Proton-Cation Translocation in Tumor Cell Mitochondria

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ABSTRACT

The capacity of mitochondria isolated from tumor cells to conserve the transmembrane electrochemical proton gradient set up by respiration has been studied. In a K⁺ medium, mitochondria from Ehrlich ascites tumor cells exhibit a capacity to conserve aerobic ΔpH⁺ comparable to that displayed by normal rat liver mitochondria. Mitochondria from Morris hepatoma 3924A show a decreased capacity to store ΔpH⁺, which is principally due to lowering of ΔpH.

In a Na⁺ medium, both species of tumor mitochondria show a significant decrease of aerobic ΔpH, while Δψ is the same, with respect to rat liver mitochondria.

Experiments on passive swelling show that mitochondria from ascites tumor cells have an enhanced permeability to chloride salts of monovalent cations and increased activity of the Na⁺(K⁺)-H⁺ exchange system of the mitochondrial membrane with respect to normal mitochondria. The enhanced activity of this system in ascites cells is also shown by the characteristics of respiration-linked proton translocation in submitochondrial particles and subsequent anaerobic proton diffusion.

It is concluded that the decreased capacity of mitochondria from tumor cells to conserve aerobic ΔpH is due to enhanced cyclic flow of Na⁺ across the membrane.

INTRODUCTION

In mitochondria, the energy made available by respiration is converted into a transmembrane thermodynamic potential difference of protons (ΔpH⁺) (3, 11, 16), which is in turn utilized for oxidative phosphorylation (3, 11) and active solute transport (5, 9, 11, 12, 20). The ΔpH component of aerobic ΔpH⁺ drives, by means of specific proton symporters (12), the uptake by mitochondria of Pi, glutamate, pyruvate, and other monocarboxylates (5, 4, 17, 20, 22). ΔpH can also be expended for extrusion of monovalent cations from mitochondria by means of a proton-monovalent alkali-metal exchange system or antiporter (11) which is reported to be particularly active with Na⁺ and other monocarboxylates (5, 4, 17, 20, 22). ΔpH can also be expended for extrusion of monovalent cations from mitochondria by means of a proton-monovalent alkali-metal exchange system or antiporter (11) which is reported to be particularly active with Na⁺ and the function of which is not clearly understood (4, 7, 13, 15, 19). It has been proposed that this system may play a role in regulating intracellular pH (24) and Na⁺ concentration (31).

Recently Papa et al. (8, 18) have described a deficiency of the pyruvate transport system in tumor mitochondria. Their results indicate that a relative insufficiency of mitochondria to take up and utilize (see also Ref. 10) pyruvate may account, at least in part, for the high rate of aerobic glycolysis characteristics of rapidly growing tumors (23, 30).

In this paper, a detailed study of proton and cation translocation in tumor cell mitochondria is presented. The results show an increased passive permeability of mitochondria from tumor cells to chloride salts of monovalent cations and enhanced activity of the Na⁺(K⁺)-H⁺ exchange system as compared to normal rat liver mitochondria. This results in a decreased capacity of mitochondria from Ehrlich ascites tumor cells and Morris hepatoma 3924A to conserve, especially in the presence of Na⁺, the chemical component, or ΔpH, of the aerobic transmembrane electrochemical proton gradient as compared to normal rat liver mitochondria.

MATERIALS AND METHODS

Preparation of Mitochondria and Submitochondrial Particles. Ehrlich hyperdiploid ascites tumor cells were maintained by weekly i.p. transplantation in albino Swiss mice. Mitochondria were isolated as described in Ref. 6. Morris hepatoma 3924A tumors were transplanted into both legs of inbred ACI/T rats and harvested 3 to 4 weeks after transplantation. Mitochondria were isolated according to described methods (26).

Rat liver mitochondria were prepared as described by Myers and Slater (14). Submitochondrial particles were prepared by exposing mitochondria suspended in 0.25 M sucrose, kept frozen at −30°C for 2 to 3 days, and thawed immediately before use to ultrasonic power for 60 sec at 0°C (Ultrasonic-Branson Sonifier, Model W 185, output, 70 watts) in the presence of 4 mM choline-EDTA, pH 8.5.

Determination of Mitochondrial Matrix Volume. The matrix volume was determined with a double labeling technique using 3H₂O to measure the total pellet water and [14C]sucrose to measure the extra matrix water. Matrix volume was estimated from the difference between 3H₂O and [14C]sucrose-permeable H₂O.

