Calcium Transport and Translocation of Adenine Nucleotides in Mitochondria from Morris Hepatoma 3924A

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ABSTRACT

The interaction of Ca\(^{2+}\) with Morris hepatoma 3924A mitochondria and its effect on the adenine nucleotide translocation have been studied. The characteristics of the Ca\(^{2+}\) transport process in mitochondria from Morris hepatoma are not significantly different from those of normal liver mitochondria. The Km for Ca\(^{2+}\) is 2 to 3 \(\mu\)M, and the rate versus concentration curve exhibits hyperbolic kinetics. A lower activity of the adenine nucleotide translocation was observed after isolated mitochondria had accumulated extra amounts of Ca\(^{2+}\). The total amount of adenine nucleotides in tumor mitochondria is one-half those present in control liver, and a significantly lower percentage of the pool is present as adenosine 5'-monophosphate.

INTRODUCTION

Among the various hypotheses postulated thus far for the high glycolytic rate of poorly differentiated tumors, one of the most reliable is generally considered to be that involving a lack of control of phosphofructokinase by intramitochondrial ATP compartmentation (see Ref. 27 for review). It has been reported (22) that Ca\(^{2+}\) stimulates the translocation of adenine nucleotides when added to rat liver mitochondria in the absence of a source of energy. Conversely, under the same conditions, Thorne and Bygrave (24) have found an inhibition of the translocation by Ca\(^{2+}\) in Ehrlich ascites tumor mitochondria, an observation which could be relevant for the above-mentioned problem of ATP compartmentation. However, these data are difficult to interpret owing to the fact that, in the absence of energy, Ca\(^{2+}\) is not allowed to penetrate into the mitochondrial matrix. Thus, a correct understanding of such Ca\(^{2+}\)-adenine nucleotide interaction could derive from experiments performed under conditions of active Ca\(^{2+}\) accumulation, i.e., in the presence of exogenous substrates. Indeed, Gómez-Puyou et al. (13) have recently found that adenine nucleotide translocation is inhibited in liver mitochondria that have been allowed to accumulate Ca\(^{2+}\).

In the present work, we have compared the kinetics of Ca\(^{2+}\) uptake and adenine nucleotide translocation in mitochondria isolated from rat liver and the poorly differentiated, fast-growing Morris hepatoma 3924A. The results obtained have indicated that mitochondria from Morris hepatoma react with Ca\(^{2+}\) much as do most other types of mitochondria. However, their ability to carry out the translocation of adenine nucleotides is limited. In all likelihood, this is attributable to their unusually large content of endogenous Ca\(^{2+}\).

MATERIALS AND METHODS

Morris hepatoma 3924A tumors were transplanted s.c. in the hind legs of inbred ACI/T rats and harvested between 2 and 4 weeks after transplantation. Tumor mitochondria were prepared according to the method of Schreiber et al. (20), care being taken to eliminate all necrotic portions of the tissue. Rat liver mitochondria were prepared from healthy ACI/T rats (except for the Ca\(^{2+}\) uptake experiments, in which Wistar rats were used), following a slightly modified standard procedure (19).

The endogenous Ca\(^{2+}\) content of mitochondria was measured in the supernatant extract after the mitochondrial protein had been removed by precipitation in 7% trichloroacetic acid, in the presence of 0.1% La\(^{3+}\) (to eliminate interference by phosphate), using a Perkin-Elmer Model 503 atomic absorption spectrophotometer. Mitochondrial Ca\(^{2+}\) uptake was measured by a Millipore filtration technique and radioactively labeled Ca\(^{2+}\). Mitochondrial protein (1 mg) was incubated at 25°C with 0.005 M Tris-succinate, 1 \(\mu\)M rotenone, 0.002 M P (when present) and 0.003 M Na-ATP (when present) in 1 ml of a medium composed of 0.21 M mannitol, 0.07 M sucrose, 0.01 M Tris-HCl (pH 7.4), and 0.05% fatty acid-free BSA. Samples were withdrawn at the desired times, and the mitochondria were separated from the medium on Millipore filters (0.45-\(\mu\)m pore size). The filters were washed with 1 ml of cold medium, then dissolved in Packard Instagel and counted for radioactivity in a liquid scintillation counter.

