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Effect of Nucleotides and Inhibitory Anions on Mitochondrial ATPase. Its pH Dependence

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The effect of pH on the sensitivity of F_1-ATPase as well as mitochondrial ATPase activity to nucleoside diand triphosphates and to inhibitory anions such as cyanate and thiocyanate, has been studied. The results obtained show that nucleotides could act as activators or inhibitors of the ATPase hydrolytic activity depending on pH, substrate concentration, and binding of the enzyme to the membrane. The effect of those nucleotides which activate the hydrolysis of ATP-Mg^{2+} was more pronounced beyon the optimum pH corresponding to each of the three catalytic sites of the enzyme, whereas those which are inhibitors had a lower effect above this value. The sensitivity to the inhibitory anions decreased with increasing pH values; the decrease in the inhibitory effect was sharper when approaching the optimum pH value. These data are in agreement with the existence in mitochondrial ATPase of two different regulatory sites, one being specific for binding nucleotides, and another for anions. Both of them showed a different response upon changes of pH.

Key words: ATPase and pH, ATPase and nucleotides, Mitochondrial ATPase.

Mitochondrial ATPase, the enzyme catalyzing the phosphorylation of ADP in the final step of oxidative phosphorylation, causes also the hydrolysis of ATP. The reaction is affected by a variety of factors, among them the pH of the medium (4, 5, 11, 16). It has been shown that the enzyme has three optimum pH values, each one depending on the substrate concentration used (17). The interpretation of these results indicates that each of the three catalytic sites in the enzyme with different affinities for the substrate (2) has also a different optimum pH value. Changes in pH have in turn been found to modify the sensitivity of the response to adenosine nucleotides. In F_1-ATPase free ATP has an activating effect, which is more pronounced at pH values higher than the optimum value for each catalytic site; however, the inhibitory effect of ADP is more manifest at pH values below the optimum pH for each catalytic site (17). The activating effect of

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dinitrofenol and bicarbonate on the hydrolytic activity of isolated F_1ATPase has also been found to be dependent on the pH of the medium; upon an increase of pH the activating effect diminishes, and even disappears, after a certain pH value is reached (4, 16, 17).

The present work has been extended to cover the effect of pH on the sensitivity of mitochondrial ATPase to other nucleoside di- and triphosphates and to some inhibitory anions.

Materials and Methods

Mitochondrial have been isolated by the method of HOGEBOOM (10). Protein determination was carried out following the technique of LOWRY *et al.* (14). F_1-ATPase has been prepared from rat liver mitochondria by the procedure of LAMBETH and LARDY (12).

ATPase activity has been determined essentially as described by PULLMAN *et al.* (16) in the absence of an ATP regenerating system. Hundred μl aliquots of the F_1-ATPase were preincubated for 5 minutes at 30°C in 0.7 ml of a medium containing 70 μmoles of Tris-acetate at the indicated pH values and 0.1 ml of the appropriate nucleotide or anion. The reaction was initiated by the addition of ATP-Mg^{2+}, pH 7.4, dissolved in 0.2 ml of distilled water. The Mg^{2+}/ATP ratio was in all cases kept equal to 1. The incubation was continued for 3 minutes and stopped by the addition of 0.1 ml of 50% trichloroacetic acid. At each pH value controls in the absence of nucleotides or anions were carried out. Inorganic phosphorous has been determined according to FISKE and SUBBAROW (6). Reagent and enzyme blanks were carried out in each experiment. Doubling the amount of protein under those conditions was always matched by a doubled amount of liberated Pi.

The disodium salt of adenosine-5'-triphosphate (ATP) was obtained from Merck. Other nucleoside di- and triphosphates were purchased from Boehringer Mannheim. All other chemicals were reagent grade unless specified.

Results

To study whether the effect of nucleoside diphosphates on the different catalytic sites of ATPase is affected by pH, the hydrolytic activity has been determined in the presence of the appropriate concentration of GDP, IDP, UDP and CDP, since a previous study (1) showed that the nucleotide concentration required for an optimum effect was dependent on the nucleotide itself as well as on the substrate concentrations and on the nature of the enzymatic preparation (membrane bound ATPase or as F_1). The range of pH has varied from 6 to 9.7, and the substrate concentrations fixed at three different values: 3, 0.6 and 0.06 mM. With 3 mM ATP-Mg^{2+} (fig. 1) nucleo-

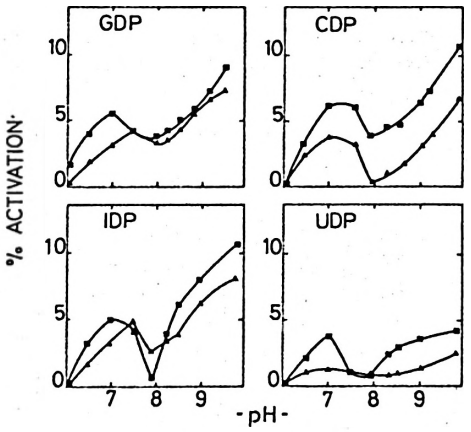

Fig. 1. *Sensitivity of ATPase to free nucleoside diphosphates as a function of pH.*
Substrate concentration, 3 mM ATP-Mg^{2+}. Membrane bound enzyme (■): GDP 0.01 mM; CDP, 0.5 mM; IDP, 0.5 mM and UDP, 0.25 mM. F_1-ATPase (▲): GDP, 0.5 mM; CDP, 0.5 mM; IDP, 0.1 mM; UDP, 0.01 mM. Number of experiments, 6.

