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Effect of Bicarbonate and Other Anions on the Oxidized and Reduced Forms of F_1-ATPase

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The effect of activating anions on the hydrolysis of ATP catalyzed by mitochondrial ATPase was higher on the oxidized than on the reduced form of the enzyme. On the contrary the effect of inhibitory anions on this reaction was more manifest on the reduced form of the enzyme. Kinetic data show that both activating and inhibitory anions compete for the same sites of the ATPase. A unifying mechanism of action is suggested according to which the anions could establish coordination bonds with the suggested iron atoms of the catalytic site. The preferential displacement of electrons of such bonds towards the ligand, or towards the metal atom, would lead respectively to an inhibition or to an activation of the enzyme.

A great variety of anions are known to affect the hydrolysis of ATP when catalyzed by mitochondrial ATPase. Some anions activate the hydrolytic rate whereas others inhibit this rate. EBEL and LARDY (2) and PEDERSEN (9) have studied the kinetics of the hydrolytic reaction catalyzed by ATPase in the presence of some of these anions. EBEL and LARDY (2) have shown that both activating and inhibitory anions compete for the same site or sites of the enzyme; however, no mechanism has so far been suggested to explain the opposite effect of these two groups of anions. SANTIAGO *et al.* (14) have recently proposed a mechanism to explain the effect of uncouplers and inhibitors of oxidative phosphorylation on the hydrolytic activity of mitochondrial ATPase. According to their proposal both types of compounds would exert their effects on ATPase through binding to metals present at the catalytic site. The relative influence of the ligands offers at a molecular level a coherent basis to their possible mechanism of action. The mechanism proposed may also be valid to explain the activating or the inhibitory effect of anions on the hydrolytic activity of ATPase.

Abbreviations: DNP, 2,4-dinitrophenol.

Materials and Methods

Rat liver mitochondria were isolated as described by HOGEBOOM (5). F_1-ATPase was prepared from rat liver mitochondria by the procedure of LAMBETH and LARDY (6). ATPase was assayed measuring the release of inorganic phosphate essentially as described by PULLMAN *et al.* (10) in the absence of an ATP-regenerating system. Aliquots of the F_1-ATPase were preincubated for 5 minutes at 30° C in 0.8 ml of a medium containing 50 μmoles of Tris-acetate, pH 7.4. The reaction was initiated by the addition of substrate (MgATP) at the appropriate concentrations. The incubation was continued for 2 minutes and stopped by the addition of 0.1 ml of 50 % trichloroacetic acid. Inorganic phosphorus was determined according to FISKE and SUBBAROW (4). Reagent and enzyme blanks were determined in each experiment.

Protein determination was carried out following the reaction of LOWRY *et al.* (8). Crystalline bovine serum albumin was used as standard.

Results

The effect of different anions on ATPase activity has been studied at different fixed MgATP concentrations in the presence or in the absence of the reducing agent dithionite (table I). In the absence of other anions dithionite caused an elevation of the hydrolytic rate which was more pronounced at 3 mM MgATP; this rate was lower at 0.6 MgATP, and negligible at 0.06 mM MgATP.

Table I also shows that the activating effect of bicarbonate, DNP and KCN appeared mainly in the absence of dithionite. However, it should be noticed that this activating effect was negligible when low substrate concentrations were used. Other anions such as SCN^- and OCN^- had an inhibitory effect on the hydrolytic activity of ATPase in agreement with EBEL and LARDY (2). It may also be seen that the inhibition caused by these anions was more marked in the presence of dithionite. This effect was more manifest in the presence of high substrate concentrations, both on the native form of the enzyme,

Table I. *Effect of anions on the reduced and oxidized forms of ATPase.*
When present the concentrations of the different anions were as follows: 4×10^{-5} M dithionite, 10^{-2} M HCO_3, 5×10^{-4} M DNP, 10^{-2} M KCN, 2×10^{-4} M KOCN and 10^{-3} M KSCN. All values are given as averages ± standard error. Each value in the table represents the average of at least eight experiments. The results have been referred to 5 μg of enzyme protein.

