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Inhibition of steady-state mitochondrial ATP synthesis by bicarbonate, an activating anion of ATP hydrolysis

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Abstract

Bicarbonate, an activating anion of ATP hydrolysis, inhibited ATP synthesis coupled to succinate oxidation in beef heart submitochondrial particles but diminished the lag time and increased the steady-state velocity of the ^{32}Pi–ATP exchange reaction. The latter effects exclude the possibility that bicarbonate is inducing an intrinsic uncoupling between ATP hydrolysis and proton translocation at the level of F_1F_o ATPase. The inhibition of ATP synthesis was competitive with respect to ADP at low fixed [Pi], mixed at high [Pi] and non-competitive towards Pi at any fixed [ADP]. From these results we can conclude that (i) bicarbonate does not bind to a Pi site in the mitochondrial F_1; (ii) it competes with the binding of ADP to a low-affinity site, likely the low-affinity non-catalytic nucleotide binding site. It is postulated that bicarbonate stimulates ATP hydrolysis and inhibits ATP synthesis by modulating the relative affinities of the catalytic site for ATP and ADP.

Keywords: F_1F_o ATP synthase; Mitochondrion; ATP synthesis; Inhibition; Activating anion; Bicarbonate

1. Introduction

F_1F_o ATPase/synthase catalyzes $\Delta\mu_{H^+}$-driven ATP synthesis and $\Delta\mu_{H^+}$-generating ATP hydrolysis. It is located in the inner mitochondrial membrane as well as in other energy-transducing membranes. The F_1 sector can be isolated from the membrane, retaining only its ability to hydrolyze ATP. It is composed of five different subunits with a stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. Its structure has been elucidated [1–3]. It has six nucleotide binding sites that differ in their binding affinity, exchangeability, catalytic involvement and localization [4]. All the sites are located in α/β interfaces. Three of them are built mainly with the contribution of β subunits (catalytic sites), the other three with the main contribution of α subunits (non-catalytic sites) [1]. The catalytic sites exhibit strong cooperativity and the fact that ATP hydrolysis and ATP synthesis follow a binding change mechanism [5,6] is accepted. It is still a matter of controversy whether two [7] or three [8] catalytic sites should be filled for rapid ATP synthesis and hydrolysis. It is also widely accepted that ATP hydrolysis and ATP synthesis are coupled to proton translocation by ro-

Abbreviations: F_1F_o, F_1F_o ATPase/synthase; SMP, submitochondrial particles

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tation of the γ and ε subunits [9] connected to another rotatory device in F_o [10]. The feasibility of such rotatory mechanism has been discussed by Hartog and Berden [11] who have reported that one of the three catalytic sites is continuously occupied by a non-exchangeable nucleotide.

Ebel and Lardy [12] have shown that the ATPase activity of isolated mitochondrial F_1 and membrane-bound F_1F_o is stimulated by several anions (bicarbonate and sulfite among them) and is inhibited by others, such as azide. They also claimed that the activating anions abolished the apparent negative cooperativity exhibited by F_1. However, more recent studies have shown that even in the presence of bicarbonate the negative cooperativity can still be observed [13,14]. This discrepancy may be due to a third apparent high-affinity K_m that has not been detected by the former authors.

Although the activation of the ATPase activity by anions has been known for almost three decades, the mechanism of action of the activating anions is still a matter of controversy. Recktenwald and Hess [15] and Berden and co-workers [16] have postulated that the binding of ATP to a 'regulatory' non-catalytic nucleotide binding site would be the reason for the apparent negative cooperativity. Sulfite would compete with the binding of ATP to that site and stabilize the enzyme in its low K_m mode abolishing the apparent negative cooperativity. An alternative mechanism has been proposed by Moyle and Mitchell [17] who postulated that the anions modulate active/inactive state transitions of F_1. Vasilyeva et al. [18] have suggested that the anions modulate the equilibrium between 'slowly interconvertible' active and inactive E–ADP complexes. Du and Boyer [19] have proposed that binding of sulfite to an inorganic phosphate (Pi) binding site in the E–ADP–Mg^{2+} complex gives a form more readily activated by the binding of ATP to an alternative site. Walker [20] has considered the possibility that an intrinsic uncoupling of ATP hydrolysis from proton translocation, similar to that observed in the yeast vacuolar ATPase [21], could be responsible for the anion activation in the structurally related F_1F_o ATPases.

