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Flux-dependent increase in the stoichiometry of charge translocation by mitochondrial ATPase/ATP synthase induced by almitrine

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After studying the effects of almitrine, a new kind of ATPase/ATP synthase inhibitor, on two kinds of isolated mammalian mitochondrion, we have observed that: (1) Almitrine inhibits oligomycin-sensitive ATPase; it decreases the ATP/O value of oxidative phosphorylations without any change in the magnitude of $\Delta\bar{\mu}_{H^+}$. (2) Almitrine increases the mechanistic H^+/ATP stoichiometry of ATPase as shown by measuring either (i) the extent of potassium acetate and of potassium phosphate accumulation sustained by ATP utilisation, or (ii) the electrical charge/ATP (K^+/ATP) ratio at steady-state of ATPase activity. (3) Rat liver mitochondria are at least 10-times more sensitive to almitrine than beef heart mitochondria. (4) The change in H^+/ATP stoichiometry induced by almitrine depends on the magnitude of the flux through ATPase. The inhibitory effect of almitrine on ATPase/ATP synthase complex, as a consequence of such an H^+/ATP stoichiometry change, is discussed.

Introduction

We have previously shown that almitrine is a new kind of energy transduction inhibitor acting on yeast mitochondria ATP synthase [1]. The interest for studying this drug is based on the peculiarities of its effects: (i) almitrine inhibits ATPase activity and decreases the ATP/O ratio of oxidative phosphorylation without any change in the magnitude of $\Delta\bar{\mu}_{H^+}$; (ii) no direct effect on respiratory chain can be evidenced; and (iii) the higher the ATP synthesis and respiratory fluxes, the larger is the decrease in the ATP/O ratio induced by almitrine.

From these results, it has been concluded that almitrine acts essentially on the ATP synthase complex. However, its effects are very different from those of classical ATP synthase inhibitors, such as oligomycin or aurovertin D, which decrease ATP synthesis and respiratory rates in such a way that the ATP/O ratio remains constant [1]. The mechanism by which almitrine decreases the ATP/O ratio is different from those invoked to explain the action of protonophoric [2] or non-protonophoric [3,4] uncouplers, since in contrast to almitrine, uncouplers stimulate both ATPase activity and state 4 respiration. Two hypotheses have been suggested to explain the mechanism whereby almitrine decreases the ATP/O ratio (see Ref. 1): either (i) it increases the intrinsic uncoupling of H^+-ATPase also called slip [5,6] or (ii) it changes the real H^+-ATP ratio of ATPase/ATP synthase.

In this paper, the mechanism of action of almitrine is further investigated, leading to the observation that the effects previously described for yeast mitochondria are also available both for beef heart and rat liver mitochondria. Moreover, by studying H^+-ATPase, we show that almitrine increases specifically the H^+/ATP ratio in a flux-dependent manner. The inhibitory effect of almitrine on the ATPase/ATP synthase complex, as a possible consequence of the H^+/ATP ratio change, is discussed.

Abbreviations: BHM, beef heart mitochondria; CCCP, carboxyl cyanide *m*-chlorophenylhydrazone; ΔpH, transmembrane difference of pH; $\Delta\psi$, transmembrane difference of electrical potential; $\Delta\bar{\mu}_{H^+}$, transmembrane difference of proton electrochemical potential; HPLC, high-performance liquid chromatography; RLM, rat liver mitochondria; TET, triethyltin chloride.

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Materials and Methods

Beef heart mitochondria

Mitochondria were isolated from slaughterhouse bovine hearts according to slight modifications of a mechanical method [7]. Removed hearts were placed in a cold medium containing 0.25 M sucrose, 20 mM KCl, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4) during transportation (about 15 min) from the slaughterhouse to the laboratory. Other experimental differences were the following: the homogenate was obtained in a medium containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.4); mitochondria were washed twice and suspended finally at a concentration of 80–90 mg protein/ml in a medium similar in composition to that of the homogenate.