Additions	nmoles of ATP hydrolyzed × min^{-1}					
	3 mM MgATP		0.6 mM MgATP		0.06 mM MgATP	
	—	+ Dithionite	—	+ Dithionite	—	+ Dithionite
None	65.2±2.1	166.2±3.1	45.0±1.8	104.2±2.8	18.0±0.5	25.5±0.9
HCO_3^-	131.4±2.8	179.0±3.2	70.9±2.0	115.1±3.1	21.5±0.9	24.2±0.9
DNP	127.5±3.1	191.0±1.8	67.5±1.8	109.2±2.5	21.2±0.9	24.0±0.9
KCN	82.1±1.9	156.7±3.1	55.1±1.4	95.1±2.3	20.0±0.8	23.0±1.0
KOCN	31.1±1.2	62.4±1.8	30.5±1.1	50.7±1.3	15.0±0.8	17.5±0.7
KSCN	37.8±1.3	71.3±1.7	31.9±1.0	56.4±1.3	16.0±0.8	17.9±0.9
DNP+HCO_3^-	172.1±3.2		90.1±2.0		24.7±1.0	
KOCN+HCO_3^-	79.3±1.8		50.2±1.7		18.0±0.8	
KSCN+HCO_3^-	83.2±1.9		53.4±1.8		19.1±0.7	

which is assumed to be its oxidized form, as well as on its reduced form.

The effect of activating and inhibitory anions on the native enzyme has also been studied in the presence of bicarbonate. It may be seen in table I that in the presence of this latter anion, the effect of inhibitory anions did not increase, contrary to what happened when dithionite was present.

In order to elucidate whether the effects

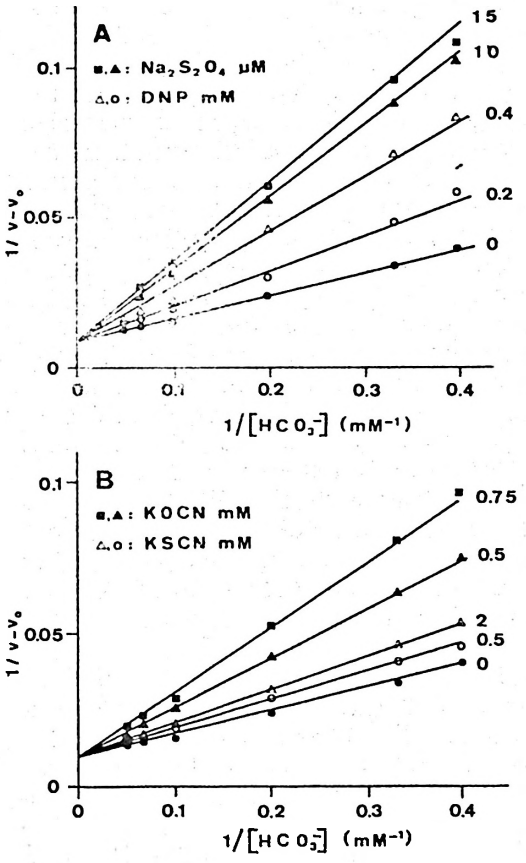

Fig. 1. *Activation by DNP and dithionite (A) and inhibition by SCN^- and OCN^- (B) of ATPase hydrolytic activity at 3 mM MgATP and varying HCO_3^- concentration.*
v, velocity in the presence and v_o, velocity in the absence of bicarbonate. The results have been referred to 5 µg of enzyme protein.

