# RESPIRATION-DEPENDENT ANION UPTAKE BY RAT LIVER MITOCHONDRIA

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## 1. Introduction

Evidence has been produced that in rat liver mitochondria, respiration and ATP hydrolysis catalyse an electrogenic outwards directed translocation of protons and that the energy provided either by the respiratory chain or ATP is conserved in the form of $\Delta pH$ and $\Delta\Psi$ [1–3].

In previous papers, in which anion uptake was studied under conditions where energy-driven ion transport (from respiration or ATP) was excluded, it was shown that the distribution of several anionic substrates across the mitochondrial membrane is regulated by the pH difference between the inner and outer phase [4–9].

The object of the work described in this paper is to investigate whether the proton-translocation respiratory activity causes an increase in anion uptake, not dependent on the energy-linked accumulation of cations. The results show that the anion distribution ratio between the inner and outer mitochondrial phase increases during respiratory activity, due to the $\Delta pH$ change created by the oxidoreduction reaction.

## 2. Materials and methods

Rat liver mitochondria were isolated as described by Klingenberg and Slenczka [10], using a medium consisting of 0.25 M sucrose, 1 mM EGTA–tris and 20 mM tris-HCl, pH 7.2. In the experiment reported in table 3, the medium used for the third wash and resuspension contained 1 mM tris-HCl instead of 20 mM.

The distribution of added anions between the intra- and the extramitochondrial space was determined using labelled compounds. These were all obtained from the Radiochemical centre (Amersham, England) except U-$^{14}$C-L-glutamic acid and 2-$^{14}$C-5,5-dimethyl-oxazolidine-2,4-dione (DMO), which were purchased from the New England Biochemical Corporation. Mitochondria were incubated with the labelled anionic substrates under the conditions specified in the legends, and, after 30 sec, they were separated from the incubation mixture by rapid centrifugation in a microcentrifuge (Misco) [11]. 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 [9].

The pH difference between the inner and outer mitochondrial phase ($\Delta pH = pH_i - pH_o$) was determined according to the method described by Addanki et al. [12], except that the extramitochondrial water was determined by using $^{14}$C-sucrose, which measures also the intermembrane space. In this manner it was possible to calculate the concentration of DMO only in the water of the mitochondrial matrix. The mitochondrial protein was determined by a modified biuret method [13].

## 3. Results

All the experiments were carried out in a $K^+$-free

Table 1
Effect of respiration on the anion distribution ratio between the inner and outer mitochondrial phase.

| Additions | [Intramitochondrial]/[Extramitochondrial] | | | | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | | Expt. 2 | | | |
| | Glutamate | Pi | Acetate | Pyruvate | Lactate | Glutamate |
| Olig + rot + AA | 5.4 | 45.6 | 7.8 | 5.4 | 5.2 | 3.3 |
| Olig | – | 61.8 | 9.6 | 7.9 | 6.1 | – |
| Olig + rot + AA + TMPD + asc | 7.3 | 64.0 | 8.9 | 9.3 | 6.6 | 4.7 |
| Olig + $K^+$ + non | – | 338.5 | 24.1 | 23.5 | 9.7 | – |
| Olig + rot + AA + TMPD + asc + $K^+$ + non | 14.1 | 375.2 | 26.8 | 27.5 | 10.9 | 8.4 |
| Olig + rot + AA + $K^+$ + non | 3.8 | 16.1 | 4.0 | 6.1 | 3.1 | 2.9 |

In addition to the oxygenated medium consisting of 0.25 M sucrose, 1 mM EGTA and 20 mM tris-HCl, pH 7.2, the reaction mixture contained (where indicated) 1 μg rotenone, 1 μg antimycin, 10 μg oligomycin, 0.2 mM TMPD plus 1 mM ascorbate, 2.5 mM KCl, 0.25 μg nonactin, 0.2 mM $^{14}$C-acetate, 0.2 mM $^{14}$C-pyruvate, 0.2 mM $^{14}$C-lactate, 0.2 mM $^{14}$C-L-glutamate and 0.2 mM $^{32}$P-orthophosphate. All the anions were neutralized with tris. Mitochondrial protein was 2.6 mg in expt. 1 and 3.0 mg in expt. 2. Final volume: 1 ml. Time: 30 sec. Temperature: 20°. –, not determined, olig = oligomycin; rot = rotenone; AA = antimycin; TMPD = *NNN'N'*-tetramethyl-*p*-phenylenediamine; asc = ascorbate; non = nonactin. Other conditions as indicated in Methods.

