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Effect of $FADH_2$ on the Hydrolytic Activity of Mitochondrial ATPase

M. Corroza*, N. López-Moratalla and E. Santiago**

Departamento de Bioquímica
Universidad de Navarra
31008 Pamplona (Spain)

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$FADH_2$ affected mitochondrial ATPase activity of both F_1 and the membrane bound enzyme. F_1-ATPase activity increased in its presence, whereas that of the membrane bound enzyme decreased. These effects were more marked at low substrate concentrations. The inhibition of membrane bound ATPase activity was not reversed by ADP. However, the simultaneous presence of free ATP and $FADH_2$ produced a stimulation of the membrane bound enzyme activity. The varying effect of $FADH_2$ on the membrane bound ATPase activity, depending on ATP concentration, could be the expression of a physiological role in mitochondria.

Key words: $FADH_2$, F_1-ATPase, AS-SMP, ATPase activity, Mitochondrial membranes.

Mitochondrial ATPase interacts with a great variety of ligands affecting its activity. Some of the nucleotides can bind to catalytic or regulatory sites giving rise to interactions which may be physiologically important. ATPase plays a key role in the electron flow linked to the oxidative phosphorylation and in the management of the energy of the cell. Gluconeogenesis, for example, depends largely on the availability of reduced coenzymes and on the energy derived from ATP.

The succinate-linked NAD^+ reduction has been studied by using spectroscopic and fluorimetric techniques both in mitochondria and submitochondrial particles (SMP) (3-8, 14, 15), which show that flavoproteins might be the electron donors for NAD^+ reduction, provided energy from ATP is available (3). ADP is an inhibitor of that process (5).

This report describes the effect of $FADH_2$ on mitochondrial ATPase as membrane free F_1, and on submitochon-

* Departamento de Química, Universidad Pública de Navarra, Edificio «El Sario», Pamplona (Spain).
** To whom all correspondence should be addressed.

drial particles, and also the effect of the simultaneous presence of $FADH_2$ or free ATP and ADP. A possible physiological role of $FADH_2$ in the modulation of ATPase is discussed.

Materials and Methods

Materials. — FAD and ADP were obtained from Boehringer Mannheim. Pd on active charcoal and ATP were purchased from Merck.

Bovine heart mitochondria were isolated by the method of LOW and VALLIN (15). SMP were prepared by the procedure of RACKER and HORSTMAN (18). F_1-ATPase was obtained as described by TUENA DE GÓMEZ-PUYOU and GÓMEZ-PUYOU (20). Protein determination in SMP and F_1-ATPase was carried out following the technique of GORNALL *et al.* (11) and LOWRY *et al.* (16), respectively.

ATPase activity was determined essentially as described by PULLMAN *et al.* (17) in the presence of an ATP generating system. Inorganic phosphorous was determined according to FISKE and SUBBAROW (9). FAD hydrogenation was carried out with hydrogen under pressure in the presence of Pt on active charcoal during 24 hours (10). Concentration of hydrogenated FAD was determined by using the molar extinction coefficient of $FADH_2$ at 295 nm ($\varepsilon = 6.7 \times 10^2\ M^{-1}\ cm^{-1}$).

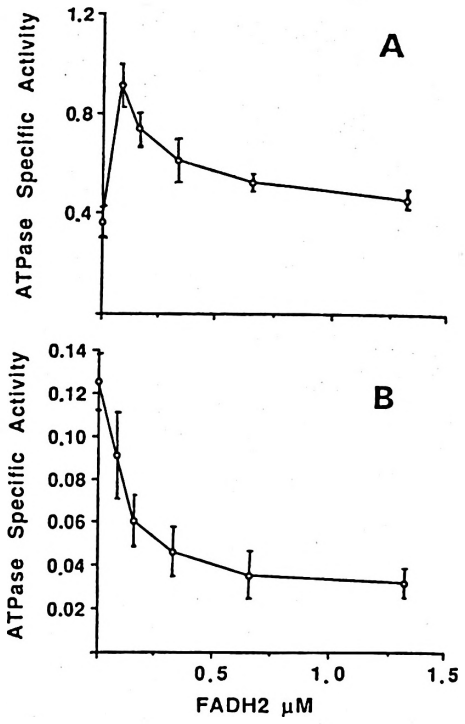

Fig. 1. *Effect of hydrogenated FAD on the catalytic activity of the enzyme both in F_1-ATPase (A) and in submitochondrial particles (B).* Concentration of ATP · Mg^{2+} : 0.06 mM. Specific activity has been expressed as µmol of ATP × min^{-1} × mg^{-1}. Total amount of protein in each assay was 8.5 µg of purified enzyme (A) or 50 µg of membrane protein (B) in a total volume of 1.1 ml. Number of assays in (A) was 9 and in (B) was 6.

