REVISTA ESPAÑOLA DE FISIOLOGIA, 35, 201-208. 1979

Effect of Uncouplers and Inhibitors of Oxidative Phosphorylation on the Reduced and Oxidized Forms of Mitochondrial ATPase

E. Santiago, N. López-Moratalla, M. J. López-Zabalza, A. J. Iriarte and J. Huamán

Departamento de Bioquímica
Universidad de Navarra
Pamplona (España)

(Received on December 18, 1978)

E. SANTIAGO, N. LOPEZ-MORATALLA, M. J. LOPEZ-ZABALZA, A. J. IRIARTE and J. HUAMAN. *Effect of Uncouplers and Inhibitors of Oxidative Phosphorylation on the Reduced and Oxidized Forms of Mitochondrial ATPase.* Rev. esp. Fisiol., 35, 201-208. 1979.

A series of uncouplers and inhibitors of oxidative phosphorylation have been studied with regard to their effect on the hydrolytic activity of the reduced and oxidized forms of isolated or membrane-bound mitochondrial ATPase. Uncouplers (2,4-dinitrophenol, dicoumarol), which are also activators of the hydrolytic activity of ATPase, were more potent activators on the oxidized form of the enzyme. Inhibitors of oxidative phosphorylation (oligomycin, azide and amytal) had a more potent inhibitory effect on the hydrolytic activity of ATPase in its reduced form. Purified F_1-ATPase, oligomycin insensitive in the oxidized form of the enzyme, became sensitive to oligomycin in the reduced form. An interpretation of the results suggests the presence of a mechanism that unifies the action of these different compounds on the synthesis and hydrolysis of ATP catalyzed by mitochondrial ATPase.

A large series of compounds capable of interfering with the synthesis of ATP associated to electron transport have been used in the past years as tools to explore the mechanisms of oxidative phosphorylation. Some of these compounds, those known as uncoupling agents, do not inhibit respiration while preventing phosphorylation. Compounds belonging to this group — among them DNP and dicoumarol — behave also as activators of mitochondrial ATPase. Other compounds, such as oligomycin and azide, inhibit oxidative phosphorylation in such a way that the electron transfer is also inhibited. Compounds belonging to this latter group are also known to inhibit the hydrolytic activity of mitochondrial ATPase (16).

A property shared by all those compounds, independently of whether they

Abbreviations: DNP, 2,4-dinitrophenol. DCPIP, dichlorophenolindophenol.

belong to the first or to the second group, is that of inhibiting the different exchange reactions —ATP:ADP, ATP:Pi, Pi:$H_2{}^{18}O$, ATP:$H_2{}^{18}O$— catalyzed by the ATPase complex (3).

Different mechanisms have been proposed to explain the action of uncouplers and of inhibitors of oxidative phosphorylation. For the supporters of the chemical-coupling hypothesis (15) inhibitors of oxidative phosphorylation would act by preventing the phosphorylation of the energy transfer carriers, whereas uncouplers would catalyze the breakdown of a nonphosphorylated high energy intermediate (for a review, see ref. 3). For the supporters of the chemiosmotic hypothesis headed by MITCHELL (10) the uncoupling is explained by making the membrane permeable to protons.

A model for oxidative phosphorylation has been recently proposed by SANTIAGO and LÓPEZ-MORATALLA (13) based on the presence of iron in the enzyme ATPase (14), and on the interconversion of the ATPase complex between two forms by a reversible redox reaction (8). According to this model the synthesis of ATP would take place on a catalytic site of ATPase constituted by a pair of iron atoms with the possibility of binding a series of different ligands. The phosphorylation reaction would be possible through a redox cycle of ATPase between two potentials and the exchange of ATP with ADP and Pi (13).

The sensitivity of the oxidized and reduced forms of ATPase towards the different inhibitors of ATP synthesis has now been studied with the idea of gaining some insight into the mechanism of action of these agents. The data here reported seem to point out that compounds belonging to the group of uncouplers, such as dinitrophenol or dicoumarol, which are known to be activators of ATPase, were less active when ATPase was in its reduced form. Compounds belonging to the group of inhibitors of oxidative phosphorylation, which at the same time behave as inhibitors of ATPase. exhibited a much stronger inhibitory effect on the reduced form of ATPase than on its oxidized form. Oligomycin has been described to behave as an inhibitor of ATPase (7) only when this enzyme is bound to the membrane or in the presence of OSCP (oligomycin sensitivity conferring protein), and not when the enzyme is obtained free as F_1 (17); the observation has now been made that purified F_1 acquired some sensitivity to oligomycin when the enzyme was in its reduced form.

