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Inhibition of the ATPase activity of *Escherichia coli* ATP synthase by magnesium fluoride

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Abstract Inhibition of ATPase activity of *Escherichia coli* ATP synthase by magnesium fluoride (MgFx) was studied. Wild-type F_1-ATPase was inhibited potently, albeit slowly, when incubated with $MgCl_2$, NaF, and NaADP. The combination of all three components was required. Reactivation of ATPase activity, after removal of unbound ligands, occurred with half-time of ~14 h at 22 °C and was quasi-irreversible at 4 °C. Mutant F_1-ATPases, in which catalytic site residues involved in transition state formation were modified, were found to be resistant to inhibition by MgFx. The data demonstrate that MgFx in combination with MgADP behaves as a tight-binding transition state analog in *E. coli* ATP synthase.
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1. Introduction

ATP synthase is the enzyme responsible for ATP synthesis in oxidative and photophosphorylation in mitochondria, chloroplasts and bacteria. It operates as a molecular motor [1] in which *trans*-membrane movement of protons (or Na^+ ions) down an electrochemical gradient, between *a* and *c* subunits, drives rotation of a group of subunits called the rotor, and the energy of rotation is thereby transferred to three catalytic sites, which are immobilised by stator subunits, resulting in efficient synthesis of ATP. In *Escherichia coli*, representing the simplest structural example, the rotor subunits consist of γ, ε, and a c_{10} ring, the three catalytic sites are located at interfaces of α and β subunits in the $\alpha_3\beta_3$ hexagon [2], and the stator consists of $b_2\delta$ [3]. ATP hydrolysis drives proton movement and rotation of the rotor in the opposite directions to those occurring in ATP synthesis [4,5]. Recent reviews of ATP synthase structure and function may be found in [6,7].

Reaction mechanisms of ATP synthesis and hydrolysis, and their relationship to mechanical rotation of subunits are therefore topics of current interest and study. Understanding the structure and characteristics of the chemical transition state is clearly of importance for advancing the field. Two general approaches to study the transition state have been used so far, and both took advantage of MgADP-fluorometal complexes such as MgADP-AlFx and MgADP-ScFx. Walker, Leslie, and colleagues have presented high-resolution X-ray structures of the "F_1" portion of mitochondrial ATP synthase ($\alpha_3\beta_3\gamma\delta\varepsilon$ subunits) in complex with catalytic site bound MgADP-AlF_3 and MgADP-AlF_4^- [8,9]. The latter appeared to represent a true transition state structure, and the former was interpreted as representing a late-transition state/early ground state structure. In addition, an X-ray structure of the ground state MgADP-BeFx complex bound in catalytic sites provided important further, comparative information [10]. Also, biochemical and kinetic studies of inhibition of ATPase activity by such fluorometal complexes were widely reported and documented in earlier years, and have been refined recently in the *E. coli* enzyme by combination with mutagenesis of catalytic site side-chains and use of engineered tryptophan fluorescence to assess catalytic site occupancy and affinities for MgADP-fluorometal complexes, leading to extensive functional characterisation of the transition state [11,12].

From its behavior as an activator of G-proteins [13,14] and as an inhibitor of myosin ATPase [15] it appeared that magnesium fluoride, specifically MgF_3^-, in combination with GDP or ADP, could also mimic a transition state complex. Direct confirmation of this came from an X-ray structure showing RhoA.GDP bound to p50RhoGAP in complex with MgF_3^- [16]. While there is as yet no published report of use of magnesium fluoride with ATP synthase, in a personal communication M.W. Bowler and colleagues [1] recently informed us that MgF_3^- does inhibit bovine heart mitochondrial F_1-ATPase activity and binds at catalytic sites in a transition state-like structure. In this report, we present studies of magnesium fluoride as an inhibitor of *E. coli* F_1-ATPase activity in both wild-type and several catalytic site mutant enzymes.

