Journal of Bioenergetics and Biomembranes, Vol. 34, No. 4, August 2002 (© 2002)

Sulfite Inhibits the F_1F_0-ATP Synthase and Activates the F_1F_0-ATPase of *Paracoccus denitrificans*

Fermín Pacheco-Moisés,[1] Fernando Minauro-Sanmiguel,[1] Concepción Bravo,[1] and José J. García[1,2]

Received June 5, 2002; accepted June 19, 2002

The F_1F_0 complex of *Paracoccus denitrificans* (PdF_1F_0) is the fastest ATP synthase but the slowest ATPase. Sulfite exerts maximal activation of the PdF_1F_0-ATPase (Pacheco-Moisés, F., García, J. J., Rodríguez-Zavala, J. S., and Moreno-Sánchez, R. (2000). *Eur. J. Biochem.* **267**, 993–1000) but its effect on the PdF_1F_0-ATP synthase activity remains unknown. Therefore, we studied the effect of sulfite on ATP synthesis and $^{32}Pi \leftrightarrow ATP$ exchange reactions of inside-out membrane vesicles of *P. denitrificans*. Sulfite inhibited both reactions under conditions of maximal ΔpH and normal sensitivity to dicyclohexylcarbodiimide. Sulfite increased by 10- and 5-fold the $K_{0.5}$ for Mg^{2+}-ADP and Pi during ATP synthesis, respectively, and by 4-fold the IC_{50} of Mg^{2+}-ADP for inhibition of the PdF_1F_0-ATPase activity. Thus, sulfite exerts opposite effects on the forward and reverse functioning of the PdF_1F_0 complex. These effects are not due to membrane or PdF_1F_0 uncoupling. Kinetic and structural modifications that could account for these results are discussed.

KEY WORDS: ATP synthase; *Paracoccus denitrificans*; sulfite; membrane vesicles; inhibition; activation.

INTRODUCTION

The F_1F_0-ATP synthases of energy-transducing membranes couple ATP synthesis or hydrolysis to a transmembrane proton transport. The F_0 portion is a proton channel and the F_1 part is the catalytic moiety where ATP synthesis and hydrolysis take place. When detached from F_0, the soluble F_1-ATPase contains five different proteins with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. It has three catalytic and three noncatalytic nucleotide binding sites alternated in the six interfaces of the hexagon formed by three α/β dimers $(\alpha/\beta)_3$ (Bianchet *et al.*, 1991). The F_0 proton channel contains three essential subunits with a stoichiometry $a_1b_2c_{9-12}$ (*Escherichia coli* nomenclature). F_0 and F_1 are connected through two stalks (Bottcher *et al.*, 1998; Karrasch and Walker, 1999; Wilkens and Capaldi, 1998). In *E. coli*, the central stalk contains subunits γ and ε that are connected to a c_{9-12} ring of F_0. Both the central stalk and the c_{9-12} ring rotate all together in relation to a stator that anchors the complex to the membrane (For recent reviews, see Capaldi *et al.*, 2000; García, 2000; Kinosita *et al.*, 2000; Leslie and Walker, 2000; Nakamoto *et al.*, 2000; Oster and Wang, 1999). In the stator of the *E. coli* enzyme, the $(\alpha/\beta)_3$ hexagon of F_1 is linked to subunit a of F_0 through a peripheral δ/b_2 second stalk (McLachlin, 2000; Rodgers and Capaldi, 1998).

In coupled conditions, the F_1F_0 complex can work reversibly as a synthase or hydrolase of ATP, consuming or pumping protons, respectively. It is assumed that this reversibility occurs through a single catalytic pathway working "forward" and "backward" (Al-Shawi *et al.*, 1997). Crystallographic structures (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998), and molecular modeling (Wang and Oster, 1998), together with kinetic and thermodynamic analyses (García, 2000), predict that this reversible pathway is associated with an inverse rotation of the $\gamma/\varepsilon/c_{9-12}$

Key to abbreviations: ACMA, 9-amino-6-chloro-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicylohexylcarbodiimide; EDTA, ethylenediamineteraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethylether); IC_{50}, half-maximal inhibitory concentration; PdF_0, PdF_1, and PdF_1F_0, the F_0, F_1, and F_1F_0 complexes of *Paracoccus denitrificans*.

[1]Departamento de Bioquímica, Instituto Nacional de Cardiología, "Ignacio Chávez," México, D.F., México.

[2]To whom correspondence should be addressed; e-mail: jjgarcia_trejo@yahoo.com.

0145-479X/02/0800-0269/0

domain. A single catalytic pathway working in both directions implies that inhibitors and activators of one reaction should exert the same effect on its reversal. However, there is evidence that shows that a number of inhibitors of the enzyme (García *et al.*, 1995; Matsuno-Yagi and Hatefi, 1984; Syroeshkin *et al.*, 1995; Bald *et al.*, 1998; Vinogradov, 1999) show different affinities or effects on ATP hydrolysis and synthesis carried out by the F_1F_0 complex. To date, the most dramatic of these differential effects is that of sodium azide. It does not inhibit ATP synthesis at all, although it inhibits by more than 90% the F_1 or F_1F_0 ATPases (Syroeshkin *et al.*, 1995; Bald *et al.*, 1998). These results reflect that in the coupled F_1F_0 complex, ATP synthesis is not a simple reversal of ATP hydrolysis (Syroeshkin *et al.*, 1995; Vinogradov, 1999; Bald *et al.*, 1998; Weber and Senior, 2000). Therefore, there seems to be an intrinsic asymmetry in the forward and backward catalytic pathway that may confer different sensitivity of ATP synthesis and hydrolysis to some inhibitors or activators.

This asymmetry is more evident in the F_1F_0-ATPase of *P. denitrificans* since it has the highest ratio of ATP synthase/ATPase activities found so far. This is the result of expressing the fastest ATP synthase rates reported, i.e., about 1–2 μmol/(min mg) (Pérez and Ferguson, 1990a,b), and the slowest ATPase activity that is generally below 0.1 μmol/(min mg) (Pacheco-Moisés *et al.*, 2000; Pérez and Ferguson, 1990a). Therefore, the ATP synthase/ATPase ratio of the *P. denitrificans* F_1F_0 complex is usually above 1, and it can be 20 or higher. In comparison, this ration for the enzymes of *E. coli* and bovine heart mitochondria is about 1 or lower in situ, when their intrinsic inhibitors are present, i.e. the subunit ε and the inhibitor protein (IF_1), respectively. The ATPase activity of the F_1F_0 complex from *P. denitrificans* is only evident after activation by physiological and nonphysiological factors such as temperature, membrane energization, and oxyanions. Among the latter, sulfite is the best activator for F_1F_0-ATPase and F_1-ATPases from various species (Bakels *et al.*, 1994, 1996; Capellini *et al.*, 1997; Pacheco-Moisés *et al.*, 2000). It induces the highest activation of the *P. denitrificans* F_1F_0-ATPase (PdF_1F_0-ATPase) (Pacheco-Moisés *et al.*, 2000). However, the effect of sulfite on the high ATP synthase rates of the F_1F_0 complex of *P. denitrificans* remains unknown. In other ATP synthases like that of cyanobacterial (Bakels *et al.*, 1996), and *R. capsulatus* inside-out vesicles (Capellini *et al.*, 1997), sulfite inhibits the F_1F_0-ATP synthase activity. Recently, it has been shown that bicarbonate inhibits ATP synthesis in bovine heart submitochondrial particles (Lodeyro *et al.*, 2001). Thus, our present study investigates whether ATP synthesis is inhibited by another oxyanion like sulfite.

