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Catalytic Sites of Mitochondrial ATPase with Different Properties. Effect of Citrate, Free ATP and ADP in the Presence of Dithionite

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The extent of stimulation of the hydrolytic activity of mitochondrial ATPase by the reducing agent dithionite has been found to depend on substrate concentration both for the membrane bound enzyme and for the isolated and purified F_1ATPase. The results suggest the existence of three catalytic sites differing in their standard reduction potential.

The activating effect of free ATP on the hydrolytic activity of rat liver F_1-ATPase has been found to be more pronounced on the reduced form of the enzyme. On the contrary, the inhibitory effect of ADP was higher on the oxidized form of F_1-ATPase. Citrate has also been found to be an inhibitor of F_1-ATPase; its effect was more pronounced on the reduced form of the enzyme, and exhibited a competitive pattern of inhibition with respect to free ATP. The results obtained have been interpreted in the sense that free ATP and ADP may be modifying the standard reduction potential of the enzyme, and suggest the existence of three independent redox cycles in ATPase governed by the exchange of ADP and Pi for the newly synthesized ATP.

The report from PENEFSKY *et al.* (8) that mitochondrial ATPase catalyzed not only the hydrolysis of ATP but also its synthesis, when properly coupled to electron flow, gave a strong support to the general belief that even the same catalytic site could participate in both reactions if the appropriate conditions are met. Therefore, it seems that any deepening in our knowledge of the hydrolytic reactions might be at the same time of usefulness in our understanding of the process of oxidative phosphorylation.

A recent observation (5) that the reducing agent dithionite leads to a change in properties of F_1-ATPase, as revealed by an increase of its hydrolytic activity together with a disappearance of the bicarbonate stimulation, prompted us to study how the kinetic properties of the enzyme are affected by its reduction with dithionite. The results now reported suggest the

existence of three catalytic sites with hydrolytic activity having not only a different affinity for the substrate ATP-Mg but also a different sensitivity to the activation by reduction with dithionite.

It has also been reported (10) that the original form, the oxidized form of ATPase, may be modified by ATP and citrate; the extent of these effects were dependent on substrate concentration.

The present report shows that the activating effect of free ATP was more pronounced in the reduced form of ATPase. ADP, a well known inhibitor of the hydrolytic activity of ATPase (2), was found to be less efficient on the reduced than on the oxidized form of ATPase. These results have been interpreted in the sense that ATP and ADP modify the standard reduction potential of each of the three postulated catalytic sites of the enzyme. It has also been shown that citrate competed with ATP when the enzyme was in its reduced form, similarly to what had already been reported for the native form of ATPase (10).

Materials and Methods

Rat liver mitochondrial were isolated as described by HOGEBOOM (3) in 250 mM sucrose.

F_1-ATPase was prepared from rat liver mitochondria by the procedure of LAMBETH and LARDY (4). It was verified that this preparation was oligomycin insensitive in agreement with these authors.

ATPase was assayed by measuring the release of inorganic phosphate essentially as described by PULLMAN *et al.* (9) in the absence of an ATP-regenerating system. Aliquots of the F_1-ATPase containing 5 μg of enzyme protein 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 ($ATP\text{-}Mg^{++}$) 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 (1). Reagent and enzyme blanks were determined in each experiment.

Protein determination was carried out following the reaction of LOWRY *et al.* (6).

Results and Discussion

Reduction of ATPase. The effect of dithionite concentration on the hydrolyzing rate of F_1-ATPase has been studied at different fixed substrate ATP-Mg concentrations (fig. 1). It was observed that the activating effect of dithionite was more pronounced at high concentrations of ATP-Mg than at low concentrations. Three distinctive slopes were obtained which corresponded essentially to the different ranges of the substrate concentra-

