FEBS Letters 580 (2006) 517–520

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

Zulfiqar Ahmad, Alan E. Senior*

Department of Biochemistry and Biophysics, Box 712, University of Rochester Medical Center, Rochester, NY 14642, USA

Received 25 October 2005; revised 5 December 2005; accepted 16 December 2005

Available online 28 December 2005

Edited by Stuart Ferguson

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.**

Keywords: Oxidative phosphorylation; ATP synthase; ATP synthesis mechanism; Magnesium fluoride; ATPase inhibition; Transition state analog

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

*Corresponding author. Fax: +1 585 271 2683.
E-mail address: alan_senior@urmc.rochester.edu (A.E. Senior).

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.

0014-5793/$32.00
doi:10.1016/j.febslet.2005.12.057

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.

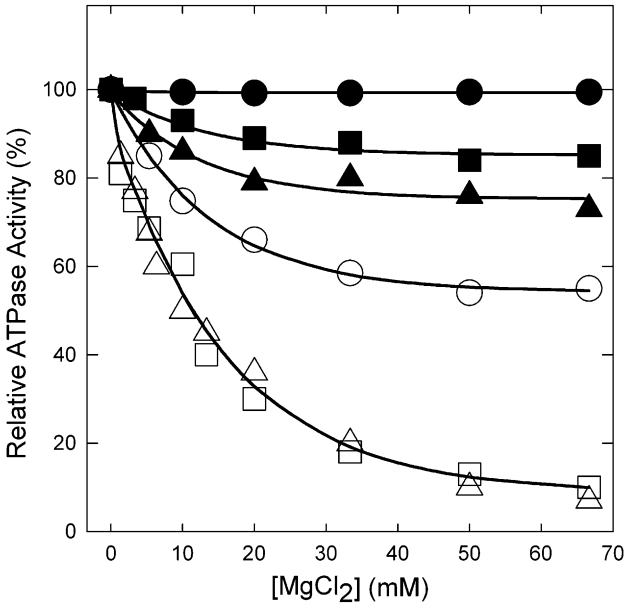

Fig. 1. Inhibition of wild-type *E. coli* F_1-ATPase by MgFx. F_1 was preincubated at 22 °C with 1 mM NaADP, 12 mM NaF and varied concentration of $MgCl_2$ as shown on the horizontal axis. (For further details see Section 2). After removal of unbound ligand by passage through centrifuge columns, ATPase 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.

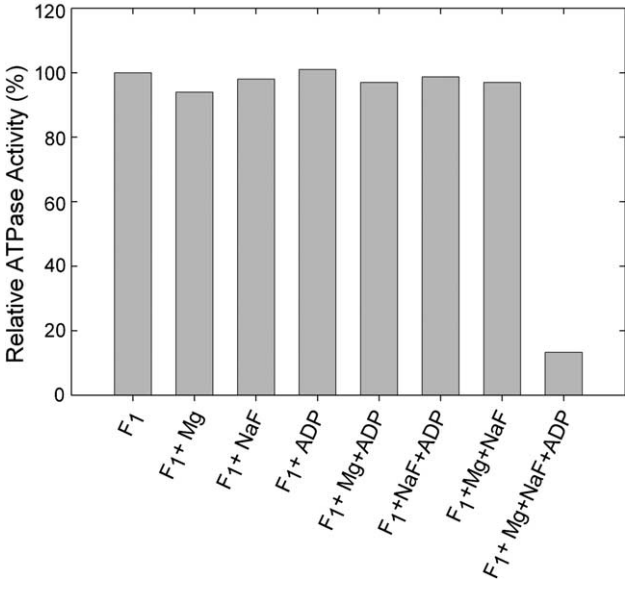

Fig. 2. Demonstration of requirements for inhibition of ATPase activity of wild-type *E. coli* F_1 by MgFx. F_1 was preincubated for 5 h at 22 °C in presence of 1 mM NaADP ("ADP") or 12 mM NaF or 50 mM $MgCl_2$ ("Mg") or combinations thereof as shown. ATPase activity was measured after passage through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