The kinetic experiments on Ca\(^{2+}\) uptake were carried out with a Ca\(^{2+}\)-specific electrode (Philips IS-561-Ca\(^{2+}\)) that had a response time of less than 2 sec and a detection limit of about 1 \(\mu\)M Ca\(^{2+}\). The medium containing 0.12 M KCl, 0.02 M Tris-HCl (pH 7.4), and 0.05% fatty acid-free BSA was decalcified with Chelex X-100 (potassium form). The mitochondria (0.04 to 0.4 and 0.1 to 0.5 mg of protein for tumor and control liver, respectively) were incubated with 1 \(\mu\)M rotenone for 3 min in a final volume of 2 ml, at 25°C, to facilitate the release of the endogenous Ca\(^{2+}\). Then small amounts of exogenous Ca\(^{2+}\) were introduced to the system and, after 30 sec, the uptake was initiated by the addition of 0.001 M Tris-succinate. The Ca\(^{2+}\) uptake was determined with the help of a calibration

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The abbreviation used is: BSA, bovine serum albumin.
The adenine nucleotide exchange experiments were performed, using carrier-free [3H]ATP or [3H]ADP, by the following procedure. Mitochondria (30 mg of protein), suspended in a basic medium containing 0.21 M mannitol, 0.07 M sucrose, and 0.01 M Tris-HCl (pH 7.4), were equilibrated with 0.4 μCi carrier-free adenine nucleotide for 50 min at 0°. The mitochondria were washed in 40 ml of the medium and centrifuged at 10,000 × g for 10 min. The supernatant extract was started with cold ADP (0.4 mM). When substrates (0.01 M glutamate plus 0.01 M malate or 0.01 M succinate) and Ca²⁺ (200 μM) were used, mitochondria were preincubated for 2.5 min at room temperature before the exchange was begun. Aliquots (0.5 ml) were removed at desired times and quickly transferred to centrifuge tubes containing 0.1 ml of 0.1 M trichloroacetic acid. The mixture was stirred and centrifuged for 2.5 min in an Eppendorf centrifuge. Aliquots of the supernatant extract were used to measure the radioactivity. A sample of the mitochondrial suspension was removed before the addition of ADP to determine the intramitochondrial radioactivity. Fatty acid-free BSA was present throughout the experiment at a concentration of 1 mg/ml mitochondrial protein for both rat liver and hepatoma mitochondria.

The endogenous content of adenine nucleotides was determined enzymatically, using hexokinase for ATP, pyruvate kinase for ADP, and myokinase for AMP (2, 3). The binding of atractyloside to mitochondria was carried out in 5 ml 0.21 M mannitol, 0.07 M sucrose, 0.01 M Tris-HCl (pH 7.4), 0.1 mM Tris-EDTA, 0.5 mg mitochondrial protein per ml, and different concentrations of [3H]atractyloside, at 0°. Samples were withdrawn at predetermined times, and mitochondria were separated from the medium by rapid centrifugation at 0°. Aliquots of the supernatant were used for the counting of radioactivity.

Cytochrome oxidase activity was measured polarographically at 30°, in a medium consisting of 0.033 M potassium phosphate (pH 7.4), 0.07 mM cytochrome c, 0.1 to 0.2 mg mitochondrial protein per ml, and 1 mg Lubrol per ml, to solubilize the mitochondrial structure. The oxidation was initiated by the addition of 10 μM sodium ascorbate which, at the same time, kept the cytochrome c reduced. The cytochrome oxidase activity is given as nanomols of oxygen per min per mg of protein.

The respiratory control index was calculated as described by Chance and Williams (9).

Protein was estimated by the biuret method (14) using BSA as standard.

The chemicals used were of the highest purity standards commercially available.