Rat liver mitochondria

Male Wistar rats (250 g) were killed by cervical dislocation and their livers were rapidly put into an ice-cold isolation medium containing 225 mM sucrose, 1 mM EGTA and 10 mM Tris-HCl (pH 7.2). Mitochondria were isolated in the same medium as described in Ref. 8. Mitochondrial pellet was finally resuspended in isolation medium at a protein concentration of 40–50 mg/ml. Protein concentration was estimated by the biuret method by using bovine serum albumin as a standard.

Respiration assay and ATP / O measurement

The oxygen consumption rate was measured polarographically at 30 °C by using a Clark electrode connected to a microcomputer giving an on-line display of rate values. Two different respiratory media were used. (i) For beef heart mitochondria (medium (A), 0.25 M sucrose, 1 mM EGTA, 5 μM rotenone, 3 mM Tris-P_i, 10 μM RbCl, 0.2 nmol valinomycin/mg protein and 10 mM Tris-HCl (pH 7.2) was used. (ii) For rat liver mitochondria (medium B), isolation medium (see above) supplied with 5 μM rotenone, 10 mM Tris-P_i, 10 μM RbCl and 0.2 nmol valinomycin/mg protein (pH 7.2) was used.

ATP/O ratio stoichiometries with succinate as a respiratory substrate were determined from the average of phosphorylation rates vs. respiratory rates in three different systems: ATP production was monitored either (i) by glucose 6-phosphate formation in the presence of a nonlimiting amount of hexokinase, 1 mM $MgCl_2$ and 10 mM glucose, or (ii) by ^{32}P incorporation in adenine nucleotides as described in Ref. 9, or (iii) by nucleotide measurement using HPLC. These three methods gave identical results, indicating that neither ATPase contaminating activity nor adenylate kinase activity significantly changed the ATP synthesis rate estimations.

Isocratic separation by HPLC

Aliquots (10 μl) were directly injected into the HPLC system (Beckman) and nucleotides separated on a Spheri 5 Amino,Aminopropyl column (0.4 M KH_2PO_4 (pH 4.5), 1 ml · min^{-1} flow rate). Nucleotides were detected at 259 nm.

Measurements of $\Delta\psi$ and ΔpH

Matrix space was determined by using [^{3}H]water and inner-membrane impermeable [^{14}C]sucrose, $\Delta\psi$ and ΔpH by distribution of ^{86}Rb (in the presence of valinomycin) and [^{14}C]DMO (5,5-dimethyl[2-^{14}C]oxazolidine-2,4 dione), respectively [10]. A slight correction was made for removing the undissociated acid concentration, according to Ref. 11. Routinely, after equilibration (3 min), mitochondria were separated from the medium by rapid centrifugation (30 s) through a silicone oil layer (silicone AR 200 fluid).

Determination of K^+/ATP

The technique for measuring electrical charge/ATP (q^+/ATP) ratios at steady state was as described by Murphy and Brand [12]. This method is based on strict equality of the rate of charge efflux from mitochondria, catalyzed by a proton pump and the involved carriers (i.e., ADP/ATP exchange) to the charge influx, catalyzed by valinomycin, at a given steady-state. Beef heart mitochondria (8 mg protein · ml^{-1}) were incubated in 7 ml of the following medium: 0.25 M sucrose, 1 mM EGTA, 3 mM $MgCl_2$, 5 μM rotenone, 4.2 mM potassium acetate or 10 mM potassium phosphate, 5 mM ATP, 16 nmol valinomycin · ml^{-1} and 10 mM Tris-HCl (pH 7.2) at 30 °C. The potassium concentration was monitored with a potassium-sensitive electrode. At a given steady state, ATPase activity was measured and the charge efflux catalyzed by this pump was determined from the initial rate of K^+ efflux following the addition of an inhibitor; either 100 μM oligomycin or 100 μM TET, an organotin [13]. Since the potassium-sensitive electrode gave a logarithmic response to K^+, the initial rate of efflux was determined from linearized replots as described in [12]. Almitrine (see Fig. 1) was a gift from Servier Laboratory, France.