Vinogradov and co-workers [22] have shown that azide, an inhibitory anion of ATP hydrolysis, does not affect ATP synthesis in submitochondrial particles (SMP). This result supports their proposal [23,24] that ATP synthesis and ATP hydrolysis are catalyzed by different F_1 states or catalytic pathways.

Few studies have been carried out on the effects of activating anions on ATP synthesis. Malyan et al. [25] have reported that sulfite is a competitive inhibitor with respect to Pi of ATP synthesis in pea chloroplasts.

We decided to study the effect of bicarbonate on ATP synthesis catalyzed by the mitochondrial F_1F_o in order to test whether the above mentioned inhibition by sulfite can be generalized to other activating anions. We show here that bicarbonate inhibits ATP synthesis in SMP whereas it stimulates the ^{32}Pi–ATP exchange reaction, suggesting that bicarbonate does not induce an intrinsic uncoupling in F_1F_o. The kinetic behavior of the inhibition of ATP synthesis leads us to exclude the possibility that bicarbonate binds to a Pi binding site as has been suggested. We also discuss the action mechanism of bicarbonate and the possible role of the low-affinity non-catalytic nucleotide binding site on the regulation of the H^+-reversible ATPase.

2. Materials and methods

2.1. Preparation of phosphorylating submitochondrial particles

Heavy bovine heart mitochondria were prepared as described [26]. Phosphorylating submitochondrial Mg^{2+}-ATP particles were prepared from heavy bovine heart mitochondria according to Low and Vallin [27].

2.2. Assays

Succinate oxidation and ^{32}Pi-ATP exchange reaction were measured as previously described [28]. ATP synthesis was measured as described [28] with the following modifications: 10 mM succinate in the presence of 3 μM rotenone was used as oxidizable substrate, the reaction time was 5 min and 0.2 mg protein was used. Under these conditions product formation was linear with time and reaction velocity was linear with [SMP] at low and high [Pi] and [ADP-Mg], in the absence and in the presence of bicarbonate. The reactions were carried out at 30°C.

Table 1
Apparent kinetic parameters for the ATP synthesis in Mg-ATP particles

Kinetic parameter	[ADP-Mg] = variable	
	[Pi] = 0.05 mM	[Pi] = 5 mM
$K_{ADP\ (1)}$	0.100 ± 0.007 mM	0.002 ± 0.001 mM
$K_{ADP\ (2)}$	0.430 ± 0.015 mM	0.150 ± 0.020 mM
$V_{max\ (1)}$	1.1 ± 0.9 nmol min^{-1} mg^{-1}	40 ± 10 nmol min^{-1} mg^{-1}
$V_{max\ (2)}$	40 ± 2 nmol min^{-1} mg^{-1}	350 ± 10 nmol min^{-1} mg^{-1}
	[Pi] = variable	
	[ADP-Mg] = 0.004 mM	[ADP-Mg] = 1 mM
$K_{Pi\ (1)}$	0.037 ± 0.002 mM	0.080 ± 0.020 mM
$K_{Pi\ (2)}$	6.0 ± 0.7 mM	3.8 ± 0.7 mM
$V_{max\ (1)}$	2 ± 0.1 nmol min^{-1} mg^{-1}	72 ± 1 nmol min^{-1} mg^{-1}
$V_{max\ (2)}$	32 ± 1 nmol min^{-1} mg^{-1}	340 ± 20 nmol min^{-1} mg^{-1}

ATP synthesis was determined as described in Section 2. The experimentally determined ATP synthesis velocities obtained varying either [ADP-Mg] or [Pi] at fixed [Pi] and [ADP-Mg], respectively, were fitted by non-linear regression analysis to the sum of two hyperbolas.

Rates which are reported here are the average of duplicate determinations that agreed within 10%. The studies have been repeated with at least two SMP preparations.

2.3. General

Protein concentration was determined by a modified biuret procedure [29], using bovine serum albumin as standard, the concentration of which was determined spectrophotometrically (A_{279} = 6.67 cm^{-1} for 1% solution [30]).