Mitochondria were incubated at 25°C with succinate as respiratory substrate (see legends to the tables) and bubbled with oxygen. The suspension was then centrifuged for 1 min at 20,000 x g. Samples of HCIO₄ extract of the mitochondrial pellet and of the supernatant were counted for radioactivity.

Determination of Transmembrane Electrochemical Proton Gradient. Δψ component of the aerobic proton electrochemical gradient was determined by the distribution of [3H]methyltriphenylphosphonium bromide between supernatant and matrix, as described by Schuldiner and Kaback (27). Δψ was calculated by the Nernst equation.

ΔPH was determined by the distribution of [14C]dimethylxalazonedione across the mitochondrial membrane and calculated according to described methods (1).

Measurement of Mitochondrial Swelling. Mitochondrial swelling was monitored by following changes in absorbance at 520 nm in a Varian spectrophotometer at room temperature.

Measurement of Proton Translocation in Submitochondrial Particles. Submitochondrial particles were incubated (for the reaction mixture, see legend to Chart 2) in the presence of catalase (0.2 mg/ml). Incubation was carried out in a glass vessel, under a stream of N₂, thermostated at 25°C. Once the particle suspension was made anaerobic by succinate, oxidation-respiration-driven proton translocation was activated by repetitive pulses of 8 μl of 0.5% HC₂O₃.

The pH of the suspension was monitored potentiometrically with a Beckman combination electrode (No. 39030; Instrument International, Geneva, Switzerland) connected to a Keithley differential electrometer.
amplifier (Model 604; Keithley Instruments) and from this to a strip chart recorder (Leeds and Northrup).

**Determination of Protein.** Mitochondrial proteins were determined by the biuret method, with bovine serum albumin as standard.

**Chemicals.** H2O, [3H]Methyltriphenylphosphonium bromide, and [14C]-5,5-dimethyl-2,4-dione were obtained from New England Nuclear, Dreieich, West Germany. [14C]Sucrose was obtained from the Radiochemical Centre, Amersham, England. All other reagents were purchased from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim Co., Mannheim, West Germany. The reagents were of the highest purity commercially available.

**RESULTS**

**Aerobic ΔpH* in Mitochondria.** Respiring mitochondria from rat liver and tumor cells were supplemented with [3H]Methyltriphenylphosphonium bromide or [14C]-5,5-dimethyl-2,4-dione to measure, respectively, the aerobic transmembrane Δψ and ΔpH from the distribution across the mitochondrial membrane of these radioactive tracers.

In Table 1, the statistical analysis of a set of measurements of aerobic transmembrane Δψ and ΔpH in mitochondria from rat liver, Ehrlich ascites cells, and Morris hepatoma 3924A is presented. A number of points emerge from this analysis: (a) replacement in the incubation medium of K+ with Na+ resulted in a significant decrease of ΔpH. This decrease of ΔpH was more marked in mitochondria from ascites cells and hepatomas than in mitochondria from rat liver; (b) in the Na+ medium, the aerobic ΔpH was considerably smaller in both types of tumor mitochondria than in rat liver mitochondria. In the case of hepatoma mitochondria, also in the K+ medium, the transmembrane ΔpH was smaller than in rat liver mitochondria.

The marked decrease of aerobic ΔpH observed in tumor mitochondria, when K+ was replaced by Na+, was accompanied by a small but significant increase of Δψ. Loss of a component of aerobic ΔpH is, in fact, expected to be compensated by enhancement of the other (11); (c) enhancement of the pH of the incubation medium from 7 to 8 resulted in a considerable decrease of ΔpH; on the other hand, Δψ was unchanged or even increased (see also Ref. 25). The decrease of ΔpH was larger in the Na+ than in the K+ medium and more evident in tumor mitochondria than in rat liver mitochondria.

In Chart 1, data are presented on the water content of mitochondria from rat liver and tumor cells. It can be noted that the water content of mitochondria from tumor cells was lower than that of rat liver mitochondria. This lowering was more pronounced in hepatoma mitochondria which exhibited a more marked decrease of ΔpH with respect to rat liver mitochondria. The water content of mitochondria seems to be directly correlated with the magnitude of the aerobic ΔpH.

Enhancement of the pH of the medium from 7 to 8 resulted in decrease of the water content in both normal and tumor cell mitochondria.