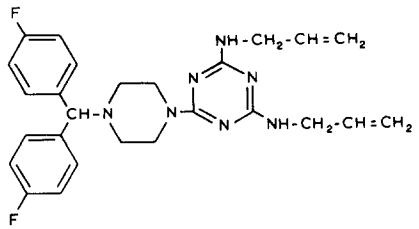

Fig. 1. Chemical structure of almitrine.

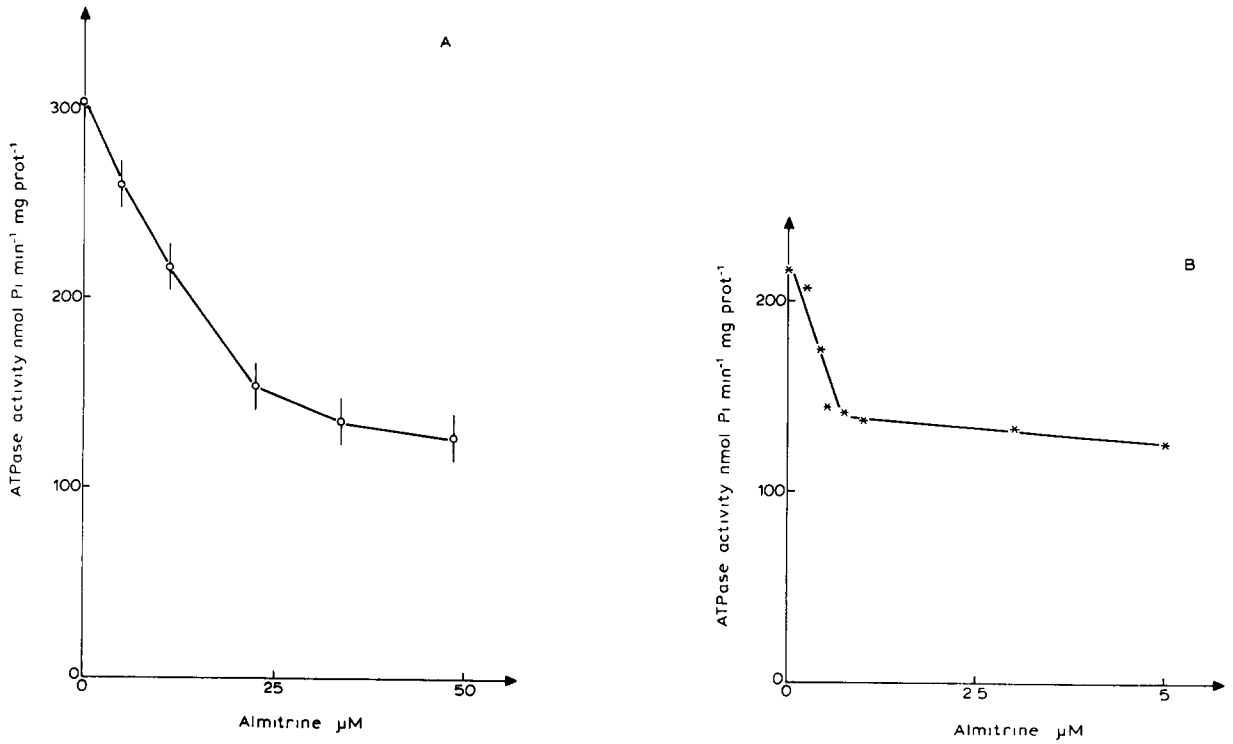

Fig. 2. Effect of almitrine on oligomycin-sensitive ATPase. Mitochondria (3 mg protein) from beef heart (medium A) and rat liver (medium B) were incubated in 3 ml of the following media. Medium A contained 80 mM sucrose, 85 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.2), 1 mM EGTA, 1 µM CCCP and in the absence or in the presence of different concentrations of almitrine. Medium B contained 125 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.2) and 1 µM CCCP. The reaction was started by addition of 1 mM ATP. The oligomycin-sensitive ATPase was the difference between the rates of ATP hydrolysis observed in the absence and in the presence of 10 $\mu g \cdot ml^{-1}$ oligomycin.

