ATP hydrolysis-driven H⁺ translocation is stimulated by sulfate, a strong inhibitor of mitochondrial ATP synthesis

Anabella F. Lodeyro · María V. Castelli · Oscar A. Roveri

Abstract Sulfate is a partial inhibitor at low and a non-essential activator at high [ATP] of the ATPase activity of F_1. Therefore, a catalytically-competent ternary $F_1 \cdot ATP \cdot sulfate$ complex can be formed. In addition, the ANS fluorescence enhancement driven by ATP hydrolysis in submitochondrial particles is also stimulated by sulfate, clearly showing that the ATP hydrolysis in its presence is coupled to H⁺ translocation. However, sulfate is a strong linear inhibitor of the mitochondrial ATP synthesis. The inhibition was competitive (K_i=0.46 mM) with respect to Pi and mixed (K_i=0.60 and K'_i=5.6 mM) towards ADP. Since it is likely that sulfate exerts its effects by binding at the Pi binding subdomain of the catalytic site, we suggest that the catalytic site involved in the H⁺ translocation driven by ATP hydrolysis has a more open conformation than the half-closed one (β_{HC}), which is an intermediate in ATP synthesis. Accordingly, ATP hydrolysis is not necessarily the exact reversal of ATP synthesis.

Keywords ATP synthesis inhibition · ATPase activation · Bovine mitochondria · Sulfate · H⁺ translocation · Reaction intermediates

Abbreviations

ANS	8-anilino-1-naphtalene sulfonate
SMP	phosphorylating Mg-ATP submitochondrial particles
FCCP	carbonyl cyanide p-trifluoromethoxyphenyl hydrazone
AMP-PNP	adenosine-5′-(β,γ-imido) triphosphate
EPPS	N-hydroxyethyl)piperazine-N′-3-propane sulphonic acid

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Introduction

The mitochondrial F_1F_o-ATPase/synthase is located in energy-transducing membranes where it catalyzes $\Delta\mu_H^+$-driven ATP synthesis and $\Delta\mu_H^+$-generating ATP hydrolysis. It has a membrane sector (F_o) attached by central and peripheral stalks to a membrane extrinsic oligomeric complex (F_1), that contains the catalytic sites for ATP synthesis and hydrolysis (Walker and Dickson 2006, and references quoted in there). F_1 can be isolated from the membrane retaining only its ability to hydrolyze ATP. It is composed of five different subunits with a stoichiometry

$\alpha_3\beta_3\gamma\delta\epsilon$. The three α subunits and the three β subunits are arranged alternately around the γ subunit (Abrahams et al. 1994) that constitutes, together with subunits δ and ϵ, the central stalk (Gibbons et al. 2000). Three catalytic and three non-catalytic sites are located in the α/β subunit interfaces. The catalytic sites are predominantly made up by β-subunit residues, whereas the non-catalytic sites belong mostly to an α-subunit domain (Abrahams et al. 1994). The catalytic sites exhibit strong cooperativity and it is widely accepted that ATP hydrolysis and ATP synthesis follow Boyer's binding-change mechanism (Boyer 1993). According to this mechanism the three catalytic sites exist at any given time in three states that differ in their affinities for reactants and/or products (O, open; L, low; T, tight). During the catalytic cycle, a particular catalytic site passes sequentially through these three states. It has been proposed that this sequential conversion of conformational states is induced by the rotation—within the $(\alpha\beta)_3$ subcomplex—of the central stalk connected to a rotary c subunit oligomer of F_o (Boyer 1997; Cross 2000). This proposal has been further supported by direct observation of ATP hydrolysis-driven counterclockwise rotation when viewed from the membrane: (1) of the central γ subunit in immobilized $(\alpha\beta)_3\gamma$ subcomplex of the thermophilic bacterium *Bacillus* PS3 (Noji et al. 1997) and in liposome-reconstituted F_1F_o (Börsch et al. 2002); and (2) of the c subunit oligomer in immobilized ATP synthase (F_oF_1) from *Escherichia coli* (Pänke et al. 2000). More recently, it has not only been shown that the proton gradient-driven ATP synthesis is accompanied by rotation of the γ subunit (Diez et al. 2004) but also that the clockwise rotation forced by external electromagnets of the γ subunit in immobilized $(\alpha\beta)_3\gamma$ subcomplex drives the synthesis of ATP from ADP and Pi (Rondelez et al. 2005). More detailed studies have shown that a complete revolution occurs in three 120° steps, further divided in 80° and 40° substeps (see Nakamoto et al. 2008 for a recent review on the rotary mechanism).

The structure of several enzyme forms has been resolved and it has been proposed that they correspond to different intermediates and/or transition states in the reaction pathway (Abrahams et al. 1994; Bianchet et al. 1998; Kagawa et al. 2004; Chen et al. 2006; Bowler et al. 2007). An electron density consistent with a bound sulfate was observed in the β_E catalytic site of the high AMP-PNP and frozen-native F_1 structures (Menz et al. 2001a), that have only two catalytic sites occupied with nucleotides. Menz et al. have also succeeded in crystallizing F_1 with three catalytic sites occupied by nucleotides: two of them by MgADP-fluoroaluminate and the third one by "ADP and what is assumed to be a sulfate ion" (*sic*) (Menz et al. 2001b). This latter site has a half-closed conformation (β_{HC}), intermediate between the closed (β_{DP} and β_{TP}) and the open (β_E) conformations. These authors postulated that

this conformation represents the posthydrolysis state of the reaction. However, a thorough understanding of the catalytic cycle has not been obtained and a generally accepted enzymatic mechanism has not emerged yet (Weber and Senior 2003). Moreover, there are still controversies whether two (Milgrom and Cross 2005) or three (Bianchet et al. 1998; Senior et al. 2002; Gao et al. 2005) catalytic sites must be occupied for steady-state turnover.

It is also matter of discussion if ATP hydrolysis and ATP synthesis follow a single unique catalytic pathway (Vinogradov 2000; Weber and Senior 2003; Gao et al. 2005). Bicarbonate and sulfite, among other compounds, exhibit opposite effects on ATP synthesis and ATP hydrolysis (see Vinogradov 2000 and references quoted in Lodeyro et al. 2001). The effects of these anions on ATP synthesis and hydrolysis have been thoroughly studied (Lodeyro et al. 2001; Pacheco-Moisés et al. 2002). The ATPase activity of isolated mitochondrial F_1 and of membrane bound F_1F_o is stimulated by bicarbonate. Ebel and Lardy (1975) have reported that bicarbonate is an activator of the ATPase activity and claimed that it abolishes the apparent negative cooperativity exhibited by F_1. However, later studies have shown that even in the presence of this anion the negative cooperativity can still be observed (Wong et al. 1984; Roveri and Calcaterra 1985). We have shown that a significant increase in the affinity for ATP of the catalytic site operating at the high $K_{m(ATP)}$ region and a slight promotion of the departure of ADP were required to satisfactorily explain the effect of bicarbonate (Roveri and Calcaterra 1985). More recently, we have also shown that bicarbonate completely inhibits ATP synthesis in submitochondrial particles (Lodeyro et al. 2001). The inhibition was described as linear non-competitive with respect to Pi and as linear competitive towards ADP. On the basis of these results we concluded that: (1) bicarbonate does not bind to a Pi binding site in the mitochondrial F_1; and (2) it competes with ADP for a low-affinity site, most likely the low affinity non-catalytic nucleotide binding site or site 6 according to Berden and Hartog's nomenclature (Berden and Hartog 2000). In addition, we have suggested that the binding of bicarbonate to this latter site increases the affinity of ATP and decreases that of ADP for the catalytic site (Lodeyro et al. 2001).

