Interaction between the mitochondrial ATP synthetase and ATPase inhibitor protein

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

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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_1) discovered by Pullman and Monroy [1] in preparations of beef heart mitochondrial coupling factor F_1. Subsequent studies (review [2]) have shown that IF_1 or similar low-M_r peptides are present in a number of membrane-bound ATP synthetase complexes [3–6]. An intriguing feature of IF_1 which is not clearly understood is that it inhibits the ATP hydrolytic activity of the soluble [1,7,8] or membrane-bound F_1 [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 $IF_1 \cdot F_1$ complex, which explains the apparently unidirectional inhibition of the reversible H^+-ATPase by IF_1. The kinetics and thermodynamics of ATPase-IF_1 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_1, namely the slow

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Abbreviations: F_1, beef heart mitochondrial coupling factor; IF_1, beef heart ATPase protein inhibitor; AS-particles, submitochondrial ultrasonic particles depleted of IF_1 by slow filtration through Sephadex; Mops, 3-(*N*-morpholino)propanesulphonate; Mes, 4-morpholineethanesulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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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 μM 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 μM EDTA, 0.1 M sucrose, 2.5 mM $KHSO_3$, 2 mM KCN, 10^{-6} M FCCP, 1 mM potassium phospho*enol*pyruvate, 1 mM ATP/Mg^{2+}, 0.16 mM NAD·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 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.

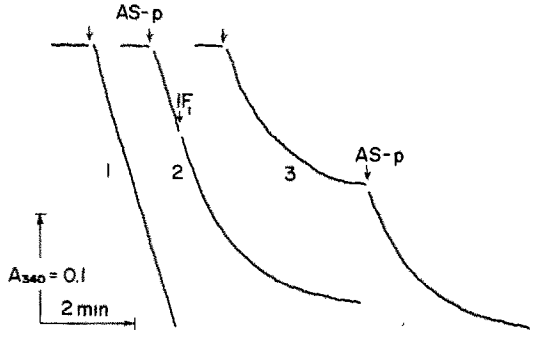

Fig.1. Time course of ATPase inhibition by IF_1. The reaction was started by the addition of activated AS-particles (AS-p) (14 μg) to 1.5 ml of the standard assay mixture (see section 2) at pH 8.0. 16 μg IF_1 dissolved in a mixture containing 50 mM sucrose, 50 μM EDTA, 5 mM Tris, and 5 mM Mes (pH 8.2) 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 μmol ATP hydrolyzed/min per mg protein.

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

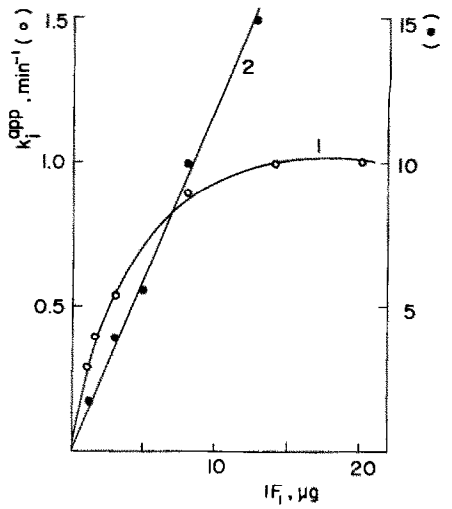

Fig.2. Dependence of the inhibition apparent first-order rate constant on the amount of IF_1 added. Experimental conditions as in fig.1. Curve 1 (left ordinate), IF_1 was added to the assay mixture (pH 8.0) from the solution adjusted to pH 8.2. Curve 2 (right ordinate), IF_1 was added to the assay mixture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

