ELUCIDATION BY IONOPHORES OF THE ΔpH CONTROL OF ANION DISTRIBUTION ACROSS THE MITOCHONDRIAL MEMBRANE

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1. Introduction

It is still matter of debate whether anions such as acetate, phosphate and the Krebs cycle intermediates distribute across the mitochondrial membrane according to the ΔpH [1, 2] or the membrane potential [3].

In an attempt to differentiate between these two possibilities, the distribution of anions across the mitochondrial membrane was studied in the presence of different ionophores [4, 5] which are expected to abolish either the membrane potential or/and the ΔpH. The results show that the anion distribution is controlled by the pH difference across the mitochondrial membrane.

2. Methods

Rat liver mitochondria were isolated as described by Klingenberg and Slenczka [6], using a medium consisting of 0.25 M sucrose, 1 mM EDTA and 20 mM tris-HCl, pH 7.3. The third wash and resuspension were carried out in 0.25 M sucrose at pH 7.4–7.5.

The distribution of added anions between the intra- and the extramitochondrial space was determined using labelled compounds, obtained from the Radiochemical center (Amersham, England). Mitochondria were incubated with the labelled anionic substrates in the presence of rotenone and oligomycin, under the conditions specified in the legends, and, after the equilibrium was reached, they were separated from the incubation mixture by rapid centrifugation in a microcentrifuge (Misco) [7]. The radioactivity in the extracts of the sediments and in the supernatants was measured in a scintillation counter (Tri-carb). ^{3}H-H_2O and ^{14}C-sucrose were added in parallel experiments to determine the total water of the pellet and the sucrose-permeable space, in order to account for the external anion in the pellet. This was subtracted from the total anion in order to obtain the internal anion concentration [1, 8].

The pH difference between the inner and outer mitochondrial phase (ΔpH) was determined according to the method described by Mitchell and Moyle [9]

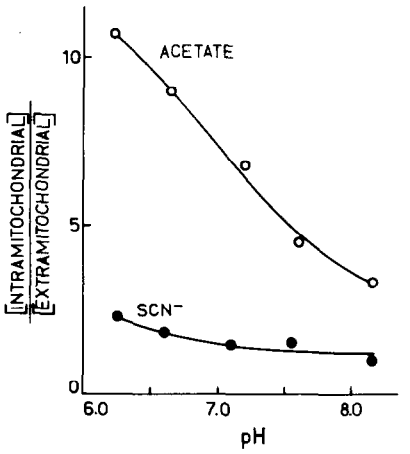

Fig. 1. pH Dependence of the distribution ratio of acetate and thiocyanate between the inner and outer mitochondrial phase. The incubation mixture contained 125 mM KCl and 20 mM tris-HCl, 2 μg rotenone, 10 μg oligomycin, 2.4 mg protein and (as indicated) 0.5 mM ^{14}C-Na-acetate or 0.5 mM ^{14}C-K-thiocyanate (SCN^-). Before the addition of the mitochondria, the external pH was adjusted in order to obtain, at the end of the incubation time, the pH indicated. Time : 1 min. Final volume: 1 ml. Temperature: 22°.

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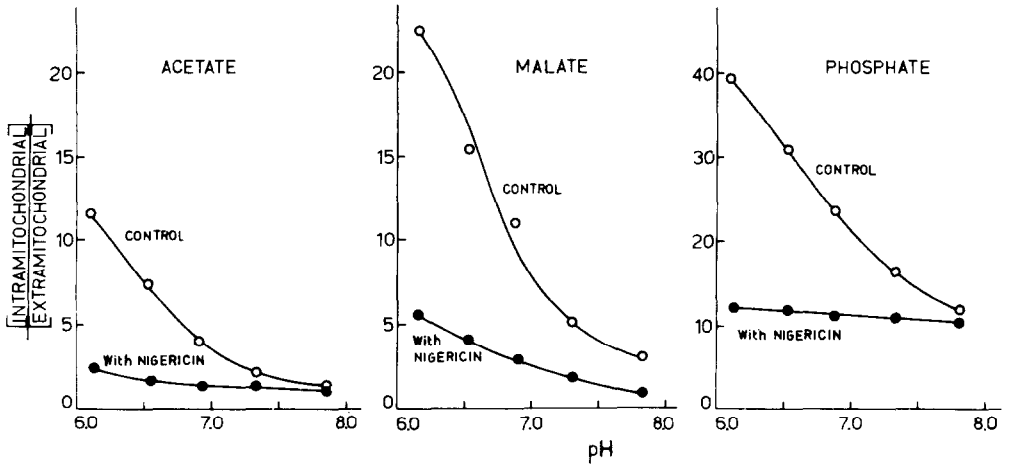

