MOLECULAR PHARMACOLOGY, 11, 166-173

Antibiotics as Tools for Metabolic Studies

XVIII. Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase

JOHN B. SUSA[1] AND HENRY A. LARDY

Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

(Received July 2, 1974)

SUMMARY

SUSA, JOHN B. & LARDY, HENRY A. (1975) Antibiotics as tools for metabolic studies. XVIII. Inhibition of sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.*, **11**, 166-173.

Antibiotics that inhibit oxidative phosphorylation or the uncoupler-induced mitochondrial ATPase activity were tested as inhibitors of rat brain ($Na^+ + K^+$)-dependent ATPase. Peliomycin, oligomycin B, and rutamycin in micromolar concentrations were found to be potent inhibitors of the ($Na^+ + K^+$)-dependent ATPase. At millimolar concentrations venturicidin X, venturicidin, and, to a lesser extent, ossamycin were also inhibitory. Even at high concentrations, aurovertin, A20668B (leucinostatin), and A23871 (efrastatin) were not inhibitory. All the antibiotics that were effective inhibitors of the over-all reaction had negligible effects on the K^+-dependent phosphatase activity in the absence of Na^+, with *p*-nitrophenyl phosphate as substrate. Inhibition by antibiotics could be relieved by washing. The Mg^{++} concentration was found to be important in regulating the degree of inhibition. The ATP-ADP exchange reaction characteristic of the ($Na^+ + K^+$)-dependent activity was stimulated by the inhibitors. The pattern of inhibition by the antibiotics tested suggests that the mitochondrial uncoupler-induced ATPase and the ($Na^+ + K^+$)-dependent ATPase possess some mechanistic similarity.

INTRODUCTION

The membrane-bound ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) and the uncoupler-induced mitochondrial ATPase have a number of common properties. They can couple the hydrolysis of ATP to the transport of cations across the membranes in which they are located. They are both capable of catalyzing an ATP-ADP exchange reaction. Under some conditions they can generate ATP from ADP and inorganic phosphate. Both require phospholipids for full complement of their activities. Finally, when included in the membrane, both are sensitive to the antibiotic oligomycin.

The oligomycin sensitivity of these two enzyme systems has been studied extensively (1-3). Since the initial observation that oligomycin inhibits the ($Na^+ + K^+$)-dependent ATPase (4), oligomycin has been used to help elucidate the mechanism of action of this enzyme system (3, 5). It therefore became of interest to study the

This investigation was supported by Grant AM10-334 from the National Institutes of Health and by the American Cancer Society.

[1] Postdoctoral Fellow of the National Institutes of Health.

effects on $(Na^+ + K^+)$-dependent ATPase of antibiotics, such as aurovertin, peliomycin, ossamycin, and venturicidin, that are known to inhibit the mitochondrial ATPase (6–8).

METHODS

Deoxycholate–sodium iodide-extracted microsomal $(Na^+ + K^+)$-dependent ATPase was prepared from rat brain by the method of Robinson (9), except that all enzyme preparations also contained 1.5 mM dithiothreitol. Aliquots of the final enzyme suspension were quick-frozen at liquid nitrogen temperature and stored at −25°. No appreciable loss of activity was observed even after 1 month of storage, by which time all samples had usually been consumed. All the data reported were obtained with the deoxycholate–sodium-iodide extracted enzyme preparation.

$(Na^+ + K^+)$-dependent ATPase activity was determined in terms of the release of P_i by the method of Parvin and Smith (10). The assay system used was that described by Robinson (11), which consists of 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 10 mM KCl, 90 mM NaCl, and microsomal protein in a 1.0-ml total volume. The reaction was started by adding enzyme and incubated for 8 min at 37° with gentle shaking. P_i production was linear with time under these conditions. The addition of up to 5 μl of an ethanol–dimethylformamide (3:1) solution had no effect on the activity of the enzyme; consequently all inhibitors were introduced in 5 μl of this solvent. The Mg^{++}-dependent ATPase activity (activity in the absence of Na^+ and K^+) was measured concurrently and subtracted from the total activity to give the $(Na^+ + K^+)$-dependent activity.