RESULTS

Ca²⁺ Content of Morris Hepatoma Mitochondria. Mitochondria from most of the mammalian tissues studied contain between 5 and 20 nmol of Ca²⁺ per mg of protein. Some mitochondrial types which have been recently studied, notably from vascular (25) and myometrial (17) smooth muscle and from chondrocytes (21), contain considerably higher amounts of Ca²⁺, ranging between 50 and 400 nmol per mg of protein. The problem of whether the Ca²⁺ pool found in isolated mitochondria truly reflects the in vivo situation or represents a Ca²⁺ redistribution among organelles during the homogenization of the tissues has been debated at length in the past. However, several pieces of evidence [see Carafoli and Crompton (6) for a comprehensive discussion] support the first alternative. The mitochondria from Morris hepatoma in the present study contained an average of 123 ± 15 nmol of Ca²⁺ per mg of protein (extreme values in a series of experiments were 227 and 73 nmol/mg of protein). It must be emphasized that these are probably minimal figures with respect to the in vivo situation, since the homogenization and isolation media used contained 0.5 mM Tris-EDTA. Thus, the endogenous Ca²⁺ content in these mitochondria is higher than in most other types of mitochondria. An important point is whether this abnormally high Ca²⁺ content is compatible with the normal functioning of the organelle. In a series of experiments, ADP-dependent respiratory control indices around 2.5 were routinely observed, using succinate as respiratory substrate, as compared with values of between 4 and 6 for control livers. Thus, the Morris hepatoma mitochondria used in the present study have a lowered energy-coupling efficiency.

Energy-linked Ca²⁺ Transport in Morris Hepatoma Mitochondria. Chart 1 shows that Morris hepatoma mitochondria accumulate Ca²⁺ when added to medium supplemented with respiratory substrates. P, is not essential for the energy-linked uptake although, in its presence, as expected, higher levels of loading can be attained. Massive loading (up to 900 nmol/mg of protein) requires the presence in the medium [and very probably (8) the penetration into mitochondria] of ATP (or ADP) in addition to P. It is important to point out that the penetration of adenine nucleotides under these experimental conditions probably is, in large part, independent of the ATP-ADP translocator. Carafoli et al. (6) have found that atractyloside inhibits the penetration of adenine nucleotides under conditions of massive Ca²⁺ loading by maximally 36%. The uptake of Ca²⁺ is inhibited completely by La³⁺ and by ruthenium red (not shown), although both compounds had to be used in concentrations 5- to 10-fold higher than those active in liver and heart mitochondria. Mg²⁺ also was found to inhibit the energy-linked uptake when added to the medium in concentrations higher than 10 mM.
Calcium and Adenine Nucleotides in Hepatoma Mitochondria

The rate of the Ca\(^{2+}\) uptake process was studied with a Ca\(^{2+}\)-specific electrode. Chart 2 shows the results of a typical experiment on tumor mitochondria and, for comparison, on mitochondria from rat liver. In both cases, the process has hyperbolic kinetics. From the points obtained with tumor mitochondria, one can derive a \(K_m\) between 2 and 3 \(\mu M\) and a \(V_{max}\) of 0.5 to 1.2 nmol Ca\(^{2+}\) per mg protein per sec, as compared to figures of 2 to 3 \(\mu M\) and 1.5 to 2 nmol Ca\(^{2+}\) per mg of protein per sec, respectively, for normal liver mitochondria. A more precise estimate of the \(K_m\) of tumor mitochondria is prevented by the difficulty of obtaining low concentrations of Ca\(^{2+}\), due to "leakage" of the large content of endogenous Ca\(^{2+}\) from these mitochondria. The fact that the process exhibits hyperbolic kinetics is in agreement with the findings of Crompton et al. (12) on heart mitochondria, and at variance with those by others (5, 26) on liver and heart mitochondria. Crompton et al. (12) have attributed the sigmoidal kinetics, which sometimes can be observed, to the incomplete removal of inhibitor traces of Mg\(^{2+}\) from the medium.

The accumulated Ca\(^{2+}\) could be released from tumor mitochondria by interrupting the energy flow, for example with uncouplers. It could not, however, be released by the addition of Na\(^{+}\), which has been shown to induce a specific release of Ca\(^{2+}\) in mitochondria from heart (10) and other excitable tissues (11), but not in liver mitochondria (11).