Fig. 2. Effect of nigericin on the pH dependence of acetate, malate and phosphate uptake. Experimental conditions as in fig. 1 except that the anion was either 0.5 mM ^{14}C-Na-acetate or 0.5 mM ^{14}C-malate or 0.5 mM ^{32}P-phosphate. Where indicated, 1.2 nmoles nigericin was also present. Mitochondrial protein was 1.6 mg with acetate and malate, and 1.35 mg with phosphate.

except that ΔpH was defined as the difference between the intra- and the extramitochondrial pH ($\Delta pH = pH_i - pH_o$) and the value of the buffering power of the inner mitochondrial phase, obtained as the difference between the total and the outer buffering powers, was not corrected by successive approximations. [H^+] was monitored using a Beckman 39030 combination electrode and an E.I.L. pH meter and Vibron electrometer, connected to the Speedomax W Azar recorder. The mitochondrial protein was determined by a modified biuret method [10].

3. Results

The dependence of the distribution of acetate and thiocyanate (SCN^-) between the inner and outer mitochondrial phase on the external pH is illustrated in fig. 1. The ratio of the intra- to the extramitochondrial

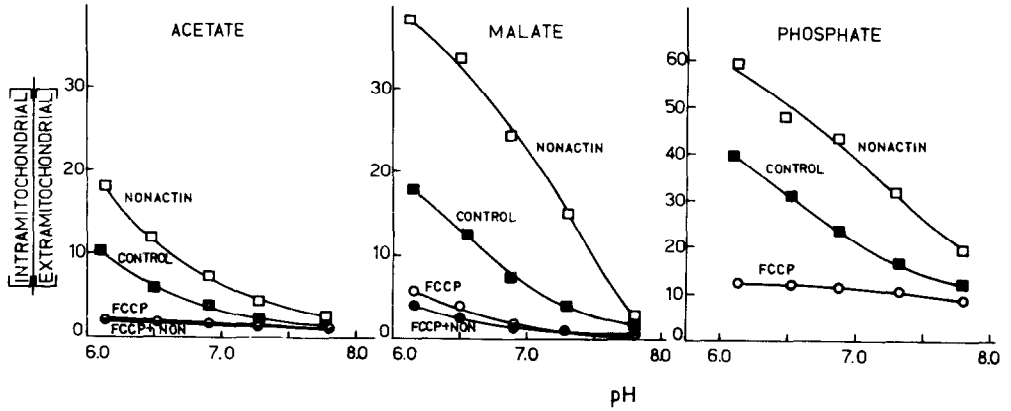

Fig. 3. Effect of nonactin, FCCP and nonactin plus FCCP on the pH dependence of anion uptake. Experimental conditions as in fig. 2. Where indicated, 0.25 μg nonactin and/or 1 μM FCCP were also added. Mitochondrial protein was 1.4 mg with acetate, 1.9 mg with malate and 1.35 mg with phosphate.

Table 1
pH Difference between the inner and outer mitochondrial phase at different external pH, in the absence or in the presence of cation- and H^+ conductors.

