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Effect of pH on the Sensitivity of Mitochondrial ATPase to Free ATP, ADP and Anions

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The pH activity curves of the hydrolyzing activity of ATPase obtained with rat liver mitochondria or with the isolated F_1-ATPase at three different substrate concentrations exhibited different optimum pH values. At 0.06 mM $ATPMg^{2+}$ maximal activity was reached at pH 7; at 0.6 mM $ATPMg^{2+}$ the optimum value was around 8; and at 3 mM $ATPMg^{2+}$ between pH 8.2 and 9. These results suggest the presence of different catalytic sites in the enzyme with different affinities for the substrate and different optimum pH values.

The sensitivity to the activating anions dinitrophenol and bicarbonate decreased with increasing pH values; the decrease in the activating effect was sharper when approaching the optimum pH value at any of the three substrate concentrations tested. These results might indicate that either OH^-, dinitrophenol, or bicarbonate could compete for a regulatory site or sites in ATPase.

The activating effect of free ATP on the hydrolyzing activity of isolated F_1-ATPase was found to be dependent on the pH of the medium. The activating effect was more pronounced above the optimum corresponding to each of the three $ATPMg^{2+}$ concentrations used, whereas the inhibitory effect of ADP was more manifest at pH values below that optimum point.

A great deal of attention has been focused on the properties of mitochondrial ATPase and on the conditions required for the variety of activities ascribed to this enzyme. One of the points meriting such an attention by different groups has been the effect of pH on its hydrolytic activity (4, 6, 11, 15 y 17). More recently HARRIS *et al.* (9) have found that pH greatly affects the exchange of tightly bound nucleotides. MYERS and SLATER (15) found also that the ATP hydrolyzing activity of isolated mitochondria exhibited four separate optimum pH values; the results were interpreted in terms of the presence of four different ATPase systems in mitochondria, three of them sensitive and one insensitive to the activating agent

dinitrophenol. It was also shown that the sensitivity to dinitrophenol stimulation was higher at pH values below the pH values needed to reach maximal hydrolytic activity in the absence of this agent (4, 15, 17). FANESTIL *et al.* (6) have also described the effect of pH on the bicarbonate stimulation of mitochondrial ATPase; they found that at high pH values the activating effect of this anion was greatly reduced, similarly to what had been observed with dinitrophenol. Recent kinetic studies suggesting the presence of more than one catalytic site in ATPase (18), as well as data reported by PEDERSEN (16) and by EBEL and LARDY (5) showing the existence of two different Km values for the enzyme prompted us to study the effect of pH on the hydrolytic activity at different substrate concentrations both in the presence or in the absence of either dinitrophenol or bicarbonate. The results obtained would be compatible with the existence of several catalytic sites with different optimum pH values. The sensitivity of ATPase to be stimulated by either dinitrophenol or by bicarbonate was always much higher at pH values below the optimum pH values found for the hydrolytic activity at each of the three substrate concentrations tested.

The activating effect of free ATP and the inhibitory effect of ADP have also been studied at different pH values. The results obtained show that the activating effect of free ATP on the hydrolytic activity increased considerably at pH values higher than the pH values corresponding to the optimum found for each of the $ATPMg^{2+}$ concentrations tested. On the contrary, ADP ceased to be an inhibitor at pH values equal to or higher than the optimum corresponding to each substrate concentration. However, its inhibitory effect was manifest at pH values below the pH optimum corresponding to each $ATPMg^{2+}$ concentration.

Materials and Methods

Mitochondria were isolated by the method of HOGEBOOM (10). Protein determination was carried out following the technique of LOWRY *et al.* (13). F_1-ATPase was prepared from rat liver mitochondria by the procedure of LAMBERTH and LARDY (12).

ATPase activity was determined essentially as described by PULMAN *et al.* (17) in the absence of an ATP generating 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 at the indicated pH values. The reaction was initiated by the addition of $ATPMg^{2+}$, pH 7.4, dissolved in 0.2 ml of distilled water. The incubation was continued for 2 minutes and stopped by the addition of 0.1 ml of 50% trichloroacetic acid. Inorganic phosphorus was according to FISKE and SUBBAROW (7). Reagent and enzyme blanks were determined in each experiment. It was always verified that under those conditions doubling the amount of protein the amount of P_i liberated also doubled.