Results and Discussion

The effects of $FADH_2$ on ATPase with soluble F1 preparations or the membrane-bound enzyme are shown in fig. 1. With F_1-ATPase, $FADH_2$ provoked an increase in the activity when present at 0.080 µM. Higher concentrations of the reduced flavine produced lower activations. The effect of hydrogenated FAD on ATPase activity of SMP differed substantially. As shown in figure 1B, $FADH_2$ behaved as an inhibitor of the enzyme at all concentrations tested. The inhibition increased with increasing concentrations of $FADH_2$.

The results obtained when hydrogenated FAD and ADP, or free ATP, were simultaneously present in the medium are shown in figs. 2A and 2B. ADP inhibited the ATPase activity of the enzyme as F_1, i.e. its soluble form. When ADP was added together with hydrogenated FAD to the soluble preparations of the enzyme,

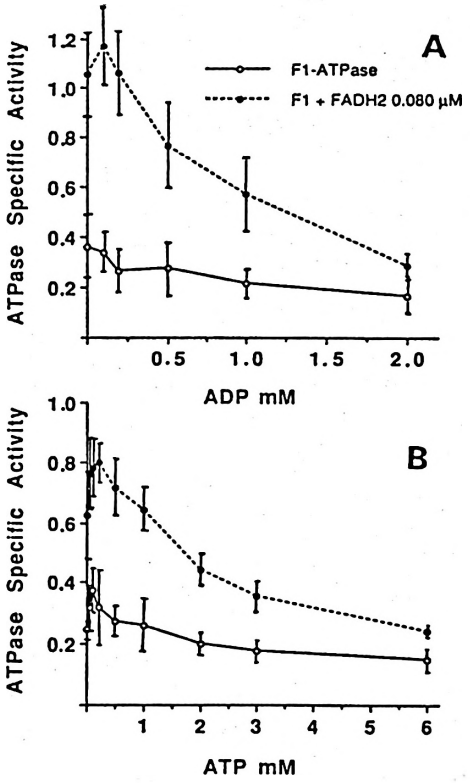

Fig. 2. *Effect of ADP (A) or free ATP (B) on the catalytic activity of F_1-ATPase in the absence or in the presence of hydrogenated FAD.*
Concentration of ATP · Mg^{2+}: 0.06 mM. Specific activity has been expressed as µmol of ATP × min^{-1} × mg^{-1}. Total amount of protein in each assay was 8.5 µg of purified enzyme in a total volume of 1.1 ml. Number of assays in (A) and in (B) was 5.

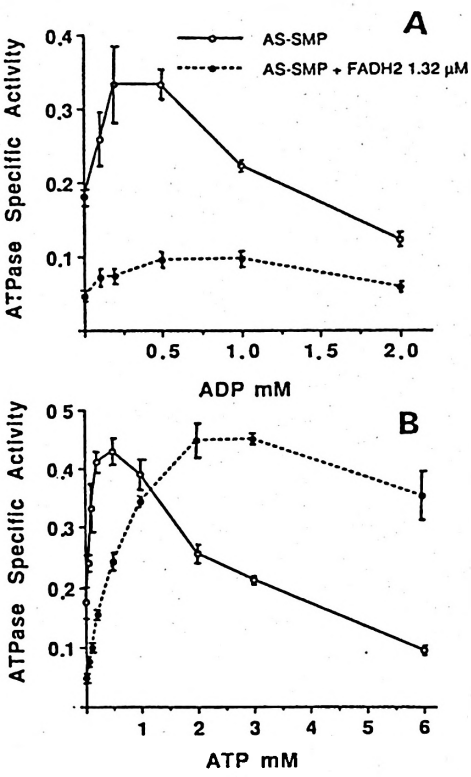

Fig. 3. *Effect of ADP (A) or free ATP (B) on the catalytic activity of ATPase in submitochondrial particles in the absence or in the presence of hydrogenated FAD.*
Concentration of ATP · Mg^{2+}: 0.06 mM. Specific activity has been expressed as µml ATP × min^{-1} × mg^{-1}. Total amount of protein in each assay was 50 µg in a total volume of 1.1 ml. Number of assays in (A) and in (B) was 4.

the stimulatory effect of flavine was markedly decreased. Increasing concentrations of ADP gradually abolished the stimulation of $FADH_2$.

