PRELIMINARY NOTES

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Possible regulatory function of a mitochondrial ATPase inhibitor in respiratory chain-linked energy transfer

In 1963 PULLMAN AND MONROY[1] reported the purification of an ATPase inhibitor from beef-heart mitochondria. The inhibitor, a highly trypsin-sensitive protein of a molecular weight of approx. 15000, potently suppressed the ATPase activity of both submitochondrial particles ("N-particles") and purified F_1, but had no effect on the F_1-restored oxidative phosphorylation catalyzed by the particles. It was suggested that the ATPase inhibitor may act as a "respiratory control factor", but its functional role in the respiratory chain-linked energy-transfer system remained undefined.

In this paper it is shown that the ATPase inhibitor efficiently blocks ATP-driven energy-transfer reactions, such as succinate-linked NAD^+ reduction, nicotinamide–nucleotide transhydrogenase, and an increase in 8-anilino-naphthalene-1-sulfonate (ANS) fluorescence[2] (an indicator of membrane energization[3]), while it leaves the same reactions when driven by the respiratory chain unaffected. It also does not inhibit, and may even stimulate, oxidative phosphorylation. The results suggest that the ATPase inhibitor may function as a directional regulator of the respiratory chain-linked energy-transfer system.

The experiments were performed with "EDTA particles", *i.e.*, particles derived from "heavy" beef-heart mitochondria by sonication in a medium containing EDTA as described by LEE AND ERNSTER[4]. These particles possess only weak capacities for oxidative phosphorylation and for ATP-driven succinate-linked NAD^+ reduction[5–7], nicotinamide–nucleotide transhydrogenase reaction[5–7] and ANS fluorescence increase[8], but these can be substantially increased by the addition of an appropriate amount of oligomycin. EDTA particles exhibit an oligomycin-sensitive ATPase activity which is not stimulated by trypsin[9]; in this respect they are different from the "S-" and "U-particles" described by RACKER[10] or the phosphorylating "Mg^{2+}–ATP-particles"[9].

ATPase inhibitor was prepared from "light" beef-heart mitochondria as described by PULLMAN AND MONROY[1]. The yield and purity of the preparation were approximately the same as those reported by these authors. To obtain maximal inhibitory effect, the ATPase inhibitor, diluted with 0.25 M sucrose containing 0.3 mM imidazole sulfate (pH 6.75), was mixed with an equal volume of particles in 0.25 M sucrose (10 mg particle protein per ml) and preincubated for 10 min at 30°, all according to the method of PULLMAN AND MONROY[1]. Aliquots of the mixture were then removed and used for the assay of various enzyme activities. Alternatively, the particles could be reisolated by centrifugation of the preincubation mixture, without loss of the effect of ATPase inhibitor (*cf.* ref. 1), and used for the enzyme assays.

Abbreviation: ANS, 8-anilino-naphthalene-1-sulfonate.

ATPase activity was assayed[11] by linking the reaction to the pyruvate kinase and lactate dehydrogenase systems and using the oxidation of NADH, measured spectrophotometrically at 340 nm, as a measure of the amount of ADP formed. Succinate-linked NAD^+ reduction[12] and nicotinamide–nucleotide transhydrogenase[13] activities, the latter coupled to the glutathione reductase system, were assayed as previously described, in both cases spectrophotometrically at 340 nm. Oxidative phosphorylation was measured in the presence or absence of hexokinase and glucose, by recording O_2 uptake with a Clark oxygen electrode and determining the amount of P_i esterified, after approx. 70 % of the O_2 was consumed, by the isotope distribution method[14]; or, alternatively, by supplementing the system with hexokinase, glucose, glucose-6-phosphate dehydrogenase, and $NADP^+$, and recording the appearance of NADPH spectrophotometrically at 340 nm (*cf.* ref. 15). In the latter case, the system also contained AMP, in order to minimize the rate of adenylate kinase, reducing it to less than 10 % of the rate of oxidative phosphorylation. P/O was estimated by allowing the system to become anaerobic and dividing the increase in NADPH concentration due to oxidative phosphorylation by the concentration of oxygen originally present in the medium. ANS fluorescence was measured at 470 nm, with an excitation wavelength of 366 nm. The composition of the assay systems is specified in the table and figure legends.