In those experiments in which the ATPase activity was determined in terms of the liberation of $^{32}P_i$ the same assay system was used, but with tracer amounts of [γ-^{32}P]ATP present. The $^{32}P_i$ was extracted by using the Parvin and Smith method, and aliquots of the 1-butanol phase, containing the phosphomolybdovanadate complex, were counted in a liquid scintillation counter using a toluene-ethanol scintillation fluid containing 700 ml of toluene, 300 ml of absolute ethanol, 4.0 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (12). [γ-^{32}P]ATP was prepared by the method of Penefsky (13) and converted to the Tris salt as described by Ulrich (14).

The K^+-dependent phosphatase activity of the $(Na^+ + K^+)$-dependent ATPase was determined by measuring the amount of *p*-nitrophenol produced from the hydrolysis of *p*-nitrophenyl phosphate (11, 15). The assay system contained 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate (Tris salt), 10 mM KCl, and microsomal protein in a final volume of 1 ml. The assay was carried out at 37° for 15 min with gentle shaking. The release of *p*-nitrophenol was linear with time under these conditions. The activity in the absence of K^+ was subtracted from activity in the presence of K^+ to obtain the K^+-dependent phosphatase activity.

The ATP-ADP exchange reaction of the $(Na^+ + K^+)$-dependent ATPase was assayed as described by Fahn *et al.* (16). The assay system contained 50 mM Tris-HCl (pH 7.8), 2.5 mM $MgCl_2$, 5.0 mM ATP (Tris salt), 1.5 mM [^{14}C]ADP (Tris salt, 14,000 cpm), 90 mM NaCl, microsomal protein, and up to 5 μl of inhibitor in ethanol-dimethylformamide. The reaction was started by adding enzyme and run for 15 min at 37° with gentle shaking. The reaction was stopped by immersing the reaction tubes in a boiling water bath for 2 min. Aliquots were removed and spotted on diethylaminoethyl cellulose paper which had been spotted with 10 μl each of 1 mM ATP, ADP, and AMP carrier. The chromatograms were run and the nucleotides separated as described by McClure *et al.* (17).

The reversibility of inhibition by the various antibiotics was assessed as described by Robinson (11).

Protein was determined by the Lowry method on trichloracetic acid-precipitated microsomal protein with bovine serum albumin as standard (18).

ATP, ADP, and *p*-nitrophenyl phosphate, all as the Tris salts, were purchased from Sigma Chemical Company.

Chromatographically pure antibiotics were the generous gifts of the following: oligomycin B, Professor F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison; rutamycin, A20668B (leucinostatin), and A23871 (efrastatin), Dr. Robert Hamill, Eli Lilly & Company; peliomycin and ossamycin, Dr. H. Schmitz, Bristol-Myers Company; aurovertin, Dr. C. L. Baldwin, Pitman Moore Division of Dow Chemical Company; venturicidin, venturicidin X, and narbomycin, Dr. W. Keller-Schierlein, E.T.H., Zurich; pikromycin, Dr. Hans Brockmann, Organic Chemistry Institute, University of Göttingen.

Because of the variability in the specific activity of the various enzyme preparations, ranging from 1.9 to 4.6 μmoles of P_i liberated per milligram of protein per minute, the $(Na^+ + K^+)$-dependent ATPase activity is expressed relative to the activity of concurrent control reactions, defined as 1.0.

RESULTS

Among the antibiotics used, peliomycin was the most effective inhibitor of the $(Na^+ + K^+)$-dependent ATPase (Fig. 1). Inhibition of ATPase activity by 50% occurred at 2 μM peliomycin, 4 μM oligomycin B, or 7 μM rutamycin. Venturicidin X, venturicidin, and ossaymcin were also inhibitory, but only at much higher concentrations. At approximately 0.5 mM venturicidin X, ATPase activity was inhibited 50%, while only 35% inhibition was attained with this concentration of venturicidin. Ossamycin at 0.5 mM inhibited only 15%. Even at 0.5 mM concentrations, the other antibiotics were found to inhibit very poorly, if at all, and can be considered noninhibitory. As was the case with inhibition by oligomycin, total inhibition of the $(Na^+ + K^+)$-dependent ATPase could not be attained; instead inhibition appeared to reach a maximal level of 75–90% (see ref. 11).