Results and Discussion

Effect of pH on the hydrolytic activity of ATPase at different substrate concentrations. Figures 1 and 2 show the effect of pH on the ATPase hydrolytic activity of F_1-ATPase from rat liver mitochondria in the presence of different $ATPMg^{2+}$ concentrations. It may be seen that at 0.06 mM $ATPMg^{2+}$ the hydrolytic rate increased with increasing pH, a plateau being reached at pH 7. At 0.6 mM $ATPMg^{2+}$ the hydrolytic rate reached a maximum at pH values from 7.7 to 8.2. A shoulder between pH 6.5 and 7 preceded the peak of maximum activity. At 3 mM $ATPMg^{2+}$ a peak of maximum activity was observed at pH values from 8.2 to 9. Two shoulders were also observed at pH values from 7.5 to 8 and

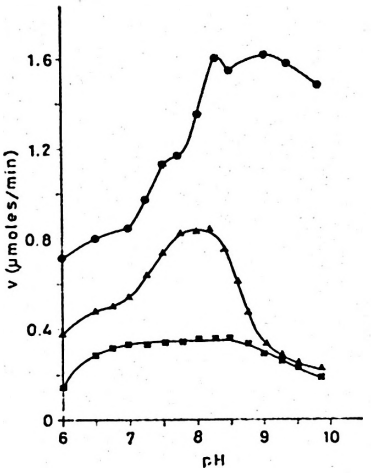

Fig. 1. *Effect of pH on the hydrolyzing activity of ATPase in isolated mitochondria at different substrate concentrations.*
(■) 0.06 mM $ATPMg^{2+}$; (▲) 0.6 mM $ATPMg^{2+}$; (●) 3 mM $ATPMg^{2+}$. The results have been referred to 1 mg protein. Number of experiments, 9.

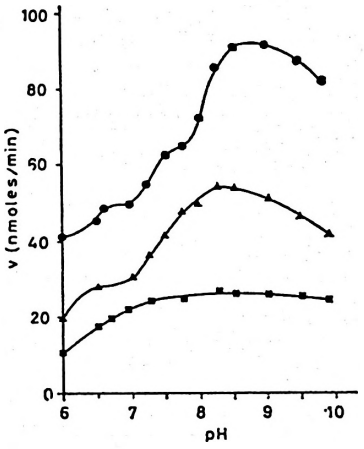

Fig. 2. *Effect of pH on the hydrolyzing activity of F_1-ATPase at different substrate concentrations.*
(■) 0.06 mM $ATPMg^{2+}$; (▲) 0.6 mM $ATPMg^{2+}$; (●) 3 mM $ATPMg^{2+}$. The results have been referred to 5 μg of enzyme protein. Number of experiments, 9.

from 6.5 to 7. It may be seen that the behavior of ATPase activity with respect to pH was quite similar both for mitochondria and for F_1-ATPase with only minor differences consisting in a decrease in activity beyond pH 8.5 in mitochondria at 3 mM $ATPMg^{2+}$, and a much sharper decrease in activity beyond pH 8.5, when concentrations of either 0.6 mM or 0.6 mM $ATPMg^{2+}$ were used with the purified enzyme. The pH activity curve at 3 mM $ATPMg^{2+}$ agrees with those already published in the literature and obtained with 1 mM or higher substrate concentrations (4, 6, 11, 15, 17).

The results reported might suggest the presence of three catalytic sites in ATPase with different affinity for the substrate and a different pH optimum. The site with the highest affinity for the substrate would reach maximal activity around pH 7; the site with intermediate affinity, at pH 8; and the third site, that of the lowest affinity for the substrate, would reach maximal activity between pH 8.2 and 9. The appearance of shoulders in the activity curves at pH values below the optimum pH for these latter two sites could be attributed to the activity of the sites with higher affinity for the substrate. The presence of at least two catalytic sites in ATPase could be suggested after the kinetic studies carried out by PEDERSEN (16), EBEL and LARDY (5), and SANTIAGO *et al.* (18).

The effects observed regarding the existence of three optimum pH values, depending on the $ATPMg^{2+}$ concentrations used, could not be ascribed to possible changes in the proportion of ionic species of ATP; changes in pH would give rise to identical proportions of each of those species independently of the initial concentrations of $ATPMg^{2+}$ used. From a theoretical point of view a correction of the actual $ATPMg^{2+}$ complex concentration at different pH values should be introduced. However, applying the equations proposed by FROMM (8) to calculate

the concentrations of the $ATPMg^{2+}$ complex at different pH values and concentrations of ATP and Mg^{2+} the corrections to be introduced are only negligible and in no way could explain the shape of the pH-activity curves.

