptic digestion [1,7] seems to be relevant to the proposed denatured state of the protein inhibitor preparations. The central finding of this report is that the pH-dependent structural changes of IF<sub>1</sub> are slow as compared to the rate of the active IF<sub>1</sub>-F<sub>1</sub> interaction, and the kinetics of the inhibition are strongly dependent on the original state of the protein. This may cause serious errors in kinetic studies of the F<sub>1</sub> inactivation/reactivation process. A more important, though less explicit implication of the model is that the slowly reversible denaturation/renaturation process should be taken into account, when the physiological 'regulatory' role of IF<sub>1</sub> is being considered [9,11,13,16,17]. At the moment, no clear-cut evidence exists for  $\Delta\mu H^+$ -dependent activation/deactivation of the ATP hydrolytic activity of F<sub>1</sub> synchronous with its ATP synthetase activity in submitochondrial particles, although some correlation between IF<sub>1</sub> release and phosphorylating activity has been reported [14,17,18]. The experimentally supported hypothesis on two states of F<sub>1</sub> (hydrolase and synthetase) [30-32] also seems relevant to the functional role of IF<sub>1</sub>. We believe that under physiological conditions IF<sub>1</sub> serves as a stabilizer of the F<sub>1</sub>-synthetase state, thus preventing a possible leakage of the phosphorylating potential of ATP in mitochondria.

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