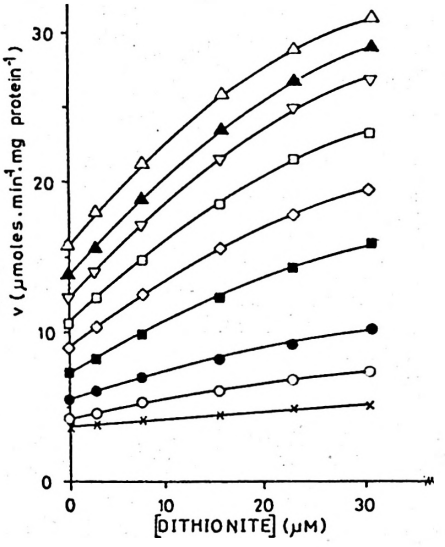

Fig. 1. *Effect of dithionite concentration on the reaction rate of ATP-Mg hydrolysis catalyzed by membrane bound ATPase.*
Concentrations of ATP-Mg: △, 4 mM; ▲, 3 mM; ▽, 2 mM; □, 1 mM; ◇, 0.6 mM; ■, 0.4 mM; ●, 0.2 mM; ○, 0.1 mM; ×, 0.06 mM. Number of experiments, 10.

tions used. For concentrations above 1 mM ATP-Mg the slope was rather steep; within the concentration range between 0.2 mM and 1 mM ATP-Mg the slope was less pronounced; at concentrations below 0.2 mM ATP-Mg the slope was only very slight. Figure 2 shows the hydrolytic activity of the membrane bound enzyme. It may be seen that the effect of increasing dithionite concentrations gave a very similar picture to that obtained with F_1-ATPase for the different substrate concentrations tested.

It might be argued that the effect of dithionite could be due not to the fact of being a reducing agent but to a generic property shared in common with other stimulatory anions. Besides the fact already reported (2) that the stimulatory effect caused by dithionite could be abolished by the oxidizing agent dichlorophenolindophenol, other experiments have been carried out in order to dispel the doubts in this respect. Table I shows that dithionite, besides stimulating the hydrolytic activity of F_1-ATPase, conferred some oligomycin sensitivity on the enzyme, and an inhibition of 35 % was observed. This rather surprising and unexpected effect was not observed if stimulations similar to those elicited by dithionite had been obtained with the non-reducing anions bicarbonate or dinitrophenol. In these latter cases the enzyme remained completely insensitivy to oligomycin.

The fact that the enzyme exhibited a different sensitivity to the stimulation by

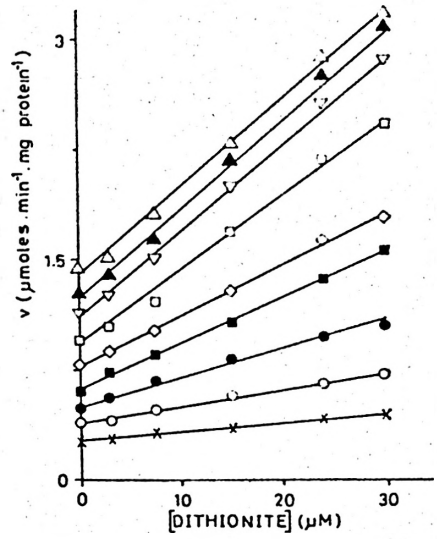

Fig. 2. *Effect of dithionite concentration on the reaction rate of ATP-Mg hydrolysis catalyzed by membrane bound ATPase.* Concentrations of ATP-Mg: △, 4 mM; ▲, 3 mM; ▽, 2 mM; □, 1 mM; ◇, 0.6 mM; ■, 0.4 mM; ●, 0.2 mM; ○, 0.1 mM; ×, 0.06 mM. Number of experiments, 8.

dithionite within the three ranges of substrate concentrations used might suggest the existence of three catalytic sites in ATPase with different affinities for its substrate and also with different standard reduction potential. The results now reported would not exclude the existence of only two catalytic sites with different standard reduction potentials, one with high affinity for the substrate and a very nega-

Table I. *Oligomycin sensitivity of F_1-ATPase after stimulation of its hydrolytic activity with dithionite, bicarbonate or dinitrophenol.* Number of experiments, 10. Data represent ± S.D. ATP-Mg concentration, 3 mM.