Effect of pH on the sensitivity of ATPase to bicarbonate and dinitrophenol. The effect of pH on the sensitivity of ATPase to be stimulated by dinitrophenol or by bicarbonate has been studied at 0.06, 0.6 and 3 mM $ATPMg^{2+}$. Table I shows the results obtained. A gradual loss of sensitivity to these activating anions observed when the pH was raised from low (pH 6.5) to high values (pH 10). It may also be seen that the decrease in percent stimulation was much sharper when approaching the optimum pH values for each of the substrate concentrations tested. These results might indicate that either OH^- or any of the other activating anions, dinitrophenol or bicarbonate, could compete for the same regulatory site in ATPase.

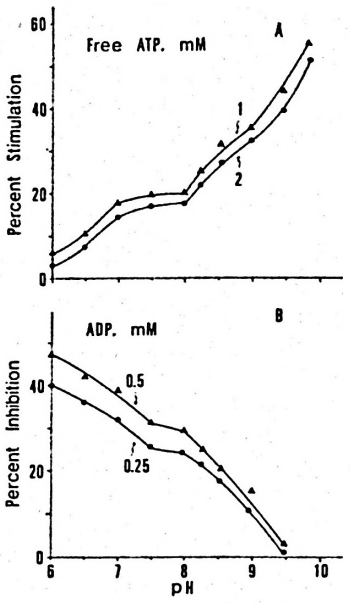

Fig. 3. *Sensitivity of ATPase to free ATP (A) and ADP (B) as a function of pH.* Substrate concentration, 3 mM $ATPMg^{2+}$. Number of experiments, 5.

Effect of pH on the sensitivity of ATPase to free ATP and ADP. The activating effect of free ATP and the inhibitory effect of ADP on F_1-ATPase have been studied at three different fixed $ATPMg^{2+}$ concentrations within the range from pH 6 to pH 10. The sensitivity of ATPase to be stimulated by free ATP increased with increasing pH values (figures 3 A, 4 A and 5 A); on the other hand, the activating effect of free ATP was always more clearly elicited at pH values higher than those corresponding to the pH optimum for each of the substrate concentrations used. On the contrary, the sensitivity to the inhibitory effect of ADP,

Table I. *Effect of pH on the sensitivity of F_1-ATPase to dinitrophenol and bicarbonate.* When present the concentration of bicarbonate was 10^{-2} M, and that of dinitrophenol (DNP) 5×10^{-4} M. Number of experiments, 5. Data represent ± S.D.

pH	Percent stimulation of F_1-ATPase hydrolyzing activity					
	3 mM ATP-Mg		0.6 mM ATP-Mg		0.06 mM ATP-Mg	
	+ HCO_3^-	+ DNP	+ HCO_3^-	+ DNP	+ HCO_3^-	+ DNP
6.5	137.2±2.1	125.3±1.9	115.2±2.0	111.2±1.9	75.5±1.1	73.0±0.9
7	136.8±1.9	121.2±1.9	88.1±1.4	100.7±1.8	51.2±0.9	50.3±0.8
7.7	108.2±1.5	99.3±1.5	78.3±0.9	79.2±0.9	22.9±0.9	24.1±0.6
8.2	90.5±0.9	88.1±0.8	50.3±0.7	40.9±0.8	20.2±0.8	21.7±0.6
9.8	30.9±0.8	27.5±0.8	51.2±0.7	42.0±0.7	23.9±0.9	20.1±0.7

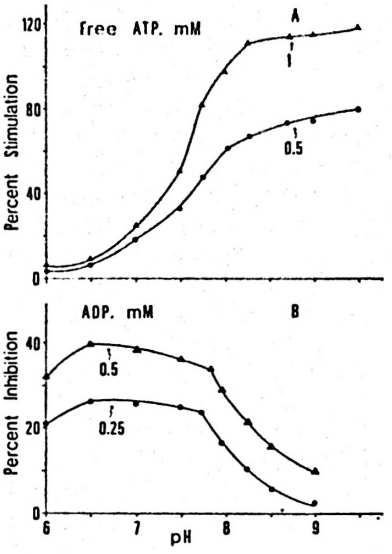

Fig. 4. *Sensitivity of ATPase to free ATP (A) and ADP (B) as a function of pH.* Substrate concentration, 0.6 mM $ATPMg^{2+}$. Number of experiments, 5.