In Table 2, the results of a set of experiments are presented in which the effect of preparing mitochondria from rat liver and Ehrlich ascites cells in the presence or absence of EDTA on the steady state respiratory ΔpH* was examined. The effect of the presence of NEM3 in the incubation mixture was also tested.

It can be seen that neither the inclusion of EDTA in the preparation medium nor the presence of NEM during the aerobic incubation had any effect on the steady state extent of aerobic Δψ and ΔpH in respiring mitochondria from rat liver and Ehrlich ascites cells. Thus, in all the conditions tested, ascites cell mitochondria exhibited at pH 8 and in presence of 30 mM NaCl the same substantial decrease of aerobic ΔpH as compared to normal rat liver mitochondria. On the other hand, the aerobic Δψ exhibited by ascites cell mitochondria was under all conditions tested equal to that of normal rat liver mitochondria.

**Passive Swelling of Mitochondria and Effect of Mg++.** Chart 2 illustrates the osmotic behavior of rat liver and tumor cell mitochondria suspended in isosmotic media. All types of mitochondria did not exhibit swelling when suspended in sucrose. On the other hand, in salt media, swelling occurred which became faster in the order KCI < NaCl, potassium acetate < sodium acetate.

In all these media, the swelling was faster in tumor cell mitochondria than in rat liver mitochondria. In tumor mitochondria, the rate of swelling in sodium acetate and potassium acetate increased when the pH of the medium was raised from 7 to 8 (Chart 2).

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3 The abbreviation used is: NEM, N-ethylmaleimide.

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**Table 1**

Proton-Cation Translocation in Tumor Mitochondria

<table>
<thead>
<tr>
<th>Component</th>
<th>pH 7</th>
<th>pH 8</th>
<th>Δψ (mV)</th>
<th>ΔpH (mV)</th>
<th>ΔH+ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+ medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>127 ± 2.5</td>
<td>131 ± 1.9</td>
<td>76 ± 4.0</td>
<td>64 ± 3.9</td>
<td>204 ± 5.7</td>
</tr>
<tr>
<td>Ehrlich ascites cells</td>
<td>119 ± 2.5</td>
<td>132 ± 3.3</td>
<td>80 ± 2.4</td>
<td>61 ± 4.1</td>
<td>200 ± 5.0</td>
</tr>
<tr>
<td>Morris hepatoma 3924A</td>
<td>112 ± 4.9</td>
<td>113 ± 4.6</td>
<td>56 ± 3.4</td>
<td>45 ± 3.7</td>
<td>167 ± 5.6</td>
</tr>
<tr>
<td>Na+ medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>132 ± 3.4</td>
<td>134 ± 3.4</td>
<td>56 ± 4.8</td>
<td>37 ± 4.5</td>
<td>188 ± 7.7</td>
</tr>
<tr>
<td>Ehrlich ascites cells</td>
<td>136 ± 3.2</td>
<td>145 ± 3.2</td>
<td>31 ± 2.8</td>
<td>16 ± 2.9</td>
<td>168 ± 6.7</td>
</tr>
<tr>
<td>Morris hepatoma 3924A</td>
<td>131 ± 4.7</td>
<td>135 ± 4.3</td>
<td>38 ± 1.7</td>
<td>16 ± 1.8</td>
<td>170 ± 7.4</td>
</tr>
</tbody>
</table>

Numbers in parentheses, number of determinations.

-mean ± S.E.
Chart 1. Intramitochondrial water of rat liver and tumor cell mitochondria. The experimental procedure and conditions are those described in the legend to Table 1. H$_2$O (10 $\mu$Ci/ml) and [1$^3$C]sucrose (10 $\mu$Ci/ml) were present instead of [3H]methyltriphenylphosphonium bromide and [1$^4$C]-5,5-dimethyloxazolidine-2,4-dione. For other details, see "Materials and Methods." ☐, rat liver; ☐, Ehrlich ascites cell; ☐, Morris hepatoma 3924A.

Table 2
Effect of EDTA and NEM on the steady state respiratory $\Delta$H$^+$ in rat liver and Ehrlich ascites cell mitochondria

Mitochondria were suspended at pH 8 in the Na$^+$ medium described in the legend to Table 1. Where indicated, 0.2 mM EDTA was present in the isolation medium, and NEM (30 nmol/mg) was present in the incubation medium. For experimental procedure and other details, see legend to Table 1 and "Materials and Methods."