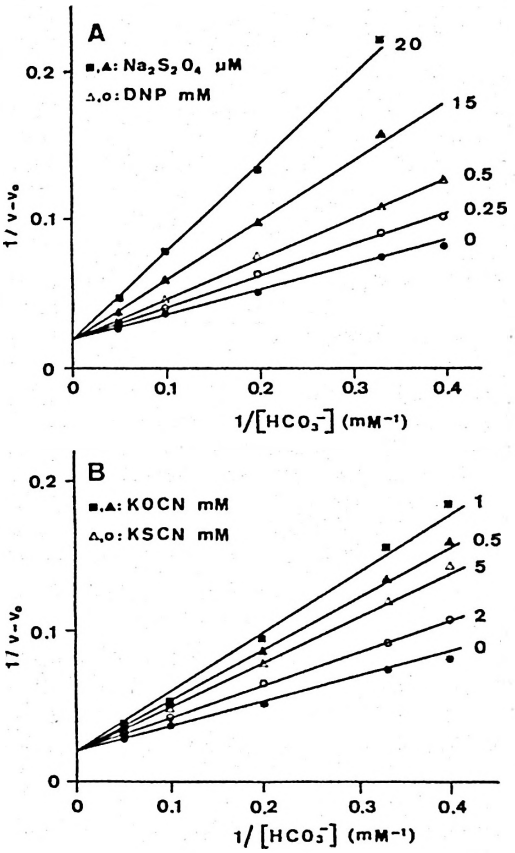

Fig. 2. *Activation by DNP and dithionite (A) and inhibition by SCN^- and OCN^- (B) of ATPase hydrolytic activity at 0.6 mM MgATP and varying HCO_3^- concentration.*
v, velocity in the presence and v_o, velocity in the absence of bicarbonate. The results have been referred to 5 µg of enzyme protein.

of the different anions were competing for the same site or sites of ATPase, plots representing $1/(v-v_0)$ versus $1/HCO_3^-$ were constructed as described by EBEL and LARDY (2). To obtain these plots three different fixed levels of MgATP were used. It is obvious from figures 1 and 2 that, at 3 and 0.6 mM MgATP, dithionite, DNP and bicarbonate exhibited a competitive pattern of interaction towards the

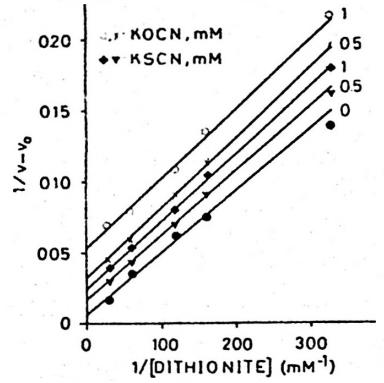

Fig. 3. *Inhibition by SCN^- or OCN^- of ATPase hydrolytic activity at 3 mM MgATP and varying dithionite concentration.*
v, velocity in the presence and v_o, velocity in the absence of dithionite. The results have been referred to 5 μg of enzyme protein.

same site or sites. Similarly the inhibitory anions SCN^- and OCN^- exhibited also a competitive pattern with respect to bicarbonate (figs. 1 and 2). This competitive pattern was also obtained at 0.06 mM MgATP.

The possible competition between the reductant anion dithionite and inhibitory anions such as SCN^- or OCN^- was also considered. Figure 3 clearly shows that both SCN^- and OCN^- gave a non-competitive pattern with respect to dithionite.

Discussion

The three different fixed concentrations of MgATP were chosen after the finding in a previous work (13) that the pH optimum of the enzyme varied with substrate concentration. The results obtained suggested the existence of three catalytic sites each with a different pH optimum and a different affinity for the substrate.

The data now reported regarding the different sensitivity to the activating anions dinitrophenol, bicarbonate and cyanide, and to the inhibitory anions SCN^- and OCN^-, at the different substrate levels used, suggest that each of the three postulated catalytic sites could differ in their sensitivity to anions.

The increasing sensitivity to bicarbonate with increasing substrate concentrations now reported is in agreement with previous findings of PEDERSEN (9) and of EBEL and LARDY (2). These authors have shown that in the absence of bicarbonate biphasic Eadie-Hofstee plots were obtained suggesting the existence of at least two catalytic sites with different affinity for the substrate. PEDERSEN (9) has also observed that the biphasic pattern of the Eadie-Hofstee plots disappears when high enough bicarbonate concentrations are used. These results could be interpreted in the sense that the Km value of the site or sites of low affinity for the substrate decreased in the presence of bicarbonate approaching the value of the Km of the high affinity site.

The results now presented also indicate that the activating anions had a higher affinity for the oxidized form of ATPase at any of the substrate concentrations tested, since their effect was more manifest in the absence than in the presence of the reducing agent dithionite. On the contrary, inhibitory anions exhibited a higher affinity for the reduced form of ATPase since their effect was more marked in the presence of dithionite.