Sulfite is considered a nonphysiological activator of the F_1F_0-ATPase. However, Wodara *et al.* (1997) cloned a sulfite dehydrogenase from *P. denitrificans* that required thiosulfate for growth. Therefore, sulfite is a toxic byproduct of thiosulfate and hydrogen sulfide oxidation (Wodara *et al.*, 1997). Consequently, the effect of sulfite on the ATPase and ATP synthase activities of the PdF_1F_0 complex might have a significant physiological relevance in this bacterium.

Therefore, this work centered on the effect of sulfite on the kinetics of ATP synthesis, and its relation to the activation of the PdF_1F_0-ATPase. Previous attempts to address this question in *P. denitrificans* and in bovine submitochondrial particles (Bakels *et al.*, 1994) were hindered by side effects of sulfite on succinate oxidation. These side effects were circumvented here by measuring the ATP driven $^{32}Pi \leftrightarrow$ ATP exchange and NADH driven ATP synthesis of *P. denitrificans* membranes. Sulfite inhibited both reactions, i.e., it induced opposing effects on ATP synthesis and hydrolysis. The correlation of these activities with the PdF_1F_0-ATPase showed that the underlying mechanism of these opposing effects of sulfite involved decreases on the affinities for ADP and Pi, i.e., products of ATP hydrolysis and substrates for ATP synthesis. These kinetic effects are likely associated with a structural shift from an ATP synthase state to another ATPase form with low affinity for ADP and Pi.

EXPERIMENTAL PROCEDURES

ATP, ADP, venturicidin, nigericin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *N,N′*-dicyclohexylcarbodiimide (DCCD), succinate, glucose dehydrogenase, and hexokinase were purchased from Sigma. Pyruvate kinase and lactate dehydrogenase were supplied from Boehringer Mannheim; 9-amino-6-chloro-methoxyacridine (ACMA) was from Molecular Probes. All other chemicals of analytical grade were from standard suppliers. ^{32}Pi was obtained from New England Nuclear and purified as described elsewhere (de Meis, 1984). Growth conditions and preparation of *P. denitrificans* membranes were described previously (Pacheco-Moisés *et al.*, 2000). The F_1-ATPase was extracted from inside-out membrane vesicles with chloroform, as described by Norling *et al.* (1998).

ATP Synthesis Measurements

ATP synthesis was assayed with an hexokinase trap at 37°C. The reaction mixture (0.5 mL) contained 125 mM

KCl, 25 mM sucrose, 40 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM NADH, 20 mM glucose, 46 units hexokinase, and variable concentrations of Mg^{2+}-ADP and of inorganic phosphate (Pi) indicated in Results. This mixture was preincubated at 37°C for 2 min in Erlenmeyer flasks under constant shaking, followed by the addition of membranes (0.12–0.8 mg of protein). The reaction was arrested with 50 μL of stop mix (25 mM EDTA, 2 μM CCCP, and 25 μg venturicidin). Afterwards, the reaction mixture was boiled for 10 min and centrifuged at $5{,}000 \times g$ for 5 min. NADP (0.5 mM) and glucose-6-phosphate dehydrogenase (30 units) were added to the supernatant. The absorbance of the samples was recorded at 340 nm. In some cases, ATP formed was quantified radioactively, as described for ^{32}Pi ↔ ATP exchange reaction (see below), except that 20 mM glucose and 46 units hexokinase were included to quantify the (^{32}P)glucose-6-phosphate formed from ^{32}Pi (1–3) $\times 10^6$ cpm/mL) added at the indicated concentrations.

ATPase Activities

ATP hydrolysis was determined by the release of Pi, according to Summer (1964). In the case of the F_1 fraction, it was measured spectrophotometrically at 37°C, using an ATP regenerating system. The assay mixture contained 125 mM KCl, 25 mM sucrose, 3 mM ATP, 40 mM Hepes–KOH (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM phospho*enol*pyruvate, pyruvate kinase (4 units), lactate dehydrogenase (4 units), and 0.2 mM NADH.

^{32}Pi ↔ ATP Exchange Measurements

The ^{32}Pi ↔ ATP exchange reaction of inside-out vesicles was carried out at 37°C. To avoid membrane energization by consumption of endogenous substrates, membranes (20 mg/mL) were incubated with 2 mM NaCN for 5 min on ice before the determination of the exchange reaction. The standard reaction medium contained 125 mM KCl, 25 mM sucrose, 40 mM Hepes–KOH (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 3 mM ATP, and the indicated concentrations of ADP and Pi in a final volume of 1 mL. The reaction mixture also included (1–3) $\times 10^6$ cpm/mL of purified [^{32}P]Pi to follow [γ^{32}P]ATP formation. The reaction was initiated with 0.8 mg of protein, and quenched with 200 μL of 30% (w/v) cold trichloroacetic acid. Thereafter, the sample was centrifuged at $5{,}000 \times g$ for 10 min, and 1 mL of ammonium molybdate (3.3% in 3.75 N HCI) was added to an aliquot of the supernatant. Inorganic phosphate was extracted six times with *n*-butyl acetate and acetone. The lower phase, which contained [γ^{32}P]ATP, was dried on filter paper and the radioactivity was counted by liquid scintillation. Control experiments showed that the radioactivity that remains in the aqueous phase was 99% [γ^{32}P]ATP. Blanks extracted with sulfite at the concentrations used showed no effect of sulfite on the efficiency of extraction of the nonincorporated [^{32}P]Pi.