An interpretation of these results permits to propose a mechanism of action of these compounds which would explain their effects not only as inhibitors of the synthesis of ATP but also as modifiers of the hydrolytic activity of ATPase.

Materials and Methods

Mitochondria were isolated by the method of HOGEBOOM (5) and inner membranes isolated by the method of PARSONS *et al.* (11). Protein determination was carried out following the reaction of LOWRY *et al.* (9). F_1-ATPase was prepared from rat liver mitochondrial by the procedure of LAMBETH and LARDY (6). ATPase activity was determined essentially as described by PULLMAN *et al.* (12) 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, pH 7.4. The reaction was initiated by the addition of appropriate amounts of ATP-Mg dissolved in 0.2 ml of distilled water to give the required final concentrations in the incubation mixture. 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 SUB-

BAROW (4). Reagent and enzyme blanks were determined in each experiment.

Results

Different compounds, all of them having in common the property of interfering with the synthesis of ATP, were studied from the point of view of their effect on the hydrolytic activity of F_1-ATPase in its original, reduced and reoxidized form (table I).

The reduction of the enzyme was effected by dithionite added directly to the enzyme suspension in the buffer where the incubation for the determination of activity was to be carried out. Reoxidation of the enzyme was effected by the addition of DCPIP and a preincubation for a further period of three minutes.

Two substrate concentrations (3 mM and 0.2 mM ATP-Mg) were used in all the experiments in order to explore the effect of these compounds on each of the catalytic sites of the enzyme recently suggested (2). Reduction of the enzyme increased its hydrolytic activity; reoxidation with DCPIP practically abolished this effect in agreement with previous observations (8).

DNP activated the hydrolyzing activity of both the oxidized and reduced forms of the enzyme. However, its activating effect was greater on the oxidized than on its reduced form. These effects were observed at the two substrate concentrations tested, although they were more marked at 3 mM ATP-Mg. Dicoumarol resembled somehow DNP; but its activating effect was less apparent on the oxidized form, and completely absent on the reduced form of the enzyme. Cyanide was tested at two concentrations: at 10^{-3} M, concentration capable of inhibiting cytochrome oxidase; and at 10^{-2} M, concentration which is necessary to inhibit oxidative phosphorylation (16). Table I shows that at 10^{-2} M CN^- the activating effect was lost when the enzyme was in its reduced form.

Rotenone did not exhibit a significant effect on any of the two forms of the enzyme. Azide, a potent inhibitor of ATPase (16), showed this effect more markedly on the reduced form of the enzyme. Amytal, at concentrations which are known to exert an uncoupling effect

Table I. *Effect of compounds capable of interfering with ATP synthesis on the hydrolyzing activity of F_1 ATPase.*

The results have been referred to 5 μg enzyme protein. When present, final concentrations of dithionite and DCPIP were 25 μM and 40 μM respectively. Number of experiments, 10. Data represent ± S.D.

		ATPase activity (nmol ATP hydrolyzed × min^{-1})					
		(3 mM ATP-Mg)			(0.2 mM ATP-Mg)		
Additions	[M]	—	+ Dithionite	+ Dithionite + DCPIP	—	+ Dithionite	+ Dithionite + DCPIP
None		65.2±2.1	155.6±3.1	76.1±2.0	20.1±1.2	29.1±1.3	22.2±1.1
DNP	5×10^{-4}	125.5±3.2	190.0±3.2	131.3±3.1	28.7±1.8	32.2±1.5	27.1±1.2
CNK	10^{-3}	73.2±2.1	161.8±3.1	83.1±1.8	23.1±1.1	30.0±1.3	24.8±1.3
CNK	10^{-2}	82.1±1.9	156.2±2.1	78.9±2.9	26.2±1.2	29.1±1.2	23.0±1.2
Rotenone	5×10^{-6}	67.5±1.9	160.2±3.8	77.1±2.0	20.2±1.2	30.7±1.3	22.9±1.3
Dicoumarol	5×10^{-6}	81.3±2.1	157.3±3.1	78.2±2.0	24.3±1.2	29.8±1.3	24.3±1.4
Azide	10^{-3}	18.2±1.1	25.2±1.1	24.1±1.7	7.1±0.5	6.2±0.5	7.2±0.5
Oligomicyn	5×10^{-5}	66.2±1.8	110.1±3.0	70.1±1.8	21.2±1.1	24.1±1.1	21.3±1.2
Amytal	2×10^{-3}	57.3±1.7	117.2±2.9	63.0±2.0	18.1±1.2	23.8±1.2	20.2±1.2

Table II. *Effect of compounds capable of interfering with ATP synthesis on the hydrolyzing activity of membrane bound mitochondrial ATPase.*
The results have been referred to 1 mg of inner mitochondrial membrane protein. When present, final concentrations of dithionite and DCPIP were 25 μM and 40 μM respectively. Number of experiments, 10. Data represented ± S.D.