2. Materials and methods

2.1. Purification of F_1; depletion of catalytic-site bound nucleotide; assay of ATPase activity of purified F_1

F_1 was purified as in [17]. Prior to the experiments, F_1 samples (100 µl) were passed twice through 1 ml centrifuge columns (Sephadex G-50) equilibrated in 50 mM Tris–Cl, pH 8.0, at 22 °C to remove

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Abbreviations: AlFx, aluminum fluoride; MgFx, magnesium fluoride; ScFx, scandium fluoride complexes; where *x* is undefined

[1] Bowler, M.W., Blackburn, G.M., Leslie, A.G.W., and Walker, J.E. (2005) personal communication of unpublished data.

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catalytic site-bound nucleotide [18]. ATPase activity was measured in 1.0 ml assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, 50 mM Tris–Cl, pH 8.5, at 22 °C. Reactions were started by addition of enzyme and stopped by addition of SDS to 3.3% final concentration. Pi was assayed as in [19]. For wild-type F_1, reaction times were 3 min. For mutant enzymes reaction times were up to 30 min. All reactions were shown to be linear with time and protein concentration.

2.2. E. coli *strains*

Wild-type strain SWM1 was used [20]. Mutant strains were α R376K [2] [21], βR182Q and βR182K [22], βK155Q [23], βR246A, βR246K and βR246Q [24] and βN243A [25]. These enzymes all contained additionally the βY331W mutation to make them compatible with the previous cited work which used the inserted Trp for fluorimetric estimations of nucleotide-binding and transition-state formation. The βY331W mutation by itself does not significantly affect activity and we confirmed that it did not affect inhibition or reactivation characteristics with MgFx.

2.3. Inhibition of ATPase activity by magnesium fluoride and reactivation after inhibition

F_1 (0.2–0.5 mg/ml, 0.52–1.3 μM) was preincubated at 22 °C for varied times as indicated in 100 μl containing 50 mM Tris–Cl, pH 8.0, with 1 mM NaADP, 12 mM NaF (Fisher ACS reagent, Cat. No. S-299), and $MgCl_2$ (J.T. Baker ACS reagent, Cat. No. 2444-05) concentrations as indicated. The NaF and $MgCl_2$ reagents were essentially free (ppm range) of Sc, Al and Be. At the end of preincubation the whole sample was passed through a 1 ml centrifuge column (Sephadex G-50, 22 °C, in 50 mM Tris–Cl, pH 8.0) and 50–80 μl of the eluate was taken for ATPase assay.

3. Results

3.1. Time courses and $MgCl_2$-concentration dependence of inhibition of ATPase activity of wild-type E. coli *F_1-ATPase by magnesium fluoride*

Wild-type enzyme was preincubated at room temperature with varied $MgCl_2$ concentration together with 12 mM NaF, and 1 mM NaADP, for varied time, then passed through a centrifuge column to remove unbound ligand before assay of ATPase activity. Fig. 1 shows the data obtained. Potent inhibition by MgFx could be achieved. At least 5 h preincubation was needed to reach maximal levels of inhibition, and high concentrations of $MgCl_2$ were required. It may be noted that previously reported time-courses for inhibition of *E. coli* F_1-ATPase by ScFx and AlFx under similar conditions were much faster than seen here in Fig. 1 [11]; it is not yet clear why this is so. Further the concentration of Mg^{2+} ion required for maximal MgFx inhibition was much higher (50 mM) than was required with ScFx and AlFx (2.5 mM). In this regard, it may be noted that maximal functional effects of MgFx in G-proteins and myosin were achieved at Mg^{2+} concentrations of 1–2 mM [13–15], with NaF at 10–12 mM concentration as used in this work. The reason for the requirement for high Mg^{2+} concentration in *E. coli* F_1-ATPase is also not yet clear.