Membrane Energization Determined by ACMA Quenching

The pH gradient driven by ATP hydrolysis or NADH oxidation in the inside-out vesicles were determined by the fluorescence quenching of 1 μM ACMA, as reported previously (Pacheco-Moisés *et al.*, 2000). Briefly, membranes (1 mg/mL) were incubated at 37°C in a medium containing 125 mM KCl, 20 mM MOPS (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1 μM ACMA, and the indicated concentrations of ADP and Pi. After stabilization of the signal, membrane was energized with 3 mM ATP or with 1 mM NADH. Calibration of quenching with artificial ΔpH generated in liposomes was made according to Rottenberg and Moreno-Sánchez (1993).

Other Procedures

For covalent modification by DCCD of F_0 c-subunits, the membranes (2 mg/mL) were incubated with 1–100 μM DCCD for 1 h, with moderate shaking at room temperature. Protein was determined by the Lowry procedure in the presence of 0.66% sodium deoxycholate, using serum albumin as standard (Lowry *et al.*, 1951).

RESULTS

Sulfite Increases the ATPase Activity of Inside-Out *P. denitrificans* Membranes and the IC_{50} for Mg^{2+}-ADP

In confirmation of previous data (Pacheco-Moisés *et al.*, 2000), sulfite increased the ATPase activity of *P. denitrificans* membranes with a $K_{0.5}$ of 8 mM (Fig. 1). In other systems, this activation has been associated with a decrease in affinity for inhibitory Mg^{2+}-ADP (Du and Boyer, 1990; Mueller *et al.*, 1994). Thus, we assessed whether sulfite modifies the IC_{50} of Mg^{2+}-ADP for inhibition of the ATPase activity. Indeed, we found that the IC_{50} for Mg^{2+}-ADP was increased about threefold in the presence of 40–60 mM sulfite (Fig. 1, inset).

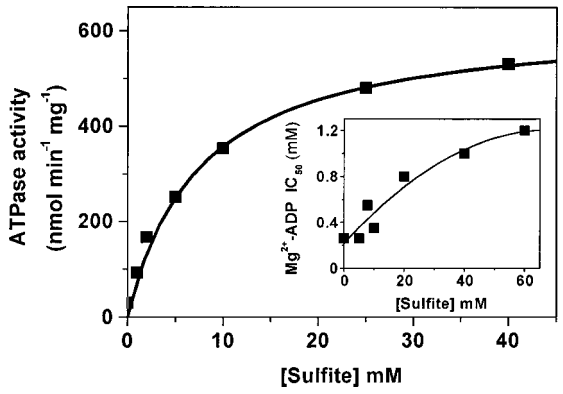

Fig. 1. Activation of the PdF_0F_1-ATPase by sulfite. ATP hydrolysis was measured at 37°C by the release of Pi, as described under Experimental Procedures. The solid line represents the best fit of the experimental points to the Michaelis–Menten equation by using the Origin computer program 5.0 (Microcal Software, Inc.). Inset: Increase of the half-maximal inhibitory concentration of Mg^{2+}-ADP for inhibition of the ATPase activity. The experimental data were connected with an exponential line.

Effect of Sulfite on the ^{32}Pi ↔ ATP Exchange of *P. denitrificans* Inside-Out Vesicles

As shown before (Pacheco-Moisés *et al.*, 2000), the activation of the PdF_1F_0-ATPase was accompanied by an increase in proton pumping activity evidenced by ACMA quenching (Fig. 2(B)). This allowed the study of the effect of sulfite on the ^{32}Pi ↔ ATP exchange in conditions of maximal membrane energization. The exchange reaction carried out by *P. denitrificans* inside-out vesicles was measured with and without added ADP. The exchange rates measured were due to membrane-integrated F_1F_0-ATP synthase, since venturicidin, a F_0 inhibitor, induced an almost complete abolition of exchange activity (not shown). Sulfite exerted either activation or inhibition of the ^{32}Pi ↔ ATP exchange reaction, depending on the concentrations of ADP and Pi in the media. In the absence of added ADP, sulfite increased the rate of ^{32}Pi incorporation into ATP (Fig. 2(A)). This increase correlated with higher ATPase activities (Fig. 1) and proton gradients (Fig. 2(B)) as induced by sulfite. However, in contrast to the at least 15-fold activation of ATPase activity (Fig. 1), the exchange rate was increased only about 2.5-fold (Fig. 2(A)). As expected from previous studies (Tuena de Gómez-Puyou *et al.*, 1983), the control exchange rate was higher in the presence of 2 mM ADP (2.6 nmol/min mg) than in its absence (0.1 nmol/min mg) (Fig. 2(A)). However, with 2 mM ADP, sulfite failed to increase the rate of ^{32}Pi ↔ ATP exchange (Fig. 2(A)). This correlated with the inability of sulfite to increase the ATP driven proton gradient (Fig. 2(B)) in the presence of saturating (inhibitory) ADP.

To explore whether there is a competition between sulfite and ADP or Pi, the exchange reactions were measured at subsaturating concentrations of ADP (50 μM) and Pi (0.5 or 0.1 mM). These concentrations are below their respective $K_{0.5}$ in the presence of sulfite during net ATP synthesis (see below, Table I). We also confirmed that in these conditions of minimal inhibition of ATPase activity by ADP, proton pumping was maximal (Fig. 2(B)). However, although membrane energization was as high as in the absence of ADP, sulfite inhibited the exchange reaction. Furthermore, the lower the concentration of Pi (and ADP), the higher the ability of sulfite to inhibit the exchange reaction (Fig. 2(A)). This indicated competition between sulfite and Pi and/or ADP. It is worth to note that the increase in proton gradient was accompanied by an increase in exchange only when ADP was not added, and Pi was present at 10 mM. The increase in exchange might occur if inhibition of the ATP synthase activity by sulfite were limited by the presence of saturating Pi. In these conditions, the endogenous or the exogenous ADP would be