<table>
<thead>
<tr>
<th>Additions</th>
<th>Rat liver</th>
<th>Ehrlich ascites cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta$V (mV)</td>
<td>$-59$ΔpH (mV)</td>
</tr>
<tr>
<td>None</td>
<td>149 ± 0.9$^a$</td>
<td>32 ± 3.6</td>
</tr>
<tr>
<td>NEM</td>
<td>147 ± 1.5</td>
<td>35 ± 2.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>152 ± 0.7</td>
<td>34 ± 1.7</td>
</tr>
<tr>
<td>EDTA and NEM</td>
<td>150 ± 0.6</td>
<td>36 ± 1.2</td>
</tr>
</tbody>
</table>

$^a$Mean ± S.E. for 4 experiments.

In Table 3, the effect of MgCl$_2$ on the aerobic $\Delta$H$^+$ and on the rate of passive swelling is presented. MgCl$_2$ added to rat liver or tumor cell mitochondria respiring in the presence of 30 mM NaCl at pH 8 caused a marked enhancement of $\Delta$pH with small if any effect on $\Delta$V. MgCl$_2$ also caused marked depression of passive swelling of both types of mitochondria suspended in isoosmotic sodium acetate.

Separate controls showed that replacement of K$^+$ with Na$^+$ and/or enhancement of the pH from 7 to 8 of the medium did not cause any significant depression of the respiratory activity with succinate in the coupled or the uncoupled state.

Respiration-linked Proton Translocation in Submitochondrial Particles. In "inside out" vesicles of the inner mitochondrial membrane, obtained by exposure of mitochondria to ultrasonic power, respiration results in proton disappearance from the medium (19).

Chart 3 shows proton translocation elicited by oxygen pulses of anaerobic particles from rat liver and ascites cells supplemented with succinate as respiratory substrate and choline-nitrate. Nitrate, which is a permeant anion, distributes in the electric field generated by aerobic proton translocation, thus collapsing the $\Delta$V component which is replaced by extra $\Delta$pH (16, 19). The cycle of proton translocation in submitochondrial particles from ascites cells was practically the same as that exhibited by rat liver submitochondrial particles except for a small decrease of the extent of aerobic proton uptake. The addition of 10 mM NaCl caused a marked depression of the extent of aerobic proton uptake by submitochondrial particles and acceleration of passive proton back flow as indicated by considerable decrease of the $t_{1/2}$ of proton appearance in the medium upon exhaustion of added oxygen. These effects of NaCl were more marked in ascites cell submitochondrial particles than in rat liver particles.

DISCUSSION

The capacity of mitochondria to conserve the energy made available by respiration in the form of $\Delta$H$^+$ depends on the intrinsic ion permeability of the mitochondrial membrane as well as on the activity of electrogenic ion translocators, which regulate the $\Delta$V component (9, 12), and electroneutral proton-anion symporters (9, 12, 20) and cation-proton antiporters (12, 16), which affect the $\Delta$pH component.
Table 3
Effect of magnesium on transmembrane $\Delta \psi$ in respiring mitochondria and on passive mitochondrial swelling

For Experiment A, transmembrane $\Delta \psi^-$, mitochondria (1.5 mg/ml) were suspended in a reaction mixture containing 80 mM sucrose, 30 mM NaCl, rotenone (1 $\mu$g/mg), oligomycin (2 $\mu$g/mg), NEM (30 nmol/mg), 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 0.5 mM ethyleneglycoltetraacetic acid. Final volume, 1 ml pH was adjusted to 8 with choline. For experimental procedure and conditions, see legends to Table 1 and Chart 1 and "Materials and Methods." Experiment B measures passive mitochondrial swelling in sodium acetate at pH 8. The experimental procedure and conditions were those described in the legend to Chart 2. Where indicated, 25 mM MgCl$_2$ was included in the medium.

<table>
<thead>
<tr>
<th>Experiment A</th>
<th>Experiment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \psi$ (mV)</td>
<td>Enhance- $\Delta \psi^-$ (%)</td>
</tr>
<tr>
<td>Rat liver cells</td>
<td>147</td>
</tr>
<tr>
<td>+ MgCl$_2$</td>
<td>158</td>
</tr>
<tr>
<td>Ehrlich ascites cells</td>
<td>132</td>
</tr>
<tr>
<td>+ MgCl$_2$</td>
<td>143</td>
</tr>
</tbody>
</table>

In a K$^+$ medium, Ehrlich cell mitochondria exhibit a capacity to conserve aerobic $\Delta \psi^+$ comparable to that displayed by rat liver mitochondria. On the other hand, hepatoma mitochondria show a decreased capacity to store $\Delta \psi^+$ which is principally due to lowering of $\Delta \psi$.