3.2. Demonstration that NaF, NaADP and $MgCl_2$ are required in combination for inhibition

Fig. 2 shows that no single component (NaF, NaADP, $MgCl_2$) nor any pair of components was sufficient to yield inhibition of ATPase activity. Rather, all three had to be present. The requirement for ADP provides evidence that magnesium fluoride is acting as a transition state analog, mimicking the γ-phosphate of ATP in the transition state, rather than as a tight binding Pi analog.

[2] *E. coli* residue numbers used throughout.

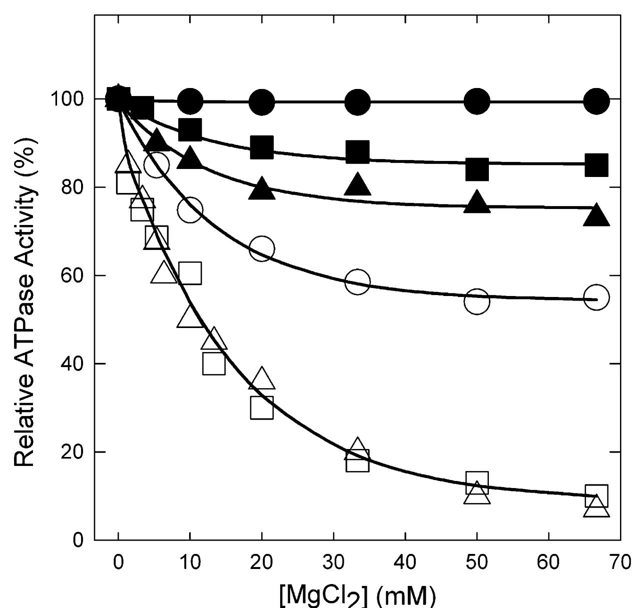

Fig. 1. Inhibition of wild-type *E. coli* F_1-ATPase by MgFx. F_1 was preincubated at 22 °C with 1 mM NaADP, 12 mM NaF and varied concentration of $MgCl_2$ as shown on the horizontal axis. (For further details see Section 2). After removal of unbound ligand by passage through centrifuge columns, ATPase activity was measured. Each point is the mean of quadruplicate experiments which agreed within ±10%. 100% represents the activity of uninhibited enzyme (Specific activity = 12 μmol/min/mg at 22 °C). ●, preincubation was for 10 min; ■, 30 min; ▲, 60 min; ○, 120 min; □, 5 h; △, 20 h.

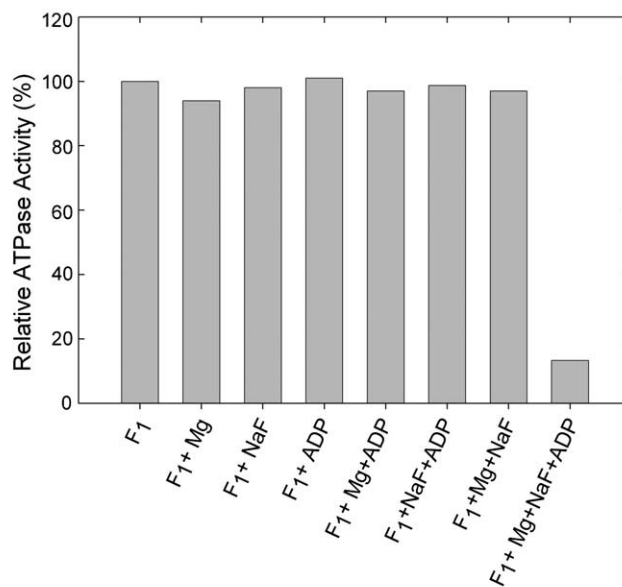

Fig. 2. Demonstration of requirements for inhibition of ATPase activity of wild-type *E. coli* F_1 by MgFx. F_1 was preincubated for 5 h at 22 °C in presence of 1 mM NaADP (“ADP”) or 12 mM NaF or 50 mM $MgCl_2$ (“Mg”) or combinations thereof as shown. ATPase activity was measured after passage through a centrifuge column to remove unbound ligand. Results are means of quadruplicate experiments which agreed within ±10%.

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