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Nucleotide Effects on Kinetic Properties of Mitochondrial ATPase

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The hydrolytic activity of mitochondrial ATPase, both in its soluble form as F_1-ATPase, or as membrane bound in whole mitochondria, was affected by the presence of free nucleoside di- or triphosphates; these effects were largely depending not only on their concentration but also on the substrate concentration. The existence of a regulatory site or sites is proposed; these sites would have a higher affinity for the free nucleoside triphosphates than for the diphosphates, and the interaction of any of these nucleotides with the proposed regulatory site or sites would lead to an activation. The nucleotide regulatory site or sites seem to be different from the anion binding sites since neither free ATP nor free GTP compete with activating or inhibitory anions.

Key words: F_1-ATPase, Mitochondrial ATPase, Regulatory sites in mitochondrial ATPase, Nucleotide binding of mitochondrial ATPase.

Mitochondrial ATPase, once released from the membrane, is only capable of catalyzing the hydrolytic reaction in which ATP-Mg^{2+} is the true substrate (31). The presence of multiple binding sites for nucleotides (3, 8, 16, 17, 33, 35, 37-39), as well as the presence of several copies of the major subunits (2, 12, 28, 32, 34), may suggest the existence of several catalytic and regulatory sites on the enzyme. Most of the work to elucidate the number of nucleotide binding sites has been carried out using ATP and ADP analogs and equilibrium binding measurements. The results obtained suggest the existence of sites strongly binding ATP and ADP which would not participate in the catalysis, as well as the existence of others identified as the catalytic sites readily exchanging nucleotides, it has also been proposed that the noncatalytic sites would be located on the α-subunits, and the catalytic sites on the β-subunits (4, 7, 9, 10, 18, 20, 24-26, 36).

After the kinetic studies carried out in different laboratories (11, 27, 30) the existence of at least two catalytic sites and of several regulatory sites (30) had also been suggested. More recent kinetic data are compatible with the presence of three catalytic sites on the rat liver enzyme (5).

The catalytic activity of mitochondrial

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ATPase is influenced by a great variety of substances, among them uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol, and a large number of anions. The existence of a common site both for activating and inhibitory anions, and for 2,4-dinitrophenol has been suggested (11, 21, 22).

This report describes the effect of free nucleoside di- and triphosphates on the hydrolytic activity of both soluble and membrane bound ATPase of rat liver mitochondria at different substrate concentrations. The kinetic data obtained are in agreement with the existence of regulatory sites capable of binding nucleotides which seem to be different from the anion sites, as suggested by a lack of competition between any of the activating or inhibitory anions and the free nucleoside triphosphates.

Materials and Methods

Rat liver mitochondria were isolated essentially as described by HOGEBOOM (19) in 250 mM sucrose. Mitoplasts were prepared according to the procedure of GREENAWALT (14). F_1-ATPase was prepared from rat liver mitochondria by the procedure of LAMBETH and LARDY (21). ATPase was assayed by measuring the release of inorganic phosphate essentially as described by PULLMAN *et al.* (29) in the absence of an ATP-regenerating system. Aliquots of the F_1-ATPase or mitochondrial suspensions, containing approximately 5 μg or 120 μg of protein respectively, were preincubated for 5 min at 30°C in 0.8 ml of a medium containing 50 μmoles of Tris-acetate, pH 7.4 and, when indicated, the free nucleotides, anions, or DNP at the amounts required. The reaction was initiated by the addition of 0.2 ml of substrate ($ATP\text{-}Mg^{2+}$) solution at the appropriate concentrations. The incubation was continued for 3 min and stopped by the addition of 0.1 ml of 50% trichloroacetic acid. Inorganic phosphorus was determined according to FISKE and SUBBAROW (13). Reagent and enzyme blanks were determined in each experiment. Adenylate kinase activity was measured according to the method described by ADAMS (1). The K_a was determined as follows: using a constant concentration of the substrate $ATP\text{-}Mg^{2+}$, plots of $1/v\text{-}v_o$ «versus» 1/concentration of activator were constructed (where v = velocity in the presence, and v_o = velocity in the absence of activator); the y intercept represents $1/V_{max}\text{-}V_o$; slope/intercept was defined as the K_a for the activator, following EBEL and LARDY (11). The different K_i values were calculated according to CLELAND (6).