Determination of ATPase activity

ATP hydrolysis was assayed under particular conditions as described in the legends to the figures. At defined times, 0.25 ml of the mitochondrial suspension was pipetted into vials containing $HClO_4$ (final concentration 10% w/v) and 0.1 M EDTA. As indicated above, ATP, ADP and AMP were measured in the protein-free neutralised extracts by HPLC and P_i was measured as according to Summer [14].

Results

ATPase activity and oxidative phosphorylation efficiency

Fig. 2 shows that almitrine inhibits oligomycin-sensitive ATPase induced by protonophore addition, with half-inhibition at 1 µM with rat liver mitochondria and at 20 µM with beef heart mitochondria, respectively. Inhibition was never higher than 60%, whatever the almitrine concentration. The very low ATPase activity

TABLE I

Almitrine effect on oxidative phosphorylation supported by succinate

Mitochondria were suspended in 1.5 ml of respiratory medium A (BHM) or B (RLM) (see Materials and Methods) and supplemented with 5 mM succinate, in the absence or in the presence of almitrine. ATP synthesis and respiratory rates in state 3 were obtained with 1 mM ADP, uncoupled respiration with 1 µM CCCP. $\Delta\bar{\mu}_{H^+}$ was obtained during state 3 respiration as indicated above.

Experimental conditions	Respiratory rate natom O/min per mg protein		ATP synthesis rate nmol ATP/min per mg protein	ATP/O	δpH (mV)	Δψ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)
	state 3	uncoupled					
BHM	164±21	178±32	254±38	1.53±0.22	23.5±1.5	152.5±11	176±12
+ almitrine 22 µM	164±16	176±22	119±16	0.74±0.12	22.5±2.5	152.5± 6	175± 8
RLM	220±25	246±16	363±37	1.65±0.18	15 ±2	169 ±12	184±12
+ almitrine 0.5 µM	220±30	250±24	209±26	0.95±0.16	14 ±2	173 ±15	187±16