Sulfate is an analog of Pi that has been observed bound at nucleotide binding sites in some of the F_1 crystal structures reported (Shirakihara et al. 1997; Menz et al. 2001a,b). Zharova and Vinogradov have recently reported that "sulfate competes with Pi when the rates of respiration-supported ATP synthesis were measured at saturating ADP concentrations" (*sic*) (Zharova and Vinogradov 2006). However a complete kinetic characterization of the effect of sulfate on ATP synthesis has not been reported as yet. Sulfate is also an effector of the ATPase activity of the

mitochondrial ATPase. Ebel and Lardy have reported that 20 mM sulfate caused a slight activation (about 20%) of the activity of rat liver mitochondrial ATPase (Ebel and Lardy 1975). Conversely, Recktenwald and Hess have shown that sulfate inhibits the ATPase activity of F_1 from yeast mitochondria at low ATP concentrations and has practically no effect at high ATP concentrations. They also claimed that 20 mM sulfate abolishes the negative cooperativity in yeast F_1. They postulated that the binding of ATP to a "regulatory" non-catalytic nucleotide binding site would be the reason of the apparent negative cooperativity and that anions like sulfate and bicarbonate would compete with ATP for that site, eliminating the negative cooperativity observed: while bicarbonate would activate the ATPase activity and stabilize the enzyme in a low $K_{m(ATP)}$ catalytic mode, sulfate would favor a high $K_{m(ATP)}$ mode (Recktenwald and Hess 1977). This model has received further support from Berden and coworkers when studying the effect of 5′-fluorosulfonybenzoyladenosine (an affinity labeling ATP analog) on the stimulatory effect of anions (Hartog et al. 1997).

In order to solve the above mentioned contradictions and to contribute to the knowledge of the enzyme intermediates that participates in $\Delta\mu_H^+$-driven ATP synthesis and $\Delta\mu_H^+$-generating ATP hydrolysis, we made a detailed kinetic study of the effect of sulfate on: (1) ATP synthesis catalyzed by bovine-heart submitochondrial particles; (2) ATP hydrolysis catalyzed by F_1; and (3) proton translocation driven by ATP hydrolysis in submitochondrial particles. We show here that sulfate is a linear inhibitor of ATP synthesis whereas it behaves as a Botts and Morales' general modifier of ATP hydrolysis (Botts and Morales 1953). ATPase activity and the ANS fluorescence enhancement driven by ATP hydrolysis were stimulated at high [ATP] by sulfate. On the basis of these results we discuss the kinetic mechanism of the F_1F_o-ATPase. We postulate that the conformation of the catalytic site of the posthydrolysis intermediate in the H^+ translocation driven by ATP hydrolysis is more open than the β_{HC} site, which is a reaction intermediate of the $\Delta\mu_H^+$-driven ATP synthesis. Accordingly, our results support the proposal that the $\Delta\mu_H^+$-generating ATP hydrolysis is not the exact reversal of $\Delta\mu_H^+$-driven ATP synthesis (Vinogradov 2000; Weber and Senior 2003; Gao et al. 2005).

Materials and methods

Phosphorylating submitochondrial Mg^+-ATP particles (SMP) were prepared from heavy bovine heart mitochondria as previously described (Lodeyro et al. 2001).

Beef heart F_1 was prepared as described (Knowles and Penefsky 1972) and stored in liquid nitrogen in 250 mM sucrose, 2 mM EDTA, 4 mM ATP and 10 mM Tris-sulfate (pH 7.5). Before use, F_1 was desalted twice by the centrifuge-column procedure as described (Penefsky 1977) using Sephadex (G-50 coarse) equilibrated with 250 mM sucrose, 2 mM EDTA and 10 mM Tris-sulfate (pH 7.5).

Electron transport

Electron transport in SMP was measured following oxygen consumption with a Clark electrode connected to a YSI Model 5300 Biological Oxygen Monitor. The reaction medium (2 ml) contained 180 mM sucrose and 50 mM Tris–HCl (pH 7.5). Before the assay, SMP were thawed and incubated as described (Kotlyar and Vinogradov 1990) to activate the succinate dehydrogenase.

ATP synthesis

The reaction was measured in a medium containing 180 mM sucrose, 1 mM $MgCl_2$, 10 mM succinate, 0.5 mM EDTA, 3 μM rotenone, 50 mM glucose, 0.01 mM ADP, 5 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris–HCl (pH 7.5). SMP (0.08 mg) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 0.1 μmol Pi, 2×10^6 cpm of carrier-free ^{32}Pi and 3 μmoles $MgCl_2$. The final volume was 1 ml. The pre-incubation and the reaction were carried out under aerobiosis obtained with a gyratory water bath shaker. After 5 min the reaction was stopped and inorganic phosphate was quantitatively precipitated as previously described (Lodeyro et al. 2001). Tubes were centrifuged for 10 min at 3,000 rpm. Aliquots were analyzed for [^{32}P]glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

ATPase activity

ATPase activity of the soluble ATPase (F_1) was determined spectrophotometrically essentially as described (Pullman et al. 1960) in a reaction medium containing 100 mM sucrose, 1 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 0.3 mM NADH, 30 IU pyruvate kinase (EC 2.7.1.40), 25 IU lactate dehydrogenase (EC 1.1.1.27) and 40 mM Tris–HCl (pH 8). The reaction was started by adding F_1 (1 μg) to the reaction media (1 ml). ATP was added as Mg-ATP, which was freshly prepared in a 1:1 Mg/ATP ratio by adding $MgCl_2$ to the ATP stock solution. The reaction velocity was determined after an acceleration phase that was only observed at [ATP] lower than 0.1 mM (Jault and Allison 1993).

The ATPase activity of SMP (0.07 mg) was similarly determined except that sucrose and $MgCl_2$ concentrations were 180 and 1.5 mM, respectively, and that 3 μM rotenone was also added in order to inhibit the NADH oxidase.

Energy-linked 8-anilino-1-naphtalene sulfonate fluorescence (ANS) enhancement

Measurements were carried out in a Kontron fluorescence spectrofluorometer SFM25 at 25 °C. SMP (0.5 mg) were added to a medium (1.5 ml) containing 180 mM sucrose, 40 mM Tris–HCl (pH 7.5) and 1 mM MgCl$_2$. ANS fluorescence was excited at 380 nm and measured at 480 nm as described (Ferguson et al. 1976).