Table 2
Comparison among the distribution of Pi, thiocyanate ($SCN^-$) and the pH difference between the inner and outer mitochondrial phase in the absence and presence of respiration.

| Additions | Without $SCN^-$ | | With $SCN^-$ | | |
|---|---|---|---|---|---|
| | $[Pi]_i/[Pi]_e$ | ΔΔpH | $[Pi]_i/[Pi]_e$ | ΔΔpH | $[SCN^-]_i/[SCN^-]_e$ |
| Olig + rot + AA | 55.5 | | 52.5 | | 1.5 |
| Olig | 72.7 | 0.06 | 66.2 | 0.05 | 0.3 |
| Olig + $K^+$ + non | 420.3 | 0.88 | 371.7 | 0.55 | 1.3 |
| Olig + rot + AA + $K^+$ + non | 35.9 | −0.33 | 39.5 | −0.21 | 0.8 |

Experimental conditions as in table 1, except that (where indicated) 0.5 mM $^{14}$C-KSCN, 0.05 μCi $^{14}$C-DMO and 7.5 mM KCl were present. Where the distribution of DMO was measured, unlabelled Pi or Pi plus KSCN were present. In order to measure the accumulation of Pi and KSCN together, parallel samples were carried out in which only one of the two substrates was radioactive. Mitochondrial protein was 3.7 mg. $[Pi]_i/[Pi]_e$ and $[SCN^-]_i/[SCN^-]_e$ are the ratios of the intra- to the extramitochondrial concentration of Pi and $SCN^-$ respectively. ΔΔpH is the difference between the ΔpH (ΔpH = $pH_i - pH_0$) measured under the conditions described in lines 2, 3 and 4 and the ΔpH measured in the presence of oligomycin, rotenone and antimycin. The ΔpH values in the presence of oligomycin, rotenone and antimycin were 0.75 in the absence of KSCN and 0.79 in its presence. Abbreviations as in table 1.

medium containing EGTA. The results reported in table 1 demonstrate that respiration either supported by endogenous substrates or by *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate causes an increase of the ratio between the intra- and extramitochondrial anion concentration as compared to that obtained in the presence of rotenone, antimycin and oligomycin. As shown in table 1, the uptake of several anions, such as Pi, acetate, glutamate, pyruvate and lactate is significantly increased by the respiratory activity. The degree of stimulation of anion uptake by respiration is, however, rather small unless $K^+$ and nonactin are also added. In this case the anion accumulation in the mitochondria is largely increased, in agreement with previous observations [14–15]. As a control, line 6 shows that, in the absence of respiration, the addition of the same amount of $K^+$ plus nonactin decreases the ratio of the intra- to the extramitochondrial

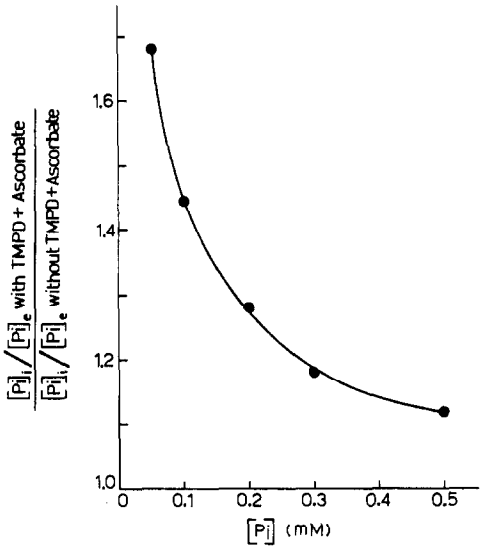


Fig. 1. Dependence on the anion concentration of the respiration-induced increase of the Pi distribution ratio between the inner and outer mitochondrial phase. In addition to the oxygenated medium consisting of 0.25 M sucrose, 1 mM EGTA and 20 mM tris-HCl, pH 7.2, the reaction mixture contained 1 μg rotenone, 1 μg antimycin, 10 μg oligomycin, $^{32}$P-orthophosphate at the concentrations indicated, 3.1 mg protein and (where indicated) 0.2 mM TMPD plus 1.5 mM ascorbate. All the anions were neutralized with tris. Other conditions as indicated in table 1 and in Methods. The Pi distribution ratio between the inner and outer mitochondrial phase ($[Pi]_i/[Pi]_e$) was measured in the absence and presence of TMPD plus ascorbate. The results are reported as $[Pi]_i/[Pi]_e$ with TMPD plus ascorbate/$[Pi]_i/[Pi]_e$ without TMPD plus ascorbate. The values of the Pi distribution ratio in the absence of respiration were 62, 80, 94, 117 and 122 at 0.5, 0.3, 0.2, 0.1 and 0.05 mM external Pi respectively. These results agree with the finding previously reported [5, 9] that the anion distribution ratio increases as the added anion concentration decreases.

anion concentration [see also 4]. The increase by respiration of malate and citrate accumulation has been recently investigated by McGivan and Klingenberg [16].

In table 2 the distribution of Pi, thiocyanate ($SCN^-$) and the pH difference between the inner and outer mitochondrial phase are compared in the presence and absence of respiration. It can be seen that the ΔpH change induced by endogenous respiration (ΔΔpH) is rather small and becomes much larger during $K^+$ uptake. The Pi distribution ratio appears to be related to the changes in ΔpH, since it increases almost as much as ΔpH and similarly diminishes when ΔpH decreases. In striking contrast to the behaviour of Pi and the other anions listed in table 1, a smaller amount of $SCN^-$, which has been shown to permeate as uncompensated anion and is therefore in passive equilibrium with the membrane potential [17–18], is taken up in the absence of respiratory inhibitors rather than in their presence. This result, indicating the formation during respiratory activity of a membrane potential negative inside, agrees with the recent finding of Bakeeva et al. [19] that synthetic penetrating anions of phenyl dicarbaundecaborane are extruded from mitochondria on transition to the energized state. When an electric potential-collapsing agent, like $K^+$ plus nonactin, is added, the uptake of $SCN^-$ increases to about the level observed in the absence of respiration.