The Exchange of Adenine Nucleotides in Morris Hepatoma Mitochondria. As mentioned above, Ca\(^{2+}\) has been shown to inhibit the ADP-ATP translocator in Ehrlich ascites cell mitochondria (24) and either to stimulate or to inhibit the translocator in liver mitochondria (13, 22), depending on the presence of Ca\(^{2+}\) outside or inside the organelle. The finding of very high levels of endogenous Ca\(^{2+}\) provided the rationale for investigating the ADP-ATP translocator in Morris hepatoma mitochondria.

Chart 3 shows that the activity of the ATP-ADP translocator, as judged by the maximal percentage of adenine nucleotides that can be exchanged, is considerably lower in Morris hepatoma mitochondria (Chart 3B) as compared to normal liver mitochondria (Chart 3A). In Chart 3C, it is also shown that the ADP-ATP exchange reaction is low in tumor mitochondria. When rat liver mitochondria accumulated Ca\(^{2+}\), the ATP-ADP exchange resulted in an inhibition of approximately 50% (Chart 3A). On the other hand, as shown in Chart 3B, no further inhibition of the translocase reaction could be induced by allowing the hepatoma mitochondria to accumulate "extra" Ca\(^{2+}\) over the high level of endogenous cation.

Table 1 shows a comparison of the composition of the adenine nucleotide pool in Morris hepatoma and liver mitochondria. Interesting differences are evident; Morris hepatoma mitochondria contain less adenine nucleotides, but a larger proportion of them is in the form of ADP and ATP. From the data of Table 1, values of 3.48 and 3.85 nmol of ADP + ATP per mg of protein have been taken for rat liver and Morris hepatoma mitochondria, respectively, and they have been used in experiments aimed at establishing the \(K_m\) and the \(V_{max}\) of the translocator. As shown in Chart 4, such parameters in tumor mitochondria (\(K_m = 17.2 \mu M\); \(V_{max} = 11.8\) nmol/min/mg protein) appear to be close to those in normal mitochondria (\(K_m = 5\) \(\mu M\); \(V_{max} = 270\) nmol/min/mg protein).
0.21 M mannitol-0.07 M sucrose-0.01 M Tris-HCl, pH 7.4, were equilibrated with [3H]ATP (A and B) or [3H]ADP (C) in the presence of 1 mg BSA per mg mitochondrial protein. For the experimental details, see "Materials and Methods." In A and B, exchange with cold ADP was performed in the presence of 0.01 M glutamate plus 0.01 M malate, with (C) or without (D) 200 μM CaCl₂ in the incubation medium. C, exchange with cold ADP performed in the presence of 0.01 N Tris-succinate.

The total level of adenine nucleotide translocase, however, is much lower than expected, being only 0.01 pmol/mg protein by adducing the specific Ca²⁺ ionophore A23187 in the presence of extramitochondrial ethylene glycol tetraacetate. The total level of adenine nucleotide translocase, however, remained unchanged. A study is being made to determine whether this was due to the incomplete removal of intra-mitochondrial Ca²⁺. Thus far, no means have been found to reduce the endogenous Ca²⁺ below the level of about 50 nmol/mg of protein.

The possibility of a lower content of translocase was investigated by measuring the maximal amount of radioactively labeled atractyloside bound to Morris hepatoma mitochondria. Chart 6 shows that about 0.006 pmol of atractyloside is bound per unit of cytochrome oxidase activity, a value very similar to that found in normal liver mitochondria (15).

DISCUSSION

The experiments presented in this article have touched on 2 different, but possibly interrelated, aspects of Morris hepatoma mitochondria: the characteristics of their energy-linked interaction with Ca²⁺; and their ability to exchange adenine nucleotides.