Materials

Enzymes and coenzymes were purchased from Sigma or ICN. The enzymes used for the ATP synthesis and ATP hydrolysis assays were exhaustively dialyzed against glycerol 50% (V/V) and afterwards stored at −20 °C. FCCP was from Du Pont de Nemours. ^{32}Pi was obtained from Perkin Elmer Life Sciences.

Rotenone, antimycin A and FCCP were dissolved in ethanol spectrophotometric grade. All other chemicals were of analytical grade.

General

Protein determinations were carried out by a modified biuret procedure (Suranyi and Avi Dor 1966) for SMP and by Lowry's procedure (Lowry et al. 1951) for F$_1$. Bovine Serum Albumin, whose concentration was determined spectrophotometrically (A_{279}=6.67 cm^{-1} for 1% solution (Foster and Sterman 1956)), was used as standard.

ATP and ADP concentrations were enzymatically determined as described (Lamprecht and Traustschold 1974; Jaworek et al. 1974).

Rates reported are the average of duplicate determinations that agreed within 10%.

Measurements, except when indicated, were carried out at 30 °C.

Results

Effect of sulfate on mitochondrial ATP synthesis

ATP synthesis was inhibited by low sulfate concentrations: 50% inhibition was attained at 1.8 mM sulfate (Fig. 1) when the reaction was measured at 0.1 mM ADP and 0.3 mM Pi. Even higher concentrations did neither affect the electron transport from succinate to oxygen (Fig. 1, inset) nor the ANS fluorescence enhancement driven by succinate oxidation (data not shown). Therefore, sulfate exerts its inhibitory action at the level of the F$_1$F$_o$-ATP synthase.

In order to characterize the inhibition, the effect of sulfate was studied at different substrate concentrations.

Linear v_0/v vs. [Na$_2$SO$_4$] plots (Roveri 1985) were obtained at any [ADP] and [Pi], indicating that the anion behaves as a full inhibitor of ATP synthesis (see Figs. 2 and 3). When the effect of sulfate was studied at fixed (0.01 mM) [ADP] and variable [Pi], the dependence of the slopes of the linear plots on [Pi] agreed (see legend to Fig. 2) with the behavior of a linear competitive inhibitor (K_i=0.45±0.05 mM). Hence, Pi and sulfate are mutually exclusive ligands of the ATP synthase and the ternary F$_1$F$_o$•Pi•sulfate complex cannot be formed. An apparent K_{Pi}=0.09±0.02 mM could be estimated for the Pi binding site involved in the competition with sulfate (Fig. 2, inset).

Conversely, when the studies were carried out at fixed (0.1 mM) [Pi] varying the [ADP] (Fig. 3), the slopes depended on [ADP] according to the equation shown in the legend to Fig. 3, that indicates that a slope significantly different than zero will be obtained at infinite [ADP], behavior that is characteristic of a linear mixed inhibitor ((K_i=0.60±0.01 mM, K'_i=5.6±1.2 mM and K_{ADP}=0.46± 0.11 mM). These results indicate that despite ADP strongly decreases the apparent affinity of sulfate, both ligands can simultaneously bind to the ATP synthase
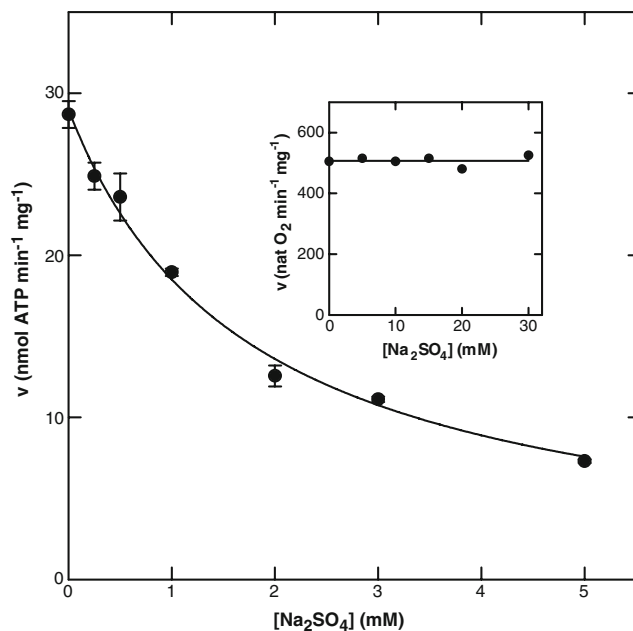

Fig. 1 Effect of sulfate on electron transport and ATP synthesis. ATP synthesis was measured as described in Materials and Methods. ADP and Pi concentrations were 0.1 and 0.3 mM respectively. The line represents the best fit of the equation $\left(v = \dfrac{v_0}{1+\dfrac{[Na_2SO_4]}{IC_{50}}} \right)$ to the experimental values. The estimated parameters were: v_0=28.9± 0.5 nmol ATP min^{-1} mg^{-1} and IC$_{50}$=1.8±0.1 mM. The inset shows the succinate oxidase activity measured as indicated in Materials and Methods in the presence of variable sulfate concentrations
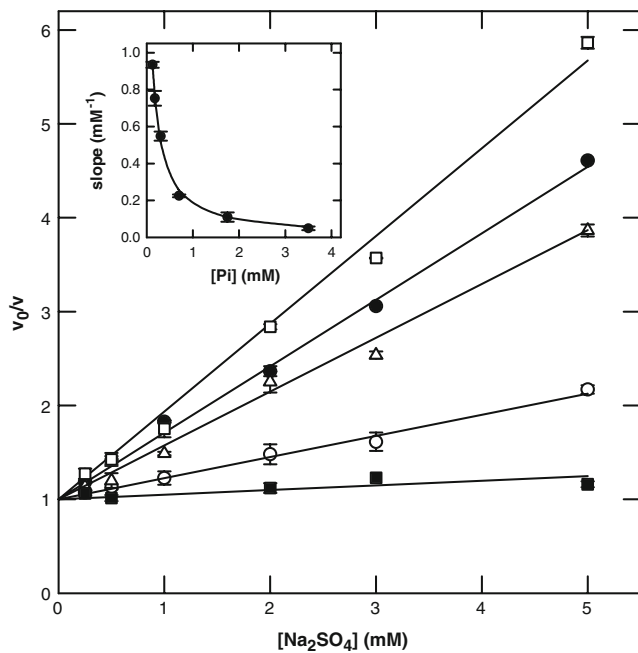
Fig. 2 Inhibition by sulfate of ATP synthesis at variable phosphate concentrations. ATP synthesis was determined as described in Materials and Methods at different [Pi] and constant [ADP]. The points are the v_0/v ratios estimated at [ADP]=0.01 mM and [Pi]= (filled square) 3.5, (empty circle) 0.7, (empty triangle) 0.3, (filled circle) 0.175 and (empty square) 0.125 mM. v_0 and v are the velocities in the absence and in the presence of sulfate, respectively. The lines are the fit of the equation $\left(\frac{v_0}{v} = 1 + \text{slope}[\text{Na}_2\text{SO}_4]\right)$ (Roveri 1985) to the experimentally determined values. The symbols in the inset are the values of the slopes estimated at the different [Pi]. The line represents the best fit of the equation $\left(\text{slope} = \frac{K_{\text{Pi}}}{K_i(K_{\text{Pi}}+[\text{Pi}])}\right)$ that describes the behavior of a linear competitive inhibitor

leading to the formation of a $F_1F_o \cdot$ADP$\cdot$sulfate dead-end ternary complex.