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

3.3. Reactivation of ATPase activity after prior inhibition of wild-type E. coli F_1-ATPase by magnesium fluoride

After preincubation to achieve inhibition and centrifuge column elution to remove unbound ligands, eluates were allowed to incubate for various times at 22, 37 and 4 °C, and assayed for recovery of ATPase activity. Fig. 3 shows the data obtained. At 22 and 37 °C, there was slow recovery of activity, up to 100% of initial activity. Both curves were fit satisfactorily by a single exponential equation, yielding half-times of 13.9 h at 22 °C and 5.7 h at 37 °C. It may be noted that the concentration of F_1 in the centrifuge column eluates was ~0.5 μM, thus re-binding of released ADP-MgFx would be negligible. These data show that magnesium fluoride in combination with ADP produces a tight binding inhibitory complex that is not covalent but is slowly reversible. For comparison, half-times for recovery of ATPase at 22 °C under similar conditions, after inhibition by MgADP-ScFx, MgADP-AlFx and MgADP-BeFx were, respectively, 12, ~100, and ≫100 h [11]. The data at 4 °C in Fig. 3 showed that there was virtually no recovery of activity even up to 60 h of incubation. This was not due to enzyme denaturation, because samples that had been incubated for 60 h at 4 °C were seen to reactivate when placed at 37 °C, with the same kinetics as seen in the 37 °C curve in Fig. 3.

3.4. Behavior of catalytic site mutant enzymes toward magnesium fluoride

The positively charged side-chains of four catalytic site residues, namely β-Lys155, β-Arg182, β-Arg246 and α-Arg376 have been shown by X-ray structure analysis and by mutagenesis studies to be engaged with the transition state complex and to be functionally required for transition state formation [9,12]. We tested whether these residues were involved in the inhibition brought about by MgFx by use of the following mutant enzymes: βK155Q; βR182Q; βR182K; βR246A; βR246Q; βR246K; αR376K. Each of these mutant enzymes has low but measurable ATPase activity [24,26]. We found that even with prolonged incubation to 20 h at 50 mM $MgCl_2$ with 12 mM NaF and 1 mM NaADP, there was no inhibition in any of these mutant enzymes (Fig. 4, open circles). This lends further support to the idea that MgFx is behaving as a transition state analog.

Residue β-Asn243 is located in the catalytic site of ATP synthase very close to bound ATP and/or Pi. Experiments using MgADP-AlFx or MgADP-ScFx as inhibitors of the βN243A mutant [25] indicated previously that the Asn side-chain does not interact with the transition state directly, but is required for correct transition state organization. Hence it was of interest to find out whether this residue is required for MgFx inhibition. For this purpose, we used mutant βN243A F_1 and the data are shown in Fig. 4, closed symbols. Inhibition of ATPase did occur, confirming that residue β-Asn243 is not directly involved in liganding the MgADP-MgFx complex. Moreover, at lower $MgCl_2$ concentrations the βN243A mutant appeared more susceptible to inhibition than wild-type, indicating that MgFx made stronger interactions with the catalytic site of the mutant. These results with MgFx mirror results found previously with AlFx and βN243A enzyme [25] confirming that residue βAsn-243 is involved indirectly in transition state organization.

