Regulation of Pyruvate Dehydrogenase in Rat Liver Mitochondria by Phosphorylation-Dephosphorylation*

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ELIZIBETA I. WALAJTYS,‡ DEMOCLEIA P. GOTTFESMAN, AND JOHN R. WILLIAMSON§
From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19174

SUMMARY

The percentage of total pyruvate dehydrogenase in the active nonphosphorylated form was measured in intact rat liver mitochondria after rapid separation of the mitochondria through silicone oil and extraction with glycerol buffer at $-10^\circ$. The contents of ATP, ADP, Mg$^{2+}$, and Ca$^{2+}$ in the mitochondrial matrix of separated mitochondria were determined in parallel experiments. Mitochondria were incubated with glutamate and malate as substrates in a variety of metabolic states. These were induced by addition of oligomycin, uncoupler, the divalent cation ionophore A23187, and other inhibitors in order to alter the phosphorylation state of the intramitochondrial adenine nucleotides and the contents of magnesium and calcium.

Interconversion between the active nonphosphorylated form of pyruvate dehydrogenase and the inactive phosphorylated form appeared to be dominated by regulation of pyruvate dehydrogenase kinase activity. Under conditions of controlled respiration, the percentage of pyruvate dehydrogenase in the active form varied directly with the ADP content and inversely with the ATP:ADP ratio. Regulation of pyruvate dehydrogenase interconversion by pyruvate dehydrogenase phosphatase activity could only be demonstrated under severe conditions of magnesium and calcium depletion. It is concluded that the normal content of Mg$^{2+}$ and Ca$^{2+}$ in isolated mitochondria is sufficient to provide optimal activation of pyruvate dehydrogenase phosphatase and that release of Mg$^{2+}$ by conversion of MgATP$^{2-}$ to ADP, P$_i$, and Mg$^{2+}$ during active respiration has a negligible effect on pyruvate dehydrogenase phosphatase activity. The over-all steady state activity of pyruvate dehydrogenase appears to be determined by pyruvate and ADP which inhibit pyruvate dehydrogenase kinase, and thereby prevent phosphorylation and inactivation of pyruvate dehydrogenase. Regulation of pyruvate dehydrogenase activity is thus achieved without an unnecessary expenditure of ATP for enzyme phosphorylation and dephosphorylation.

The pyruvate dehydrogenase complex contains three catalytic components. Pyruvate dehydrogenase itself and flavin containing dihydrolipoyl dehydrogenase are bound to the dihydrolipoyl transacetetylase which plays both a catalytic and a structural role (1-4). The mammalian pyruvate dehydrogenase complex also contains two regulatory enzymes, a kinase and a phosphatase (5, 6). Pyruvate dehydrogenase kinase is tightly bound to the transacetetylase, while pyruvate dehydrogenase phosphatase appears to be loosely associated with the complex (7, 8). It is now well established that the activity of the pyruvate dehydrogenase complex in a number of mammalian tissues (heart, liver, kidney) are regulated by a phosphorylation-dephosphorylation cycle (9-11). Pyruvate dehydrogenase kinase utilizes MgATP$^{2-}$ as substrate and inactivates pyruvate dehydrogenase by phosphorylation of a seryl residue in one of the two subunits of pyruvate dehydrogenase (7, 9, 12). Pyruvate dehydrogenase phosphatase reactivates the enzyme by removing the covalently bound phosphate (9, 10, 12). Similar observations have been made with the enzyme from brain (13) and epididymal fat cells (14-16).

According to available data obtained from the literature, the following scheme is proposed to represent the interaction of these two regulatory enzymes (Fig. 1). ADP is a competitive inhibitor, with MgATP$^{2-}$, for pyruvate dehydrogenase kinase (5, 7, 16), the apparent $K_i$ value being about 0.1 mM for the enzyme from both bovine kidney and heart (17). Pyruvate protects the pyruvate dehydrogenase complex against inactivation and appears to exert its effect via inhibition of pyruvate dehydrogenase kinase (17). Apparent $K_i$ values for pyruvate of about 0.2 mM have been found for the heart complex, while values of about 1.4 mM were found for the kidney complex (17). Pyruvate dehydrogenase phosphatase requires for its activity both magnesium (9, 11, 15, 18, 19) and calcium (20, 21), the apparent $K_m$ for Mg$^{2+}$ being 2 to 3 mM (16, 17, 22). Recently Pettit et al. (20) showed that Ca$^{2+}$ acts by binding phosphatase to dihydrolipoyl transacetetylase, and lowers the apparent $K_m$ of the phosphatase for phosphorylated pyruvate dehydrogenase about 20 fold, thereby facilitating the Mg$^{2+}$-dependent dephosphorylation of pyruvate dehydrogenase.

Since ADP inhibition of pyruvate dehydrogenase kinase is competitive with ATP, and since ADP is also a weaker chelator for Mg$^{2+}$ (23-25), it has been proposed that changes of the intramitochondrial ATP:ADP ratio may regulate the activity of pyruvate dehydrogenase in two ways. First, a high ATP:ADP ratio may be associated with an active pyruvate dehydrogenase kinase due to a low degree of inhibition by ADP, thereby result-
ing in a high proportion of the total pyruvate dehydrogenase being in the inactive phosphorylated form. Second, when the mitochondrial adenine nucleotides are predominantly present as ATP, a higher proportion of the intramitochondrial Mg²⁺ content will be chelated. Thus, the free Mg²⁺ concentration may be decreased sufficiently to limit the activity of pyruvate dehydrogenase phosphatase and therefore retain pyruvate dehydrogenase in the inactive phosphorylated state.