The nature of the inhibition by peliomycin, rutamycin, venturicidin, and venturicidin X was studied in greater detail. Prior incubation of inhibitor with enzyme for as long as 30 min had no additional

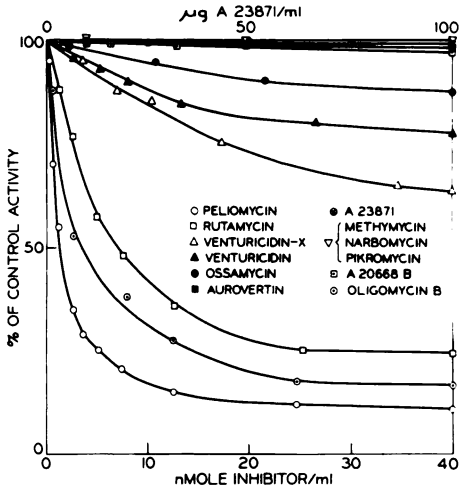

FIG. 1. *Inhibition of $(Na^+ + K^+)$-dependent ATPase by antibiotics*

Activity was determined in 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, and 10 mM KCl, with antibiotic and microsomal protein in a final volume of 1.0 ml at 37°. The peliomycin, oligomycin B, and rutamycin studies were carried out with 78 μg of microsomal protein per milliliter. The venturicidin, venturicidin X, and ossamycin studies contained 91 μg of microsomal protein per milliliter. The remainder of the studies were carried out with 65 μg of microsomal protein per milliliter. Concentrations of antibiotics are expressed as micromolar units except for A23871, which is given as micrograms per milliliter because its molecular weight is not known.

effect on the activity, regardless of the omission of the various components of the assay system. Washing the inhibited enzyme with buffer reversed the inhibition (Table 1).

Although the ATPase activity was inhibited by peliomycin, rutamycin, and high concentrations of the venturicidins, the K^+-dependent phosphatase activity was inhibited only slightly by peliomycin and rutamycin, and not at all by venturicidin and venturicidin X (Fig. 2).

The ATP-ADP exchange reaction catalyzed by the $(Na^+ + K^+)$-dependent ATPase has been shown to be stimulated by oligomycin (16). Peliomycin and rutamycin at low concentrations were found to be as effective as oligomycin in stimulating the exchange rate. The venturicidins also stimulated the rate of exchange, but even high concentrations did not stimulate as

TABLE 1

Reversibility of antibiotic inhibition of $(Na^+ + K^+)$-dependent ATPase.

Incubations were performed in the presence and absence of inhibitor in the standard medium scaled up to 5.0 ml with 465 μg of microsomal protein. After incubation for 8 min at 37°, a 0.5-ml aliquot was removed and assayed for P_i. To the remaining assay mixture 30 ml of ice-cold 50 mM Tris-HCl (pH 7.8), which was also 1% in ethanol–dimethylformamide (3:1) and 1.5 mM dithiothreitol, was added, and the mixture was centrifuged at 32,000 × *g* for 25 min. The pellet was washed again as described and then resuspended in 2.5 ml of 10 mM Tris-HCl (pH 7.3) with 1.5 mM dithiothreitol. This suspension, with 310 μg of microsomal protein remaining, was then assayed for activity by the standard ATPase assay.

Inhibitor	Initial activity	Inhibition	Activity after washing	Inhibition
	μmoles P_i/mg/min	%	*μmoles P_i/mg/min*	%
None (control)	2.93		1.54	
Peliomycin (3 μM)	0.44	85	1.25	19
Rutamycin (3 μM)	1.14	61	1.22	21
Venturicidin X (200 μM)	1.50	49	1.33	14
Venturicidin (200 μM)	2.17	26	1.37	11

much as peliomycin and rutamycin did (Table 2).

The effect of the inhibitors on the ATPase activity was dependent on the concentration of magnesium and ATP present. The antibiotics were all more effective at low Mg^{++} concentrations than at the higher concentrations (Table 3). The ratio of Mg^{++} to ATP also affected the inhibition by the antibiotics. The inhibition produced in the presence of 3 μM Mg^{++} and 3 μM ATP could be relieved by increasing the Mg^{++} concentration to 3 mM. Inhibition was completely relieved, in the presence of peliomycin or rutamycin, but only partially in the presence of the venturicidins.

The activation of the ATPase by monovalent cations was affected by the antibiotics that were inhibitory. Hill plots of kinetic data revealed that, in addition to lowering the V_{max}, the antibiotics influenced the $K_{0.5}$ values (the concentrations of metal ion which produced half-maximal activation) (Figs. 3 and 4). Peliomycin and rutamycin decreased the $K_{0.5}$ for Na^+ and had little effect on the $K_{0.5}$ for K^+. The venturicidins, on the other hand, increased the $K_{0.5}$ for both Na^+ and K^+.

