ACRIFLAVINE: ANISOTROPIC INHIBITOR OF ENERGY TRANSDUCTION IN OXIDATIVE PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA

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1. Introduction

Recently we found that ethidium inhibited energy transduction in oxidative phosphorylation by acting on the outer side (C-side) of the inner mitochondrial membranes, perhaps by neutralizing negative charges created on this side. It had no inhibitory activity on the inner side (M-side) of the membranes [1]. We also showed that the energy-dependent binding of ethidium to the membranes of mitochondria is not due to electrophoretic transport down the membrane potential [2–6]. We proposed that this type of inhibitor should be called an 'anisotropic inhibitor of energy transduction' [1].

The present paper shows that the energization of inner mitochondrial membranes caused with either succinate or ATP induced an increase in the affinity of the membranes for acriflavine, which is a positively-charged amphipathic molecule. The present paper also shows that like ethidium, acriflavine inhibited energy transduction of oxidative phosphorylation in intact mitochondria, but not in sonicated submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [7–10]. Acriflavine incorporated inside the submitochondrial particles inhibited ATP synthesis in the particles. Addition of acriflavine to mitochondria energized with ATP or succinate caused ejection of protons into the suspending medium. The present study clearly shows that, like ethidium bromide, acriflavine acts as an anisotropic inhibitor of energy transduction in mitochondria.

2. Materials and methods

Acriflavine hydrochloride was purchased from Tokyokasei, Tokyo (Japan). Other reagents were as in [1].

Rat liver mitochondria were isolated by the method [11], as in [12], except that 0.25 M sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and 2 washings [13]. Submitochondrial particles were prepared by a modification of the method [14], as in [1]. Protein was estimated from the contents of cytochromes $a + a_3$ in intact mitochondria and submitochondrial particles, as in [13,15].

To measure binding of acriflavine to mitochondria, the mitochondria (1 mg protein/ml) were incubated for 5 min with a known concentration of acriflavine in the presence of 10 mM succinate (+ 2 μg rotenone) or 2 mM ATP (+ 1 μg antimycin A)/5 mM $MgCl_2$/ 2 mM EDTA/15 mM KCl/25 mM Tris/50 mM sucrose, at pH 7.4. Then the mixture was rapidly cooled to about 0°C and centrifuged at 8000 × g for 2 min in an Eppendorf, Model 3200, microcentrifuge, and the remaining dye was estimated by measuring its A_{452} with a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer.

The amount of [^{32}P]P_i-labeled substances was determined by the method [16] as modified [17].

3. Results

ATP decreased the fluorescence of acriflavine in

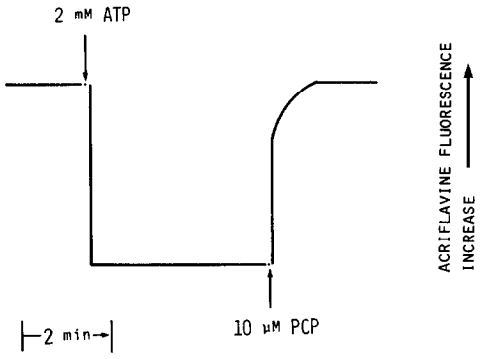

Fig.1. Effect of ATP on acriflavine fluorescence in anaerobic mitochondria. Rat liver mitochondria (1 mg protein/ml) were suspended in medium consisting of: 25 mM Tris; 50 mM sucrose; 5 mM $MgCl_2$; 2 mM EDTA; 15 mM KCl; 12 μM acriflavine. ATP, 2 mM, was added to anaerobic mitochondria induced with 10 mM succinate (+ 2 μg rotenone). Change in fluorescence of acriflavine was determined with a Hitachi, Model MPF 3, spectrofluorometer, using the wavelength of 452 nm for excitation and measuring fluorescence at 503 nm.

anaerobic mitochondria, and subsequent addition of 10 μM pentachlorophenol (uncoupler) reversed this effect (fig.1). Figure 2 shows that when intact mitochondria were energized with either succinate or ATP the amount of energy-dependent binding of acriflavine to the membranes increased to a saturation level with

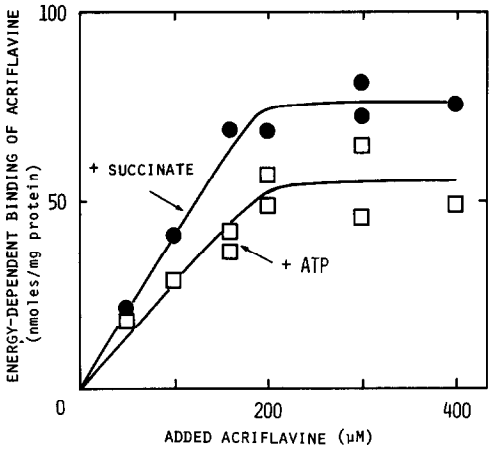

Fig.2. Energy-dependent binding of acriflavine to intact mitochondria. The conditions were as described in section 2. The amount of acriflavine bound to energized mitochondria at a dye concentration of 200 μM was 134 nmol/mg protein.