3. Results

3.1. Effect of bicarbonate on ATP synthesis

ATP synthesis catalyzed by SMP exhibited non-hyperbolic steady-state kinetics when either [ADP] or [Pi] was varied – in agreement with previous reports by Matsuno-Yagi and Hatefi [31,32]. However, the preparation we used (Mg^{2+}-ATP particles) behaved slightly differently from that used by these authors, since the more important contribution to the reaction velocity was that corresponding to the low-affinity K_{ms} when succinate was the oxidizable substrate (Table 1).

Bicarbonate inhibited ATP synthesis at concentrations that did not affect succinate oxidation (Fig. 1). The anion did not modify the apparent negative cooperativity observed in its absence (data not shown). Therefore, the inhibition was characterized using Pi or ADP as fixed substrate at concentrations that correspond to their low- and high-K_m regions. At any [ADP] and [Pi] bicarbonate behaved as a linear inhibitor (Figs. 2 and 3), clearly indicating that (i) the complex F_1F_o–$NaHCO_3$ is unable to catalyze steady-state ATP synthesis; and (ii) one molecule of bicarbonate binds to one or more intermediates in the mechanism of this multisubstrate enzyme [33].

When the inhibition was studied at variable [ADP] and at 0.1 mM Pi, v_0/v_i vs. [$NaHCO_3$] plots gave straight lines (Fig. 2A) whose slopes depended on [ADP] (Fig. 2A, inset) according to the behavior of a linear competitive inhibitor [34]. Bicarbonate and ADP behaved as mutually exclusive ligands. An ap-

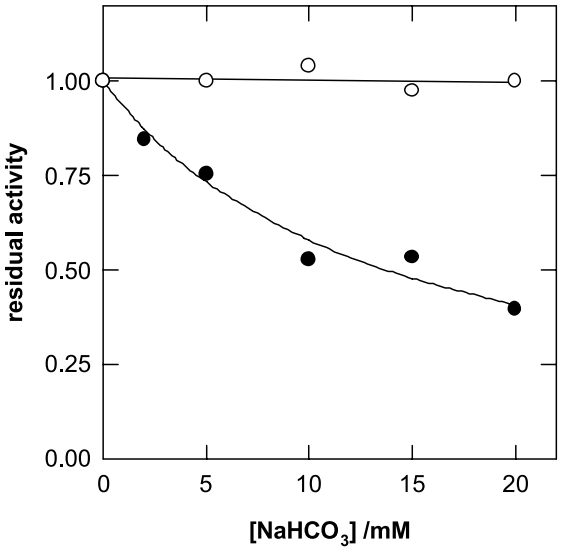

Fig. 1. Effect of bicarbonate on electron transport and ATP synthesis. Succinate oxidation (○) and ATP synthesis (●) were measured as indicated in Section 2 at different [$NaHCO_3$]. [ADP-Mg] and [Pi] were 0.01 and 5.0 mM, respectively. The curve line represents the fit of the experimentally determined ATP synthesis residual activities to the equation of a linear inhibitor ($v/v_0 = 1-[NaHCO_3]/([NaHCO_3]_{0.5}+[NaHCO_3])$, with $[NaHCO_3]_{0.5}$ = 13.8 ± 1.1 mM. Activities of controls (v_0) were 244 nat oxygen min^{-1} mg^{-1} and 73 nmol min^{-1} mg^{-1} for succinate oxidation and ATP synthesis respectively.