DISCUSSION

Table 4 summarizes the inhibitory effects of the antibiotics used in this study on

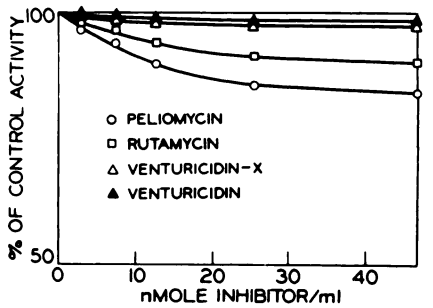

FIG. 2. *Effect of antibiotics on K^+-dependent phosphatase activity of $(Na^+ + K^+)$-dependent ATPase*

The 1.0-ml assay mixture contained 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate (as the Tris salt), 10 mM KCl, antibiotic, and 88 μg of microsomal protein at 37°.

TABLE 2

Effect of antibiotics on $(Na^+ + K^+)$-dependent ATPase activity and ATP-ADP exchange reaction

$(Na^+ + K^+)$-dependent ATPase activity was measured in 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as Tris salt), 90 mM NaCl, 10 mM KCl, and 90 μg of microsomal protein in 1.0 ml at 37°. The ATP-ADP exchange reaction was determined in 50 mM Tris-HCl (pH 7.8), 2.5 mM $MgCl_2$, 5 mM ATP (as Tris salt), 1.5 mM[^{14}C]ADP (as Tris salt), 90 mM NaCl, and 84 μg of microsomal protein in 1.0 ml at 37°.

Inhibitor	$(Na^+ + K^+)$-ATPase[a]	Exchange reaction[b]
	% control	
None	100	100
Peliomycin (15 μM)	15	162
Rutamycin (15 μM)	37	156
Venturicidin X (200 μM)	45	142
Venturicidin (200 μM)	70	117

[a]Control specific activity was 3.9 μmoles of P_i per minute per milligram of protein.

[b]Control specific activity was 3.2 μmoles of P_i per minute per milligram of protein.

TABLE 3

Effect of varying concentrations of ATP and Mg^{++} on inhibition of (Na^+ + K^+)-dependent ATPase

In the first three experiments the (Na^+ + K^+)-dependent ATPase activity was determined by measuring the liberation of $^{32}P_i$ from [γ-^{32}P]ATP as described under METHODS. In the remaining five experiments the standard assay was used. The activities are expressed relative to controls without inhibitor. The reaction mixture had a 1-ml total volume with 66 μg of protein and the amount of inhibitor indicated.

$MgCl_2$ mM	ATP mM	Control activity μmoles P_i/mg/min	Percentage of control activity			
			Peliomycin (5 μM)	Rutamycin (10 μM)	Venturicidin X (500 μM)	Venturicidin (500 μM)
0.003	0.003	0.4	56	63	32	33
3	0.003	1.6	97	100	68	63
3	3	2.9	35	48	45	70
0.1	3	0.9	18	26	24	36
0.3	3	1.03	24	32	30	47
1.0	3	1.55	29	40	39	56
3.0	3	3.40	40	51	48	73
6.0	3	3.56	48	53	49	79

the (Na^+ + K^+)-dependent ATPase and the mitochondrial uncoupler-induced ATPase. Oligomycin, rutamycin, peliomycin, and ossamycin are qualitatively alike in their inhibition of oxidative phosphorylation and the uncoupler-induced ATPase in mitochondria (7). On a weight basis oligomycin, rutamycin, and peliomycin are equally effective inhibitors, while ossamycin is one-half to one-fourth as effective. Venturicidin has inhibitory properties similar to those of this group, but is less effective. Venturicidin X, the aglycone of venturicidin, is a more potent inhibitor of the mitochondrial ATPase, but is similar to venturicidin in all other respects.[2]