Effect of sulfate on ATP hydrolysis

When the ATPase activity of mitochondrial F_1 was determined at variable ATP concentrations, a non-linear Hanes plot was obtained (see Fig. 4a, inset). The sum of at least two hyperbolas ($K_{m1}=3.4\pm0.4\times10^{-3}$ mM; $V_{max1}=3.9\pm0.3$ μmol min^{-1} mg^{-1} and $K_{m2}=0.20\pm0.01$ mM; $V_{max2}=65\pm1$ μmol min^{-1} mg^{-1}) was needed to fit the experimentally determined values. A qualitatively similar behavior was obtained in the presence of sulfate. However a significant increase in K_m and in V_{max} values ($K_{m1}=12\pm3\times10^{-3}$ mM; $V_{max1}=9.6\pm1.0$ μmol min^{-1} mg^{-1} and $K_{m2}=1.45\pm0.21$ mM; $V_{max2}=88\pm6$ μmol min^{-1} mg^{-1}) was observed (Fig. 4a). The increases in V_{max1} and V_{max2} were smaller than those in K_m values. Therefore, it is expected that sulfate would behave as a partial inhibitor at low [ATP] and as non-essential activator at high [ATP]. This prediction was confirmed when the ATPase activity of F_1 was determined at different fixed

[ATP] and variable sulfate concentrations (Fig. 4b). The following equation was fitted to the experimental values:

$$\frac{v}{v_0} = 1 + \frac{\left(\left(\frac{v}{v_0}\right)_{max} - 1\right)[\text{Na}_2\text{SO}_4]}{C_{0.5} + [\text{Na}_2\text{SO}_4]} \qquad (1)$$

where v and v_0 are the velocity of ATP hydrolysis determined in the presence of sulfate concentration and in its absence, respectively; $C_{0.5}$ is the anion concentration that produces half of the maximal effect and $\left(\frac{v}{v_0}\right)_{max}$ is the relative velocity attained at saturating anion concentration. Values of $\left(\frac{v}{v_0}\right)_{max}$ smaller than 1—indicating inhibition— were estimated at [ATP] <2 mM and bigger than 1— meaning activation—at [ATP] > 2 mM (see Table 1). This behavior resembles that of a generalized modifier as described by Botts and Morales (1953) (Scheme 1) with α and β values bigger than 1. Therefore, a ternary $F_1 \cdot$ATP$\cdot$ sulfate complex that is kinetically competent for the catalysis of ATP hydrolysis is formed.

Similar activation at high [ATP] was observed for ATP hydrolysis catalyzed by the membrane bound F_1F_o under coupled conditions in SMP. Sulfate increases the amplitude
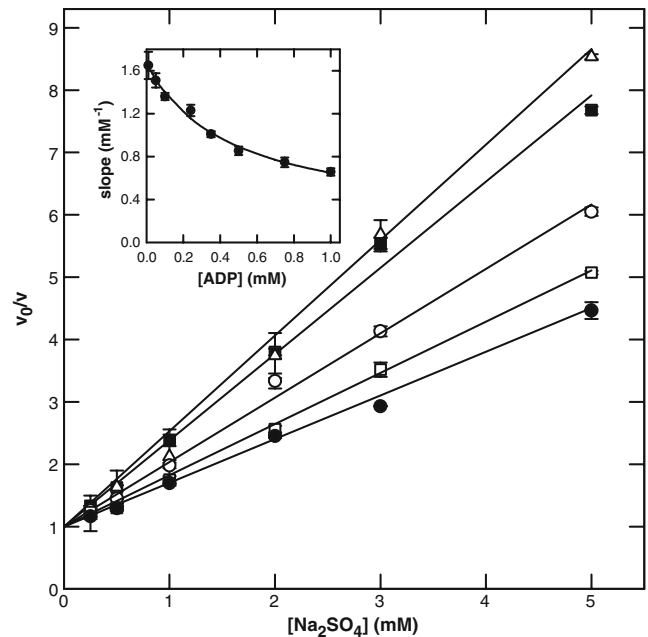

Fig. 3 Inhibition of ATP synthesis by sulfate at variable ADP concentrations. ATP synthesis was determined as described in Materials and Methods at 0.1 mM Pi and filled circle 1, empty square 0.75, empty circle 0.35, filled square 0.1 and empty triangle 0.05 mM ADP. The experimentally determined data are shown as described in the caption to Fig. 2. The slopes estimated from the linear primary plot are shown in the inset. The line represents the best fit of the equation $\left(\text{slope} = \frac{\frac{K_{\text{ADP}}}{K_i} + \frac{[\text{ADP}]}{K_i'}}{K_{\text{ADP}}+[\text{ADP}]}\right)$ that describes the behavior of a linear mixed inhibitor (Roveri 1985)
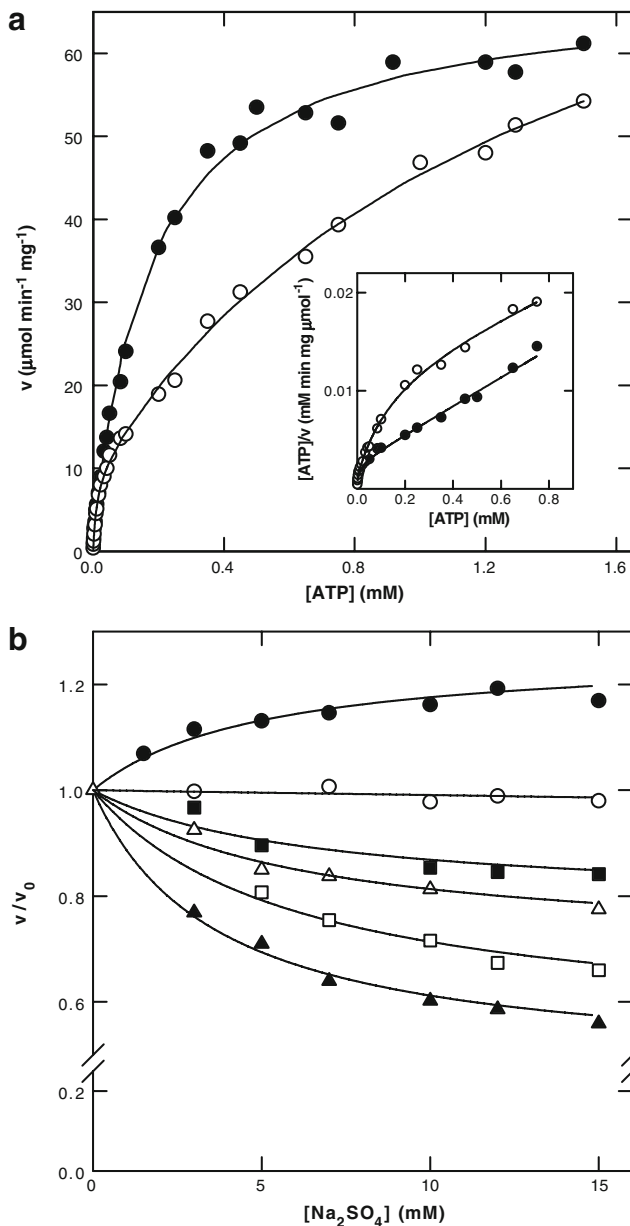
a