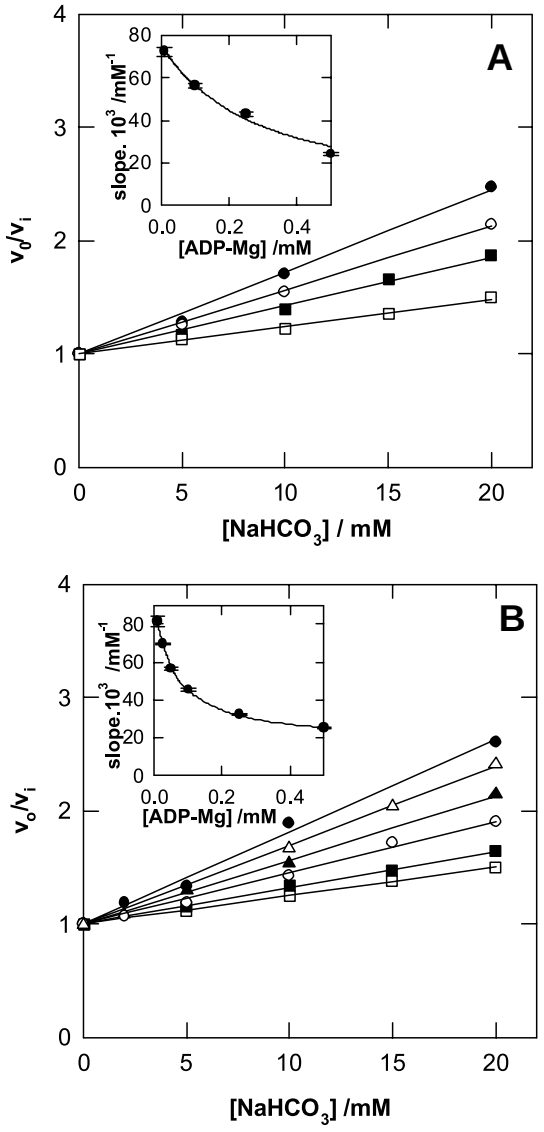

Fig. 2. Inhibition of ATP synthesis by bicarbonate at fixed [Pi]. ATP synthesis was determined as described in Section 2 at fixed [Pi] and variable [ADP-Mg]. v_0/v_i values are plotted against [$NaHCO_3$], as has been previously described [34]. Slope values, estimated from the primary plots by linear regression, are plotted in the insets as a function of [ADP-Mg]. The vertical bars indicate the corresponding standard deviations. (A) [Pi] was 0.1 mM and [ADP-Mg] was 0.01 (●), 0.1 (○), 0.25 (■) or 0.5 (□) mM. The line in the inset is the best fit obtained by non-linear regression analysis using the equation: slope = $K_{ADP}/\{K_i(K_{ADP}+[ADP\text{-}Mg])\}$ that describes the behavior of a linear competitive inhibitor [34] ($K_i = 13.2 \pm 0.5$ mM and $K_{ADP} = 0.29 \pm 0.04$ mM). (B) [Pi] was 5.0 mM and [ADP-Mg] was 0.01 (●), 0.025 (△), 0.05 (▲), 0.1 (○), 0.25 (■), or 0.5 (□) mM. The line in the inset is the best fit of the slope values estimated from the primary plots to the equation that corresponds to a linear mixed inhibitor [34]: slope = $(K_{ADP}/K_i+[ADP\text{-}Mg]/K'_i)/(K_{ADP}+[ADP\text{-}Mg])$. The estimated parameters were $K_i = 10.8 \pm 0.1$ mM, $K'_i = 56.2 \pm 2.2$ mM and $K_{ADP} = 0.056 \pm 0.003$ mM. ←

parent $K_{d(ADP)} = 0.29$ mM for the ADP binding site could be estimated.

At 5 mM Pi, v_0/v_i vs. [$NaHCO_3$] linear plots were also obtained (Fig. 2B), but the dependence of their slopes on [ADP-Mg] corresponded to a linear mixed inhibitor (Fig. 2B, inset). Apparent dissociation constants estimated for the F_1F_o–$NaHCO_3$ and F_1F_o–$NaHCO_3$–ADP complexes were 10.8 and 56.2 mM, indicating that binding of ADP strongly decreased the apparent affinity for bicarbonate. However, the competition was not complete and a ternary dead-end complex could be formed at high [Pi].

On the other hand, when the studies were carried out at variable [Pi] and at 0.010 mM ADP, the slopes of the linear v_0/v_i vs. [$NaHCO_3$] plots did not depend significantly on [Pi] (Fig. 3 and inset). This behavior is characteristic of a linear non-competitive inhibitor [34] and indicates that a ternary F_1F_o–Pi–$NaHCO_3$ complex can be formed. Similar results were obtained when 1 mM ADP was used as fixed substrate (data not shown).