Protein determination was carried out following the reaction of LOWRY *et al.* (23). Crystalline bovine serum albumin was used as a reference standard.

Results

Effect of ADP on the catalytic sites of ATPase. — To study if the competitive inhibitor ADP (15) affects each of the three proposed catalytic sites of ATPase to the same extent (5), the hydrolytic activity of F_1-ATPase was determined at $ATP\text{-}Mg^{2+}$ concentrations ranging from 0.06 to 3 mM. The concentrations of ADP ranged from 0.1 to 1 mM. The inset of figure 1 shows the Eadie-Hofstee plot in the absence and in the presence of 0.5 mM ADP. The graph may be seen to be triphasic both in the absence and in the presence of ADP; in the presence of ADP the slopes were affected, but their intercepts at the y-axis remained unaltered as corresponding to a competitive inhibition. From the Dixon plot, (figure 1) the K_i values of ADP for the high, intermediate, and low affinity sites were calculated yielding values of 0.80, 0.64 and 0.46 mM respectively.

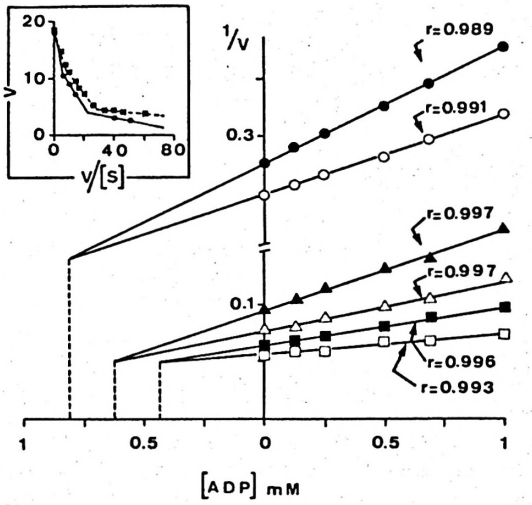

Fig. 1. *Effect of ADP on the catalytic activity of F_1–ATPase at different substrate concentrations.* The inset shows the Eadie-Hofstee plot in the presence (———) and in the absence (— — —) of 0.5 mM ADP. In the Dixon plot which appears in the figure the concentrations of the substrate ATP–Mg^{2+} were 0.06 (●), 0.1 (○), 0.6 (▲), 1 (△), 2.4 (■) and 3 (□) mM. Velocity has been expressed as μmol of ATP × min^{-1} × mg^{-1}. Number of experiments, 6.

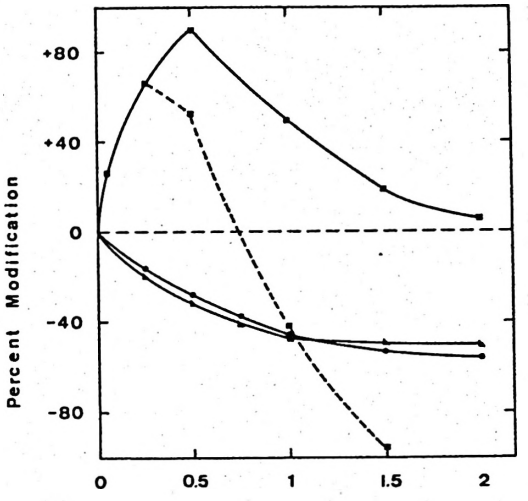

Fig. 2. *Effect od ADP on the catalytic activity of mitochondrial ATPase bound to membrane at different substrate concentrations.* Concentration of ATP-Mg^{2+}: 0.06 (■), 0.6 (▲) and 3 (●) mM. Dashed line represents the effect of ADP at 0.06 mM ATP-mg^{2+} as corrected for adenylate kinase activity (n = 8).