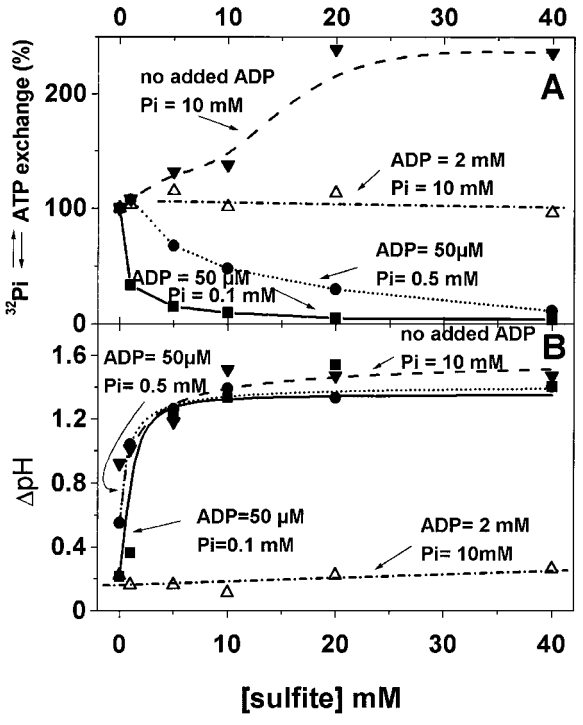

Fig. 2. Effect of sulfite on the ^{32}Pi ↔ ATP exchange and ATP driven pH gradients. (A) ^{32}Pi ↔ ATP exchange was measured at 37°C, as described under Experimental Procedures, in the presence of the following conditions: (▼) 10 mM Pi (0.1); (△) 10 mM Pi, 2 mM ADP (2.6); (■) 0.1 mM Pi, 50 μM ADP (0.1); (●) 0.5 mM Pi, 50 μM ADP (0.3). The control 100% activities are indicated here in parentheses in nmol/min mg. (B) ATP driven ΔpH determined by ACMA quenching (see Experimental Procedures) carried out under the same conditions as in (A) indicated with the same symbols.

Table I. Effect of Sulfite on the Affinities for Mg^{2+}-ADP and Pi During NADH Driven ATP Synthesis of Inside-Out *P. denitrificans* Membranes

	Control ($n = 4$)	5 mM sulfite ($n = 3$)	10 mM sulfite ($n = 3$)
Mg^{2+}-ADP varied			
V_{max}	280 ± 18	94 ± 21	46 ± 6
$K_{0.5}$ for Mg^{2+}-ADP	33 ± 5	243 ± 135	327 ± 75
n (Hill value)	1	1.3 ± 0.5	2.3 ± 0.8
Pi varied			
V_{max}	414 ± 23	251 ± 11	190 ± 26
$K_{0.5}$ for Pi	340 ± 69	1440 ± 140	1410 ± 489
n (Hill value)	1	1.6 ± 0.2	1.6 ± 0.5

Note. Apparent $K_{0.5}$, V_{max}, and Hill values (n) were determined with a nonlinear square fit of the Hill equation to the experimental data, using the Microcal Origin 5.0 software (Fig. 1). ATP synthesis was measured as in Fig. 4, with an hexokinase trap at 37°C (see Experimental Procedures), with 2 mM Mg^{2+}-ADP when Pi was varied, and with 10 mM Pi when Mg^{2+}-ADP was varied. The values are mean ± standard deviation of three to four different membrane preparations. Titration of Mg^{2+}-ADP was made with equimolar amounts of $MgCl_2$ and ADP.

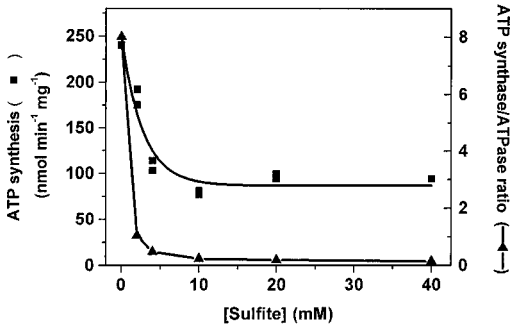

Fig. 3. Effect of sulfite on the rate of ATP synthesis and the ATP synthase/ATPase ratio. ATP synthesis was measured at 37°C, as described under Experimental Procedures, with 10 mM Pi and 2 mM Mg^{2+}-ADP. The ATP synthase/ATPase ratio was calculated by dividing the values of ATP synthase activity by those of ATPase activity of Fig. 1. The experimental data were connected by exponential lines.

prone to a faster exchange driven by increased proton gradients. In contrast, when ADP and Pi are subsaturating, sulfite becomes an effective inhibitor of exchange since both competitors are at low concentrations although ATP hydrolysis and proton gradients are maximal.

Effect of Sulfite on the NADH Driven ATP Synthesis Carried Out by *P. denitrificans* Inside-Out Membranes

Although sulfite impairs succinate oxidation, it does not inhibit significantly the NADH-sustained respiration or proton pumping in *P. denitrificans* membranes (Pacheco-Moisés *et al.*, 2000). Hence, we measured the intensity of the NADH-induced ΔpH by calibration of ACMA fluorescence quenching under conditions of net ATP synthesis (see Experimental Procedures). The data showed that 1 mM NADH generated a ΔpH of 1.6 in *P. denitrificans* vesicles, similar to that formed with Mg^{2+}-ATP (Fig. 2(B)). Upon addition of 20 mM sulfite, the ΔpH decreased by about 0.4 ΔpH units. However, at the sulfite concentrations required for inhibition of ATP synthesis (5–10 mM), the decrease of ΔpH was only 0.2 ΔpH units, i.e., 12% of the total ΔpH (data not shown).

Without sulfite, the basal ATP synthase activity was much higher than the ATPase one (Fig. 3), producing an ATPsynthase/ATPase ratio of 8. In the presence of sulfite, the rate of ATP synthesis diminished with a half-maximal inhibition (IC_{50}) of 3.5 mM. The maximal inhibitory effect (70%) was attained with 10 mM sulfite (Fig. 3). In consequence, the ratio of ATP synthase/ATPase declined progressively with increasing concentrations of sulfite from a value of 8 down to 0.14.

Effect of Sulfite on the $K_{0.5}$ for ADP and Pi During ATP Synthesis

Under the conditions described above, the affinities for both substrates (ADP and Pi) were determined during NADH driven ATP synthesis with and without sulfite. As observed before (Pérez and Ferguson, 1990a,b), the enzyme showed a Michaelis–Menten type kinetics for ADP and Pi. The apparent $K_{0.5}$ for Mg^{2+}-ADP and Pi were 33 ± 5 and 340 ± 69 μM, respectively (Table I), which are in the range of those reported earlier by Pérez and Ferguson (1990a,b). As shown in Fig. 4(A) and Table I, sulfite reduced dramatically the affinity for Mg^{2+}-ADP, i.e., 10 mM sulfite increased 10-fold the apparent $K_{0.5}$ for Mg^{2+}-ADP. Likewise, sulfite increased about fivefold the $K_{0.5}$ for Pi (Fig. 4(B), Table I). Computer-assisted curve fitting to a Hill equation showed that with sulfite, Mg^{2+}-ADP saturation acquired sigmoidal kinetics; the Hill coefficient increased from 1 in the controls to 2–3 with 5 mM and 10 mM sulfite. In addition, sulfite also decreased V_{max} (Table I), indicating that sulfite works as a mixed-type inhibitor of ATP synthesis with ADP and Pi.