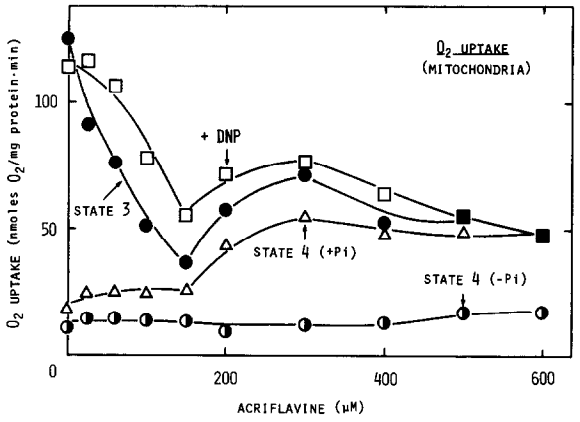

Fig.3. Effect of acriflavine concentration on oxygen uptake by intact mitochondria. Intact mitochondria (1 mg protein/ml) were preincubated for 5 min 25°C in the presence of: 10 mM succinate; 2 μg rotenone; 5 mM $MgCl_2$; 2 mM EDTA; 15 mM KCl; 50 mM sucrose; 25 mM Tris; the indicated concentration of acriflavine in final vol. 4.5 ml, pH 7.4 (state 4 ($-P_i$)), then 20 mM potassium phosphate (state 4 (+ P_i)), 200 μM ADP (state 3) and 50 μM 2,4-dinitrophenol (DNP) were added.

a concentration of about 200 μM acriflavine. Figure 3 shows that concentrations of up to 150 μM acriflavine inhibited ADP-stimulated respiration (state 3) in intact mitochondria. This inhibition was only partially released by addition of the uncoupler DNP (2,4-dinitrophenol) (fig.3). The inhibition was maximal about 3 min after addition of the dye to the mitochondrial suspension in the presence of succinate. At concentrations of above 150 μM it stimulated state 4 respiration in the membranes. This stimulation was dependent on the presence of inorganic phosphate and ADP (fig.3). Figures 2 and 3 clearly show that acriflavine does not inhibit electron transport of mitochondria but inhibits energy transduction in oxidative phosphorylation of the membranes, and suggest that it may inhibit the action of uncoupler. Figure 4 shows that 200 μM acriflavine completely inhibited ATP synthesis in intact mitochondria. Figures 2 and 4 show that the concentration of acriflavine required for saturation of energy-dependent binding of the dye on mitochondria approximately coincided with the amount of the dye required for complete inhibition of ATP synthesis in the membranes. Figure 4 also shows that acriflavine only

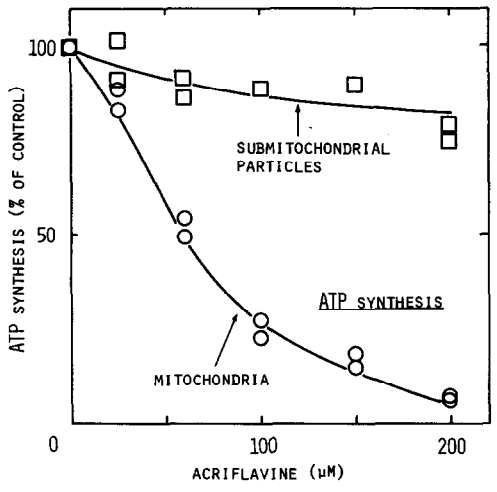

Fig.4. Effect of acriflavine concentration on ATP synthesis in intact mitochondria and submitochondrial particles. Intact mitochondria (1 mg protein/ml) were preincubated for 5 min at 25°C in the same medium as for fig.3 except that 0.5 mM ADP, 10 mM glucose, 0.1 mg hexokinase and various amounts of acriflavine were included in final vol. 1.5 ml, pH 7.4. Submitochondrial particles (1 mg protein/ml) were preincubated for 5 min at 25°C in the presence of: 10 mM succinate; 2 μg rotenone; 0.5 mM ADP; 10 mM glucose; 0.1 mg hexokinase; 3 mM $MgCl_2$; 1 mM EDTA; 5 mM GSH; 10 mM Tris; various amounts of acriflavine, in final vol. 1.5 ml, pH 7.4. The reaction was started by adding 30 μmol potassium phosphate, containing about 5×10^5 cpm $[^{32}P]P_i$, and was stopped 6 min later by adding 0.5 ml 40% trichloroacetic acid.

partially inhibited ATP synthesis in submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [9–12].