Some aspects of energy-linked uptake of Ca²⁺ in tumor mitochondria already have been studied. Reynafarje and Lehninger (18) have used mitochondria from L1210 mouse ascites tumor cells, whereas Bygrave (4) and Thorne and Bygrave (24) have used Ehrlich ascites tumor cells. The former authors have found maximal accumulation of about 1.5 μmol Ca²⁺/mg of protein in the presence of Pi, Mg²⁺, and adenine nucleotides. The Ca²⁺-stimulated respiration had a normal stoichiometry in the presence of phosphate, but in its absence the calcium-oxygen accumulation ratios became "superstoichiometric." Using the stimulation of O₂ uptake by Ca²⁺, Reynafarje and Lehninger (18) found half-maximal stimulation rates at 8 μM external Ca²⁺; they also discovered a second energy-dependent type of interaction with Ca²⁺, having a much lower affinity for Ca²⁺ (Kₘ = 120 μM), and probably corresponding to the exchange of Ca²⁺ with previously stored membrane protons. Bygrave (4) and Thorne and Bygrave (24) found, on the other hand, that the respiring ascites mitochondria respond cyclically to Ca²⁺ only in the presence of phosphate, with normal calcium-oxygen stoichiometries.

The first interesting result of the studies in this paper is the very high content of endogenous Ca²⁺ in mitochondria of the Morris hepatoma 3924A. Its interest resides in the fact that mitochondria from normal livers contain very small amounts of Ca²⁺ (10 to 20 nmol/mg of protein) (7) and are easily damaged by excess Ca²⁺. In contrast, the hepatoma mitochondria are apparently still able to create and maintain a membrane potential, as evidenced by the fact that the accumulated Ca²⁺ is rapidly released by uncouplers (see above), in spite of their high Ca²⁺ content. Therefore, as has already emerged from the above-mentioned studies on smooth muscle (17, 25) and chondrocytes (21), the presence of large amounts of Ca²⁺ inside mitochondria is not necessarily linked to complete and irreversible functional damage of the organelle.

The use of a Ca²⁺-specific electrode in this study has permitted us to measure the affinity of Morris hepatoma mitochondria for Ca²⁺. The Kₘ of 2 to 3 μM, which has been measured, is in the same range as that of normal liver mitochondria and of the same order as that estimated by Reynafarje and Lehninger (18) for ascites tumor mitochondria. Other characteristics of the process of energy-linked uptake are also similar to those of normal liver, including the inhibition of the process by Mg²⁺ (1). Of some interest are the high levels of ruthenium red and La³⁺ which are necessary to inhibit completely the uptake process in Morris hepatoma mitochondria. This finding is not easily explained but could perhaps reflect a different (phospholipid) composition of the membrane and/or (in the case of

Table 1
Adenine nucleotide content in rat liver and Morris hepatoma mitochondria

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>0.52 ± 0.08 (3)</td>
<td>2.96 ± 0.55 (3)</td>
<td>3.85 ± 0.35 (3)</td>
</tr>
<tr>
<td>Morris hepatoma</td>
<td>0.92 ± 0.20 (7)</td>
<td>2.93 ± 0.50 (7)</td>
<td>0.70 ± 0.30 (4)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

Numbers in parentheses, number of observations.

= 20.8 μM; Vₓₓₓ = 7.1 nmol/min/mg protein). This is better documented in Table 2, where it can be seen that the differences between the 2 types of mitochondria are not statistically significant.

Attempts were made to restore a normal level of translocase activity by depleting Morris hepatoma mitochondria of their excessive endogenous Ca²⁺. The attempts, however, have thus far been unsuccessful. In the experiment of Chart 5, the endogenous Ca²⁺ pool was reduced from 300 to 46 nmol/mg protein by adding the specific Ca²⁺ ionophore A23187 in the presence of extramitochondrial ethylene glycol tetraacetate. The total level of adenine nucleotide translocase, however, remained unchanged. A study is being made to determine whether this was due to the incomplete removal of intra-mitochondrial Ca²⁺. Thus far, no means have been found to reduce the endogenous Ca²⁺ below the level of about 50 nmol/mg of protein.

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Mitochondria were equilibrated with [3H]ATP, as described in "Materials and Methods," and allowed to exchange, in the absence of exogenous substrates, with different concentrations of cold ADP during 10 sec of incubation. The values presented here for rat liver mitochondria are consistent with those reported previously by other authors (22).