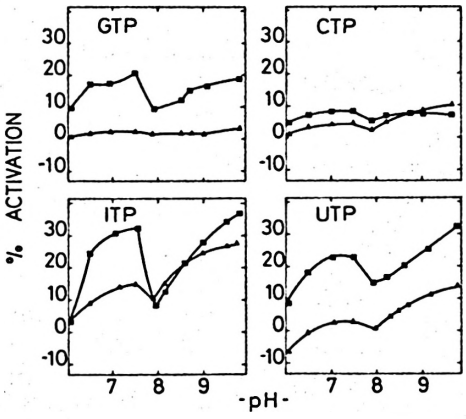

Fig. 2. *Sensitivity of ATPase to free nucleoside triphosphates as a function of pH.*
Substrate concentration: 3 mM ATP-Mg^{2+}. Membrane bound enzyme (■): GTP, 0.1 mM; CTP, 0.05 mM; ITP, 0.5 mM and UTP, 0.5 mM. F_1-ATPase (▲): GTP, 0.1 mM; CTP, 0.5 mM; ITP, 0.5 mM and UTP, 0.5 mM. Number of experiments, 8.

side diphosphates behaved as weack activators both on the F_1 and on the membrane bound enzyme. The activating effect reached a maximum at pH 7, and a minimum at pH 7.9 rising again beyond that value. In mitochondria at 0.6 mM substrate concentration (data not shown) nucleoside diphosphates CDP, IDP and UDP behaved similarly; however, no effect was observed on F_1-ATPase. GDP showed no effect on either enzymatic preparation. When the substrate concentration was 0.06 mM, the effect of these nucleotides was even weaker, and a maximum was observed around pH 7.9. Again, GDP showed no effect on either enzymatic preparations.

A similar study has been carried out with nucleoside triphosphates. In mitochondria at 3 mM substrate concentration (fig. 2) all nucleotides except CTP, behaved as activators, reaching a maximum at pH 7.5, and a minimum at pH 7.9. Beyond this pH value an increase on the activating effect of the nucleotides was

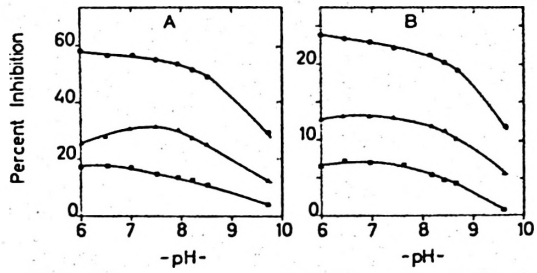

Fig. 3. *Effect of KOCN (A) and KSCN (B) on the hydrolytic activity of F_1-ATPase.*
Substrate concentration: (●) 3 mM; (▲) 0.6 mM; (■) 0.06 mM. KOCN, 0.5 mM; KSCN, 0.5 mM. Number of experiments, 6.

observed. In F_1-ATPase the effect of ITP and UTP was similar, althorugh less pronounced; GTP and CTP showed no effect on the purified enzyme. Similar results were obtained in the presence of 0.6 and 0.06 mM substrate concentrations (data not shown).

The effect of inhibitory anions, such as cyanate and thiocyanate, has been tested at three different substrate concentrations: 3, 0.6 and 0.06 mM ATP-Mg^{2+}, varying the pH from 6 to 10. Figure 3 shows that the anions exhibited an inhibitory effect, which decreased when increasing pH. At 0.06 mM ATP-Mg^{2+} the decrease was more marked at higher than values 7 pH; at 0.6 mM and 3 mM ATP-Mg^{2+} a similar effect was observed with pH values higher than 7.8 and 8.5 respectively.

Discussion

The presence of three catalytic sites in ATPase with different affinity for the substrate (2, 3, 7, 20, 21) and different optimum pH values (7) has been previously reported. The site with the highest affinity for the substrate reaches maximal activity around pH 7; the site with intermediate affinity at pH 8, and the one with the lowest affinity for the substrate between pH 8.2 and 9 (17). It is known

that the true substrate for the hydrolytic ATPase activity is the ATP-Mg^{2+} complex (18, 19), although in the presence of Mg^{2+}, GTP and ITP can also be hydrolytic although to a lower extent (15). On the contrary, the complexes of Mg^{2+} with nucleoside diphosphates cannot be hydrolyzed by the enzyme (18).