Additions	ATPase activity (μmoles of ATP hydrolyzed × min⁻¹ × mg⁻¹)			
		+ HCO_3^- (10 mM)	+ Dinitrophenol (5×10^{-4} M)	+ Dithionite (25 μM)
None	13.0 ± 0.4	26.2 ± 0.5	25.1 ± 0.6	31.0 ± 0.6
Oligomycin (5×10^{-5} M)	13.2 ± 0.4	26.3 ± 0.5	25.1 ± 0.6	22.1 ± 0.6

tive standard reduction potential, and another site with low affinity and a more positive standard reduction potential; in the range of intermediate substrate concentrations, at which the two sites might be catalyzing the reaction, values of intermediate affinity for the substrate and sensitivity to the reduction by dithionite could be obtained. However, the suggestion of three catalytic sites with different properties would be consistent with the results of MYERS and SLATER (7) which led them to propose the existence of four different mitochondrial ATPase systems, three of them sensitive to dinitrophenol stimulation.

The results now reported would also be compatible with the recent suggestion of SANTIAGO and LÓPEZ MORATALLA (11) according to which each of the three gaps of potential in the respiratory chain would be filled by three independent redox cycles in cascade fashion with the flow of two electrons.

Since similar results were obtained both with F_1-ATPase and the membrane-bound enzyme the properties of the suggested active catalytic sites seem to have been somehow preserved throughout the purification procedure.

Effect of free ATP on the reduced form of ATPase. Figure 3 shows the effect of free ATP on the hydrolyzing rate of ATPase at different dithionite concentrations and fixed substrate MgATP. In the absence of dithionite free ATP, at three different fixed MgATP concentrations (3 mM, 0.6 mM and 0.06 mM), had a clear activating effect. With increasing dithionite concentration this activation effect was more noticeable; this effect was even more pronounced at the lowest MgATP concentration tested. A possible interpretation of these data might be that free ATP, due to a higher affinity for the reduced form, would cause the standard reduction potential to become more positive, threrefore favoring the reduction of

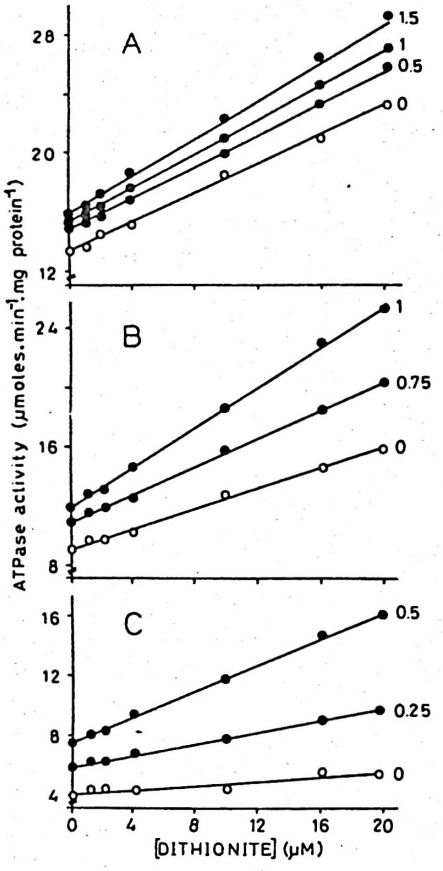

Fig. 3. *Activating effect of free ATP on F_1-ATPase at different dithionite concentrations.* A, 3 mM MgATP; B, 0.6 mM MgATP; C, 0.06 mM MgATP. mM concentrations of free ATP are indicated on the graphs. The order of additions was a follows: enzyme suspension, dithionite, and after 3 min at room temperature free ATP. A further 10 min preincubation at 30° C preceded the addition of MgATP to give the final concentrations indicated. Number of experiments, 8.

each of the catalytic sites of ATPase. It is remarkable that the slope obtained at 0.06 mM MgATP, which had only a very small value in the absence of free ATP, in the presence of free ATP its value exhibited an increase higher than those obtained at 3 mM or 0.6 mM MgATP. The site with the highest affinity for the

substrate, that with the lowest standard reduction potential as suggested by the lowest value of the slope in the absence of free ATP, would also be more sensitive to the reduction in the presence of free ATP indicating that the free nucleotide is rendering the potential more positive; another explanation could be that ATPase, in its reduced form, was more sensitive to the activating effect of free ATP. However, this latter interpretation would fail to explain why the site with the highest affinity for the substrate, and at the same time with the lowest capacity to be reduced by dithionite, exhibited the highest sensitivity to be activated by free ATP when increasing dithionite concentration.