DCCD Inhibition of the PdF_1F_0-ATPase in the Presence of Sulfite

Inhibition of ATP synthesis and activation of hydrolysis, as induced by sulfite, are classical features of an uncoupler. The previous results show that membrane uncoupling does not contribute significantly to the inhibition of ATP synthesis by sulfite. However, uncoupling

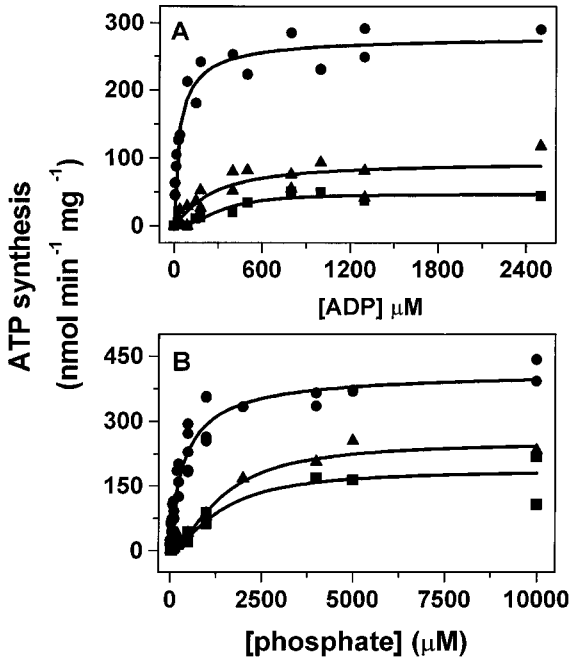

Fig. 4. Effect of sulfite on the affinity for Mg^{2+}-ADP and Pi. ATP synthesis was measured at 37°C with an hexokinase trap, either spectrophotometrically (A) or radioactively (B), as described under Experimental Procedures. (●) Controls without sulfite; (▲) 5 mM sulfite; (■) 10 mM sulfite. The solid lines are the best fit of the experimental points to the Hill equation. In (A), $MgCl_2$ was added in equimolar amounts to ADP in addition to the 5 mM $MgCl_2$ of the reaction mixture.

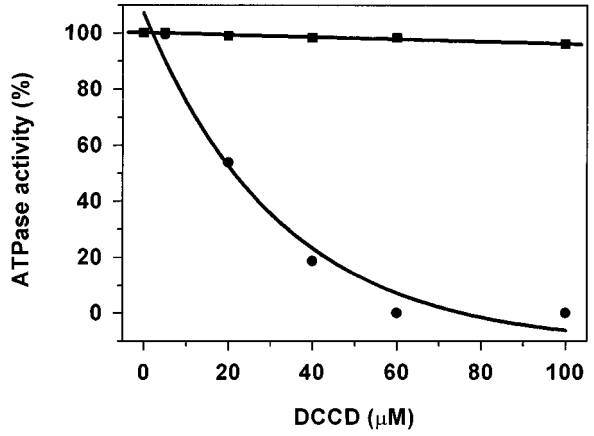

Fig. 5. Effect of preincubation of inside-out vesicles with DCCD on the F_1F_0-ATPase and F_1-ATPase activities. (●) Membranes were preincubated at room temperature with DCCD at the indicated concentrations, and the ATPase activity of inside-out vesicles was assayed in the presence of 100 mM sulfite, as described under Experimental Procedures. The 100% activity was 750 nmol/(min mg). (■) The PdF_1-ATPase was extracted with chloroform, as described under Experimental Procedures, after modification of membranes with DCCD as indicated above. The 100% F_1-ATPase activity corresponded to 400 nmol/(min mg).

may not only be membranal, but also intramolecular when F_1 and F_0 are separated physically or kinetically. Thus, intramolecular coupling between F_1 and F_0 was studied in the presence of sulfite. Previous evidence that the sulfite-stimulated ATP hydrolysis was inhibited by venturicidin and oligomycin (Capellini *et al.*, 1997; Pacheco-Moisés *et al.*, 2000), argued against sulfite-induced uncoupling. However, a more conclusive test for intramolecular F_1F_0 coupling is the sensitivity of the ATPase activity to micromolar concentrations of the covalent F_0 inhibitor dicyclohexilcarbodiimide (DCCD). At these concentrations, DCCD modifies specifically an essential carboxyl residue of the c subunit of F_0. Modification of a single c-subunit of the c_{9-12} ring by DCCD suffices to induce almost total and irreversible inhibition of the tightly coupled F_1F_0-ATPase (Hermolin and Fillingame, 1989). Therefore, *P. denitrificans* inside-out vesicles were preincubated with 1–100 μM DCCD, and the ATPase activity was assayed afterwards. DCCD inhibited progressively the sulfite-activated ATPase activity of the vesicles, until almost complete inhibition was attained (Fig. 5). To discard that DCCD could inhibit the ATPase activity through modification of the F_1 β subunit, two different controls were made. In the first one, the soluble PdF_1 was released with chloroform (see Experimental Procedures) after DCCD modification of the vesicles (Fig. 5). In the second, DCCD was added, in the same concentration range used in vesicles, to the previously released PdF_1-ATPase. In none of these conditions, the soluble PdF_1-ATPase activity was affected by preincubation with DCCD.

DISCUSSION

The novel information provided by this work is that the most effective activator of the PdF_1F_0-ATPase is also an inhibitor of its reversal, the PdF_0F_1-ATP synthase. Together with the unidirectional inhibition of the F_1F_0 complex by azide (Syroeshkin *et al.*, 1995; Bald *et al.*, 1998; Vinogradov, 1999), these data raised the question of how a molecule exerts different effects on the forward and reverse functioning of the F_1F_0 molecular motor. As discussed below, this work shows that the mechanism of sulfite to induce opposing effects on the F_1F_0 complex could involve changes in affinity for ADP and Pi, probably reflecting structural shifts of the enzyme.

Mechanism of PdF_1F_0-ATPase Activation by Sulfite

According to similar findings in the chloroplast (Du and Boyer, 1990) and yeast F_1-ATPases (Mueller *et al.*,

1994), activation of the PdF_0F_1-ATPase by sulfite was associated with a decrease in the affinity for inhibitory Mg^{2+}-ADP (Fig. 1). The data of Capellini *et al.* (1997) working with *R. capsulatus*, and our own previous studies with *P. denitrificans* (Pacheco-Moisés *et al.*, 2000) have shown that sulfite and Pi compete with each other during ATP hydrolysis. These overall results indicate that activation of the PdF_1F_0-ATPase is the result of a decrease in affinity for inhibitory ADP, and a faster release of products (ADP and Pi). Recently, Mg^{2+}-ADP inhibition has been associated with binding of nucleotides into noncatalytic sites (Matsui *et al.*, 1997). This makes possible that the increase in Ki for Mg^{2+}-ADP exerted by sulfite could be associated with its binding to noncatalytic sites, as suggested by Recktenwald and Hess (1977).