However, in mitochondria the effect of ADP on the hydrolytic activity of the membrane bound enzyme ATPase differed substantially from that on the activity of F_1-ATPase. Figure 2 shows the percent modification of the hydrolytic activity as a function of ADP concentration, at three different substrate concentrations. At 3 and 0.6 mM ATP-Mg^{2+}, ADP was always an inhibitor. On the other hand, ADP behaved, surprisingly, as an activator of the hydrolytic reaction when a substrate concentration of 0.06 mM was used. At this concentration the activating effect of ADP increased with its concentration up to a maximum being reached at 0.5 mM. However, it migth be argued that the activating effect of ADP could be due to the presence of mitochondrial adenylate kinase, which would convert the added ADP to ATP and AMP, raising consequently the actual substrate concentration. To rule out this possibility adenylate kinase activity was measured, and its specific activity was found to be 0.022 μmoles ATP/min × mg protein. According to these results the corrections to be introduced are negligible for the high and intermediate substrate concentrations. A dashed line in figure 2 shows the effect of ADP at low substrate concentration, corrected for adenylate kinase activity. Maximum activation was reached at 0.25 mM ADP. Moreover, exactly the same results were obtained with mitoplasts (mitochondria devoid of outer membrane and therefore with no adenylate kinase activity).

Effect of free ATP on the catalytic sites of ATPase. — The hydrolytic activity of ATPase has been determined at different substrate concentrations (3, 0.6 and 0.06

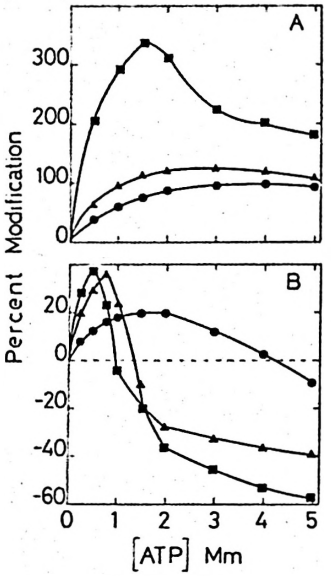

Fig. 3. *Effect of free ATP on the catalytic activity of ATPase at different substrate concentrations.* A. Mitochondrial preparations. B. F_1-ATPase. Concentration of ATP-Mg^{2+}: 0.06 (■), 0.6 (▲) and 3 (●) mM (n = 8).

mM ATP-Mg^{2+}) in the presence of free ATP (ATP in excess of ATP-Mg^{2+}). In mitochondria free ATP was an activator in the whole range of free ATP concentrations used (0.5-5 mM). On F_1-ATPase free ATP had an activating effect (fig. 3B) which increased with its concentration to reach a maximum at a concentration which in turn depended on the substrate concentration used. With further increases in the concentration of free ATP the activating effect decreased and even became an inhibitor. The inhibitory effect observed under those conditions was of a competitive nature with respect to the substrate (data not shown).

Effects of the simultaneous presence of free ATP and ADP on the hydrolytic activity of ATPase. — The hydrolytic activity of ATPase was determined in mitochondria at the fixed substrate concentration 0.06 mM ATP-Mg^{2+} in the presence of varying concentrations of ADP, at two different fixed concentrations of free ATP. Double reciprocal plots, $\frac{1}{v-v_o}$ versus $\frac{1}{APD}$, indicate that free ATP and ADP, both activators under those conditions, were mutually competitive (diagrams not shown).

Effect of nucleoside diphosphates on the hydrolytic activity of ATPase. — The effect of nucleoside diphosphates GDP, CDP, IDP and UDP was only very limited on either membrane bound ATPase or F_1-ATPase preparations. A slight activating effect on the hydrolytic activity of F_1-ATPase was observed when these nucleotides were present at concentrations below 10^{-4} M and they behaved as very weak inhibitors of the membrane bound enzyme under the same conditions.