Effect of ADP on the reduced form of ATPase. The inhibitory effect of ADP on the hydrolytic activity of ATPase has been studied at three different fixed levels of MgATP in the absence or in the presence of increasing concentrations of the reducing agent dithionite. Plots representing v versus 1/dithionite concentrations were obtained. Figure 4 shows that at low concentrating of ADP an increase of the slope was observed; this seems to indicate that the affinity of ADP was higher for the oxidized than for the reduced form of ATPase; the higher affinity for the oxidized form of ATPase should tend to make the standard reduction potential of the enzyme more negative, hindering consequently its reduction. The hindrance of the reduction seems also to be clearly indicated by the fact that at higher ADP concentrations the straight lines representing the hydrolytic rate as a function of dithionite concentration exhibited slopes considerably lower. This behavior of ATPase towards ADP was observed at each of the three fixed substrate concentrations tested.

Three possible redox cycles in ATPase. It has been suggested (11) that the mechanism of oxidative phosphorylation at a

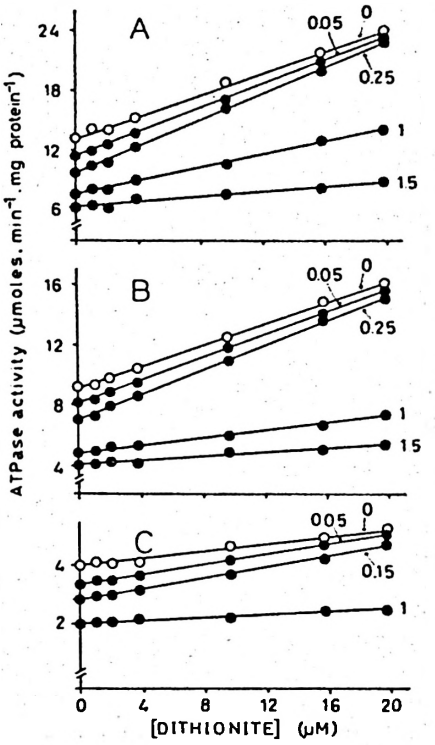

Fig. 4. *Inhibitory effect of ADP on F_1-ATPase at different dithionite concentrations.* A, 3 mM MgATP; B, 0.6 mM MgATP; C, 0.06 mM MgATP. mMM concentrations of ADP are indicated on the graphs. The order of addition was a follows: enzyme suspension, dithionite, and after 3 min at room temperature free ADP. A further 10 min preincubation at 30° C preceded the addition of MgATP to give the final concentrations indicated. Number of experiments, 7.

molecular level might imply the operation of a cycle of the catalytic site of ATPase governed by the nucleotides ADP and ATP. ADP would turn the standard reduction potential of the active site more negative allowing its isopotential reduction with adequate electron donors of the respiratory chain. In its reduced state the catalytic site would be in an energized state ready to give its electrons to an adequate acceptor with the concomitant

synthesis of ATP, nucleotide which would keep the standard reduction potential of the active site more positive. Moreover, it has also been shown that the catalytic site in its oxidized form has a higher affinity for ADP than for ATP, thus allowing the exchange of the newly synthesized ATP for a new molecule of ADP. This interplay of potentials and affinities, which in turn are the expression of the same physicochemical phenomenon, would keep the iron redox cycle turning, and synthesizing ATP, if electrons, ADP and phosphate are available.

The results now reported support the idea of the existence of three independent redox cycles in ATPase, each one of them governed by the exchange of ADP and Pi for the newly synthesized ATP. Since the standard reduction potential of the three catalytic sites was also different it might be suggested that they could be filling the three gaps of potential in the mitochondrial respiratory chain allowing the energy transduction in a cascade fashion.

Effect of citrate on the reduced form of ATPase. Figure 5 shows the effect of citrate at different dithionite concentrations in the presence of two fixed substrate concentrations. It may be seen that the inhibitory effect of citrate was pronounced in the presence than in the absence of dithionite.