The fact that anions are potential ligands of the atoms of iron may give a clue regarding their mechanism of action on ATPase activity. A possible interpretation of the effect of activators and inhibitors of the hydrolytic activity of ATPase has already been reported (14) based on the presence of iron as a constituent of the enzyme (12). It was suggested that the activating effect of uncouplers, and the inhibitory effect on ATPase of inhibitors of oxidative phosphorylation of the ATPase hydrolytic activity, could be due to the displacement of electrons shared in

the coordination bond towards the ligand or towards the metal. The hydrolytic activity of the enzyme is known to be enhanced by reduction (7); the reduction of ATPase would imply the transformation of Fe^{3+} into Fe^{2+}. The suggestion could be made that ligands allowing the electrons shared in the coordination bond to be preferentially displaced towards the atom of iron, mainly when in the form of Fe (III), should behave as activators. On the contrary, ligands attracting the electrons more strongly than the atoms of iron, mainly in the form of Fe (II), should behave as inhibitors of ATPase activity. A consideration of the molecular structure of the different anions now studied seems to offer support to this idea. Bicarbonate could allow the electron density belonging to the delocalized bonds between the atom of carbon and the three oxygens to be displaced towards the coordination bond O-Fe (III) under the attracting influence of the metal. In the case of dinitrophenol the electron cloud around the nitrogen atom belongind to the $-NO_2$ group establishing the coordination bond through its negative charge with the atom of iron could be displaced towards the metal (14). On the contrary, the inhibitory anions would exert an attracting effect on the electrons of the coordination bond due to the presence of a partial positive charge near the atom involved in the bond with the metal.

$$\overset{\delta^- \quad \delta^+}{S = C = N^-} \qquad \overset{\delta^- \quad \delta^+}{O = C = N^-}$$

The competitive pattern regarding the action of the different anions as reflected in figures 1 and 2, seems to suggest that these potential ligands of iron compete among them for the formation of a coordination bond. The reduction of the anion binding site with dithionite could explain the competitive pattern of this reducing agent with respect to activating anions such as bicarbonate through a decrease in affinity for them. An increase in affinity of the enzyme in its reduced form for the inhibitory anions would account for the absence of a competitive pattern of these anions with respect to dithionite. According to this interpretation dithionite would exert its effect on ATPase through a reduction of the anion binding site, and leaving this site free to interact with other anions. The idea that the enzyme undergoes a reduction with dithionite is also supported by the recent observation in our laboratory (15) that physiological electron donors increased the sensitivity to inhibitory anions and decreased that of activating anions. Interestingly, BUTOW and RACKER (1) have found that a series of uncouplers were competitive with o-phenantroline and released the inhibition of respiration of submitochondrial particles provoked by this latter compound. These authors have suggested that non-heme iron may play a key role in the phenomenon of respiratory control and that this iron may be the site at which dinitrophenol and other uncouplers interact. Their observation that o-phenantroline decreases the ^{32}P-ATP exchange when dithionite was present might be interpreted as an indication of a higher affinity of the chelating agent for the reduced form of the enzyme.

This unifying mechanism of action of the different anions, activating or inhibitory, coincides with that given previously for uncouplers and inhibitors of oxidative phosphorylation (14). In agreement with this, bicarbonate or any of the other activating anions having a higher affinity for the oxidized form of the enzyme would consequently turn the standard reduction potential of ATPase more negative, a property shared in common with uncouplers (12). An early suggestion of FANESTIL *et al.* (3) that bicarbonate could be an uncoupler of oxidative phosphorylation finds now a new support. Furthermore, bicarbonate, being a physiological anion, could play some role in the control of

the process of oxidative phosphorylation as suggested by RACKER (11).

Resumen

El efecto de aniones activadores sobre la hidrólisis de ATP, catalizada por la ATPasa mitocondrial, es mayor cuando la enzima está en la forma oxidada que cuando está en la forma reducida. Por el contrario, el efecto de aniones inhibidores sobre esta reacción es más pronunciado sobre la forma reducida de la enzima. Los datos cinéticos muestran que los aniones, tanto activadores como inhibidores, compiten por el mismo centro de la ATPasa. Se sugiere un mecanismo de acción de acuerdo con el cual los aniones establecerían enlaces de coordinación con los átomos de hierro del centro catalítico. El desplazamiento de electrones de tales enlaces de forma preferente hacia el ligando o hacia el átomo metálico daría lugar respectivamente a una inhibición o a una activación de la enzima.

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