Effect of nucleoside triphosphates on the hydrolytic activity of ATPase. — Figure 4 shows the effect of nucleoside triphosphates GTP, ITP, CTP and UTP on the hydrolytic activity of membrane bound

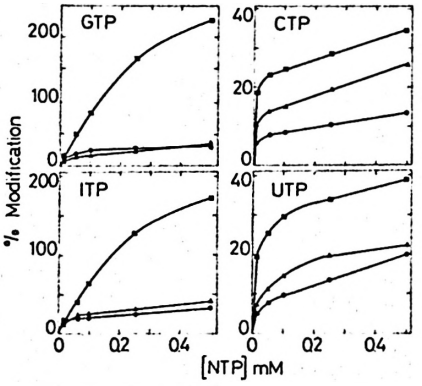

Fig. 4. *Effect of nucleoside triphosphates on ATPase activity of mitochondria.* Substrate concentration: 3 mM (●), 0.6 mM (▲) and 0.06 mM (■) ATP-Mg^{2+} (n = 8).

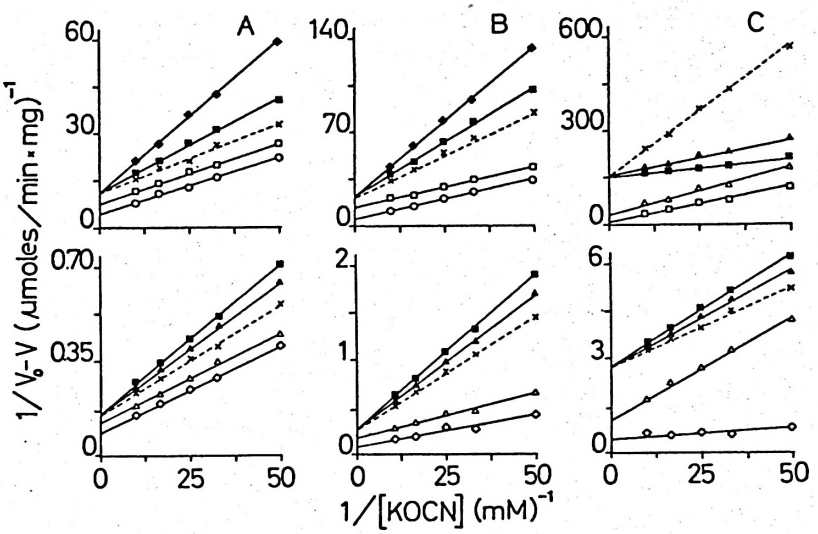

Fig. 5. *Effect of KOCN on the hydrolytic activity of ATPase in the absence or in the presence of free ATP or ADP.*

Upper diagrams, mitochondria; lower diagrams, F_1-ATPase. Substrate concentration: 3 mM (A): 0.6 mM (B); 0.06 mM ATP-Mg^{2+} (C). ADP concentration: 0.75 mM (◆); 0.5 mM (■); 1 mM (●); 0.25 mM (▲). Free ATP concentration: 1 mM (O); 0.75 mM (◇); 0.5 mM (□); 0.25 (△). Control: (X). The lines drawn are based on a linear regression analysis.

ATPase. GTP and ITP behaved as weak activators, except at 0.06 mM; at this concentration the activating effect of both ITP and GTP on the membrane bound enzyme was outstanding. CTP and UTP behaved also as activators on membrane bound ATPase; however, with F_1-ATPase (data not shown) the GTP and ITP behaved as activators, but the effect of CTP and UTP was negligible.