b

Fig. 4 Effect of sulfate on the ATP hydrolysis catalyzed by the soluble ATPase (F_1). **a** ATPase activity was measured at variable ATP-Mg concentrations as indicated under "Materials and methods" in the absence (*filled circle*) and in the presence (*empty circle*) of 50 mM Na_2SO_4. The *lines* are the best fit of the sum of two hyperbolas to the experimental data. In the *inset* it is shown the Hanes representation of the same data. Values obtained at ATP concentrations from 0.8 to 1.5 mM (not shown) belong to the same straight line that those obtained at ATP concentrations from 0.2 to 0.8 mM. **b** ATPase activity was measured at variable sulfate concentrations and at the following ATP concentrations: *empty triangle* 0.05, *filled triangle* 0.10, *empty square* 0.50, *filled square* 1, *empty circle* 2 and *filled circle* 4 mM. The ionic strength was kept constant by the addition of appropriate concentrations of EPPS (*N*-hydroxyethyl)piperazine-N'-3-propane sulphonic acid). The *lines* represent the best fit of the equation described in the text to the experimental values

Table 1 Effect of sulfate on ATP hydrolysis catalyzed by F_1

[ATP-Mg] mM	$\left(\dfrac{v}{v_0}\right)_{max}$	$C_{0.5}$ mM
4	1.26 ± 0.02	4.9 ± 1.3
1	0.79 ± 0.04	6.5 ± 3.8
0.5	0.54 ± 0.03	6.1 ± 1.1
0.1	0.47 ± 0.02	3.7 ± 0.4
0.05	0.70 ± 0.03	6.0 ± 1.8
0.025	0.67 ± 0.03	6.3 ± 1.7

Equation 1 (see text) was fitted to the experimental values shown in Fig. 6b and $\left(\dfrac{v}{v_0}\right)_{max}$ and $C_{0.5}$ were estimated by a non-linear regression procedure

of ANS fluorescence enhancement induced by ATP hydrolysis (Fig. 5a) and its rate constant (k) from 2.7 to 4.2 min^{-1}, with a $C_{0.5}$ equal to 2.4 mM (Fig. 5b). These results indicate that the ATP hydrolysis stimulated by sulfate in SMP is coupled to H$^+$ translocation.

Does sulfate bind to the bicarbonate site?

Inhibition of ATP synthesis

In order to determine if sulfate and bicarbonate inhibit ATP synthesis by binding to the same site, we studied the effect of mixtures of both anions. The results were analyzed by means of the Yonetani-Theorell plot (Yonetani and Theorell 1964). Plots of $1/v$ vs. [Na_2SO_4] yielded intersecting lines at different fixed [$NaHCO_3$] (Fig. 6) that corresponds to nonexclusive ligands. Therefore, the F_1F_o•sulfate•bicarbonate ternary complex can be formed, clearly indicating that both anions bind to different sites.

Activation of ATP hydrolysis

To determine whether both anions bind to the same site in F_1 to exert the activation of ATP hydrolysis, we adapted the Yagi and Osawa's procedure for partial inhibitors (Yagi and Osawa 1960) to our case of non-essential activators. The method consists in studying the effect of mixtures of both anions such that:

$$[J] = r[I] \tag{2}$$

where J=bicarbonate and I=sulfate.
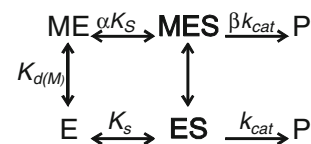
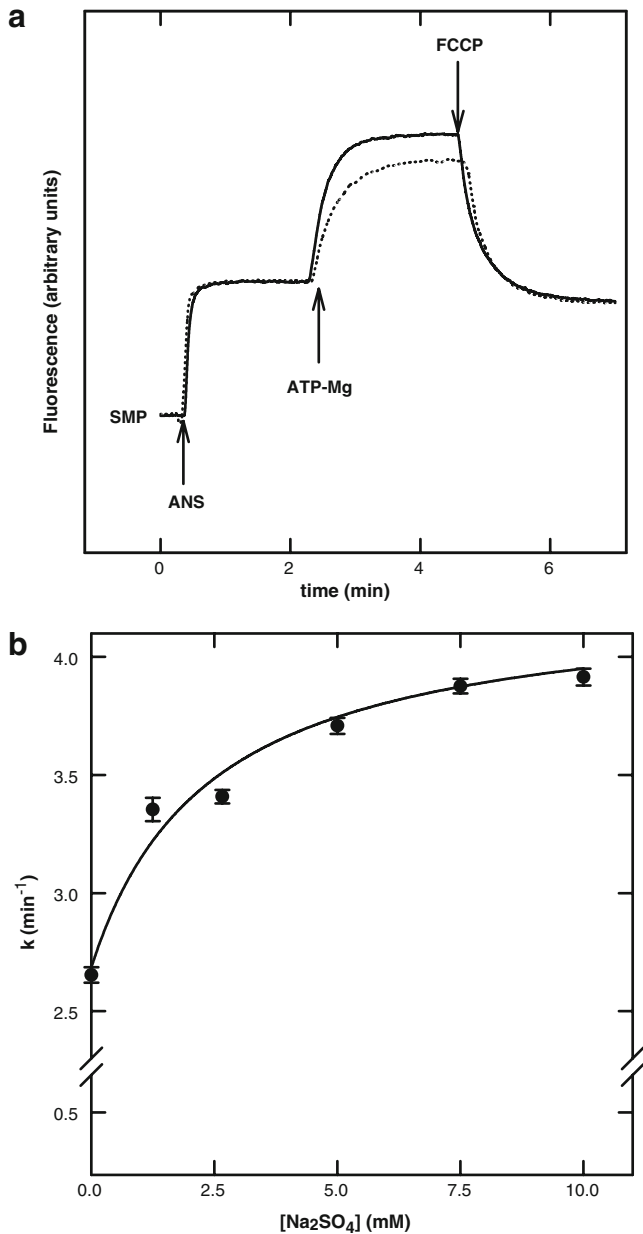**Scheme 1** Botts and Morales' model. $E=F_1$; $S=$ATP; $M=$sulfate; $P=$ product (ADP+Pi)