		ATPase activity (μmol ATP hydrolyzed × min^{-1})					
		(3 mM ATP-Mg)			(0.2 mM ATP-Mg)		
Additions	[M]	—	+ Dithionite	+ Dithionite + DCPIP	—	+ Dithionite	+ Dithionite + DCPIP
None		1.13±0.07	2.34±0.11	1.41±0.06	0.41±0.01	0.61±0.02	0.49±0.01
DNP	5×10^{-4}	2.03±0.07	2.93±0.12	2.05±0.08	0.58±0.02	0.68±0.02	0.58±0.02
CNK	10^{-2}	1.30±0.07	2.33±0.10	1.65±0.07	0.46±0.01	0.61±0.02	0.54±0.02
Rotenone	5×10^{-6}	1.22±0.07	2.33±0.10	1.49±0.06	0.42±0.01	0.60±0.02	0.50±0.01
Dicoumarol	5×10^{-6}	1.31±0.07	2.32±0.12	1.51±0.05	0.46±0.01	0.61±0.02	0.51±0.01
Azide	10^{-3}	0.23±0.02	0.43±0.03	0.28±0.02	0.12±0.01	0.18±0.01	0.21±0.01
Oligomicyn	5×10^{-5}	0.42±0.03	0.78±0.04	0.51±0.03	0.24±0.01	0.34±0.01	0.28±0.01
Amytal	2×10^{-3}	0.98±0.04	1.82±0.07	1.21±0.05	0.38±0.01	0.50±0.02	0.43±0.02

(16), behaved as a weak inhibitor of the oxidized form of the enzyme; its inhibitory effect was more manifest on the reduced form of ATPase. Oligomycin deserves a special commentary. This compound, so far considered as an inhibitor of coupled respiration, and without effect on uncoupled respiration, has also been known as an inhibitor of membrane-bound ATPase (7) but without any effect on isolated F_1-ATPase (17). Table I clearly shows that, whereas no effect was observed on the original form of F_1-ATPase, a noticeable inhibitory effect was already apparent on the reduced form of the enzyme.

Table II shows the effect of the same series of compounds on the hydrolyzing activity of the original, reduced and reoxidized form of membrane-bound mitochondrial ATPase. The results were parallel to those found with F_1-ATPase. The activators DNP, CN^- and dicoumarol had a more potent activating effect on the original and on the reoxidized form of the enzyme. Azide, amytal and oligomycin were potent inhibitors on the reduced form of the enzyme. Rotenone had no effect either on the oxidized or on the reduced form. At high concentrations of ATP-Mg the activating or inhibitory effects were more marked than at low substrate concentrations.

DCPIP had a negligible effect on the original form of either F_1-ATPase or on the membrane-bound enzyme.

Discussion

A consideration of the effects on ATPase activity of the different compounds listed in table I and II allows a classification of them into two groups. That of the activators, and that of the inhibitors of mitochondrial ATPase. The activating effect of compounds belonging to the first group was always more pronounced on the oxidized than on the reduced form of the enzyme; this seems to imply that they had a higher affinity for the oxidized than for the reduced form of ATPase. It is obvious that the higher affinity for the oxidized form of the enzyme would consequently imply that the interaction of any of these compounds with the enzyme made the standard reduction potential of ATPase more negative.

Inhibitors of the hydrolyzing activity

of ATPase showed a much higher inhibitory effect on the reduced than on the oxidized form of the enzyme. This higher affinity for the reduced form would lead to a more positive standard reduction potential of ATPase.

SANTIAGO and LÓPEZ-MORATALLA (13) have recently suggested a model to explain the ATP synthesis at a molecular level through the conjoint operation of a pair of iron atoms undergoing a redox cycle between two potentials depending on the presence of ligands ATP or ADP. It was also suggested that the same catalytic site constituted by a set of two iron atoms could also catalyze the hydrolysis of ATP under appropriate conditions (13).

An examination of the chemical structure of all the compounds tested and capable both of interfering with ATP synthesis and of modifying the rate of ATP hydrolysis led us to think that a possible mechanism of action could be considered. It is clear that all those compounds might form coordination bonds with the atoms of iron of the catalytic site of ATPase through unshared pairs of electrons.