Effect of anions and dinitrophenol on the hydrolytic activity of ATPase in the presence of free ATP or ADP. — To study the possible interrelation of regulatory nucleotide binding sites and anion binding sites, the hydrolytic activity in F_1 and in the membrane bound enzyme was determined in the presence of varying concentrations of KOCN between 0.02 and 0.1 mM both in the absence and in the presence of either free ATP or ADP, at two fixed concentrations. In the concentrations of free ATP used, the nucleotide behaved as an activator of the hydrolytic activity. ADP was an inhibitor of the hydrolytic activity of the enzyme at any of the concentrations used, except in mitochondria at 0.06 mM ATP-Mg^{2+} where it behaved as an activator, as indicated above.

The results have been plotted as a double reciprocal diagram. Figure 5 shows that the lines representing $1/v_o - v$ versus 1/[KOCN], in the absence or in the presence of two fixed ADP concentrations, intersect with the y axis at the same point, which indicates that KOCN and ADP compete fot the same regulatory site. However, the lines representing $1/v_o - v$ versus 1/[KOCN], in the absence or in the presence of different free ATP concentrations, intersect with the y axis at different points, thus indicating that free ATP does not interact with the anion site.

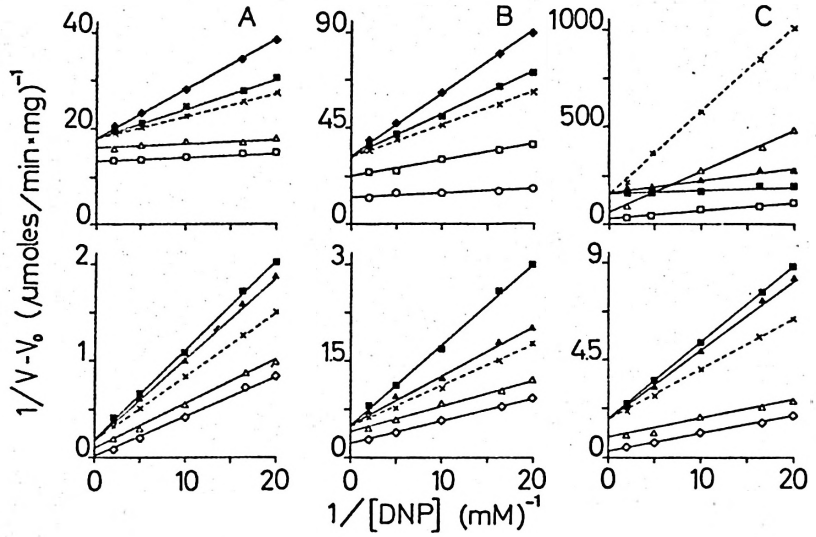

Fig. 6. *Effect of DNP on the hydrolytic activity of ATPase in the absence or in the presence of free ATP or ADP.*
Legends as in figure 5.

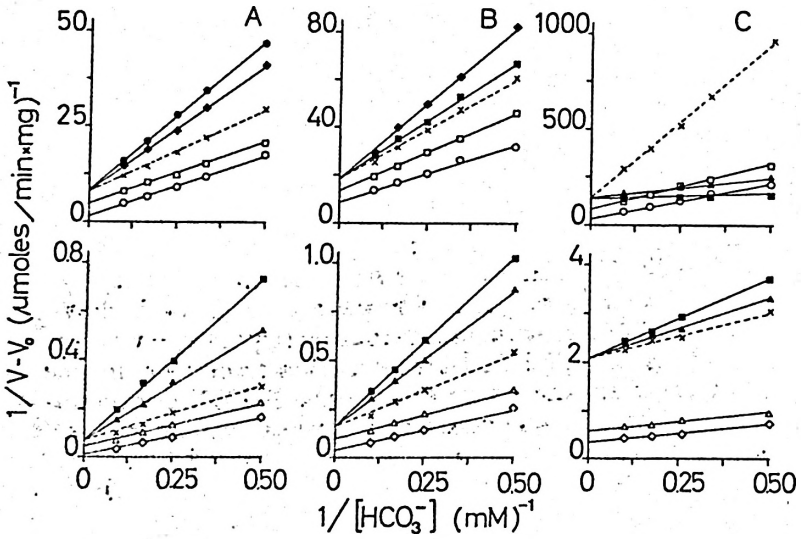

Fig. 7. *Effect of bicarbonate on the hydrolytic activity of ATPase in the absence or in the presence of free ATP or ADP.*
Legends as in figure 5.