As shown in Fig. 1, the ATPase inhibitor suppressed the ATPase, ATP-driven succinate-linked NAD^+ reduction and ATP-driven nicotinamide–nucleotide transhydrogenase reactions (the two latter reactions measured in the presence of an optimal amount of oligomycin) with approximately equal efficiencies; half-inhibition occurred at about 5 μg inhibitor per mg particle protein. In contrast, the respiratory chain-driven succinate-linked NAD^+ reduction and nicotinamide–nucleotide transhydrogenase reactions were unaffected by the ATPase inhibitor. Similarly, the ATPase inhibitor blocked the ATP-induced increase in ANS fluorescence (measured in the presence of an optimal amount of oligomycin), without affecting the succinate (*plus* oligomycin)-induced fluorescence increase (Fig. 2).

Oxidative phosphorylation with NADH or succinate as substrate, measured in the presence of an optimal amount of oligomycin, was likewise not suppressed by the ATPase inhibitor (Table I), in agreement with earlier findings of PULLMAN AND MONROY[1] with the F_1-induced oxidative phosphorylation of "N-particles". As expected, a decrease in P/O occurred when hexokinase and glucose were omitted, due to the hydrolysis of ATP, and this effect was counteracted by the ATPase inhibitor (Table I, Expt. 2). With succinate as substrate, the ATPase inhibitor repeatedly caused an increase in P/O, in spite of the presence of an ATP-trapping system (Table I, Expts. 3 and 4)—a phenomenon that will require further investigation. The ATPase inhibitor did not replace oligomycin in promoting oxidative phosphorylation (*cf.* Table I, Expt. 1), nor did it alter the respiratory rate in either the absence or presence of oligomycin (not shown), leaving the extent of oligomycin-induced "respiratory control" unaffected. These findings thus fail to support the suggestion[1] that the ATPase inhibitor may function as a "respiratory control factor".

It is evident from the data presented above that the ATPase inhibitor, besides blocking ATPase, also suppresses ATP-driven energy-transfer reactions. PULLMAN AND MONROY[1] have noticed an inhibition of the P_i–ATP exchange reaction, catalyzed by F_1-supplemented "N-particles", when ATPase inhibitor was present, but this effect

was overcome, and even turned into a stimulation, by the addition of an excess of ATP. They have also found[1] that F_1–ATPase inhibitor complex, when added to "N-particles", supported ATP-driven succinate-linked NAD^+ reduction equally as