Two interpretations of the effect of citrate could be given. It might mean that citrate had a higher affinity for the reduced form of the enzyme, showing then more markedly its inhibitory effect; or it might also be that it had a higher affinity for the oxidized form of the enzyme rendering its potential more negative, thus hindering the increase in activity which was observed in the presence of dithionite alone. Further experimentation would be needed to establish which of these two interpretations is the correct one. Figure 6 shows that similarly to what happened with the native form of ATPase (10) citrate

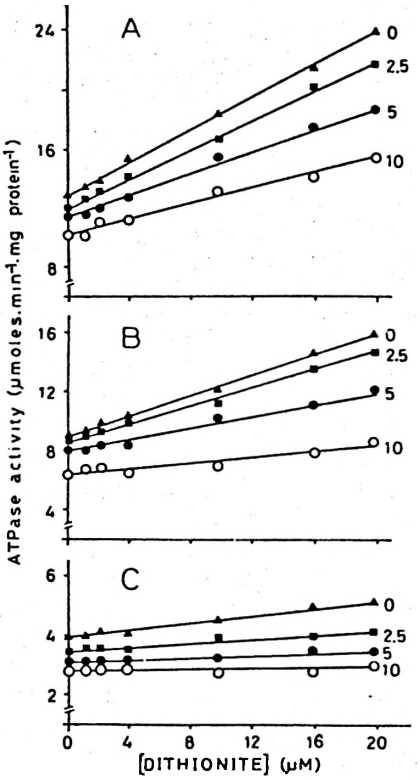

Fig. 5. *Inhibitory effect of citrate on F_1-ATPase at different dithionite concentrations.* A, 3 mM Mg ATP; B, 0.6 mM MgATP; C, 0.06 mM MgATP. mM concentrations of citrate are indicated on the graphs. The order of addition was a follows: enzyme suspension, dithionite, and after 3 min at room temperature free citrate. A further 10 min preincubation at 30° C preceded the addition of MgATP to give the final concentrations indicated. Number of experiments, 8.

inhibited also the reduced form of ATPase in a competitive fashion towards free ATP. The possible effect of citrate on the standard reduction potential of ATPase, together with its competition with ATP, might be the expression of some physiological control of the flow of electrons and the hydrolysis or the synthesis of ATP by this anion.

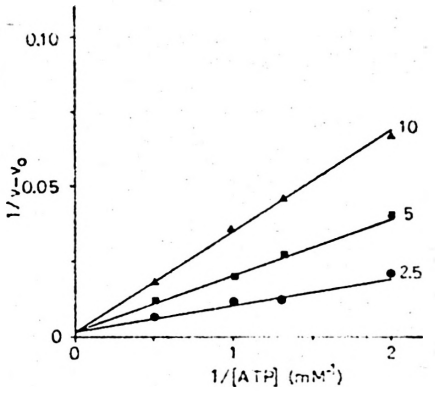

Fig. 6. *Inhibitory effect of citrate on the reduced form of F_1-ATPase at different concentrations of ATP.*
Results have been referred to 5 μg of enzyme protein. The order of additions was a follows. Enzyme suspension, dithionite (final concentration, 35 μM), and after 3 min at room temperature citrate and free ATP. A further 10 min preincubation at 30° C preceded the addition of 3 mM MgATP. Citrate concentrations are indicated on the graphs. Number of experiments, 4.

Resumen

El grado de estimulación de la actividad hidrolítica de la ATPasa mitocondrial por el agente reductor ditionito depende de la concentración de sustrato, tanto si el enzima está ligado a la membrana, como si está aislado y purificado en forma de F_1-ATPasa. Se sugiere la existencia de tres centros catalíticos que difieren en su potencial redox.

El efecto activador del ATP libre sobre la actividad hidrolítica de la F_1-ATPasa de hígado de rata es mucho más pronunciado cuando el enzima se encuentra en su forma reducida. Por el contrario, el efecto inhibidor del ADP es mucho mayor sobre la forma oxidada de la F_1-ATPasa. El citrato es también un inhibidor del enzima y su efecto es más pronunciado cuando éste se encuentra en la forma reducida: este efecto inhibidor exhibe un carácter competitivo con respecto al ATP libre. Los resultados obtenidos se interpretan en el sentido de que el ATP libre y el ADP pueden modificar el potencial standard de la enzima y sugieren la existencia de tres ciclos redox independientes en la ATPasa mitocondrial de hígado dirigidos por el intercambio de ATP por ADP y Pi.

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