KSCN, another inhibitor of ATPase activity, behaved in a similar way when either free ATP or ADP were present (fig. 6), thus indicating that it was competitive with ADP, and noncompetitive with free ATP.

The coincidence of the y intercepts both in the presence or in the absence of ADP suggests that this nucleotide competed with the activators 2,4-dinitrophenol (DNP) (fig. 6) and bicarbonate (fig. 7); however, the lack of coincidence of the y intercepts in the presence and in the absence of free ATP seems to exclude a competition between activators and this free nucleotide.

Effect of activators or inhibitors on the hydrolytic activity of ATPase in the presence of GTP or GDP. — The effect of activators DNP and bicarbonate and inhibitors KOCN and KSCN, on the hydrolytic activity of F_1—ATPase and membrane bound enzyme has been determined at three fixed substrate concentrations in the presence or in the absence of free GTP or GDP.

The concentrations of KOCN and KSCN ranged from 0.02 to 0.1 mM; those of DNP, from 0.05 to 0.5 mM; and those of bicarbonate from 2 to 10 mM. GTP the concentrations ranged from 0.02 to 0.1 mM in which GTP was an activator; the GDP concentrations ranged also from 0.02 to 0.1 mM. In all cases GTP did not compete with either activators or inhibitors, whereas GDP competed with all of them (data not shown).

Discussion

Previous results suggested the existence of three catalytic sites with different affinity for the substrate ATP-Mg^{2+} on rat liver mitochondrial ATPase (5). As shown in the results, the free nucleoside di- and triphosphates modified the hydrolytic activity of ATPase, further dependent on the substrate concentration used. The study of the effect of different free nucleotides, at substrate concentrations within the ranges corresponding to the three slopes of the Eadie-Hofstee plot, should discriminate the possible selective modification of each catalytic site by the free nucleotides. Figure 1 shows that ADP inhibited the hydrolysis of ATP-Mg^{2+} catalyzed by F_1—ATPase in a competitive fashion; three K_i values were obtained, one for each range of substrate concentration used. It should be kept in mind that the assumption of «simple» kinetics to calculate inhibition and activation constants in a complex enzyme such as ATPase should be taken with some reservations. The values of these constants should be taken only as an approximation since all the catalytic sites would be fully participating in the hydrolytic reaction, especially at the highest substrate concentration used. In any case, these results could be considered as an indication that the proposed catalytic sites, besides differing in affinity for the substrate would also differ, although slightly, in affinity for the inhibitor ADP.

Figure 3 deserves also a special comment. It confirms previous results (30) which showed that free ATP, ATP in excess of ATP-Mg^{2+}, behaved as an activator, or as an inhibitor of the hydrolytic reaction catalyzed by F_1—ATPase, depending on the concentration of the free nucleotide; the activating effects were observed at concentrations below a certain value; at higher concentrations free ATP behaved as an inhibitor. Those results were interpreted as a consequence of possible interactions of the free nucleotide with a regulatory site, where it would cause an activation of the hydrolytic reaction, but also with a lower affinity, with the catalytic site. In the light of the results suggesting the existence of three catalytic sites, the study of this striking effect of free ATP at substrate concentra-

tions corresponding to the three slopes of the Eadie-Hofstee plot was undertaken. Figure 3 shows that this double behavior of free ATP on F_1–ATPase could be observed at any of the substrate concentrations tested. The K_a's values have been calculated for three fixed substrate concentrations, one for each of the three slopes of the Eadie-Hofstee plot, and using exclusively concentrations of free ATP below those required to reach a maximum of activation. Those values were similar and therefore they do not allow to discriminate between the existence of one or several regulatory sites for free ATP.