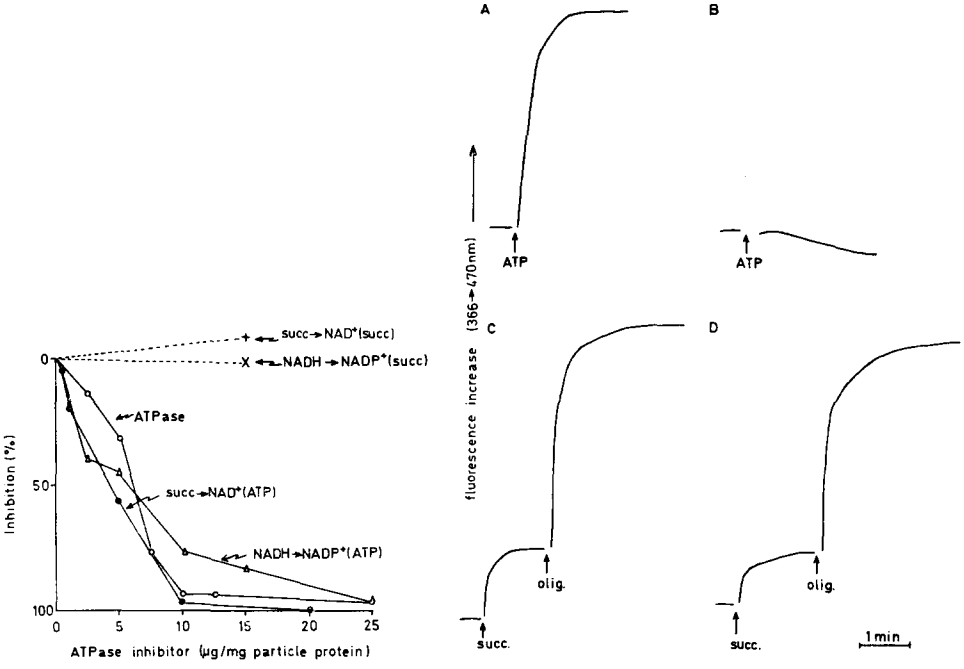

Fig. 1. Effect of ATPase inhibitor on various energy-transfer reactions. The assay systems contained, in a final volume of 1 ml: *ATPase*: 10 mM Tris–acetate (pH 7.5), 30 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 10 µg pyruvate kinase, 2.5 mM phosphoenolpyruvate, 15 µg lactate dehydrogenase, 0.18 mM NADH, 1 µM rotenone and 0.02 mg particle protein. The activity in the absence of ATPase inhibitor was 3.04 µmoles ADP formed per min per mg protein. *ATP-driven succinate-linked NAD^+ reduction* (succ → NAD^+ (ATP)): 50 mM Tris–acetate (pH 7.5), 170 mM sucrose, 5 mM succinate, 0.15 mM NAD^+, 3 mM ATP, 2 mM $MgSO_4$, 2 mM KCN, 0.3 mg particle protein and 0.1 µg oligomycin per mg particle protein. The activity in the absence of ATPase inhibitor was 56.3 nmoles NAD^+ reduced per min per mg protein. *Respiratory chain-driven succinate-linked NAD^+ reduction* (succ → NAD^+ (succ)): 50 mM Tris–acetate (pH 7.5), 170 mM sucrose, 5 mM succinate, 5 mM NAD^+, 0.3 mg particle protein and 2 µg oligomycin per mg particle protein. The activity in the absence of ATPase inhibitor was 4.5 nmoles/min per mg protein. *ATP-driven transhydrogenase* (NADH → $NADP^+$ (ATP)): 50 mM Tris–acetate (pH 7.5), 170 mM sucrose, 0.2 mM NADH, 0.15 mM $NADP^+$, 1 mM oxidized glutathione, 2 µg glutathione reductase in 1% bovine serum albumin, 1 µM rotenone, 2 mM KCN, 5 mM $MgSO_4$, 4.5 mM ATP, 0.1 µg oligomycin per mg protein, and 0.2 mg particle protein. The activity in the absence of ATPase inhibitor was 141 nmoles $NADP^+$ per min per mg protein. *Respiratory chain-driven transhydrogenase* (NADH → $NADP^+$ (succ)): 50 mM Tris–acetate (pH 7.5), 170 mM sucrose, 0.2 mM NADH, 0.15 mM $NADP^+$, 1 mM oxidized glutathione, 2 µg glutathione reductase in 1% bovine serum albumin, 1 µM rotenone, 5 mM succinate, 0.2 mg particle protein and 2 µg oligomycin per mg particle protein. The activity in the absence of ATPase inhibitor was 214 nmoles $NADP^+$ reduced per min per mg protein. Temp., 30°.

Fig. 2. Effect of ATPase inhibitor on the energy-dependent enhancement of ANS fluorescence. The assay system contained, in a final volume of 3 ml, 10 mM Tris–acetate (pH 8.8), 170 mM sucrose, 20 µM ANS and 1 mg particle protein. In A and B, the medium also contained 2 mM $MgSO_4$ and 0.1 µg oligomycin per mg particle protein and, in C and D, 1.7 µM rotenone. Additions at the times indicated were: 2 mM ATP (A and B); 5 mM succinate and 5 µg oligomycin per mg protein (C and D). Temp., 24°. A and C: no ATPase inhibitor. B and D: 30 µg ATPase inhibitor per mg protein.