Effect of Sulfite on $^{32}Pi \leftrightarrow$ ATP Exchange Reaction Carried Out by the PdF_1F_0 Complex

The effects of sulfite on the $^{32}Pi \leftrightarrow$ ATP exchange paralleled its effects on membrane energization when added ADP was absent or present at saturating concentrations (2 mM) (Fig. 2). The increase in $^{32}Pi \leftrightarrow$ ATP exchange observed in the absence of added ADP (Fig. 2) resembled a similar increase induced by bicarbonate in the same reaction carried out by bovine heart submitochondrial particles (Lodeyro *et al.*, 2001). Our data therefore indicate that the increase in $^{32}Pi \leftrightarrow$ ATP exchange observed by Lodeyro *et al.* (2001) could also result from the higher ATPase activities and proton gradients possibly induced by bicarbonate. On the other hand, when ADP (50 μM) and Pi (0.1 or 0.5 mM) were present at subsaturating concentrations, the effect of sulfite on exchange and membrane energization were opposite; i.e., sulfite inhibited the $^{32}Pi \leftrightarrow$ ATP exchange although it induced a maximal membrane energization (Fig. 2). In other words, we found conditions where the effects of sulfite on the $^{32}Pi \leftrightarrow$ ATP exchange reaction were independent of the extent of membrane energization (Fig. 2). This indicates that sulfite exerts a direct effect on the ATP synthase that can be separated from its effects on the respiratory chain of *P. denitrificans* (Bakels *et al.*, 1994). Furthermore, the data also indicated a competition between sulfite and ADP or Pi as confirmed by the measurements of net ATP synthesis.

Inhibition of PdF_0F_1-ATP Synthase Activity by Sulfite

The kinetic mechanism of sulfite to inhibit ATP synthesis in *P. denitrificans* involves a noncompetitive effect on V_{max}, and a decrease in affinity for ADP as well as for Pi. The contribution of sulfite-induced membrane or intramolecular uncoupling to these effects can be ruled out with the following evidence:

I. The sulfite activated ATPase was inhibited by DCCD (Fig. 5), oligomycin, and venturicidin (Capellini *et al.*, 1997; Pacheco-Moisés *et al.*, 2000).
II. The ATP driven membrane energization, which requires membrane coupling, was maximized by sulfite.
III. The $^{32}Pi \leftrightarrow$ ATP exchange reaction was inhibited by sulfite at low concentrations of ADP and Pi although membrane energization was maximal (Fig. 2).
IV. The NADH energized ΔpH of inside-out vesicles was marginally decreased (12%), whereas ATP synthesis was inhibited by 70% with 5–10 mM sulfite.

Taken together, these data show that sulfite interacts directly with the F_1F_0 complex to inhibit ATP synthesis. Similar conclusions have been derived from studies of inhibition of ATP synthesis by bicarbonate (Lodeyro *et al.*, 2001).

In previous studies with *P. denitrificans* membranes, lower ΔpH values (Mc Carthy and Ferguson, 1983a,b) but higher rates of ATP synthesis and $^{32}Pi \leftrightarrow$ ATP exchange (Pérez and Ferguson, 1990a,b) were reported. These discrepancies could be due to differences in conditions for growth (aerobic vs. anaerobic) or ΔpH measurements, or the different strains used (see Experimental Procedures). However, other results obtained here are similar to those observed before (Pérez and Ferguson, 1990a,b). For example, the rate of $^{32}Pi \leftrightarrow$ ATP exchange carried out by *P. denitrificans* membrane vesicles is slower than expected from the rates of ATP hydrolysis and synthesis observed. This indicates that the PdF_1F_0-ATP synthase has a limited capacity for $^{32}Pi \leftrightarrow$ ATP exchange compared, for example, to that of the mitochondrial enzyme (Tuena de Gómez-Puyou *et al.*, 1983). Regardless of the differences in net rates, the affinities of the enzyme for substrates and for sulfite show the same trends reported before, therefore, supporting the conclusions derived from these data.

Sulfite Works as a Mixed Type Inhibitor With ADP and Pi During ATP Synthesis Carried Out by the PdF_1F_0 Complex

As described, membranal or intramolecular uncoupling cannot explain the inhibition of ATP synthesis exerted by sulfite in *P. denitrificans*. Therefore, a direct

competition between sulfite, ADP, and Pi for the catalytic sites has been proposed. In fact, competition between sulfite and ADP or Pi has been observed during ATPase assays in the chloroplast ATPase (Larson and Jagendorf, 1989; Malyan and Vitseva, 2001), in *R. capsulatus* (Capellini *et al.*, 1997), and in *P. denitrificans* vesicles (Pacheco-Moises *et al.*, 2000). It has also been suggested that sulfite vs. Pi competition accounts for the inhibition of ATP synthesis in membrane vesicles of the cyanobacteria *Synechococcus* 6716 (Bakels *et al.*, 1996). However, the present data show that the inhibition of ATP synthesis exerted by sulfite is not purely competitive with ADP and Pi (Table I), but it is a mixed type inhibition. Furthermore, the increase in $K_{0.5}$ induced by sulfite was higher for ADP than for Pi during net ATP synthesis (Table I). Similar results have been recently obtained with bicarbonate which inhibited ATP synthesis competitively with ADP but noncompetitively with Pi (Lodeyro *et al.*, 2001). The higher increase in K_m for Pi with sulfite than with bicarbonate could be related to the closer structural similarity of Pi with sulfite (both tetrahedral) than with bicarbonate (triangular). A direct interaction of sulfite with the catalytic sites of F_1 predicts a competitive inhibition of the ATPase activity. However, sulfite is an activator of F_1 and F_1F_0-ATPases that does not modify significantly the $K_{0.5}$ for ATP (Capellini *et al.*, 1997; Murataliev and Boyer, 1992; Pacheco-Moisés *et al.*, 2000). This evidence shows that sulfite could bind not only to catalytic sites but also to other sites to inhibit ATP synthesis and simultaneously activate ATP hydrolysis. As mentioned before, noncatalytic sites are good candidates to bind sulfite and to exert ATPase activation (Recktenwald and Hess, 1977) and ATP synthase inhibition (Lodeyro *et al.*, 2001). Similar conclusions have been derived from the inhibition of ATP synthesis by bicarbonate (Lodeyro *et al.*, 2001). This explanation would fit with the rotational catalysis as well as with evidence incompatible with the rotational model (reviewed in Berden and Hartog, 2000).