Additions	pH	$\Delta pH = pH_i - pH_o$	
		Acetate	Malate
None	7.6	−0.12	−0.05
None	6.1	+ 0.82	+ 0.59
Nonactin + FCCP	6.1	+ 0.16	+ 0.14
FCCP	6.1	+ 0.20	+ 0.22
Nigericin	6.1	+ 0.32	+ 0.19
Nonactin	6.1	+ 1.07	+ 0.63

The incubation mixture contained 125 mM KCl, 25 mM sucrose, 2 μg rotenone, 10 μg oligomycin, 2.8 mg protein and 0.5 mM acetate or 0.5 mM malate. Where indicated 1.2 nmoles nigericin, 1 μM FCCP and/or 0.25 μg nonactin were also present. Other conditions as in fig. 1. After 2 min incubation, 0.5% (final concentration) Triton X-114 was added. The pH change was recorded and used to estimate ΔpH (see methods).

concentration of acetate increases as the external pH decreases. The distribution ratios of malate, phosphate, malonate and, in the presence of malate, citrate were also found to increase on lowering the pH. These results extend our previous observation [1] that the uptake of succinate has a similar pH dependence [cf. also ref. 11]. On the other hand, the uptake of SCN^-, which has been indicated to permeate into the mitochondria as an uncompensated anion [12], is virtually pH independent.

In figs. 2 and 3, the effect of cation- and H^+ conductors on the pH dependence of anion distribution is shown. Nigericin, which catalyses a strictly coupled H^+/K^+ exchange [13] and is therefore expected, in KCl medium, to collapse the ΔpH without affecting the membrane potential [2, 14], strongly inhibits the increase of acetate, malate and phosphate uptake obtained on lowering the pH (fig. 2). The H^+ conductor FCCP, or the combination of nonactin and FCCP, also abolishes the pH dependence of anion uptake (fig. 3). On the other hand, the addition of nonactin alone, which is expected to collapse the membrane potential but not the ΔpH [2, 14], stimulates anion uptake on lowering the pH (fig. 3).

Table 1 reports the values of the pH difference across the mitochondrial membrane at an external pH 7.6 and 6.1 in the presence of acetate or malate. The effect of cation- and H^+ conductors on ΔpH is also shown. On lowering the external pH from 7.6 to 6.1, the ΔpH increases from values slightly negative to + 0.82 and + 0.59 with acetate and malate respectively. These sensible pH differences, at external pH 6.1, are strongly diminished in the presence of nigericin, FCCP, or the combination of nonactin and FCCP, and are still higher in the presence of nonactin alone. From these data it is apparent that there is a clear correlation between the effects of cation- and H^+ conductors on ΔpH and on anion uptake.

4. Discussion

The above results show that the distribution of acetate, phosphate and the Krebs cycle intermediates is dependent on ΔpH [1, 2] and not on the membrane potential [3]. Thus, as the external pH is decreased from 7.6 to 6.1, both the ratio between $[H^+]_o$ and $[H^+]_i$ and the uptake of acetate, phosphate and malate increase. If the $[H^+]_o/[H^+]_i$ ratio is further increased by addition of nonactin in KCl medium, also the distribution ratio of acetate, malate and phosphate increases. Vice versa, the increase of anion uptake on lowering the pH is abolished in the presence of nigericin, FCCP or nonactin plus FCCP, which collapse the ΔpH (table 1). Furthermore, SCN^- was found to be the only anion, among those tested, whose distribution is pH independent. Since SCN^- is permeable through the mitochondrial membrane as uncompensated anion [12] and is therefore in passive equilibrium with the membrane potential, this finding shows that no significant membrane potential developes on lowering the pH. It is possible therefore to conclude that the pH dependence of the uptake of acetate, phosphate and the Krebs cycle intermediates is not due to a membrane potential and that these anions do not cross the mitochondrial membrane as uncompensated anions [c.f. ref. 3]. The results presented rather support the hypothesis that acetate, phosphate and the Krebs cycle intermediates are transported into the mitochondria associated with an equivalent amount of H^+ [1, 2]. In favour of this view we have also observed a quantitative cor-

relation between the distribution of anions of varied charge and the ΔpH [11, 15].

Acknowledgements

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