Nucleoside diphosphates slightly modify the hydrolytic ATPase activity and only ADP has a significant effect. Although ADP has been considered as a competitive inhibitor both on F_1-ATPase (8) and on the membrane bound enzyme (9), we have recently found that at pH 7.4 in the catalytic site with the highest affinity for the substrate, this nucleotide acts as an activator in whole mitochondria (1).

For each catalytic site, the effect of pH on the inhibition of mitochondrial F_1-ATPase by ADP is practically lost at pH values higher than those corresponding to the optimum in the absence of free nucleotides (17). Our results obtained in the presence of nucleoside diphosphates were also dependent on pH (fig. 1) and the variation of the effect was more manifest at alkaline pH values.

The inhibitory effect that nucleoside diphosphates exhibit under determined conditions is due to its binding to the catalytic site (8, 9). On the other hand the activating effect has been interpreted as a result of their interaction with a nucleotide regulatory site (1). In this sense, our results suggest that the increase of pH could modify the extent of interaction of the nucleotides, either with the catalytic or regulatory binding sites. This interpretation would explain the change from an inhibitory to an activating effect as well as the increase in activation observed with pH. In other words, ^-OH anions could diminish the affinity of the catalytic site for nucleotides and or raise the affinity of the catalytic site. This variation in the affinity could be due to a change of the ionic form both in the binding groups of regulatory and catalytic sites and in the nucleotide itself; in addition, probably, some conformational changes could be implied.

The effect of nucleoside diphosphates is found to be slightly higher in whole mitochondria than in the purified enzyme (fig. 1). This is probably due to an increase of the real substrate concentration caused by the nucleoside diphosphate-kinase activity and the endogenous mitochondrial Mg^{2+} concentration.

Regarding the nucleoside triphosphates, previous studies showed that in F_1-ATPase the activating effect of free ATP was more manifest at pH values higher than those corresponding to the pH optimum for each substrate concentration (17). The effect of GTP, ITP, CTP and UTP resembled that of ATP suggested above. Nucleoside triphosphates can be expected to interact with the same regulatory site proposed for nucleoside diphosphates, so that the effect of pH on the sensitivity of ATPase to both nucleoside di- and triphosphates would be the same. However, taking into account that nucleoside triphosphates can also act as substrates (15), and that ^-OH concentration may as well affect the interaction with the catalytic sites, the differences observed coul be attributed to an overlapping between the effects of a particular nucleoside triphosphate on both the catalytic and the regulatory sites. This is especially true in the catalytic site that shows the highest affinity for the substrate. Besides, in mitochondria the possibility exists that nucleosides interact with Mg^{2+} giving rise to complexes which are more active as substrates. That would explain the slightly higher hydrolytic rates observed in this enzymatic preparation.

Both activating and inhibitory anions have been reported compete for the same regulatory ATPase (13) site which is different from that of nucleosides (1). Resembling the results found for activating

anions such as bicarbonate and dinitrophenol (17), the inhibitory effect of cyanate and thiocyanate became lower when increasing the pH values; once the optimum pH for each catalytic site had been reached this effect was more manifest (fig. 3). This fact may be explained as a result of a competition between ^-OH and activating or inhibitory anions.

The results here reported together with previous data from our laboratory support the existence in ATPase of two different regulatory sites. One of them would be specific for binding nucleotides, whereas the other would specifically interact with both inhibitory and activating anions. The effect of pH on the nucleotide interaction with its regulatory site was opposite to that corresponding to anions, thus suggesting that ^-OH could compete with the anions for their specific site, and not with the specific regulatory site for the nucleotides.

Resumen

Se estudia el efecto del pH sobre la sensibilidad de la ATPasa mitocondrial a los nucleósidos di- y trifosfato, así como a los aniones inhibidores cianato y tiocianato. Los resultados obtenidos muestran que un nucleótido puede actuar como activador o inhibidor de la actividad hidrolítica ATPasa dependiendo del pH, de la concentración de sustrato y de que la enzima se encuentre purificada o unida a membrana. El efecto de los nucleótidos que activan la hidrólisis de ATP-Mg es más pronunciado una vez alcanzado el pH óptimo correspondiente a cada centro catalítico, disminuyendo su efecto por encima de este valor, los inhibidores. La sensibilidad a los aniones inhibidores disminuye a medida que aumenta el pH. La disminución del efecto inhibidor se acentúa una vez alcanzado el pH óptimo. Estos datos sugieren la existencia en la ATPasa mitocondrial de dos sitios reguladores diferentes, uno específico para nucleótidos y otro específico para aniones, que muestran una respuesta distinta a los cambios de pH.

Palabras clave: ATPasa y pH, ATPasa y nucleótidos, ATPasa mitocondrial.

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