The effect of venturicidin and venturicidin X on the (Na^+ + K^+)-dependent ATPase appears to be similar to their effect on the mitochondrial ATPase. Although they inhibit, they are effective only at much higher concentrations than oligomycin. The facts that inhibition by the venturicidins at low ATP and Mg^{++} concentrations is relieved by high Mg^{++} concentrations, but not completely, as for peliomycin, and that the $K_{0.5}$ values for Na^+ and K^+ are increased by the venturicidins and decreased by peliomycin indicate that there is some difference in the way in which the venturicidins and the oligomycins affect the enzyme. A23871 is an inhibitor of the purified mitochondrial F_1 ATPase (19). The antibiotic A20668B (leucinostatin) is qualitatively similar to oligomycin as an inhibitor of the mitochondrial ATPase, but probably acts at a different site (20). The antibiotics methymycin, narbomycin, and pikromycin are macrolides structurally similar to oligomycin, but do not inhibit oxidative phosphorylation or the mitochondrial ATPase.[2]

Rutamycin is a homologue[3] of oligomycin B and has similar effects on mitochondria (8). The fact that the effects of rutamycin and oligomycin on the (Na^+ + K^+)-dependent ATPase system are identical has been reported (21).

The structure of peliomycin has been reported to be different from that of oligomycin (22). The structure reported, however, is open to question, since it does not agree with other reports of the molecular weight and elemental composition (23, 24). A more recent preliminary study of the structure of peliomycin by techniques used to elucidate the structure of oligomycin disclosed no structural differences between oligomycin B and peliomycin.[3] The similar effects on the two ATPase systems by these two inhibitors suggest that they are indeed very closely related, but the much greater inhibition of (Na^+ + K^+)-dependent ATPase by peliomycin as compared to oligomycin B indicates that these two antibiotics may be stereoisomers.

All the inhibitors that inhibit both enzyme systems are macrolides. Some function is obviously common to oligomycin, peliomycin, rutamycin, and, to a lesser extent, the venturicidins. Whether this common function is ring size, juxtaposition of functional groups, or conformation is not known. Inturrisi and Titus (21) suggested that the size of the ring may be a critical

[2] H. A. Lardy, unpublished observations.

[3] H. Schnoes, unpublished observations.

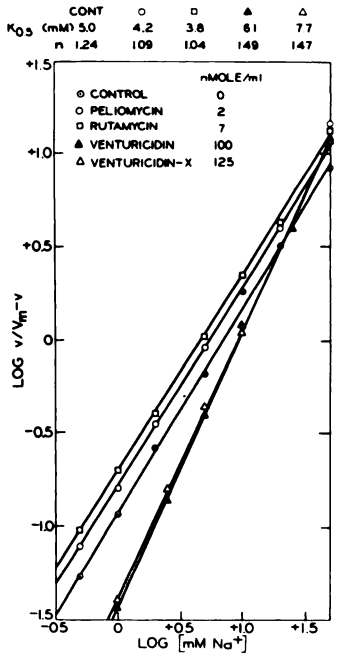

FIG. 3. *Effect of inhibitors on activation of (Na⁺ + K⁺)-dependent ATPase by NaCl*

Activity in the presence of inhibitors was determined by the standard method with 10 mM KCl and varying concentrations of NaCl in the presence of 74 μg of microsomal protein. The data are presented in the form of a Hill plot, with velocities expressed relative to that in the complete assay medium. The values for n and $K_{0.5}$ are included.

factor in determining whether the macrolide is or is not an inhibitor of the (Na⁺ + K⁺)-dependent ATPase. Our observations support this hypothesis. The venturicidins have a ring size of 20 atoms and are in an intermediate position between oligomycin, with 26 ring atoms, and narbomycin, with 14 ring atoms. They are also intermediate in inhibitory potency between narbomycin, which does not inhibit, and oligomycin, which is a good inhibitor. Substitution of carbohydrate moieties on the macrolide ring plays a role in determining potency, as seen by comparing venturicidin with venturicidin X, the aglycone, which is more effective. Ossamycin, which is also a glycosidic macrolide of unknown structure, is an even poorer inhibitor than venturicidin.

Besides the common, previously described properties of the (Na⁺ + K⁺)-dependent ATPase and the mitochondrial uncoupler-induced ATPase—(a) coupling of transport of cations to ATP hydrolysis, (b) catalyzing ATP-ADP exchange, (c) under appropriate conditions, generating ATP from ADP and P_i, and (d) requiring phospholipid for complete expression of activity—the two enzyme systems manifest similar responses to a number of antibiotics. This is suggestive of some similarity between their mechanisms of action.