It would be then easily understood that the formation of a coordination bond between the iron atoms of the suggested catalytic site of ATPase (13) and any of these agents would affect not only the process of ATP synthesis but also the exchange reactions, reactions which are considered to be part of the machinery of oxidative phosphorylation (3). As suggested by the model referred above (13) the synthesis of ATP which requires the presence of the bidentate ligands ADP and Pi would be clearly hindered by any extraneous ligand interfering with the formation of the bidentate ligands (fig. 1). Since, in order to display the hydrolytic activity, it would suffice the presence of ATP as a bidentate ligand forming a coordination bond with each of the two irons, it is clear that the actual hydrolytic reaction would still be feasible, although modified by the presence of extraneous ligands.

A possible interpretation of the effect of activators or inhibitors of the hydrolytic activity of ATPase could be based on the relative electronegativity of them as ligands of the atoms of iron in ATPase. We refer here to the term electronegativity in the sense of electron attracting or electron releasing forces on the atom participating in the coordination bond with iron, forces due to resonance or inductive effects of the rest of the molecule of the ligand. The hydrolytic activity of the enzyme is known to be enhanced by reduction (8); the reduction

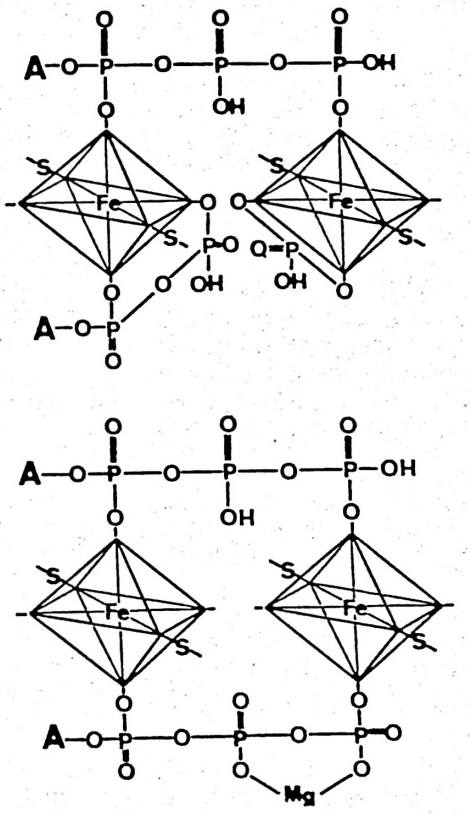
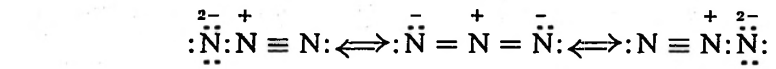
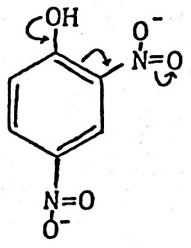


Fig. 1. *Catalytic site of ATPase with ligands committed to ATP synthesis (upper part), or to ATP hydrolysis (lower part). A stands for adenosine.*

of ATPase would imply the transformation of the atoms of Fe^{+++} into Fe^{++}. This would also suggest that ligands of iron which might allow the electrons shared in the coordination bond to be displaced preferentially towards the atom of iron, mainly when in the form of Fe (III), should behave as activators. On the contrary, ligands attracting the electrons more strongly than the atoms of iron, mainly when in the form of Fe (II), should behave as inhibitors of ATPase activity. These considerations would also explain why ligands allowing the electrons to be more displaced towards the atom of iron should have a much higher affinity for the oxidized form of ATPase, revealed by a more potent activating effect. However, ligands with a tendency to displace the electrons preferentially towards themselves should have a higher affinity for the reduced form of the enzyme, manifested by a more potent inhibitory effect on this form. The chemical structures of the different compounds affecting. ATase activity allows the interpretation given above. We will examine in the first place the structure of azide, one of the inhibitors. The azide may exist in different resonant forms.



It would be easy to understand that the existence of the positive charge on the azide would displace the electrons of N — Fe (II) towards the atom of nitrogen. The activator 2,4-dinitrophenol would bind the atom of iron through the oxygen with the negative charge of the $—NO_2$ in ortho position with the —OH group. It seems that the arrangement of these functional groups should permit the displacement of electrons of the oxygen with the negative charge towards the atom of Fe (III).



At the same time the displacement of electrons towards the ligand atom in the case of inhibitors, or towards the atom of iron in the case of activators, offers a reasonable interpretation of the results obtained, in the sense that inhibitors had a higher affinity towards the reduced form of ATPase, whereas activators had a much higher affinity towards the oxidized form of ATPase.