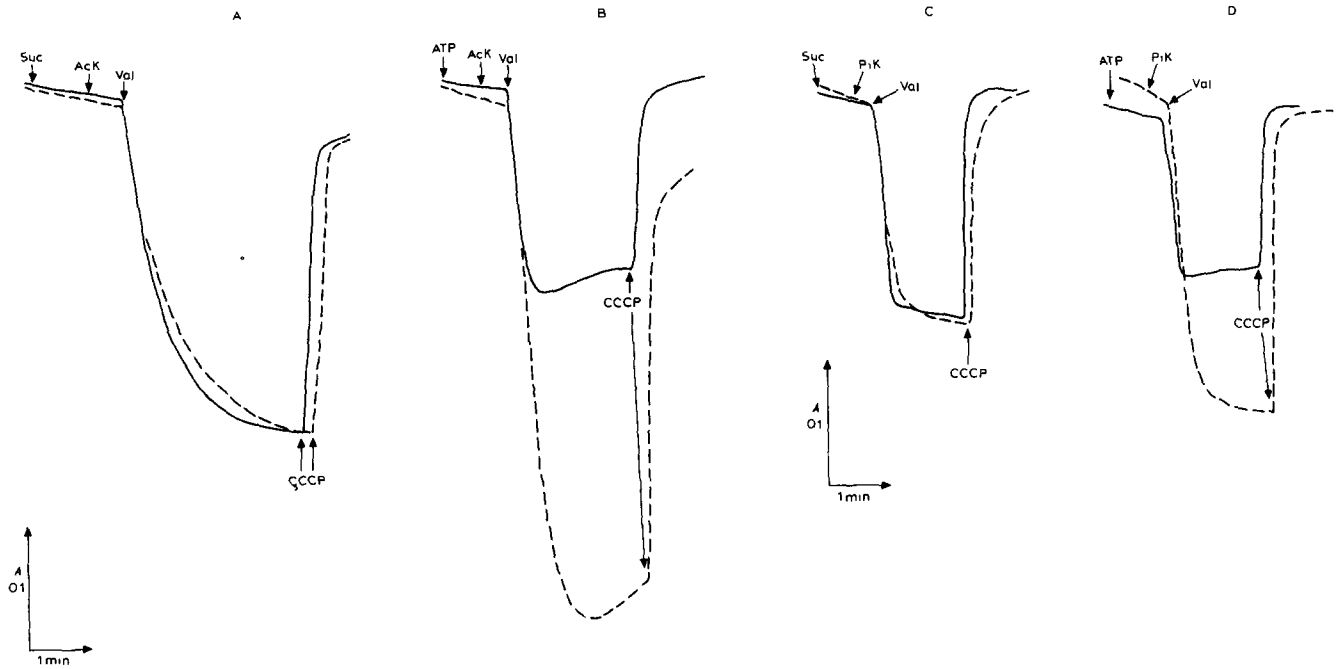

Fig. 3. Effect of almitrine on the energy-linked swelling of mitochondria in potassium acetate and phosphate salt. Swelling was monitored at 546 nm by using an Eppendorf photometer. BHM (1 mg protein) were incubated in 1 ml of the following basal medium: 0.25 M sucrose, 1 mM EGTA, 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.2). As indicated on the curves, either 5 mM succinate or 2 mM ATP, and either 10 mM potassium phosphate or 4.2 mM potassium acetate, plus 2 nmol valinomycin and 1 μM CCCP in the absence (———) or in the presence (- - - - -) of 60 μM almitrine, were added.