3.2. Effect of bicarbonate on the ^{32}Pi–ATP exchange reaction

As has been previously reported [35], the ^{32}Pi–ATP exchange reaction exhibited an acceleration phase before the steady-state velocity was reached. Bicarbonate not only shortened the lag time but also increased the steady-state velocity (Fig. 4A).

The lag time (τ) decreased with bicarbonate concentration according to the following equation:

$$\tau = \frac{\tau_0}{1 + \dfrac{\left(\dfrac{\tau_0}{\tau_\infty} - 1\right)[NaHCO_3]}{[NaHCO_3]_{0.5} + [NaHCO_3]}} \quad (1)$$

where τ_0 is the lag time in the absence of bicarbonate (4.39 ± 0.03 min), τ_∞ the lag time at saturating [$NaHCO_3$] (2.88 ± 0.08 min) and $[NaHCO_3]_{0.5}$ the concentration that exerted half of the maximal effect (13 ± 2 mM) (Fig. 4B).

The steady-state velocity increased from 51 ± 2

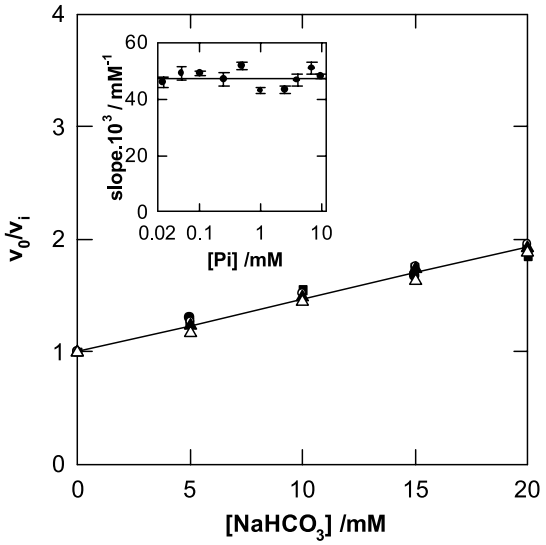

Fig. 3. Inhibition of ATP synthesis by bicarbonate at fixed [ADP]. ATP synthesis was determined as described in Section 2 at fixed [ADP-Mg] (0.01 mM) and variable [Pi] ranging from 0.025 to 10 mM. Shown are the experimentally determined data plotted as indicated in the legend to Fig. 2 for the following [Pi]: 0.025 (●), 0.1 (○), 1.0 (△), 4.0 (■) and 10.0 (▲) mM. The closed circles in the inset are the slope values estimated by linear regression from primary plots drawn at different [Pi] and the vertical bars are their standard deviations. Note that on the *x*-axis a logarithmic scale is used because of the wide range of [Pi]. The lines in the primary plot and in the inset identify [34] a linear non-competitive inhibitor with $K_i = 21.1 \pm 1.3$ mM (slope = $1/K_i$).

nmol min^{-1} in the absence of bicarbonate (v_0) to 112 ± 11 nmol min^{-1} at saturation with the anion (v_∞). Half-maximal effect was produced by 17 ± 6 mM bicarbonate (Fig. 4C). These parameters were estimated by fitting the experimentally determined data to the following equation:

$$v = v_0 + \frac{(v_\infty - v_0)\,[\mathrm{NaHCO_3}]}{[\mathrm{NaHCO_3}]_{0.5} + [\mathrm{NaHCO_3}]} \qquad (2)$$

The $[NaHCO_3]_{0.5}$ values estimated for the effects of the anion on the ^{32}Pi–ATP exchange reaction are similar to the apparent K_i values for the inhibition of ATP synthesis (see legends to Figs. 2 and 3).