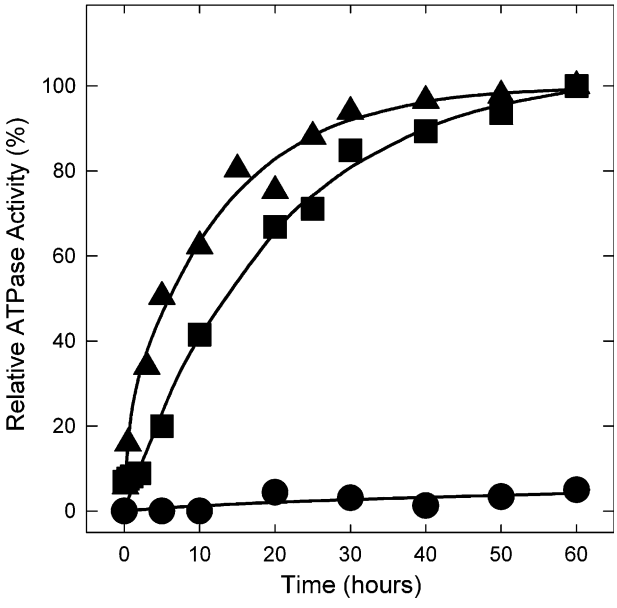

Fig. 3. Reactivation of ATPase activity in wild-type *E. coli* F_1 after inhibition by MgFx. Inhibition was achieved as in Fig. 1 by preincubation for 5 h at 50 mM $MgCl_2$. After centrifuge column elution the eluates were further incubated at varying temperature and assayed at times shown. ▲, 37 °C; ■, 22 °C; ●, 4 °C. Each point is the mean of quadruplicate experiments which agreed within ±10%. Lines are best fits using a single exponential equation (except at 4 °C where a line was simply drawn through the points).

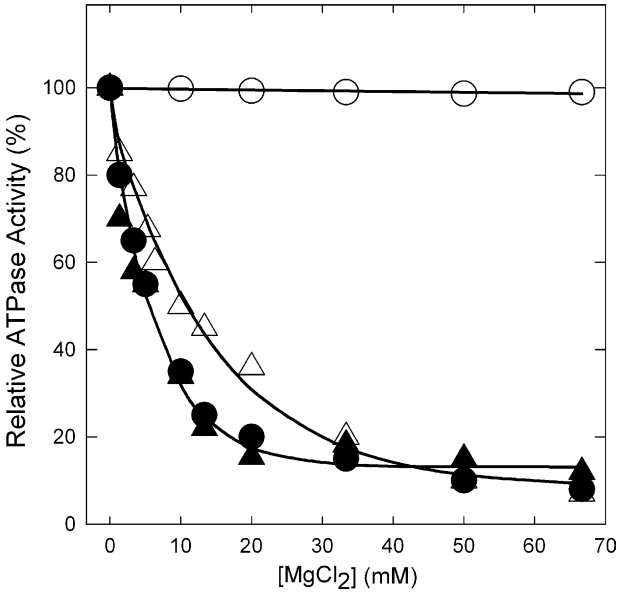

Fig. 4. Effect of MgFx on mutant *E. coli* F_1-ATPase enzymes. The experimental procedure was as in Fig. 1. ○, preincubation of βR246A mutant enzyme for 20 h at 22 °C with 1 mM NaADP, 12 mM NaF and varied concentration of $MgCl_2$ as shown on the horizontal axis. The same result was seen with βR246Q, βR246K, βR182Q, βR182K, βK155Q and αR376K mutant enzymes, but these data are omitted to avoid congestion. △, wild-type enzyme preincubated 20 h (same data as in Fig. 1). ●, ▲, βN243A enzyme preincubated for 5 and 20 h, respectively. Each point is the mean of at least six experiments which agreed within ±10%. 100% represents the activity of uninhibited enzyme. Specific ATPase activities (i.e. 100% values) of the F_1-ATPase enzymes at 22 °C in μmol/mg/min were as follows: Wild-type, 12.1; βN243A, 0.87; βR246A, 0.24; βR246Q, 0.28; βR246K, 0.26; βR182Q, 0.023; βR182K, 0.24; βK155Q, 0.026; αR376K, 0.12.

4. Discussion

The goals of this study were to find out whether magnesium fluoride (MgFx) inhibited F_1-ATPase from *E. coli* and to characterize the inhibition mechanism. The data presented show that MgFx is a slow-acting yet potent inhibitor of wild-type enzyme, that ADP is required for inhibition, and that reactivation occurs slowly but with full recovery to initial uninhibited level. All of these data point to the conclusion that in *E. coli* ATP synthase MgADP-MgFx acts as a tight-binding transition state analog that brings about inhibition by tenaciously binding to catalytic sites, and is released slowly concomitant with reactivation.