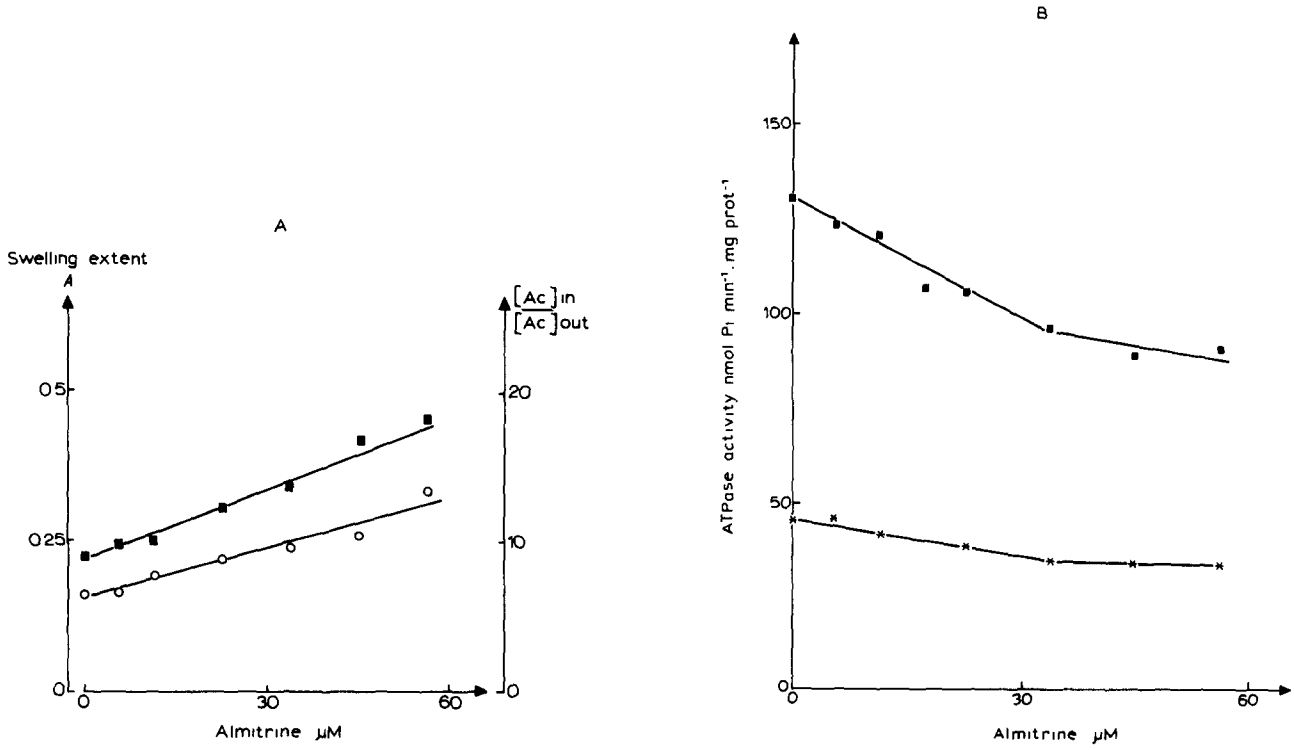

Fig. 4. Dependence of the swelling extent, acetate accumulation and ATPase activity on almitrine concentration. Swelling measurements (○) were obtained as indicated in the legend to Fig. 3B. Volume and acetate accumulation (■) estimations were as described in Materials and Methods. ATPase activity was measured either when mitochondrial volume increased (■) or when swelling was maximum and stable (★).

observed in the absence of CCCP or valinomycin was not modified by almitrine (not shown). It is worth noting that rat liver mitochondria (RLM) are at least 20-times more sensitive to almitrine than beef heart mitochondria (BHM).

As previously shown for yeast mitochondria [1], almitrine does not inhibit state 3 or uncoupled respiration of mammalian mitochondria (Table I). However, a large decrease in the ATP synthesis rate and consequently a drop in the ATP/O ratio are seen, whereas the $\Delta\bar{\mu}_{H^+}$ is not affected (Table I). Since almitrine does not increase state 4 respiration (not shown but see Ref. 1), the decrease of the ATP synthesis rate is not related to the uncoupling effect of this drug.

Energy-linked swelling

In order to investigate the possibility that almitrine modifies the q^+/ATP ratio of the ATPase/ATP synthase complex, we tested the action of this drug on an ATP consuming process. A very simple system is the energy-dependent potassium salt uptake, which can be followed by valinomycin-induced mitochondrial swelling [15–17]. The H^+ efflux catalysed by an H^+ pump sustained a $\Delta\psi$-dependent K^+ and a ΔpH-dependent acetate or phosphate accumulation. Therefore, energy supply (redox or ATP) was converted into salt accumulation.

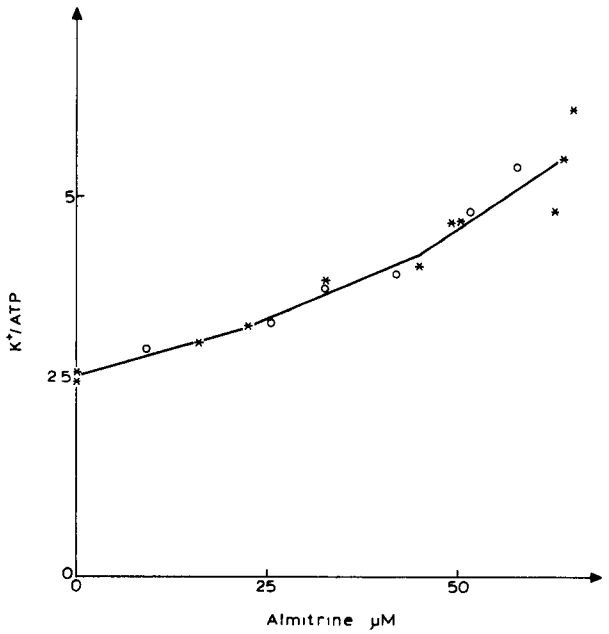

Fig. 5. Effect of almitrine on K^+/ATP stoichiometry of mitochondrial ATPase. Experimental procedure was as described in Materials and Methods. In the absence or in the presence of different concentrations of almitrine, J_{ATP} was determined from samples taken at the steady state of swelling and J_{in} was estimated from the rate of K^+ efflux following the addition either of 100 µM oligomycin (★) or of 100 µM TET (○). The rate of efflux was determined from linearized replots calculated from the logarithmic response of a potassium-sensitive electrode.