4. Discussion

Since the ^{32}Pi–ATP exchange reaction is stimulated by bicarbonate, the possibility that the anion

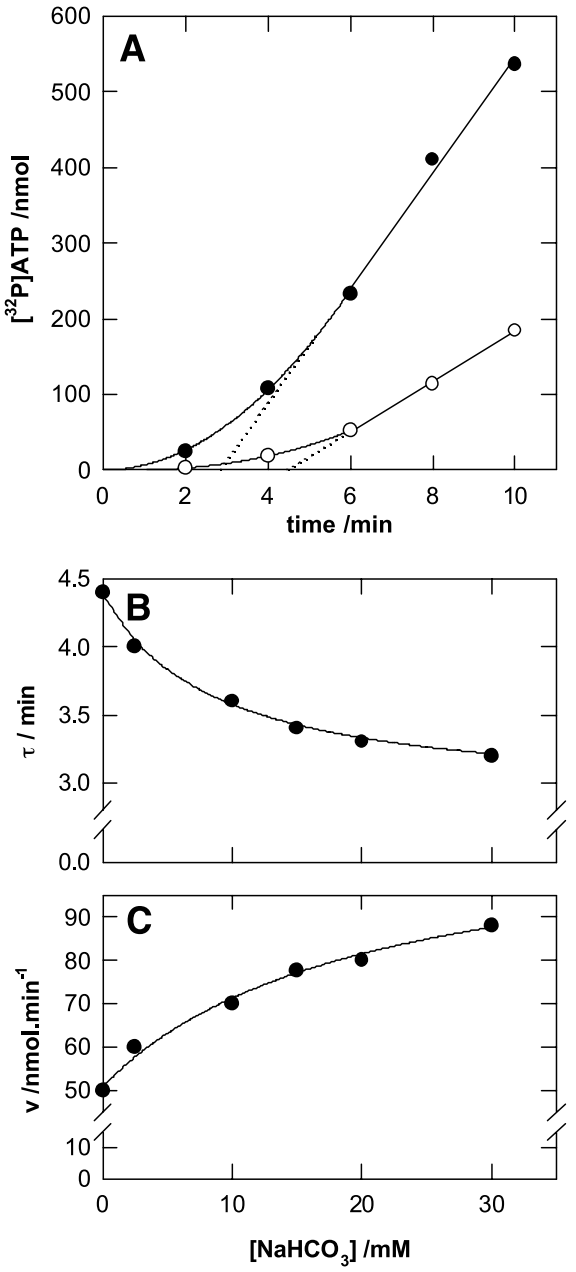

Fig. 4. Effect of bicarbonate on the ^{32}Pi–ATP exchange reaction. The reaction, measured as indicated in Section 2, was started by the addition of 0.7 mg SMP to the reaction medium and stopped at different reaction times. (A) Typical time courses of the reaction in the absence (○) and in the presence (●) of 30 mM $NaHCO_3$ are shown. The extrapolation to the *x*-axis of the time course in the steady state yielded the respective lag times (τ). Lag times (B) and steady-state velocities (C) were determined at different $[NaHCO_3]$ from plots similar to those shown in A. The lines are the fit by non-linear regression analysis of the experimentally determined data to the equations given in the text.

either behaves as a typical uncoupler or induces an intrinsic uncoupling of proton translocation from ATP hydrolysis – as has been shown for sulfite [21] – can be excluded. In addition, bicarbonate does not inhibit electron transport from succinate to oxygen. Consequently, its site of action as inhibitor of ATP synthesis can be located at the level of the chemical reaction catalyzed by F_1F_o.

Bicarbonate inhibited steady-state ATP synthesis and stimulated ATP hydrolysis in SMP. It has been previously shown that the stimulus of the ATPase activity by bicarbonate in mitochondrial F_1 could be explained by a decrease in the $k_{off(ATP)}$ and an increase in the $k_{off(ADP)}$ (see Table 1 in [14]) from the low-affinity catalytic site. Then, the simplest explanation for the opposite effects of bicarbonate on ATP synthesis and hydrolysis is that by binding to F_1 it increases ATP and decreases ADP affinities for the catalytic site under multi-site catalysis conditions.

The above-mentioned effects, as well as the stimulus of the ^{32}Pi–ATP exchange reaction and of the ATPase activity in SMP, are produced by similar bicarbonate concentrations. Therefore, a reasonable working hypothesis is that the different effects of the anion on F_1F_o could be explained by the binding of bicarbonate to a unique site in the mitochondrial F_1.