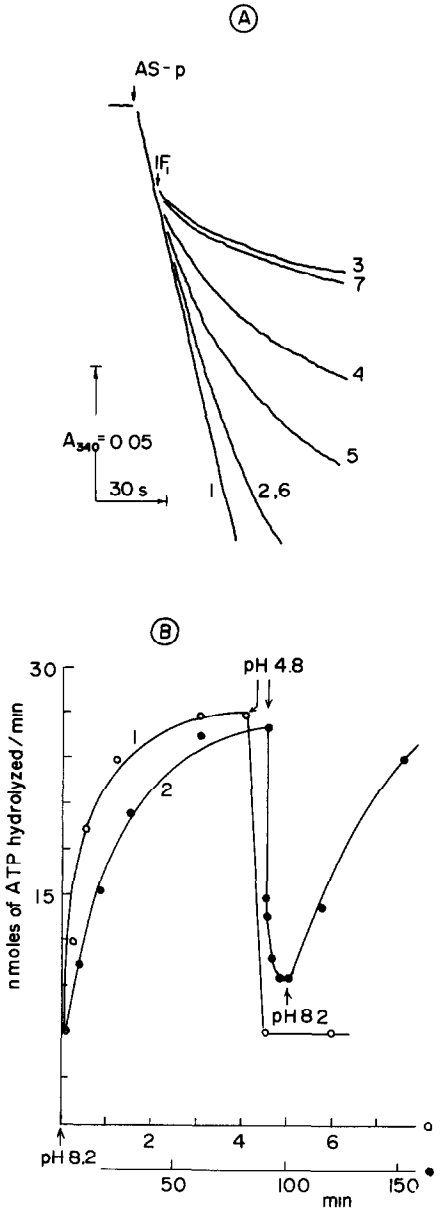

Fig.3. Time course of the pH-dependent active/inactive transitions of IF_1. (A) The actual traces of the ATPase assays at pH 8.0 after addition of IF_1. pH of the buffers where IF_1 was dissolved was adjusted to 8.2 (curve 2) or 4.8 (curve 3) and IF_1 was added to the assay mixture (pH 8.0) where indicated. The buffer containing IF_1 at pH 4.8 was then rapidly adjusted to pH 8.2, and IF_1 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_1 was added to the assay mixture (curve 7). Curve 1, no IF_1 added. (B) IF_1 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_1. At zero time, the pH of the IF_1 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_1 for the assays were taken from were incubated at 25 and 0°C, respectively. The amounts of AS-particles and IF_1 added to 1.5 ml of the assay mixture were 14 and 16 μg, respectively.

saturating curve was obtained with an upper limit of 1.0 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 (~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_1 rather than of F_1.

An unexpected finding shown in fig.3A is that when equal amounts of IF_1 were added to ATP hydrolyzing particles from acidic or alkaline solutions of the inhibitor the inhibition patterns were quite different, i.e., acidic IF_1 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_1 is slow enough to be registered at 25°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 interconversion of 'active' to 'inactive' IF_1 is about 30 s and 20 min at 25 and 0°C, respectively). When the inhibition rate was plotted as a function of acidic IF_1, a straight line was obtained (fig.2, line 2), indicating a simple second-order reaction between F_1 and acidic IF_1, which is a first-order process with respect to the inhibitor.

The pH profile of the active/inactive interconversion of IF_1 (not shown) suggested the participation of a single group with an apparent pK_a of 6.5. Although a straightforward interpretation

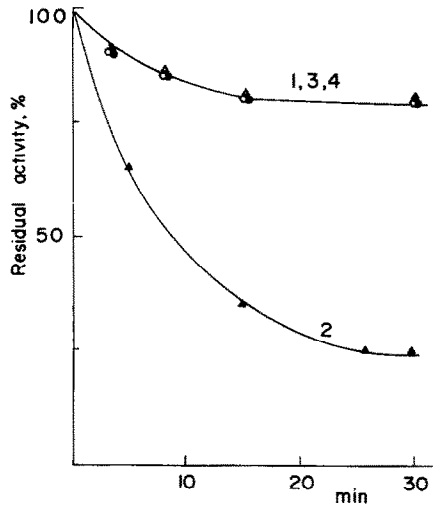

Fig.4. Inactivation of IF_1 by a histidine-specific reagent. 14 μg AS-particles were preincubated at 25°C for the time indicated on abscissa in 200 μl of the buffer used for activation (see section 2) which contained 4 μM ATP, 5 mM phospho*enol*pyruvate, 10 units of pyruvate kinase and 0.045 μg IF_1 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 μg IF_1 were preincubated at 25°C for 10 min in 100 μl 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_1 preincubated without diethyl pyrocarbonate was added to AS-particles; curve 3, IF_1 treated with diethyl pyrocarbonate was added to AS-particles; curve 4, an aliquot containing diethyl pyrocarbonate without IF_1 was added to AS-particles. The IF_1 to F_1 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_1 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_1 was treated with a histidine-specific reagent (diethyl pyrocarbonate), its inhibitory potency was lost (fig.4).

The simplest model for the IF_1-F_1 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_1·substrate (product) complexes (presumably on the β-subunit [12,27]), where IF_1 binds, thus inhibiting the ATP hydrolytic activity. The IF_1 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_1 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^{-1}; fig.2, curve 1). A very high sensitivity of IF_1 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_1 are slow as compared to the rate of the active IF_1-F_1 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_1 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_1 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_1 synchronous with its ATP synthetase activity in submitochondrial particles, although some correlation between IF_1 release and phosphorylating activity has been reported [14,17,18]. The experimentally supported hypothesis on two states of F_1 (hydrolase and synthetase) [30–32] also seems relevant to the functional role of IF_1. We believe that under physiological conditions IF_1 serves as a stabilizer of the F_1-synthetase state, thus preventing a possible leakage of the phosphorylating potential of ATP in mitochondria.

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