Possible Structural Effects of Sulfite on the PdF_1F_0 Complex

Our previous experiments of limited proteolysis (Pacheco-Moisés *et al.*, 2000) suggest that the kinetic effects of sulfite could be associated with structural modifications. This oxyanion increases the activation of the PdF_1F_0-ATPase exerted by limited proteolysis with trypsin (Pacheco-Moisés *et al.*, 2000). Therefore, it can be proposed that sulfite induces a conformation of lower affinity for Mg^{2+}-ADP, and thus a low ATP synthase activity and higher ATPase turnover, associated with a higher exposition of its intrinsic inhibitory subunit(s). Evidence for two different conformations have been recently obtained by engineered crosslinkings in the *E. coli* F_1F_0-ATP synthase (Tsunoda *et al.*, 2001). In these studies, the C-terminal domain of the inhibitory ε subunit shifted in two different positions, one facing the ring of F_0 c-subunits and another oriented toward the C-terminal portion of the α and β subunits of F_1. In the former, ATP synthesis occurred unaffected, and hydrolysis was activated. In contrast, with the latter conformation of the C-terminus of ε facing F_1, only the ATPase activity was inhibited (Tsunoda *et al.*, 2001).

Sulfite could therefore impair the binding of ADP and Pi directly by occupation of the catalytic sites, or indirectly through noncatalytic sites, or by shifting the conformation of the F_1F_0 complex to an ATPase form. In any case, the net effect of sulfite is to decrease the affinity for ADP and Pi. Together with the proton slip induced by sulfite in *R. capsulatus* (Capellini *et al.*, 1997), this mechanism would explain how a molecule may exert opposite effects on ATP synthesis and hydrolysis carried out by the PdF_1F_0 complex. In the presence of sulfite, products of hydrolysis would be released faster, whereas the binding of these substrates for ATP synthesis would be impaired.

The Effects of Sulfite and Sodium Azide on ATP Synthesis and Hydrolysis Are Interrelated

It is worth noting that together with azide (Bald *et al.*, 1998; Syroeshkin *et al.*, 1995; Vinogradov, 1999), sulfite becomes another molecule that exerts different effects on ATP synthesis and hydrolysis carried out by the F_1F_0 complex. Interestingly, there is a close relationship between sulfite, azide, and ADP binding during activation or inhibition of the F_1-ATPase. For instance, the inhibition of F_1-ATPase by azide is reverted by sulfite (Vasilyeva *et al.*, 1982). In addition, the βThr 197 $\rightarrow$ Ser mutation activates the *Saccharomyces cerevisiae* F_1-ATPase, increases the K_i for ADP, decreases azide inhibition, and abolishes activation by sulfite (Mueller, 1989; Mueller *et al.*, 1994). During ATP synthesis, azide is therefore unable to entrap the F_1-Mg^{2+}-ADP inhibited form (Hyndman *et al.*, 1994; Murataliev *et al.*, 1991; Vasilyeva *et al.*, 1982) or to affect the positive cooperativity between the catalytic sites of F_1 (García *et al.*, 1997; Harris, 1989; Noumi *et al.*, 1987; Weber and Senior, 1998) as it does during ATP hydrolysis. Therefore, the ability to entrap Mg^{2+}-ADP is an important common factor in the unidirectional inhibition by azide, and in the opposite effects of sulfite on the forward and reverse functioning of F_1F_0. Nucleotide binding fuels the rotation of the $\gamma/\varepsilon/c_{12}$ domain and the positive

cooperativity of F_1 during ATP hydrolysis (García, 2000; García and Capaldi, 1998; Wang and Oster, 1998; Yasuda *et al.*,2001). However, during ATP synthesis, rotation is fueled not only by nucleotide binding, but also by proton conduction through the F_0 motor (García, 2000; Oster and Wang, 1999). Therefore, during ATP synthesis, the presence of azide may be circumvented by F_0 proton conduction that induces positive cooperativity in F_1 through rotation of the $\gamma/\varepsilon/c_{12}$ domain. In this way, accumulation of an F_1F_0-Mg^{2+}-ADP inhibited form may be prevented. In the case of sulfite, the possible binding of this oxyanion to noncatalytic sites (Recktenwald and Hess, 1977), or the structural modifications that sulfite induce in the PdF_1F_0 complexes, might prevent accumulation of the PdF_1F_0-Mg^{2+}-ADP inhibited form.

In summary, this work shows that during ATP hydrolysis or ATP synthesis, sulfite decreases the affinity for ADP and Pi, leading to its opposing effects on ATPase and ATP synthase activities. Therefore, in the presence of sulfite, the PdF_1F_0 complex is completely shifted from its physiological ATP synthase activity to the opposite highly active ATPase. This effect of sulfite may therefore contribute significantly to the toxic effect of this oxyanion in *P. denitrificans* (Wodara *et al.*, 1997). The kinetic effects of sulfite on the PdF_1F_0 complex are likely the result of structural shifts from a basal ATP synthase state into another ATPase form that possesses a low affinity for ADP and Pi. Together with azide, sulfite provides evidence for the asymmetry between ATP synthesis and hydrolysis carried out by F_1F_0. Control factors such as the affinity for nucleotides and Pi, together with the proton gradient, may contribute to the different effects of these molecules on the forward and reverse functioning of the F_1F_0-ATP synthase.

ACKNOWLEDGMENTS

The authors thank Professors Marietta Tuena de Gómez-Puyou and Armando Gómez-Puyou from the National University of México for their critical revision of the manuscript. This work was partially supported by grants I32903-N and J34744-N from CONACyT-México.

REFERENCES

Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994). *Nature* **370**, 621–628.

Al-Shawi, M. K., Ketchum, C. J., and Nakamoto, R. K. (1997). *Biochemistry* **36**, 12961–12969.

Bakels, R. H. A., Van Walraven, H. S., Van Wielink, J. E., Der Zwet-De Graaff, I. V., Krenn, B. E., Krab, K., Berden, J. A., and Kraayenhof, R. (1994). *Biochem. Biophys. Res. Commun.* **201**, 487–492.

Bakels, R. H. A., Van Wielink, J. E., Krab, K., and Van Walraven, H. S. (1996). *Arch. Biochem. Biophys.* **332**, 170–174.

Bald, D., Amano, T., Muneyuki, E., Pitard, B., Rigaud, J. L., Kruip, J., Hisabori, T., Yoshida, M., and Shibata, M. (1998). *J. Biol. Chem.* **273**, 865–870.

Berden, J. A., and Hartog, A. F. (2000). *Biochim. Biophys. Acta* **1458**, 234–251.