The mechanism of action of uncouplers and of inhibitors of oxidative phosphorylation could be envisaged in the light of the findings now being reported. Uncouplers, which at the same time are activators of the hydrolytic activity of ATPase, would facilitate the flow of electrons in a similar way to how ADP exerts the respiratory control (1), i.e., by making the standard reduction potential more negative (13). When the uncoupler is bound to the active site, the reduced form of the enzyme would constitute an unproductive energized form which would donate electrons to an acceptor without synthesizing ATP; coordination bonds of iron to form the bidentate ligands of ADP and of Pi may be occupied by the uncoupler.

The inhibitors of oxidative phosphorylation, which at the same time behave as inhibitors of the hydrolytic activity of ATPase, would inhibit the electron flow by making the standard reduction po-

tential of the enzyme more positive, thus hindering the isopotential transfer of electrons at the more negative end of the corresponding gap of potential; under the physiological operation of the respiratory chain, the gaps of potential would be closed by the redox cycle of ATPase with the concomitant synthesis of ATP (13).

Resumen

Se ensaya el efecto de una serie de desacoplantes e inhibidores de la fosforilación oxidativa sobre la actividad hidrolítica de las formas oxidada y reducida de la ATPasa aislada y unida a la membrana mitocondrial. Los desacoplantes (2,4-dinitrofenol, dicumarol), que son también activadores de la actividad hidrolítica ATPasa, son más potentes activadores de la forma oxidada del enzima; por el contrario, los inhibidores de la fosforilación oxidativa (oligomicina, azida y amital) tienen más potente efecto inhibidor sobre la actividad hidrolítica de la ATPasa en su forma reducida. La F_1-ATPasa, que es insensible a la oligomicina en la forma oxidada del enzima, llega a ser sensible a la oligomicina en su forma reducida. Una interpretación de los resultados permite sugerir un mecanismo unificante de la acción de estos diferentes compuestos sobre la síntesis y la hidrólisis del ATP catalizadas por la ATPasa mitocondrial.

References

1. CHANCE, B. and WILLIAMS, G. R.: *Adv. Enzymol.*, **17**, 65-134, 1956.
2. EBEL, R. E. and LARDY, H. A.: *J. Biol. Chem.*, **250**, 191-196, 1975.
3. ERNSTER, L. and LEE, C. P.: *Ann. Rev. Biochem.*, **33**, 729-788, 1967.
4. FISKE, C. N. and SUBBAROW, Y.: *J. Biol. Chem.*, **66**, 375-400 1925.
5. HOGEBOOM, G. H.: In «Methods in Enzymology» (S. I. Colowick and N. O. Kaplan, eds.), Vol. I, Academic Press, New York, 1955, pp. 16-19.
6. LAMBETH, D. O. and LARDY, H. A.: *Eur. J. Biochem.*, **22**, 355-363, 1971.
7. LARDY, H. A., JONHSON, D. and MCMURRAY, W. C.: *Arch. Biochem. Biophys.*, **78**, 587-597, 1958.
8. LÓPEZ-MORATALLA, N., SANTIAGO, E., IRIARTE, A. J. and LÓPEZ-ZABALZA, M. J.: *Rev. esp. Fisiol.*, **34**, 473-476, 1978.
9. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
10. MTCHELL, P.: *Nature*, **191**, 144-148, 1961.
11. PARSON, D. F., WILLIAMS, G. R. and CHANCE, B.: *Ann. N.Y. Acad. Sci.*, **137**, 643-666, 1966.
12. PULLMAN, M. E., PENEFSKY, H. S., DATTA, A. and RACKER, E.: *J. Biol. Chem.*, **235**, 3322-3329, 1960.
13. SANTIAGO, E. and LÓPEZ-MORATALLA, N.: *Rev. esp. Fisiol.*, **34**, 477-480, 1978.
14. SANTIAGO, E., LÓPEZ-MORATALLA, N., HUAMAN, J., LÓPEZ-ZABALZA, M. J. and IRIARTE, A. J.: *Rev. esp. Fisiol.*, **34**, 481-490, 1978.
15. SLATER, E. C.: *Nature*, **172**, 975-979, 1953.
16. SLATER, E. C.: In «Methods in Enzymology» (R. W. Estabrook and M. E. Pullman, eds.), Vol. X, Academic Press, New York, 1967, pp. 48-57.
17. RACKER, E.: *Biochem. Biophys. Res. Commun.*, **10**, 435-439, 1963.