Studies with mutant enzymes further showed that residues β-Lys155, β-Arg182, β-Arg246 and α-Arg376 all interact with bound MgFx and that removal of any one side-chain abolished inhibition, supporting the idea of an extremely cooperative transition state structure. Each of these residues has previously been implicated in transition state formation by X-ray structure analysis [9] and by mutational analysis combined with MgADP-AlFx and MgADP-ScFx binding and inhibition studies [12].

Our studies do not identify the actual species of MgFx that is producing the inhibition. In previous work with G-proteins, MgF_3^- was found to be the species bound [16]. In recent, X-ray structural studies by Bowler and colleagues (see Section 1 and Footnote 1) of bovine mitochondrial F_1-ATPase complexed with magnesium fluoride, MgF_3^- was also found to be the species bound. However, in studies of sarcoplasmic reticulum Ca^{2+}-pumping ATPase MgF_4^{2-} was the species that was bound, and in that case MgF_4^{2-} was clearly mimicking a bound Pi ion [27]. It is quite reasonable to suggest that inhibition of F_1-ATPase as seen here in Fig. 1 might be achieved by MgF_4^{2-} acting as a Pi analog, and this would also be compatible with much of the mutant data (text and Fig. 4) since βK155Q, βR182Q, βR182K, and βR246A, K and Q enzymes are all deficient in Pi binding [26]. Two observations argue against this conclusion, however. One is that inhibition was fully MgADP-dependent (Fig. 2). The second is that the mutant enzyme αR376K was not inbited by MgFx, despite the fact that it retains ability to bind Pi [26]. Thus we strongly favor the interpretation that MgADP-MgF_3^- complex is the inhibitory species in *E. coli* under the conditions used here.

Acknowledgments: Supported by NIH Grant GM25349 to AES. The work described in this paper was stimulated by access to the unpublished transition state analogue crystal structure of bovine F_1-ATPase inhibited with ADP and magnesium fluoride determined by Matthew Bowler, Andrew Leslie and John Walker (MRC, Cambridge, UK). We are grateful to them for access to their data and for their advice on the conditions for inhibition.