The inhibitor effect of free ATP at concentrations higher than those capable of eliciting a stimulatory response has been found to be competitive in nature. These observations suggest that each catalytic site could also bind free ATP, but with a much lower affinity than the true substrate ATP-Mg^{2+}.

Figure 3A shows that in whole mitochondria the hydrolytic activity of ATPase exhibited a maximum response to the activation by free ATP at a given concentration of the free nucleotide; at concentrations higher than those required to reach that maximum, the activation was correspondingly lower. The shape of the curve is similar to that obtained with F_1–ATPase. Therefore the same mechanism seems to be operating both in the free enzyme and in the enzyme bound to the membrane, although in this latter case the inhibition was not apparent, at least within the range of concentrations of free ATP tested. However, the shape of the curve could be considered as indicative of a mixed effect. The actual values of the concentration of the free ATP added could be obscured in this case due to the intramitochondrial Mg^{2+}.

The effect of ADP on the ATPase hydrolytic activity in whole mitochondria at 0.06 mM ATP-Mg^{2+}, as shown in figure 2, could be interpreted as reflecting the possible binding of this nucleotide not only to the catalytic sites where it would cause an inhibition, but also to a regulatory site and leading then to an activation. That regulatory site would probably be the same regulatory site or sites binding free ATP. This suggestion is based on the competition between free ATP and ADP, under conditions where both nucleotides behaved as activators.

Different nucleoside diphosphates other than ADP caused weak stimulatory or inhibitory effects on each catalytic site both on F_1–ATPase and membrane bound ATPase. Nucleoside triphosphates other than ATP showed an activating effect (fig. 4).

The activating effects may reflect the binding of these nucleotides to regulatory sites. The weak inhibitory effect could be also due to an interaction with the catalytic site, but with much lower affinity than the adenine nucleotides. It should be noticed that the regulatory sites exhibited a higher affinity for the nucleoside triphosphates than for the diphosphates. The affinity of the different nucleotides was lower in the F_1–ATPase than in the membrane bound enzyme; these differences might be due to the conformation imposed on the enzyme when membrane bound or to a restricted passage of some nucleotides through the inner mitochondrial membrane.

The lack of competition between either activating of inhibition anions and free nucleoside triphosphates (ATP, GTP) seems to indicate that the anion binding site is different from the nucleotide regulatory sites. ADP, on the other hand, could interact with the catalytic site thus competing with the substrate, or with the nucleotide regulatory site in some instances competing then with free ATP; since in both cases ADP competes with the anions, the suggestion may be advanced that this nucleotide interacts simultaneously with the catalytic site and with the anion binding site, or simul-

taneously with the nucleotide regulatory site and the anion binding site. The fact that GDP behaved similarly to ADP suggests that this simultaneous interaction, not present in the case of the nucleoside triphosphates, might be due to the smaller molecular size of the diphosphates.

Acknowledgements

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Resumen

La actividad hidrolítica de la ATPasa mitocondrial, tanto en su forma soluble como F_1–ATPasa, o unida a la membrana en mitocondrias, se afecta por la presencia de nucleótidos di- o trifosfato libres; estos efectos dependen ampliamente no sólo de su concentración sino también de la del sustrato. Se propone la existencia de un sitio o sitios reguladores, los cuales tendrían mayor afinidad por los nucleótidos trifosfato libres que por los difosfato, y la interacción de cualquiera de estos nucleótidos con el sitio o sitios reguladores propuestos llevaría consigo una activación. El sitio o sitios reguladores para nucleótidos serían diferentes de los sitios de enlace de aniones puesto que ni el ATP libre ni el GTP libre compiten con los aniones activadores e inhibidores.

Palabras clave: F_1–ATPasa, ATPasa mitocondrial, Centros reguladores de la F_1–ATPasa, Nucleótidos y ATPasa mitocondrial.

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