Fig. 5 Effect of sulfate on the ANS fluorescence enhancement driven by ATP hydrolysis. **a** The ANS fluorescence enhancement induced by ATP hydrolysis was measured as described in "Materials and methods" in the absence (*dotted line*) and in the presence of 10 mM sulfate (*full line*). Additions were: 5 µM ANS, 2 mM ATP and 1 µM FCCP. **b** Experiments similar to that shown in **a** were performed at different sulfate concentrations. The fluorescence increase observed upon ATP addition was fitted with an exponential increase $\left(F = F_0 + \Delta F\left(1 - e^{-kt}\right)\right)$. The *symbols* indicate the values of k estimated at the different sulfate concentrations and the line represents the fit of the following equation: $k = k_0 + \frac{(k_{max} - k_0)[\text{Na}_2\text{SO}_4]}{C_{0.5} + [\text{Na}_2\text{SO}_4]}$, where k_0 and k_{max} are the values of k at zero and at saturating anion concentration, respectively. The parameters estimated by non-linear regression were: $k_0 = 2.7 \pm 0.1$ min^{-1}, $k_{max} = 4.2 \pm 0.1$ min^{-1} and $C_{0.5} = 2.4 \pm 0.6$ mM

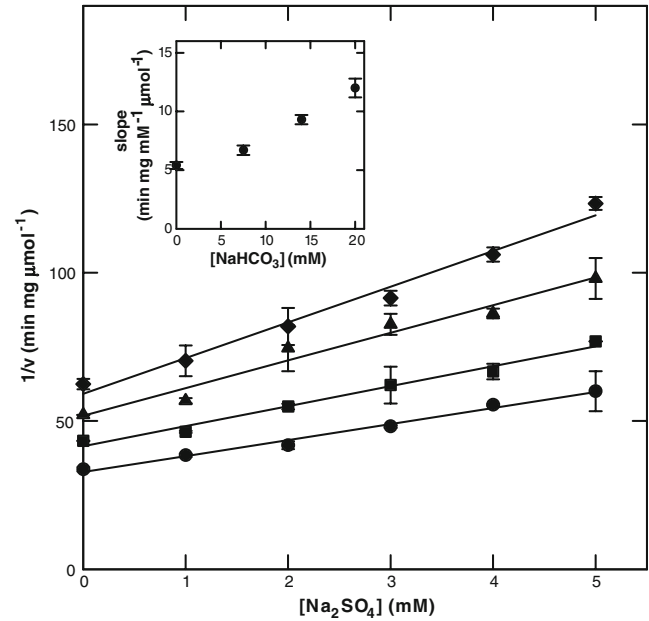

Fig. 6 Effect of mixtures of bicarbonate and sulfate on ATP synthesis. The reaction was measured at [Pi]=0.7 mM and [ADP]=0.01 mM with variable [Na$_2$SO$_4$] and fixed [NaHCO$_3$]: 0 (*circle*), 7.5 (*square*), 14 (*triangle*) and 20 mM (*diamond*). The *lines* represent the linear regression of the experimental data. The slope values estimated at the different [NaHCO$_3$] are shown in the *inset*

The dependence of the reaction velocity with [*I*] follows a first-degree modifier function for mutually exclusive ligands (Eq. 3) and a second-degree modifier function for nonexclusive ligands (Eq. 4; Wong 1975):

$$v = \frac{m_i[I] + m_j[J] + m_0}{d_i[I] + d_j[J] + d_0} = \frac{m_1[I] + m_0}{d_1[I] + d_0} \tag{3}$$

$$v = \frac{m_{ij}[I][J] + m_i[I] + m_j[J] + m_0}{d_{ij}[I][J] + d_i[I] + d_j[J] + d_0} = \frac{m_2[I]^2 + m_1[I] + m_0}{d_2[I]^2 + d_1[I] + d_0} \tag{4}$$

where

$$m_1 = m_i + r \cdot m_j \tag{5}$$

$$d_1 = d_i + r \cdot d_j \tag{6}$$

$$m_2 = r \cdot m_{ij} \tag{7}$$

$$d_2 = r \cdot d_{ij} \tag{8}$$

Therefore plots of $(v-v_0)^{-1}$ vs. $[I]^{-1}$ (being v_0 and v the velocity in the absence and in the presence of anions) yield a straight line for mutually exclusive ligands (Eq. 9) and non-linear plots for nonexclusive ligands (Eq. 10).

$$(v-v_0)^{-1} = \frac{d_1 d_0}{m_1 d_0 - m_0 d_1} + \frac{d_0^2}{m_1 d_0 - m_0 d_1}[I]^{-1} \qquad (9)$$

$$(v-v_0)^{-1} = \frac{d_0(d_2[I]+d_1)}{(m_2 d_0 - m_0 d_2)[I] + m_1 d_0 - d_1 m_0}$$
$$+ \frac{d_0^2}{(m_2 d_0 - m_0 d_2)[I] + m_1 d_0 - d_1 m_0}$$
$$\times [I]^{-1} \qquad (10)$$

The studies were performed at 4 mM ATP, substrate concentration at which both anions behaved as activators. The values of $(v-v_0)^{-1}$ calculated from the experimental data obtained in the absence and in the presence of mixtures of equal concentrations ($r=1$) of sulfate and bicarbonate, clearly showed a non-linear dependence on $[I]^{-1}$ (Fig. 7), characteristic of nonexclusive ligands. Therefore sulfate and bicarbonate bind to different sites in F_1 to activate ATP hydrolysis.
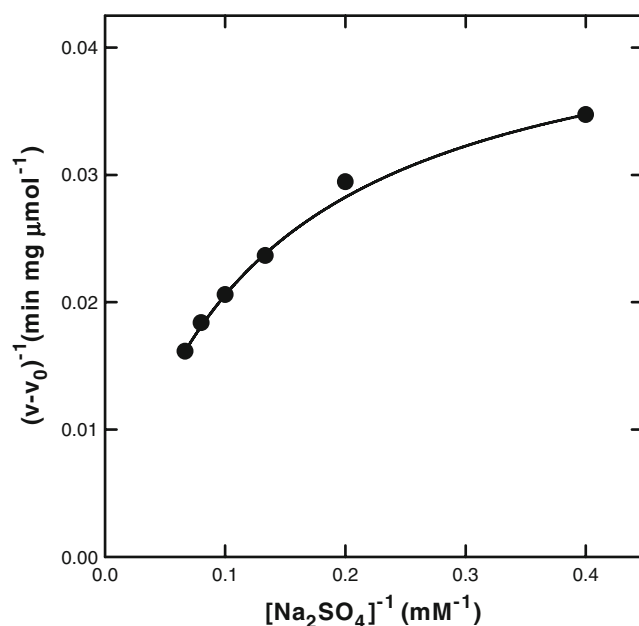