Increasing concentrations of free ATP, within the range of 0.1 mM-1.0 mM, had an activating effect on F_1-ATPase and a maximum value was observed. Further increases in the concentration of free ATP decreased the activating effect, ATP becoming actually an inhibitor (full line in figure 2B). When free ATP was added together with hydrogenated FAD (dashed line in figure 2B), the activity was similar to that obtained with ADP. The stimulation of the enzyme due to $FADH_2$ decreased in the presense of higher concentrations of ATP.

The simultaneous presence of the hydrogenated FAD and ADP, or free ATP, in the medium provoked different effects depending on whether the enzyme prep-

aration was F_1-ATPase or particulate SMP. Figure 3A shows the effect of ADP on SMP both in the absence or in the presence of the hydrogenated flavine. ADP behaved as a biphasic modulator on SMP. ATPase activity increased with increasing ADP concentrations, a maximum being reached when ADP concentration was within the range of 0.2-0.5 mM. Further increases in the concentrations of ADP inhibited ATPase activity of SMP (full line in figure 3A). When hydrogenated FAD was added at the fixed concentration of 1.32 μM, together with increasing concentrations of ADP, the stimulation practically disappeared, and ATPase activity was inhibited for the whole range of ADP concentrations used (dashed line in figure 3A).

The effect of free ATP on SMP both in the absence or in the presence of hydrogenated flavine, may be seen in figure 3B. Activity reached a maximum at 0.5 mM free ATP. Further increases in free ATP concentration caused a decrease in the activity, free ATP becoming actually an inhibitor. The simultaneous presence of 1.32 μM $FADH_2$ with increasing free ATP concentrations caused quite a different effect from that observed with ADP (figure 3B). Surprisingly, the inhibitory effect of hydrogenated flavine turned into large activation with increasing amounts of free ATP. Inhibition of ATPase activity disappeared and an activation was produced. Thus, the inhibition caused by hydrogenated flavine was transformed into an activation, when free ATP was present.

The effect of variable concentrations of $FADH_2$ (0.19-1.54 μM) on ATPase activity of SMP in the presence of two fixed concentrations of free ATP (3 and 6 mM) can be seen in figure 4. In all cases a stimulation was observed, which was found an unexpected result since at those concentrations $FADH_2$ or free ATP, when present separately, had either practically no effect (3 mM ATP), or a clear inhibitory effect (6 mM ATP or 0.19-1.54 μM

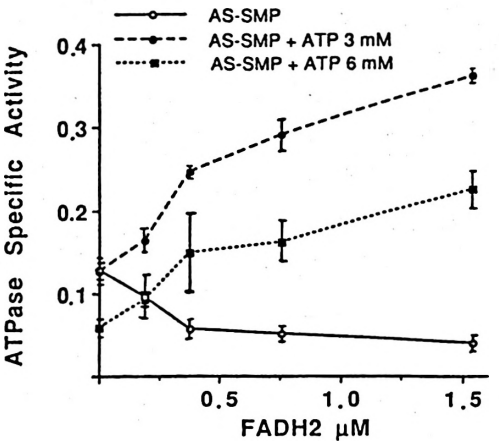

Fig. 4. *Effect of hydrogenated FAD on the catalytic activity of ATPase in submitochondrial particles in the absence or in the presence of two fixed concentrations of free ATP (3 and 6 mM).* Legend as in figure 3. Number of assays was 4.

$FADH_2$). Double reciprocal plots suggest that free ATP and hydrogenated flavine are not mutually competitive modulators (data not shown).

Previous results suggested the existence of two different binding sites for free ATP and ADP, both on the soluble F_1-ATPase, and on the enzyme in whole mitochondria (1). Free ATP, if present at low concentrations, could bind to a regulatory site on the soluble membrane-bound enzyme. At high concentrations it would also bind to a catalytic site with an affinity lower than that corresponding to the regulatory site, bringing about a competitive inhibition. ADP would bind to the same catalytic site, but not to the regulatory site on soluble F_1, due presumably to a conformational change closing this latter site to ADP (but not to free ATP). On membrane-bound ATPase ADP would bind at both sites, catalytic and regulatory, similarly to what happens with free ATP.