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>26.1 ± 3.0* (3)</td>
<td>9.5 ± 1.1 (3)</td>
</tr>
<tr>
<td>Morris hepatoma 3924A</td>
<td>22.2 ± 2.9 (6)</td>
<td>11.3 ± 1.9 (6)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.  
Numbers in parentheses, number of observations.

Chart 4. Double-reciprocal plots of the ATP$_{in}$ - ADP$_{out}$ exchange in rat liver (A) and Morris hepatoma (B) mitochondria. The translocation experiments were performed as described in "Materials and Methods." No exogenous substrate was added. The exchange reaction was stopped after 10 sec of incubation.

Table 2

<table>
<thead>
<tr>
<th>Mitochondria</th>
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La$^{3+}$ at least) the removal of some of the inhibitor by complexing agents, e.g., P$_o$.

The exchange of adenine nucleotides in Morris hepatoma mitochondria has approximately the same kinetic properties ($K_m$, $V_{max}$) as that of normal liver mitochondria. The amount of adenine nucleotide translocator, as judged by the binding of atracyloside to the membrane, was also about the same as in mitochondria from normal livers. The results on the kinetic parameters of adenine nucleotide exchange in tumor mitochondria may appear inconsistent with those published previously by other authors (16, 23). However, these data were obtained either on a Morris hepatoma of unspecified origin (23) or on the very fast-growing (1 week growth time) Zajdela hepatoma (16). Despite the similarities found in the kinetic parameters, the size of the exchangeable pool of the hepatoma 3924A was markedly reduced with respect to normal liver mitochondria, a finding that has been reported for Ehrlich ascites (24) and myometrum (17) mitochondria. One possible explanation could be the complexation of adenine nucleotides inside mitochondria by the excess Ca$^{2+}$, thus subtracting a fraction from the exchangeable pool. This possibility is also suggested by the observation that liver mitochondria have a markedly reduced exchangeable pool of adenine nucleotides after loading with Ca$^{2+}$ (see Chart 3A). However, attempts to verify this hypothesis directly by depleting tumor mitochondria of at least part of their endogenous Ca$^{2+}$ to restore the size of the exchangeable pool to normal levels have failed. Such unsuccessful results are presumably due to the still high residual concentration of Ca$^{2+}$ found in tumor mitochondria after the treatment with the ionophore (cf. the value 46 nmol Ca$^{2+}$ per mg protein found in the experiment described in Chart 5 with the value 10 to 20 nmol Ca$^{2+}$ per mg protein of rat liver mitochondria). Alternative explanations for the observed reduction in size of the exchangeable pool are, however, difficult to propose. The finding of a reduced total and relative AMP content excludes the possibility that the effect of Ca$^{2+}$ on the exchange of adenine nucleotides is due to an increased content of AMP.

Chart 5. Kinetics of ATP translocation in Morris hepatoma mitochondria after Ca$^{2+}$ depletion by the ionophore A23187. Hepatoma mitochondria were allowed to reduce their Ca$^{2+}$ content from 300 to 46 nmol/mg protein after treatment for 5 min at room temperature with the ionophore A23187, 1 nmol/mg protein, in the presence of 1.5 mM ethylene glycol tetraacetate. Mitochondria were then washed 3 times with the incubation medium (see legend to Chart 3) and loaded with labeled ATP as for control experiments. For further details, see "Materials and Methods." ○, control; ○, ionophore A23187.

Chart 6. Atractyloside binding to Morris hepatoma mitochondria. The binding of atractyloside was referred to the unit of cytochrome (cyt.) oxidase activity (measured polarographically on the same preparation) to correct for contaminating proteins of nonmitochondrial origin. For details, see "Materials and Methods."
Whether the reduced exchangeability of adenine nucleotides is a general property of tumor mitochondria is an open question, and its solution is considered of marked significance for the role that adenine nucleotides have in the control of aerobic glycolysis through their influence on the activity of key enzymes (e.g., phosphofructokinase, pyruvate kinase).

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