Fig. 7 Effect of mixtures of bicarbonate and sulfate on ATP hydrolysis. The reaction was measured as indicated in "Materials and methods" at $[ATP]=4$ mM and at a $[Na_2SO_4]=[NaHCO_3]$, following the procedure explained in the text: v_0 and v are the velocities in the absence and in the presence of the mixture of anions, respectively

Discussion

On the mechanism of action of sulfate on the reversible H^+-ATPase

Sulfate is an inhibitor of mitochondrial ATP synthesis that has a high affinity for the F_1F_o-ATPsynthase: from the kinetic studies described in "Effect of sulfate on mitochondrial ATP synthesis", an apparent dissociation constant in the submilimolar range (0.45 mM) could be estimated (Figs. 2 and 3). Our kinetic studies have also shown that the binding of Pi with an apparent dissociation constant equal to 0.09 mM (Fig. 3, inset) completely excludes sulfate from its binding site in F_1. These results qualitatively agree with those reported by Zharova and Vinogradov (2006). However, our complete kinetic study not only unequivocally shows that sulfate is a linear competitive inhibitor towards Pi of the mitochondrial ATP synthesis, but also that it is a full mixed inhibitor with respect to ADP. Hence, the following complexes can be formed in the presence of sulfate: F_1F_o•sulfate and F_1F_o•ADP•sulfate, despite the affinity of the former for ADP is much lower than that of F_1F_o free.

On the other hand, sulfate increases the V_{max} values estimated for the ATPase activity of isolated F_1 (see Fig. 4a) and the amplitude and rate of the ANS fluorescence enhancement driven by ATP hydrolysis in SMP (Fig. 5). These results conclusively show that: (1) sulfate and ATP can simultaneously bind to F_1F_o; (2) the ternary F_1•ATP•sulfate complex is catalytically active and has a higher turnover number than the binary F_1•ATP complex formed in the absence of sulfate; and (3) the ATP hydrolysis catalyzed by the F_1F_o-ATPase in the presence of sulfate is coupled to H^+ translocation.

In summary, sulfate exerts different effects on ATP hydrolysis and ATP synthesis. Bicarbonate and sulfite, among other compounds, behave similarly (Vinogradov 2000; Lodeyro et al. 2001; Pacheco-Moisés et al. 2002). We have previously postulated that bicarbonate modulates the affinity of adenine nucleotides for the catalytic site: it increases the affinity of ATP activating ATP hydrolysis and decreases that of ADP inhibiting ATP synthesis (Lodeyro et al. 2001). It has also been postulated (Pacheco-Moisés et al. 2002) that the binding of sulfite to catalytic and non-catalytic nucleotide binding sites would decrease the affinities of ADP and/or Pi for the catalytic site, being this decrease the reason for the opposite effects of the anion on ATP synthesis and hydrolysis. However, there are some relevant differences in the behavior of bicarbonate/sulfite and sulfate. Bicarbonate inhibited ATP synthesis in beef heart SMP competitively with respect to ADP and non-competitively towards Pi (Lodeyro et al. 2001) and the inhibition by sulfite in inside-out vesicles from *Paracoccus*

denitrificans is mixed with respect to both substrates (Pacheco-Moisés et al. 2002). Therefore, Pi and bicarbonate/sulfite are not mutually exclusive ligands of F_1F_o. Hence, a clear distinction in the interaction of sulfate and of bicarbonate/sulfite with F_1F_o can be made: while the formation of the ternary $F_1F_o \cdot P_i \cdot$bicarbonate and $F_1F_o \cdot P_i \cdot$sulfite complexes is feasible, that of the $F_1F_o \cdot P_i \cdot$sulfate complex is not (see above). Bicarbonate and sulfate not only differ in their kinetic behavior as inhibitors of ATP synthesis, but also in their effect as activators of ATP hydrolysis in F_1 and in their binding sites on the enzyme. Bicarbonate increases V_{max} values and decreases $K_{ms(ATP)}$ (Roveri and Calcaterra 1985) whereas sulfate increases both kinetic parameters (see Fig. 4a and "Effect of sulfate on ATP hydrolysis"). Results obtained when we studied the effect of mixtures of bicarbonate and sulfate on ATPase synthesis (Fig. 6) clearly indicated that these anions are not mutually exclusive ligands of F_1. Similar behavior (mutually non-exclusive binding) was observed when the effect of mixtures of the anions on ATP hydrolysis was studied (Fig. 7). Therefore it can be unambiguously concluded that the inhibition of ATP synthesis and the activation of ATP hydrolysis by sulfate are exerted by binding to a different site than the bicarbonate binding site. We have previously suggested that the effects of bicarbonate on ATP synthesis and hydrolysis are exerted by binding of the anion to the low-affinity non-catalytic nucleotide binding site (Lodeyro et al. 2001). Hence, it is rather unlikely that sulfate produced any of its effects by binding to a non-catalytic site. The possibility that sulfate binds to site(s) different than nucleotide binding sites can also be excluded, since sulfate bound only to nucleotide binding sites was observed in the structures derived from crystals grown in the presence of sulfate (Menz et al. 2001a,b; Shirakihara et al. 1997).

The simplest mechanism that can be postulated to explain the inhibition of ATP synthesis by sulfate is that it binds to the β_E catalytic site at or near the putative Pi site preventing neither the binding of ADP nor the proton gradient-driven conformational change ($\beta_E \rightarrow \beta_{HC}$). Such conformational change would lead to the formation of a dead-end complex with sulfate and ADP bound to a catalytic site in a conformation similar to the β_{HC} one described by Menz et al. (2001b). This suggestion is supported not only by the results described in this manuscript but also by the previous observation of an electron density consistent with sulfate in F_1 crystals in the β_{HC} (Menz et al. 2001b) or in the β_E (Menz et al. 2001a) sites.

The fact that the ATP hydrolysis is activated by sulfate, clearly indicates that a ternary $F_1 \cdot$ATP$\cdot$sulfate catalytically active complex is formed. If sulfate binds to the catalytic site, the latter must be able to simultaneously bind ATP and sulfate. However, Menz et al., who have postulated that the Michaelis-Menten complex for ATP hydrolysis has a half-open conformation similar to that of β_{HC}, have also claimed that such subunit conformation cannot accommodate ATP and sulfate at the same time (Menz et al. 2001b). Such discrepancy can be overcome by postulating that, at least in the presence of sulfate, the catalytic site conformation corresponding to the Michaelis–Menten complex for ATP hydrolysis ($F_1 \cdot$ATP$\cdot$sulfate) must be more open than the β_{HC} one. As a matter of fact, Ahmad and Senior have stated that "…a β_E site with Pi bound can partly close and still accommodate Pi along with Mg-AMP-PNP or ATP…"(*sic*) (Ahmad and Senior 2004). Moreover, it has been recently shown that yeast F_1 can be crystallized with three F_1 complexes (yF_1I, yF_1II and yF_1III) in the crystallographic asymmetric unit (Kabaleeswaran et al. 2006). These authors have also stated that the Pi (sulfate) binding site in the β_E-subunit of yF_1II is far away from the P-loop and 7.7 Å from the γ-phosphate of the ATP analog AMP-PNP in the β_{DP}-subunit of yF_1I.