The mechanism of the (Na⁺ + K⁺)-dependent ATPase was proposed by Albers (25) to proceed as follows:

$$E + \text{ATP} \underset{\text{Oligomycin}}{\overset{Na^+,\, Mg^{++}}{\rightleftharpoons}} E\text{-ATP} \rightleftharpoons E_1 \sim \text{P}$$

$$+ \text{ADP} \xrightarrow{\times} E_2\text{-P} \xrightarrow{K^+} \text{E} + \text{P}_i$$

The $E_1 \sim$ P is a high-energy phosphorylated intermediate which can react with ADP to form ATP, while the E_2-P is a low-energy intermediate which does not react with added ADP but is hydrolyzed in

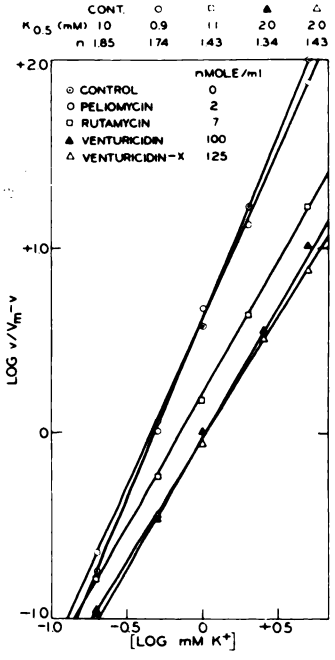

FIG. 4. *Effect of inhibitors on activation of (Na⁺ + K⁺)-dependent ATPase by KCl*

Conditions were the same as described in Fig. 3, except that the concentration of NaCl was 90 mM and that of KCl was varied in the presence of 65 μg of microsomal protein.

TABLE 4

Inhibition of ATPases by antibiotics

Inhibitor	I_{50}	
	$(Na^+ + K^+)$-ATPase	Mitochondrial ATPase
	nmoles/mg protein	
Peliomycin	20	0.08
Rutamycin	70	0.08
Oligomycin B	40	0.17
Venturicidin X	5000	0.26
Venturicidin	5000 (I_{50})	0.31
Ossamycin	5000 (I_{15})	0.29
Aurovertin	NI[a]	0.26[b]
Leucinostatin	NI	0.23
Efrastatin	NI	2[c]
Methymycin	NI	NI
Narbomycin	NI	NI
Pikromycin	NI	NI

[a] NI = no inhibition.

[b] Purified F_1. (mitochondrial ATPase).

[c] Micrograms of efrastatin per milligram of purified F_1.

the presence of K^+. The difference between these two intermediates is suggested to be conformational. $E_I \sim P$ undergoes a Mg^{++}-dependent conformational transition, and it is this conformational transition that is inhibited by oligomycin (26) and the antibiotics used in this study.

Robinson (11) proposed a scheme in which two enzyme forms, E_I and E_{II}, exist:

$$\begin{array}{ccccc} E_I + ATP & \xrightarrow{Na^+, Mg^{++}} & E_I\text{-}P & \overset{K^+}{\rightleftharpoons} & E_I + P_i \\ \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ E_{II} + ATP & \xrightarrow{Na^+, Mg^{++}} & E_{II}\text{-}P & \overset{K^+}{\rightleftharpoons} & E_{II} + P_i \end{array}$$

These two forms are in equilibrium, with one inherently more active than the other. Oligomycin in opposition to Mg^{++} could exert its effect by shifting the equilibrium from the more active to the less active form at one or more steps in the sequence. Both the Albers and Robinson schemes propose that conformational changes play key roles in the reaction sequence and that these changes are inhibited by the oligomycin class of antibiotics.

That conformational changes play key parts in the mechanism of oxidative phosphorylation has been proposed by Boyer (27, 28). Briefly, this hypothesis states that in biological membranes energy capture, transmission, and use for energy-linked reductions, active transport, photosynthetic phosphorylation, and oxidative phosphorylation occur with the involvement of conformational changes of catalytic proteins. The central role played by a conformational change in oxidative phosphorylation is described by Boyer *et al.* (29). Invoking a molecular explanation of oxygen exchange reactions, these workers suggested that the energy input for ATP synthesis is used to bring about release of previously formed, tightly bound ATP from the catalytic site.

It is clear that the $(Na^+ + K^+)$-dependent ATPase and the mitochondrial uncoupler-induced ATPase are similarly affected by the antibiotics reported in this communication. As more is learned about these two enzyme systems, it is becoming apparent that they may possess several common characteristics, even at the mechanistic level.

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