TABLE I

EFFECT OF ATPase INHIBITOR ON OXIDATIVE PHOSPHORYLATION

The assay system contained 170 mM sucrose, 0.4 mg (Expts. 1 and 2) or 0.8 mg (Expts. 3 and 4) particle protein, 50 mM Tris–acetate (pH 7.5), 3.2 mM P_i, 3 mM (Expt. 1), 5 mM (Expt. 2) or 1 mM (Expts. 3 and 4) ADP, 2 mM $MgSO_4$, 8 mM AMP (Expts. 3 and 4 only) and, when indicated, 1 mM NADH, 10 mM succinate, 0.1 μg oligomycin per mg protein, 0.1 mg (Expts. 1 and 2) or 0.2 mg (Expts. 3 and 4) hexokinase, 20 mM glucose, 9 μg glucose-6-phosphate dehydrogenase, and 1 mM $NADP^+$. The final volume was 3 ml (Expts. 1 and 2) or 1 ml (Expts. 3 and 4). The amount of ATPase inhibitor used in all experiments inhibited the ATPase activity of the same particles by more than 95%. In Expts. 3 and 4, the particles were reisolated from the preincubation with ATPase inhibitor before the assay (see text). In Expts. 1 and 2, the rate of O_2 consumption was measured polarographically, and P_i uptake was determined by the isotope distribution method[13]. In Expts. 3 and 4, the rate of P_i uptake was determined from the reduction of $NADP^+$, measured spectrophotometrically at 340 nm, and the rate of O_2 uptake was estimated by allowing the system to become anaerobic as indicated by the decline in $NADP^+$ reduction. Temperature, 30°.

Expt. No.	*Substrate*	*ATP-trapping system*	*Oligomycin*	*ATPase inhibitor (μg/mg particle protein)*	*P/O*
1	NADH	Hexokinase, glucose	−	0	0.13
			+	0	0.76
			−	30	0.08
			+	30	0.76
2	NADH	Hexokinase, glucose	+	0	0.53
		None	+	0	0.35
		None	+	30	0.49
3	Succinate	Hexokinase, glucose, glucose-6-phosphate dehydrogenase, $NADP^+$	+	0	0.19
			+	15	0.35
4	Succinate	Hexokinase, glucose, glucose-6-phosphate dehydrogenase, $NADP^+$	+	0	0.21
			+	15	0.32

well as did added F_1. This apparent discrepancy between their and our results may be explained in view of the fact, first suggested when the phosphorylation-restoring effect of oligomycin in EDTA particles was discovered[5], and subsequently demonstrated by SCHATZ *et al.*[16] with immunological techniques, that added F_1 may act not only by replacing a missing enzyme species but also by promoting the action of F_1 already present in the particles. Conceivably, this latter effect of F_1 is not abolished by the ATPase inhibitor, and thus the added F_1–ATPase inhibitor complex may have promoted the action of residual F_1 present in the particles used by PULLMAN AND MONROY[1] to mediate the ATP-driven reduction of NAD^+ by succinate.

It is noteworthy that the unidirectional effect of the ATPase inhibitor on energy-transfer reactions is the converse of that found with the antibiotic aurovertin, which has been shown to inhibit oxidative phosphorylation[17–21] but not ATPase[17,18] or ATP-driven ion translocation[19–21], succinate-linked NAD^+ reduction[7,22,23], and nicotinamide–nucleotide transhydrogenation[7,22,23]. Aurovertin forms a firm complex with F_1[24], similarly to the ATPase inhibitor[1], and recent evidence[25] suggests that it acts as a modifier of F_1, altering its relative affinities for ADP and ATP. Whether a similar though converse relationship holds for the ATPase inhibitor is the subject

of current studies in this laboratory. Whatever the precise mechanism, the present results strongly suggest that the mitochondrial protein, now known for 7 years[1] as the "ATPase inhibitor", may have the important functional role of a directional regulator of respiratory chain-linked energy transfer, controlling the back-flow of energy from ATP to the mitochondrial electron- and ion-transport systems.

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