It has also been suggested that during ATP hydrolysis the Pi is released from the fully open conformation of the β_E-subunit (Kabaleeswaran et al. 2006; Gao et al. 2005). It has been more recently suggested, from single molecule studies, that Pi release precedes (Ariga et al. 2007) and drives the 40° rotation substep (Adachi et al. 2007). Conversely, based on pre-steady-state kinetic studies, Baylis Scanlon et al. have postulated that the rate limiting step for ATP hydrolysis occurs after ATP hydrolysis and prior to Pi dissociation (Baylis Scanlon et al. 2007). Such step would consist in a conformational change leading to a decrease in the affinity for Pi and would coincide with the 40° rotation substep (Nakamoto et al. 2008). Since the catalytic site of the $F_1F_o \cdot$ADP$\cdot$Pi$\cdot$sulfate (posthydrolysis intermediate in the sulfate mode of hydrolysis) has a more open conformation than that of $F_1F_o \cdot$ADP$\cdot$Pi (β_{HC}), it is expected that the former will have a lower affinity for Pi. Therefore, the increase in V_{max} would be simply a consequence of the increase in the rate ($k_{off(Pi)}$) of Pi dissociation. Another consequence will be that the energy released upon Pi dissociation would be significantly lower in the presence of sulfate.

Sulfate also decreases the affinity of the enzyme for ADP (see Fig. 3 and "Effect of sulfate on mitochondrial ATP synthesis"). Therefore, it will also accelerate the departure of ADP and it will prevent the turnover dependent formation at low [ATP] of inhibitory MgADP (Allison 1998; Ren and Allison 2000). Its binding at or near the Pi binding site would supress the transient entrapment of inhibitory MgADP, as it has been reported for Pi (see Mitome et al. 2002).

The proton translocation driven by ATP hydrolysis is also stimulated by sulfate. Therefore, in the sulfate mode of ATP hydrolysis the γ subunit rotation is fully operative, despite: (1) the significant decrease in the energy released

upon dissociation of Pi; and (2) the difference in conformation of the catalytic site of the posthydrolysis intermediate F_1F_o•ADP•Pi•sulfate and β_{HC}. Hence, a lower Pi binding-energy could still be able to drive the 40° rotation substep. Also, the conformational change of the catalytic site that drives the 40° rotation step in the ATP hydrolysis direction, does not necessarily involves the β_{HC} conformation. Conversely, the $\Delta\mu_H^+$-driven 40° rotation substep in the reverse direction, must induce the $\beta_E \rightarrow \beta_{HC}$ conformational transition, since the β_{HC} is required in order to permit the kinetically competent binding of Pi leading to ATP synthesis.

Kinetic mechanism of ATP synthesis: inhibition by competitive inhibitors

The study of the effect of competitive inhibitors is a useful tool for the determination of the binding order of Bi substrate enzymes (Fromm 1969). The fact that sulfate, a competitive inhibitor towards Pi (Fig. 3), is noncompetitive (mixed) with respect to ADP (Fig. 4), clearly excludes the possibility of an Ordered Bi Uni mechanism with ADP as leader substrate as it has been suggested (Xing et al. 2005). A classical Ordered mechanism with Pi as leader substrate can also be excluded since bicarbonate is competitive inhibitor with respect to ADP and noncompetitive towards Pi (Lodeyro et al. 2001). Despite that these results favor the operation of a Random Bi Uni mechanism [as it has been previously suggested by others (Perez and Ferguson 1990 and references quoted in there)] the possibility of a Bi Uni Ordered-Subsite (Fromm 1969) with Pi as leader cannot be excluded.

Is ATP synthesis the reversal of ATP hydrolysis?

Several reports suggest the existence of different pathways for ATP synthesis and ATP hydrolysis. It has been shown that the non-hydrolysable analogue AMP-PNP inhibits ATP hydrolysis (Penefsky 1974) affecting neither ATP synthesis (Penefsky 1974; Pedersen 1975) nor high affinity ADP binding to F_1 (Pedersen 1975). These results were interpreted as an indication of the existence of separate catalytic sites specialized for ATP synthesis and hydrolysis. However, with the actual knowledge of the structure and mechanism of F_1 they can be rationalized as a consequence of the rotary mechanism.

The ATPase activity of a mutant F_1F_o-ATP synthase from the thermophilic *Bacillus* PS3 was completely inactivated by accumulation of a MgADP-inhibited form. Since this mutant could conversely catalyze continuous turnover of ATP synthesis, it was suggested that the MgADP inhibited form is not produced during ATP synthesis reaction (Bald et al. 1998). It has also been reported that ATP synthesis is not inhibited by azide, which inhibits ATP hydrolysis by stabilizing the MgADP-inhibited form (Syroeshkin et al 1995). However, it has been more recently postulated that azide does not inhibit ATP synthesis because the rotation of the γ-subunit in the synthetic direction would destabilize the MgADP-inhibited form (Bowler et al. 2007).

Other compounds that exhibit differential effects on ATP synthesis and ATP hydrolysis are the oxyanions discussed above. A clear cut difference must be pointed out between the cases discussed in the previous paragraph and the oxyanions. While the former affect only one of the reaction directions (inhibition of ATPase activity with no effect on ATP synthesis), the oxyanions affect both directions (stimulation of ATPase and inhibition of ATP synthesis): they inhibit ATP synthesis producing dead-end complexes (F_1F_o•ADP•sulfate or F_1F_o•Pi•bicarbonate) and stimulate ATP hydrolysis producing ternary complexes (F_1F_o•ATP•anion) with higher turnover number than F_1F_o•ATP. Therefore their differential effects cannot be easily explained by differences in the anion affinities produced by the γ-subunit rotation.

Concluding remarks

The F_1F_o•sulfate•ATP is a kinetically competent complex, that is not only able to catalyze the ATP hydrolysis faster than the F_1F_o•ATP complex, but also to couple the chemical reaction with the vectorial translocation of H^+. Since the β_{HC} conformation cannot accommodate sulfate and ATP (Menz et al. 2001a), it must be concluded that an intermediate with this conformation does not participate in the catalytic pathway coupled to proton translocation (see Fig. 5) at least in the presence of sulfate, whereas it is an obligatory intermediate in ATP synthesis. Therefore, $\Delta\mu_H^+$-generating ATP hydrolysis is not necessarily the exact reversal of $\Delta\mu_H^+$-driven ATP synthesis.

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