The binding site of bicarbonate on F_1 has not been conclusively identified yet. Dagget et al. [36] have shown that azide and bicarbonate bind to different sites on mitochondrial F_1. It is also rather unlikely that the anion binds to catalytic nucleotide binding sites since the stimulus of ATP hydrolysis is also observed at high [ATP]. It has been postulated that activating anions bind in place of Pi to an E–ADP inhibited form [19]. Accordingly, it has been reported that the inhibition of photophosphorylation by sulfite was competitive towards Pi [25]. However, the fact that the inhibition of ATP synthesis by bicarbonate in SMP was non-competitive with respect to inorganic phosphate excludes the possibility that bicarbonate binds to the Pi site in the mitochondrial ATPase. As a matter of fact, it has already been reported by Penefsky [37] that bicarbonate did not affect binding of Pi to F_1.

Slater et al. [38] have suggested that activating anions bind to non-catalytic nucleotide binding site(s), which would have a regulatory allosteric function (positive and negative effector). Recktenwald and Hess [15] have proposed that the binding of ATP to a non-catalytic site increases the $K_{m(ATP)}$ for ATP hydrolysis. Edel et al. [39] have shown that covalent modification of a low-affinity non-catalytic site (site 6 according to Berden's nomenclature [4]) by 8-nitreno-AT(D)P increased the $K_{m(ATP)}$ and abolished the negative cooperativity. It has also been proposed that activating anions would exert their effect by preventing the binding of ATP to that site [15,16]. More recently, Pacheco-Moisés et al. [40] have shown that sulfite activation of *Paracoccus denitrificans* ATPase is strongly inhibited by ADP, not excluding the possibility that sulfite binds to a non-catalytic nucleotide binding site.

The competitive inhibition of ATP synthesis at low [Pi] (Fig. 3A) suggests that bicarbonate prevents the binding of ADP to a low-affinity site. A good candidate for this site is the low-affinity non-catalytic nucleotide binding site mentioned in the previous paragraph. As an extension of the proposal of Recktenwald and Hess [15] and Berden and co-workers [16] it can be postulated as a working hypothesis that bicarbonate inhibits ATP synthesis and stimulates ATP hydrolysis by preventing the binding of AD(T)P to site 6, whose ligand state would play a role in the differential regulation of $\Delta\mu_{H^+}$-driven ATP synthesis and $\Delta\mu_{H^+}$-generating ATP hydrolysis.

One important feature in the interaction of the H^+-ATPase and bicarbonate is that the anion does not stimulate ATP hydrolysis at low [ATP]. It has been postulated that bicarbonate does not accelerate the dissociation of ADP from the high-affinity catalytic site [14,41]. In addition, Harris [42] has reported that ATP hydrolysis at a single site is not affected by azide. On that ground he suggested that azide inhibition of steady-state ATP hydrolysis is produced by abolishing the inter-subunit cooperativity that would operate in multi-site catalysis. This proposal has been recently supported by Weber and Senior [43]. Hence, an alternative explanation for the effects of bicarbonate is that the anion interferes in the interaction between subunits when the enzyme is synthesizing ATP in the steady state.

The differences between the kinetic behavior of bicarbonate as inhibitor of ATP synthesis in SMP and of sulfite in chloroplasts [25] may be due to

differences either in behavior of the two activating anions or between the respective F_1F_o ATP synthases. Bakels et al. [44] have reported that ATP synthesis in cyanobacteria was inhibited by sulfite, whereas cyclic photophosphorylation in purple bacteria and ATP synthesis coupled to succinate oxidation in SMP were not affected by the anion. To explain the reason(s) for these differences a careful comparative study of the effects of different activating anions on different F_1F_o ATPases will be required.

5. Concluding remarks

Bicarbonate stimulates ATP hydrolysis [12] while it inhibits steady-state ATP synthesis likely by increasing the affinity of ATP and decreasing that of ADP for the catalytic site. From our data it can be concluded that: (i) bicarbonate binds to a site different from the Pi binding site kinetically competent in steady-state ATP synthesis; (ii) at low [Pi] the binding of bicarbonate is mutually exclusive with the binding of ADP to a low-affinity nucleotide binding site, most likely the non-catalytic site 6. The possibility that this site would play a role in the regulation of the H^+-reversible ATPase must be considered but requires further exploring.

Studies leading to the direct identification of the bicarbonate binding site will be extremely useful for the final elucidation of its mechanism of action on the mitochondrial F_1F_o ATPase.

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