after reaching a maximum, decreased with increasing pH values; this inhibitory effect was negligible at pH values higher than the optimum pH value for the hydrolytic activity of ATPase corresponding to each of the three substrate concentrations used. At 3 mM $ATPMg^{2+}$, and in the absence of free ATP, a maximum for the hydrolytic rate was reached at the pH interval between 8.2 and 9 (fig. 1); figure 3 A shows that, in the presence of several increasing fixed concentrations of free ATP the activating effect was more pronounced at pH values higher than pH 9; figure 3 B shows that the inhibitory effect of different concentrations of ADP was more manifest at pH values below the pH optimum obtained at 3 mM $ATPMg^{2+}$ in the absence of free nucleotides. Figures 4 and 5 show a similar behavior of the hydrolytic activity of F_1-ATPase in the presence of free ATP or ADP at 0.6 and 0.06 mM $ATPMg^{2+}$. The activating effect of free ATP was more manifest at pH values higher than those corresponding to the pH optimum for each substrate concentration: pH 8 for 0.6 mM $ATPMg^{2+}$, and pH 7 for 0.06 mM $ATPMg^{2+}$. On the contrary, the inhibitory effect of ADP was practically lost at pH values higher than those corresponding to the optimum in the absence of free nucleotides. It was also observed that for each substrate concentration the inhibitory effect of ADP exhibited an optimum pH: below pH 6, at 3 mM; pH 6.5 at 0.6 mM; and pH 7 at 0.06 mM $ATPMg^{2+}$. These results would also be consistent with the existence in the ATPase complex of several catalytic sites as suggested above.

It has also been suggested (18) that under appropriate conditions the catalytic site could promote the hydrolysis of $ATPMg^{2+}$, when another molecule of ATP is also bound to the same catalytic site. Figure 6 A depicts a possible arrangement of ligands in the proposed catalytic site (19); the two atoms of iron of each catalytic site would be bridged by a bidentate ligand of $ATPMg^{2+}$ and by anoth-

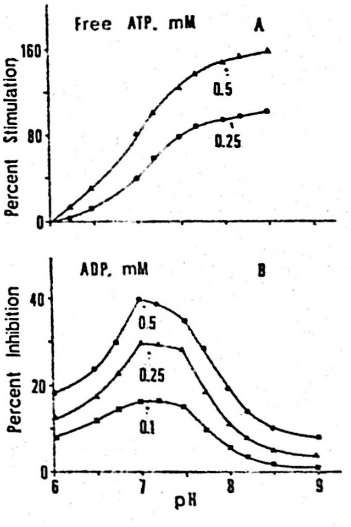

Fig. 5. *Sensitivity of ATPase to free ATP (A) and ADP (B) as a function of pH.* Substrate concentration, 0.06 mM $ATPMg^{2+}$. Number of experiments, 5.

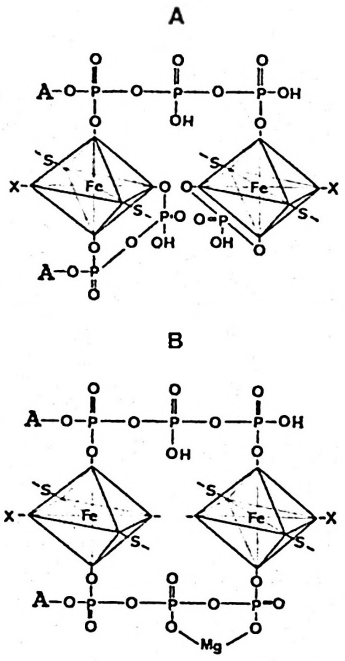

Fig. 6. *Catalytic site of ATPase with ligands committed to ATP synthesis (A), or to ATP hydrolysis (B).*

er bidentate ligand of ATP; ligand OH^- could still be allowed in that structure. Since ATPase is activated by OH^-, it would be suggested that this effect might be due to a preferential displacement of electrons of the coordination bond towards the atoms of iron — its chemical structure allowing such an interpretation— in agreement whit the proposed mechanism of action of compounds behaving as activators of the hydrolytic activity (20). Figure 6 B shows the catalytic site occupied by a molecule of ATP as a bidentate ligand bridging both iron atoms, one molecule of ADP as a bidentate ligand of one of the atoms of iron, and a molecule of Pi as a bidentate ligand of the second metal atom. It is clear that in the presence of high concentrations of OH^- the binding of ADP would be hindered. The loss of the inhibitory effect of ADP at pH values higher than the pH optimum corresponding to the different substrate concentrations could be explained by the displacement of ligand ADP by OH^-. It should be recalled that binding of ATP, ADP and Pi to the catalytic site would lead to the synthesis of ATP if conditions allowing the coupled electron flow are met, as recently suggested (19). Interestingly, COOPER and LEHNINGER (2, 3) have made the observation that the P/O ratio exhibits a pH optimum considerably lower than that of ATP hydrolysis. On the other hand HARRIS *et al.* (9) have also shown that nucleotide exchange, ATP for ADP, is favored at pH values considerably lower than those corresponding to the hydrolytic reaction. These facts, together with the results now being reported, seem to offer a coherent picture of how a proton cycle, associated with the suggested redox cycle of ATPase, and an exchange of ATP for ADP and Pi, might be operating (fig. 7). The proposed model for oxidative phosphorylation (19) suggests that the actual formation of the pyrophosphate bond between ADP and Pi,