Bianchet, M., Ysern, X., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1991). *J. Biol. Chem.* **266**, 21197–21201.

Bianchet, M. A., Hullihen, J., Pedersen, P. L., and Amzel M. L. (1998). *Proc. Natl. Acad. Sci. U.S.A.* **95**, 11065–11070.

Bottcher, B., Schwarz, L., and Graber, P. (1998). *J. Mol. Biol.* **281**, 757–762.

Capaldi, R. A., Schulenberg, B., Murray, J., and Aggeler, R. (2000). *J. Exp. Biol.* **203**, 29–33.

Capellini, P., Turina, P., Fregni, V., and Melandri, B. A. (1997). *Eur. J. Biochem.* **248**, 496–506.

de Meis, L. (1984). *J. Biol. Chem.* **259**, 6090–6097.

Du, Z., and Boyer, P. D. (1990). *Biochemistry* **29**, 402–407.

García, J. J. (2000). In *Recent Research Developments in Bioenergetics* (Pandalai, S. G., ed.), Transworld Research Network, Trivandrum, India, pp. 41–62.

García, J. J., and Capaldi, R. A. (1998). *J. Biol. Chem.* **273**, 15940–15945.

García, J. J., Gómez-Puyou, A., Maldonado, E., and Tuena de Gómez-Puyou, M. (1997). *Eur. J. Biochem.* **249**, 622–629.

García, J. J., Tuena de Gómez-Puyou, M., and Gómez-Puyou, A. (1995). *J. Bioenerg. Biomembr.* **27**, 127–136

Harris, D. A. (1989). *Biochim. Biophys. Acta* **974**, 156–162.

Hermolin, J., and Fillingame, R. H. (1989). *J. Biol. Chem.* **264**, 3896–3903.

Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., and Cross, R. L. (1994). *J. Biol. Chem.* **269**, 28871–28877.

Karrasch, S., and Walker, J. E. (1999). *J. Mol. Biol.* **290**, 379–384.

Kinosita, K., Jr., Yasuda, R., Noji, H., and Adachi, K. A. (2000). *Philos. Trans. R. Soc. London, Sex. B: Biol. Sci.* **355**, 473–489.

Larson, E. M., and Jagendorf. A. T. (1989). *Biochim. Biophys. Acta* **973**, 66–77.

Leslie, A. G., and Walker, J. E. (2000). *Philos. Trans. R. Soc. London, Sex. B: Biol. Sci.* **355**, 465–471.

Lodeyro, A. F., Calcaterra, N. B., and Roveri, O. A. (2001). *Biochim. Biophys. Acta* **1506**, 236–243.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.

Malyan, A. N., and Vitseva, O. I. (2001). *Biochemistry* (*Moscow*) **4**, 410–414.

Matsui, T., Muneyuki, E., Honda, M., Allison, W. S., Dou, C., and Yoshida, M. (1997). *J. Biol. Chem.*, **272**, 8215–8221.

Matsuno-Yagi, A., and Hatefi, Y. (1984). *Biochemistry* **23**, 3508–3514.

Mc Carthy, J. E. G., and Ferguson, S. J. (1983a). *Eur. J. Biochem.* **132**, 417–424.

Mc Carthy, J. E. G., and Ferguson, S. J. (1983b). *Eur. J. Biochem.* **132**, 425–431.

McLachlin, D. T., Coveny, A. M., Clark, S. M., and Dunn S. D. (2000). *J. Biol. Chem.* **275**, 17571–17577.

Mueller, D. M. (1989). *J. Biol. Chem.* **264**, 16552–16556.

Mueller, D. M., Indyk, V., and McGill, L. (1994). *Eur. J. Biochem.* **222**, 991–999.

Murataliev, M. B., and Boyer, P. D. (1992). *Eur. J. Biochem.* **209**, 681–687.

Murataliev, M. B., Milgrom, Y. M., and Boyer, P. D. (1991). *Biochemistry* **30**, 8305–8310.

Nakamoto, R. K., Ketchum, C. J., Kuo, P. H., Peskova, Y. B., and Al-Shawi, M. K. (2000). *Biochim. Biophys. Acta* **1458**, 289–299.

Norling, B., Strid, A., and Nyrén, P. (1988). *Biochim. Biophys. Acta* **935**, 123–129.

Noumi, T., Maeda, M., and Futai, M. (1987). *FEBS Lett.* **213**, 381–384

Oster, G., and Wang, H. (1999). *Structure* **7**, 67–72.

Pacheco-Moisés, F., Gracía, J. J., Rodríguez-Zavala, J. S., and Moreno-Sánchez, R. (2000). *Eur. J. Biochem.* **267**, 993–1000.

Pérez, J. A., and Ferguson, S. J. (1990a). *Biochemistry* **29**, 10503–10518.
Pérez, J. A., and Ferguson, S. J. (1990b). *Biochemistry* **29**, 10518–10526.
Recktenwald, D., and Hess, B. (1977). *FEBS Lett.* **76**, 25–28.
Rodgers, A. J. W., and Capaldi, R. A. (1998). *J. Biol. Chem.* **273**, 29406–29410.
Rottenberg, H., and Moreno-Sánchez, R. (1993). *Biochim. Biophys. Acta* **1183**, 161–170.
Sumner, J. B. (1964). *Science* **100**, 413–414.
Syroeshkin, A. V., Vasilyeva, E. A., and Vinogradov, A.D. (1995). *FEBS Lett.* **366**, 29–32.
Tsunoda, S., Rodgers, A. J. W., Aggeler, R., Wilce, M. C. J., Yoshida, M., and Capaldi, R. A. (2001). *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6560–6564.
Tuena de Gómez-Puyou, M., Muller, U., Dreyfus, G., Ayala, G., and Gómez-Puyou, A. (1983). *J. Biol. Chem.* **258**, 13680–13684.
Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982). *Biochem. J.* **202**, 15–23.
Vinogradov, A. D. (1999). *Biochemistry* (*Moscow*) **64**, 1219–1229.
Wang, H., and Oster, G. (1998). *Nature* (*London*) **396**, 279–282.
Weber, J., and Senior, A. E. (1998). *J. Biol. Chem.* **273**, 33210–33215
Weber, J., and Senior, A. E. (2000). *Biochim. Biophys. Acta* **1458**, 300–309.
Wilkens, S., and Capaldi, R. A. (1998). *Nature* (*London*) **393**, 29.
Wodara, C., Bardischewsky, F., and Friedrich, C. G. (1997). *J. Bacteriol.* **179**, 5014–5023.
Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., and Itoh, H. (2001). *Nature* (*London*) **410**, 898–904.