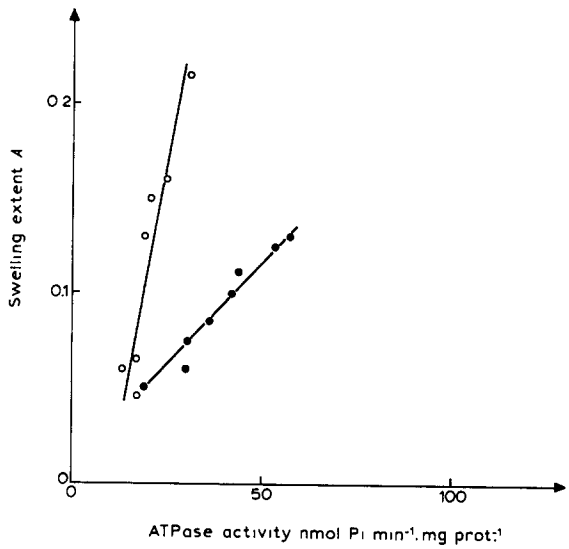

Fig. 6. Dependence of the relationship between swelling extent and ATPase activity on limitation of ATP supply in the absence or in the presence of almitrine. Experimental conditions were as described in the legend to Fig. 3B with various concentrations (between 0 and 1 µM) of carboxyatractyloside and without (●) or with (○) 56 µM almitrine.

Fig. 3A and C shows that almitrine does not change the swelling extent of beef heart mitochondria either in K^+ acetate or K^+ phosphate when the proton pump is the respiratory chain. As expected, the swelling is completely reversed by uncoupler addition. The energy-linked swelling supported by oligomycin-sensitive ATPase is largely increased by almitrine (Fig. 3B and D) although the effect is more pronounced with acetate. Similar results were obtained with rat liver mitochondria (not shown). The fact that almitrine did not modify the swelling linked to respiratory chain activity ruled out the possibility of an unspecific effect of this drug on the properties of the inner membrane. Increase in the swelling extent induced by almitrine, when the driving force is generated by ATPase, was a dose-dependent process (Fig. 4A). Moreover, there was a direct relationship between the swelling extent and the accumulation of acetate (Fig. 4A). ATPase activity was measured during the two phases of swelling: i.e., either when the volume of mitochondria was increased (phase 1) or when the swelling was maximum and stable (phase 2). Whatever the phase considered, almitrine slightly decreased ATPase activity (Fig. 4B). Thus, in the presence of almitrine, a lower rate of ATP hydrolysis leads to the maintenance of a greater transmembrane salt gradient indicating a better efficiency of this proton pump.

Determination of K^+/ATP stoichiometry

The electrical charge/ATP (K^+/ATP) ratio was measured at steady state by using the technique described by Murphy and Brand [12,18]. At a given steady

state, it is assumed that the rate of charge efflux from mitochondria (J_O), catalysed by a proton pump and its associated solute transports, is strictly equal to the rate of charge influx (J_i). In the presence of valinomycin, ATP induced potassium acetate accumulation as described above. At the steady state, the charge efflux is due to activities of both H^+-ATPase and the ADP/ATP carrier. By this latter carrier, one charge is transferred by one ATP consumed. So that $J_O = (n+1)J_{ATP}$ when n is the H^+/ATP stoichiometry of ATPase. This efflux counterbalances exactly the H^+-influx (H^+-leak). Oligomycin or TET addition, by blocking H^+-efflux, induces a K^+ efflux equal to an H^+-leak. Consequently, the initial rate of K^+ efflux reflects J_O. By measuring J_{ATP} and J_{K^+} under these conditions, we showed that K^+/ATP value increased when almitrine concentration was raised (Fig. 5). In experiments performed without almitrine, the K^+/ATP value was 2.7 ± 0.3; therefore, the stoichiometry of the ATPase proton pump can be estimated to be 1.7 ± 0.3. A 2-fold increase in this ratio was observed for about 45 μM almitrine. Similar results were obtained when potassium phosphate was used instead of potassium acetate.