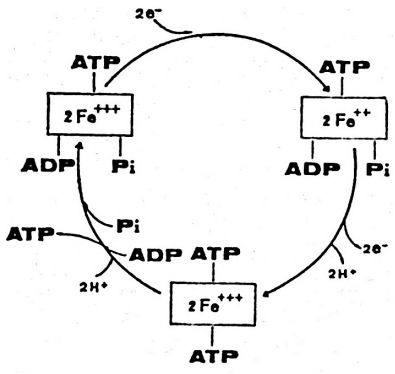

Fig. 7. *Coupling of the redox cycle of ATPase with a proton cycle.*
The exit of protons would favor de actual phosphorylation reaction coupled to the oxidation of ATPase. The return of protons would favor replacement of ATP by a new molecule of ADP.

concomitant with the oxidation of Fe (II) to Fe (III), should be favored by the ejection of protons from the mitochondria. A decrease in pH would promote the exchange of ATP and ADP by the catalytic site allowing the exit of ATP and the reentry of ADP, Pi and protons.

COOPER and LEHNINGER (2, 3) have shown that the P/O ratio with ferrocytochrome c as electron donor reaches a maximum at an acid pH (pH 5.5) relatively lower than if β-hydroxybutyrate is used as electron donor; in this latter case the P/O reaches a maximum around pH 6-7. These findings, together with the results here reported and previous data from our laboratory (21) regarding the existence of three catalytic sites with different redox potentials seem to be compatible with a tentative interpretation, which of course should need further experimentation. The catalytic site of ATPase with the highest affinity for $ATPMg^{2+}$ would correspond to coupling site I, the site with the lowest redox potential (21); this site would have maximum affinity for ADP at pH 7 (figure 5 B). Coupling site II, with more positive redox potential and low affinity for $ATPMg^{2+}$ (21) would have its maximum affinity for ADP at pH value below 6 (fig. 3 B).

This effect of pH on the affinity of the enzyme for ATP and ADP would also be compatible with the tenets of the chemiosmotic hypothesis (14) or with those of the conformational hypothesis (1).

Resumen

Las curvas de la actividad hidrolítica de la ATPasa mitocondrial, en función del pH de la enzima presente en mitocondrias de hígado de rata o en F_1-ATPasa a tres diferentes concentraciones de sustrato, exhiben diferentes valores de pH óptimo. A concentración 0,06 mM de $ATPMg^{2+}$ la actividad alcanza un máximo a pH 7; a 0,6 mM de $ATPMg^{2+}$ el valor óptimo es de alrededor de 8; y a 3 mM $ATPMg^{2+}$ entre 8,2 y 9. Estos resultados sugieren la presencia de diferentes centros catalíticos en la enzima con diferente afinidad por el sustrato y diferentes valores de pH óptimo.

La sensibilidad a los aniones activadores dinitrofenol y bicarbonato desciende con el incremento de los valores de pH; la disminución del efecto activador fue más pronunciada cuando se aproxima al valor óptimo del pH a cualquiera de las tres concentraciones de sustrato utilizadas. Estos resultados pueden indicar que tanto el OH^-, dinitrofenol o bicarbonato competirían por un mismo centro o centros reguladores en la ATPasa.

El efecto activador del ATP libre sobre la actividad hidrolítica de la F_1-ATPasa se vio que es dependiente del pH del medio. El efecto activador es más marcado por encima del óptimo correspondiente a cada una de las concentraciones de $ATPMg^{2+}$ utilizadas.

Por el contrario, el efecto inhibidor del ADP fue más manifiesto a valores de pH por debajo de cada uno de los óptimos que corresponden a las tres concentraciones de $ATPMg^{2+}$ utilizadas.

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