References

[1] Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K. (1997) Direct observation of the rotation of F_1-ATPase. Nature 386, 299–302.
[2] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature 370, 621–628.
[3] Dunn, S.D., McLachlin, D.T. and Revington, M. (2000) The second stalk of *Escherichia coli* ATP synthase. Biochim. Biophys. Acta 1458, 356–363.
[4] Diez, M., Zimmerman, B., Börsch, M., König, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekyan, S., Kudryavtsev, V., Seidel, C.A.M. and Gräber, P. (2004) Proton-powered subunit rotation in single membrane-bound F_0F_1-ATP synthase. Nature Struct. Mol. Biol. 11, 135–141.
[5] Itoh, H., Takahashi, A., Adachi, K., Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K. (2004) Mechanically driven ATP synthesis by F_1-ATPase. Nature 427, 465–468.
[6] Noji, H. and Yoshida, M. (2001) The rotary machine in the cell, ATP synthase. J. Biol. Chem. 276, 1665–1668.
[7] Weber, J. and Senior, A.E. (2003) ATP synthesis driven by proton transport in F_1F_0-ATP synthase. FEBS Lett. 545, 61–70.
[8] Braig, K., Menz, R.I., Montgomery, M.G., Leslie, A.G.W. and Walker, J.E. (2000) Structure of bovine F_1-ATPase inhibited by MgADP and aluminium fluoride. Structure 8, 567–573.
[9] Menz, R.I., Walker, J.E. and Leslie, A.G.W. (2001) Structure of bovine mitochondrial F_1-ATPase with nucleotide bound to all three catalytic sites: implications for the mechanism of rotary catalysis. Cell 106, 331–341.
[10] Kagawa, R., Montgomery, M.G., Braig, K., Leslie, A.G.W. and Walker, J.E. (2004) The structure of bovine F_1-ATPase inhibited by ADP and beryllium fluoride. EMBO J. 23, 2734–2744.
[11] Nadanaciva, S., Weber, J. and Senior, A.E. (2000) New probes of the F_1-ATPase catalytic transition state reveal that two of the three catalytic sites can assume a transition state conformation simultaneously. Biochemistry 39, 9583–9590.
[12] Senior, A.E., Weber, J. and Nadanaciva, S. (2000) The catalytic transition state in ATP synthase. J. Bioenerg. Biomemb. 32, 523–529.
[13] Antonny, B., Bigay, J. and Chabre, M. (1990) A novel magnesium-dependent mechanism for the activation of transducin by fluoride. FEBS Lett. 268, 277–280.
[14] Antonny, B., Sukumar, M., Bigay, J., Chabre, M. and Higashijima, T. (1993) The mechanism of aluminum-independent G-protein activation by fluoride and magnesium. J. Biol. Chem. 268, 2393–2402.
[15] Park, S., Ajtai, K. and Burghardt, T.P. (1999) Inhibition of myosin ATPase by metal fluoride complexes. Biochim. Biophys. Acta 1430, 127–140.
[16] Graham, D.L., Lowe, P.N., Grime, G.W., Marsh, M., Rittinger, K., Smerdon, S.J., Gamblin, S.J. and Eccleston, J.F. (2002) MgF_3^- as a transition state analog of phosphoryl transfer. Chem. Biol. 9, 375–381.
[17] Weber, J., Lee, R.S.F., Grell, E., Wise, J.G. and Senior, A.E. (1992) On the location and function of tyrosine β331 in the catalytic site of *Escherichia coli* F_1-ATPase. J. Biol. Chem. 267, 1712–1718.
[18] Weber, J., Wilke-Mounts, S. and Senior, A.E. (1994) Cooperativity and stoichiometry of substrate binding to the catalytic sites of *Escherichia coli* F_1-ATPase. Effects of magnesium, inhibitors and mutation. J. Biol. Chem. 269, 20462–20467.
[19] Taussky, H.H. and Shorr, E. (1953) A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202, 675–685.
[20] Rao, R., Al-Shawi, M.K. and Senior, A.E. (1988) Directed mutagenesis of the strongly conserved lysine 175 in the proposed nucleotide-binding domain of α-subunit from *Escherichia coli* F_1-ATPase. J. Biol. Chem. 263, 5569–5573.
[21] Nadanaciva, S., Weber, J., Wilke-Mounts, S. and Senior, A.E. (1999) Importance of F_1-ATPase residue α-Arg-376 for catalytic transition state stabilization. Biochemistry 38, 15493–15499.
[22] Nadanaciva, S., Weber, J. and Senior, A.E. (1999) The role of β-Arg-182, an essential catalytic residue in *Escherichia coli* F_1-ATPase. Biochemistry 38, 7670–7677.
[23] Löbau, S., Weber, J., Wilke-Mounts, S. and Senior, A.E. (1997) F_1-ATPase, roles of three catalytic site residues. J. Biol. Chem. 272, 3648–3656.
[24] Ahmad, Z. and Senior, A.E. (2004) Mutagenesis of residue βArg-246 in the phosphate-binding subdomain of catalytic sites of *Escherichia coli* F_1-ATPase. J. Biol. Chem. 279, 31505–31513.
[25] Ahmad, Z. and Senior, A.E. (2004) Role of βAsn-243 in the phosphate-binding subdomain of catalytic sites of *Escherichia coli* F_1-ATPase. J. Biol. Chem. 279, 46057–46064.
[26] Ahmad, Z. and Senior, A.E. (2005) Involvement of ATP synthase residues αArg-376, βArg-182, and βLys-155 in Pi binding. FEBS Lett. 579, 523–528.
[27] Toyoshima, C., Nomura, H. and Tsuda, T. (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. Nature 432, 361–368.