In a Na$^+$ medium, both species of tumor mitochondria show a significant decrease of $\Delta \psi^+$ with respect to rat liver mitochondria, which is due to substantial lowering of $\Delta \psi$.

Thus, tumor mitochondria while exhibiting a capacity to store aerobic $\Delta \psi^+$ comparable to that of mitochondria from normal cells [this is consistent with the observation that tumor mitochondria display a normal capacity for electrophoretic Ca$^+$ uptake (29)] have a decreased capacity to store the $\Delta \psi$ component.

The results on passive swelling of mitochondria show an increased passive permeability of mitochondria from tumor cells to chloride salts of monovalent cations and an enhanced activity of the Na$^+$(K$^+$)-H$^+$ exchange system as compared to normal rat liver mitochondria (see Chart 2). The enhanced rate of Na$^+$ (or K$^+$) diffusion from the cytosol to the mitochondrial matrix and the enhanced rate of Na$^+$ extrusion in exchange with protons mediated by the Na$^+$(K$^+$)-H$^+$ antiporter result in respiring of mitochondria from tumor cells in a significant cyclic flow of monovalent cations across the mitochondrial membrane, which dissipates the transmembrane $\Delta \psi$.

The decrease of aerobic $\Delta \psi$ observed in rat liver and especially in tumor cell mitochondria when K$^+$ in the medium is replaced with Na$^+$ can, in fact, be ascribed to exchange of protons extruded by the respiratory chain with Na$^+$ diffusing passively into mitochondria. It is known that the monovalent cation-proton antiporter of mitochondria and bacterial plasma membrane exhibits a relative specificity for Na$^+$ (7, 11, 16, 24, 31). The experiments on mitochondrial swelling and those on proton translocation in "inside out" submitochondrial particles provide 2 independent lines of evidence which show that the antiporter (a) has an activity much higher with Na$^+$ than with K$^+$ and (b) is more active in tumor cell mitochondria than in rat liver mitochondria.

This study confirms earlier observations that at alkaline pH there occurs a selective decrease of aerobic $\Delta \psi$; $\Delta \psi$ remains unchanged or even increases in mitochondria as well as in bacteria (25). The present observations indicate that this decrease of $\Delta \psi$ depends on the activity of the monovalent cation-proton antiporter. In fact, the decrease of $\Delta \psi$ at alkaline pH...
pH is more marked in the Na⁺ medium than in the K⁺ medium.

The critical role of Na⁺(K⁺)-H⁺ antiporter in regulating the extent of aerobic transmembrane ΔpH is documented by the effect of MgCl₂ which inhibits the antiporter (7) (see Table 3) and restores aerobic ΔpH without affecting significantly the aerobic Δψ. Although the activity of the Na⁺(K⁺)-H⁺ antiporter is depressed by Mg²⁺, the enhancement of its activity in tumor cell mitochondria as compared to rat liver mitochondria cannot be ascribed simply to lowering of the content of endogenous Mg²⁺ in tumor cell mitochondria (2). In fact, the presence of EDTA during the preparation of rat liver or Ehrlich ascites cell mitochondria had no effect whatsoever on the extent of the aerobic ΔpH.

It can also be noted that the presence of NEM during incubation of mitochondria had no effect on aerobic ΔpH and Δψ in mitochondria from rat liver or ascites tumor cells. Thus, the decreased capacity to store aerobic ΔpH reflects an intrinsic property of tumor cells mitochondria.

It seems worth recalling that tumors show an enhanced level of intracellular Na⁺ (28). This could contribute to promotion of a ΔpH-dissipating cyclic flow of Na⁺ across the mitochondrial membrane. Dissipation of ΔpH can result, in turn, in a decreased capacity of mitochondria to take up from the cytosol pyruvate, P₃, glutamate, and other metabolites which are transported across the mitochondrial membrane at the expenses of ΔpH. Impaired uptake of metabolites by mitochondria could contribute to the enhancement of aerobic glycolysis in rapidly growing tumors.

ACKNOWLEDGMENTS

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REFERENCES


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