Hydrogenated flavine showed a bipha-

sic behavior on F_1-ATPase, but not apparent on SMP. This fact could be interpreted as reflecting the possible binding of flavine to two hypothetic sites on the soluble enzyme, one corresponding to an activatory binding site and the other to an inhibitory site. This latter site would be available only on the membrane-bound ATPase. Upon binding to the catalytic site free ATP or ADP could hinder the binding of $FADH_2$ to its regulatory site (see figures 2A and 2B). On the membrane-bound ATPase, ADP would not alter the inhibitory effect of $FADH_2$ (figure 3A), but free ATP, binding at the same regulatory site as ADP, although with a much higher affinity, might prevent the binding of $FADH_2$ (or provoke its displacement), and consequently the inhibitory effect of hydrogenated FAD would disappear (figure 3B).

The contrasting effects of $FADH_2$ on ATPase, depending on the nature of the enzyme preparation, resemble those of ADP on ATPase activity of F_1 or of SMP. ADP behaves as an ATPase inhibitor on soluble F_1, but it exhibits a biphasic profile (activatory and inhibitory) on the membrane-bound ATPase (1). The reason for these contrasting effects might be the existence of two ADP binding sites on membrane-bound ATPase, but not on the soluble form of the ATPase.

The existence of three catalytic sites on ATPase, exhibiting different substrate affinity and standard reduction potential, has been previously proposed (2, 13). The effect of the reducing reagent dithionite on F_1 and membrane-bound ATPase (12, 19), together with the results of potentiometric assays on the enzyme (13), suggest that the catalytic site with the highest affinity should also be the one with the most negative standard potential. Its value would fall between the standard potentials of the NADH dehydrogenase ($E^0 = -320$ mV) and the succinate dehydrogenase complex ($E^0 = 0$ mV) of the mitochondrial respiratory chain. As described above, $FADH_2$ affected ATPase catalytic activity of F_1 or SMP with 0.06 mM ATP · Mg^{2+} (>Km corresponding to the catalytic site with the highest affinity). Interactions with free ATP and ADP were also affected. However, negligible effects were observed when 0.6 mM or 3 mM ATP · Mg^{2+} substrate concentrations were tested (data not shown). Taking this into consideration, the possibility of some physiological role of $FADH_2$, as a consequence of its interaction with the most electronegative catalytic site of the ATPase and the portion of the respiratory chain included between complex I and II, could find support in the experiments discussed above. Intramitochondrial ATP/ADP ratio increases in some physiological situations coinciding with a stimulation of fatty acid oxidation. Gluconeogenesis requires NADH as reduction power. In lypolysis $FADH_2$ and NADH are formed and NADH supplies electrons needed for gluconeogenesis. Thus $FADH_2$/NADH ratio would increase within the mitochondria. $FADH_2$ might then donate electrons to NAD^+ generating NADH, in a process requiring ATP, since electrons will have to flow from $FADH_2$ to NAD^+ through site I of the respiratory chain against a redox gradient («reverse electron flow»). The energy for such a flow would be provided by coupled ATP hydrolysis catalyzed by ATPase; $FADH_2$, stimulating the enzyme hydrolytic activity, would push the reverse electron flow.

Results represented in figure 3B could be interpreted as having a physiological meaning. $FADH_2$ is an inhibitor of membrane-bound ATPase, as reflected in figure 1B. When free ATP increases (fig. 3B), $FADH_2$ becomes an activator, a change promoting the reverse electron flow, and, together with it, anabolic processes such as gluconeogenesis. According to this interpretation, intramitochondrial ATP concentration could behave as a «key» or a «valve» allowing, or preventing, ATPase stimulation by $FADH_2$.

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Resumen

El $FADH_2$ modifica la actividad hidrolítica ATPasa tanto en F_1 como en la enzima unida a membrana. En F_1 aumenta la actividad, mientras que en la enzima unida a membrana disminuye, siendo los efectos más acusados para las concentraciones bajas de sustrato. El ADP no revierte la inhibición de la actividad ATPasa de la enzima unida a membrana. Sin embargo, dicha actividad es estimulada por la presencia simultánea de ATP y $FADH_2$ en el medio de incubación. Se sugiere una posible función para el $FADH_2$, mediada por la enzima ATPasa. El efecto variable del $FADH_2$ en la actividad ATPasa de la enzima unida a membrana, dependiendo de la concentración de ATP, podría desempeñar una función fisiológica en la mitocondria.

Palabras clave: $FADH_2$, F1-ATPasa, SMP-AS, Actividad ATPasa, Membrana mitocondrial.

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