Table 1 shows that acriflavine incorporated inside the submitochondrial particles greatly inhibited ATP synthesis in the particles. Table 1 also shows that the dye incorporated inside the particles was not released into the medium even by washing the particles (144 000 × *g* for 30 min). Figure 4 and table 1 clearly show that acriflavine inhibited energy transduction in oxidative phosphorylation by acting on the C-side of the inner mitochondrial membranes, and that it had no inhibitory activity on the M-side of the membranes. These results also show that the inhibitory effect of the dye on ATP synthesis in intact mitochondria is not due to modification of the transport system for respiratory substrates, inorganic phosphate or adenine nucleotide. This explanation is also supported by the facts that energy-dependent binding of acriflavine to the mitochondria occurs with either respiratory substrates or ATP (fig.2).

Table 1
Effect of incorporation of acriflavine inside the submitochondrial particles on ATP synthesis in the particles

Submitochondrial particles	ATP synthesis (nmol ATP /mg protein · min)	ATP synthesis (%)	Incorporated acriflavine (nmol/mg protein)
Control particles	15.4	100	–
Particles containing acriflavine	3.3	21.4	180

Acriflavine was introduced into the submitochondrial particles as follows: the mitochondria (1 mg protein/ml) were incubated for 5 min at about 25°C in the same medium as for fig.3 except that 200 μM acriflavine was also added. The resulting suspension was rapidly cooled to about 0°C and then centrifuged at 18 000 × *g* for 10 min. The precipitate was used as the starting material for preparing submitochondrial particles as described in section 2. Control particles were obtained similarly, but without acriflavine treatment. The amount of acriflavine incorporated inside the particles was estimated from its absorbance spectrum using a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer. Other experimental conditions were as for fig.4

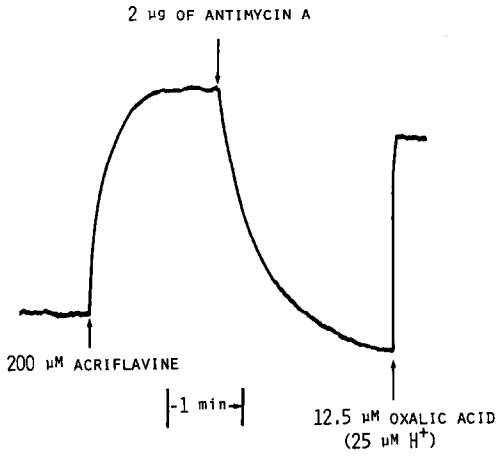

Fig.5. H^+-ejection from energized mitochondria caused by acriflavine. Intact mitochondria (1 mg protein/ml) were preincubated in the same medium as for fig.3 except that the concentrations of potassium chloride and Tris were 20 mM and 2 mM, respectively, in final vol. 3 ml, pH 7.1. Acriflavine, antimycin A and oxalic acid were added as indicated. The reaction was followed with a Hitachi-Horiba, Model F-7, expanded scale pH meter equipped with a Radiometer pH electrode (GK 2402C).

We also found that addition of acriflavine to mitochondria energized with succinate (fig.5) or ATP (data not shown) caused ejection of protons into the suspending medium.

4. Discussion

The present experiments showed that acriflavine inhibited energy transduction of oxidative phosphorylation in intact mitochondria but not in submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [7–10].

This sidedness of the inhibition could be explained by supposing that acriflavine penetrates the membranes, becoming concentrated inside the mitochondria (minus inside), and that it is extruded from the particles (plus inside) electrophoretically, i.e., in a membrane potential-dependent fashion [2–6]. Accumulation of this dye in the mitochondrial matrix inhibited ATP synthesis by acting on phosphorylative enzymes on the side of the membranes facing the matrix.

However, this explanation does not account satisfactorily for various observations, including the following:

(1) The concentration of acriflavine (200 μM) required to saturate the energy-dependent binding of the dye to mitochondria (fig.2) was much lower than the amount of potassium chloride (+ valinomycin) (20 mM) required for complete loss of the membrane potential [1].

(2) The saturation level of energy-dependent binding of acriflavine to mitochondria approximately corresponded with the amount of dye required to inhibit ATP synthesis in the membranes completely (fig.2,4).

(3) Acriflavine incorporated inside the submitochondrial particles was not released into the suspending medium through the membranes (table 1).

(4) Acriflavine incorporated inside the particles inhibited ATP synthesis in the particles, as it did in mitochondria (table 1, fig.2,4).

The present results clearly show that acriflavine inhibited energy transduction in oxidative phosphorylation by acting on the C-side of the inner mitochondrial membranes, and that it had no inhibitory activity on the M-side of the membranes. The results also show that acriflavine did not penetrate the inner mitochondrial membranes rapidly. Thus it is concluded that, like ethidium bromide [1], acriflavine acts as an anisotropic inhibitor of energy transduction in mitochondria.

Addition of acriflavine to mitochondria energized with succinate or ATP caused ejection of protons into the suspending medium. This finding could be explained as follows:

Acriflavine cation binds electrostatically to the negative charge known to be created on the C-side of the membrane [18] and then the proton drawn electrostatically to the negative charge is released into the suspending medium to maintain the electroneutrality of the medium.

This neutralization of the negative charge on the C-side by acriflavine could be the reason why acriflavine inhibits energy transduction [1].

Acknowledgements

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