Flux-dependent effect of almitrine

It has previously been shown in yeast mitochondria that the effect of almitrine on the ATP/O ratio depended on the respiratory and ATP synthesis flux values: the larger the fluxes, the lower the ATP/O value is [1]. In order to test this possibility in the ATP-induced swelling system, energy supply was limited by addition of different amounts of carboxyatractylate. ATP hydrolysis rates during phase 2 (see above) and the extent of swelling were compared in the absence or in the presence of 56 μM almitrine. Fig. 6 clearly shows that the effects of almitrine on both ATPase activity and the swelling extent decreased as a function of carboxyatractylate concentration.

Discussion

As reported in this work, the effects of almitrine, previously described in yeast mitochondria [1], were also observed both in beef heart and rat liver mitochondria Indeed, almitrine acts as an ATPase inhibitor but, in contrast to other ATPase inhibitors (i.e., oligomycin), it induces an ATP/O decrease at constant $\Delta\bar{\mu}_{H^+}$ [1]. Since the flux through the respiratory chain was not enhanced, the ATP/O decrease must be related to a change in the H^+/ATP stoichiometry of the ATP synthase. As first proposed by Pietrobon et al. [5.6], the degree of coupling between the H^+-flux and a chemical reaction can vary according to the conditions in which the H^+ pump works. For instance, some protons would be translocated by the ATP synthase-catalysed process without concomitant ATP synthesis ('proton slip'). It has been reported that some drugs, like general anaesthetics, increase the H^+ pump slipping [4].

A change in proton pump stoichiometry could be interpreted as an increase in slipping if coupling efficiency between the proton flux and the chemical reaction is decreased, whatever the direction of the reaction (i.e., ATP synthesis and ATP hydrolysis) [4].

An experimental proof of real mechanistic change in stoichiometry is given by observation of the same degree of stoichiometry in the forward as in the reverse chemical reactions. This is the case for the almitrine effect, since this drug acts by decreasing the reaction yield of ATP synthesis (measured by the ATP/O ratio) and by increasing that of ATP hydrolysis (evidenced by swelling experiments and K^+/ATP measurements). To our knowledge, this is the first time such an observation has been made.

Although RLM were more almitrine-sensitive than BHM, the different effects of this drug were similar in both types of mitochondrion. In other words, almitrine concentration, required to decrease ATP synthesis rate, increased the ATP-dependent salt accumulation. Consequently, the fact that almitrine acts in a similar way on both types of mitochondrion could mean that the difference in sensitivity is only due to a difference in accessibility to their ATPases.

The mechanism whereby almitrine induces a change in the H^+/ATP ratio remains an open question. It is generally proposed that ATP synthase works through a reaction cycle in which conformational transitions of the complex corresponding to the energy span occur (see Refs. 19–21 and 22 for review). In this view, the energy-dependent conformational cycle is coupled to a proton flux through the complex. As a working hypothesis, one may propose that almitrine modifies at least a given state of the enzyme into the cycle in such a way that the energy span between this state and the following is increased. Indeed, in the presence of almitrine, more energy is necessary to synthesize 1 mol of ATP, at a given $\Delta\bar{\mu}_{H^+}$, and reciprocally, more energy is delivered by the use of 1 mol of ATP.

Whatever the mechanism whereby almitrine acts on the complex, it is likely that inhibition of ATPase/ATP synthase activity is linked to the H^+/ATP stoichiometry change. Such a dependence between the increase in the H^+/ATP ratio and inhibition of ATPase activity could be explained in the context of the above-mentioned hypothesis, since increase in energy span between two steps of the cycle slows down the probability of a transition between the two steps.

As shown on Fig. 6, no difference in the swelling extent, in the absence or in the presence of almitrine, can be found when ATPase activity is equally depressed by lowering the ATP supply. The flux-dependent H^+/ATP change can be easily understood by hypothesizing two populations of enzyme in the presence of

almitrine: one modified and one unmodified by the drug. At low flux, the unmodified ATPase works essentially because of its high probability of transition (see above); at high flux, both populations are implicated in the flux.

In conclusion, almitrine appears as a new powerful tool for studying the mechanism of coupling between proton flux and ATP synthesis (or hydrolysis) at the molecular level.

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