Fig. 1 shows the dependence on the added Pi of the respiration-induced increase of the anion distribution ratio between the inner and outer mitochondrial phase. As the Pi concentration increases, the ratio between $[Pi]_i/[Pi]_e$ obtained in the presence of TMPD plus ascorbate and that obtained in their absence is progressively decreased. Correspondingly Mitchell and Moyle [1] have shown that the enhancement of the rate of decay of respiration-driven proton pulses in the presence of Pi, succinate or malonate is dependent on the anion concentration. Furthermore, it has been found that ΔpH decreases with increasing external anion concentrations at a fixed external $pH_0$ [see 9].

The effect of the outer buffering power on the respiration-induced changes in anion distribution ratio and in ΔpH is shown in table 3. It can be seen that respiration causes a higher ΔΔpH and a greater stimulation of the acetate distribution ratio in the low buffered medium (Medium B) than in the highly buffered medium (Medium A).

## 4. Discussion

Although it has been appreciated that respiration increases anion accumulation in as much as it gives rise to proton ejection [20, 4], no difference in the anion uptake was observed by several authors in the presence and absence of respiration, or, when an energy-linked anion uptake has been found, insufficient care was generally taken to exclude the uptake of cations [see 21 for refs.].

Under conditions in which cation uptake is unlikely,

Table 3
Dependence on the outer buffering power of the respiration-induced changes in ΔpH and in the anion distribution ratio.

| Additions | Medium | $A_i/A_e$ | Increase of $A_i/A_e$ (%) | ΔpH | ΔΔpH |
|---|---|---|---|---|---|
| Olig + rot + AA | A | 6.5 | – | 0.65(0.63–0.66) | – |
| Olig | A | 7.8 | 20 | 0.70(0.68–0.71) | 0.05 |
| Olig + rot + AA | B | 6.6 | – | 0.72(0.71–0.73) | – |
| Olig | B | 10.7 | 62 | 0.83(0.82–0.85) | 0.11 |

In addition to Medium A, consisting of 0.25 M sucrose, 1 mM EGTA, 20 mM tris-HCl, or to Medium B, consisting of 0.25 M sucrose, 1 mM EGTA, 0.1 mM tris (derived from the mitochondrial suspension), the reaction mixture contained 0.2 mM $^{14}$C-acetate or 0.05 μCi $^{14}$C-DMO plus 0.2 mM acetate and (where indicated) 1 μg rotenone, 1 μg antimycin, 10μg oligomycin. Final pH 7.2. All the anions were neutralized with tris. Mitochondrial protein was 2.5 mg. Temperature: 21°. Other conditions as indicated in table 1 and in Methods. Each value is mean of 3 measurements, with the ranges in brackets. $A_i/A_e$ = ratio of the intra- to the extramitochondrial acetate concentration. Abbreviations as in table 1.

the above results demonstrate that the respiratory activity, which creates a ΔpH change, increases the anion distribution ratio between the inner and outer mitochondrial phase, particularly in the presence of low concentrations of anionic substrates. The small degree of stimulation of anion uptake by respiration can be explained by considering that the pH difference, created by the proton-translocating oxidoreduction system, is very low, due to the formation of a high membrane potential [see also 3]. Thus, if $K^+$ plus nonactin is also present, there is entry of $K^+$ down the electric gradient causing a corresponding rise in ΔpH with a consequent increase in anion uptake. The higher stimulatory effect of respiration in a low buffered medium and in the presence of low concentrations of anions, i.e. under conditions in which ΔpH is expected to be higher, further supports the view that the respiration-induced increase of the anion distribution ratio is related to the ΔpH change.

The present results are also relevant to the mechanism of the transport of anionic substrates into the mitochondria. Since a membrane potential negative inside is created by the respiratory activity, the respiration-induced increase of anion uptake gives evidence that the permeability of the mitochondrial membrane to the anionic form of acetate, Pi, pyruvate, lactate and glutamate is very low. On the basis of recent experiments, which have shown that the uptake of glutamate, pyruvate and lactate increases as the external pH decreases and that this increase of anion uptake by lowering the pH is abolished by nigericin in a KCl medium [22], the transport of glutamate, pyruvate and lactate appears to be $H^+$-coupled, such as previously demonstrated for acetate, Pi and the Krebs cycle intermediates [4–9]. It is apparent, therefore, that the proton-linked translocation of the anionic substrates represents the coupling mechanism between the oxidoreductive metabolism and the transport of substrates into the mitochondria [cf. 23].

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