A considerable amount of work has been done with the isolated enzyme components of the pyruvate dehydrogenase complex (17, 20, 22), and the percentage of total pyruvate dehydrogenase in the active form in intact tissues (14, 15, 19, 26-30) and isolated fat cells (16) has been measured. However, very few reports have been published relating the activity of pyruvate dehydrogenase to the energy state of isolated mitochondria (16, 31), and none in which pyruvate dehydrogenase activity has been correlated with the state of phosphorylation of the intramitochondrial adenine nucleotides and the intramitochondrial content of Mg²⁺ and Ca²⁺. The purpose of the present experiments was to obtain more definitive information of these relationships in rat liver mitochondria. Use has been made of the divalent cation ionophore A23187 which was shown by Reed and Lardy (32) to dissociate mitochondria. Use has been made of the divalent cation ionophore A23187 which was shown by Reed and Lardy (32) to dissociate mitochondria. Experimental conditions have been sought which allowed regulation of pyruvate dehydrogenase interconversion between phosphorylated and nonphosphorylated forms in changes of pyruvate dehydrogenase kinase activity to be distinguished from regulation via pyruvate dehydrogenase phosphatase. Our major conclusion is that the state of activity of pyruvate dehydrogenase in liver mitochondria is regulated primarily by changes of pyruvate dehydrogenase kinase activity and that regulation is achieved by changes of the intramitochondrial ADP content, particularly when the pyruvate concentration is low.

**Experimental Procedures**

**Preparation and Incubation of Mitochondria—**Mitochondria were prepared from livers of male albino Holtzman rats (240 to 260 g) by a minor modification of the method of Schneider and Hogeboom (33). The isolation medium contained 75 mm sucrose, 225 mm mannitol, and 0.1 mm EDTA. The basic medium used for mitochondrial incubations contained 130 mm KCl, 20 mm Tris-chloride, and 5 mm KH₂PO₄ adjusted to pH 7.2. Mitochondria were incubated in different respiratory states, each of which included buffer, substrate as indicated, and 6 to 8 mg of mitochondrial protein per ml of medium contained in a water-jacketed chamber maintained at 28°C. Oxygen was blown over the surface of the stirred reaction mixture to maintain aerobic conditions. Other additions are indicated in the figure and table legends.

**Measurement of Pyruvate Dehydrogenase Activity and Mitochondrial Ca²⁺ and Mg²⁺ Content—**When pyruvate dehydrogenase activity and the mitochondrial content of Mg²⁺ and Ca²⁺ were examined, the mitochondria were separated from the incubation medium by rapid centrifugation (approximately 10 s) in a high speed Eppendorf centrifuge, model 2200, at room temperature. The supernatant solution was decanted, the insides of the tube wiped dry, and the mitochondrial pellet lysed in deionized water and subsequently extracted twice with 1.5 n perchloric acid. Calcium and magnesium contents were measured with a Varian model AA-3 atomic absorption spectrophotometer. Strontium chloride (15 mm) was included in all calcium sample tubes to provide interference from phosphate. Pyruvate dehydrogenase was extracted by diluting mitochondrial pellets, immediately after separation, to the same volume as in the initial incubation medium with cold (−20°C) buffer mixture (trisethanolamine, 40 mm; glutathione, 1 mm; glycerol 50%, v/v; pH 7.0). All samples were then sonicated at −10°C for 10 s with a Branson Sonifier cell disruptor, model 220. Aliquots were taken for measurement of enzyme activity immediately after sonication.

Pyruvate dehydrogenase activity was determined by coupling the reaction with the arylamine acetyltransferase (EC 2.3.1.3) using p-nitroaniline as acetyl acceptor, as described by Wieland et al. (29) with minor modifications. Arylamine acetyltransferase was prepared from pig liver according to Wieland et al. (34) and Wieland et al. (29), with the variations that 20 mm p-nitroanilin was used for the elution of the enzyme from the Al(OH)₃ gel, and precipitation with (NH₄)₂SO₄ was omitted. The enzyme was concentrated by lyophilization and stored at −15°C. The assay mixture contained in a final volume of 1 ml: 50 mm potassium phosphate, pH 8.0; 0.5 mm MgCl₂; 0.15 mm p-nitroaniline; 0.5 mm thiamine pyrophosphate; 1.5 mm NAD; 5 mm thioglycolate; 0.12 mm NADP; 10 mM pyruvate, 20 μg of crystalline lactate dehydrogenase and approximately 30 milliliters of arylamine acetyltransferase. The reaction was started by adding 50 μl of mitochondrial homogenate, and incubations were made at 30°C. After 20 min the reaction mixture was rapidly cooled in an ice bath and immediately centrifuged at 20,000 × g for 10 min. The decrease in absorbance at 420 nm was measured against a blank reaction mixture which had been incubated in the absence of CoA-SH and pyruvate. All assays were run in triplicate. Pyruvate dehydrogenase activity was expressed as a fraction of total activity. Total pyruvate dehydrogenase activity was routinely measured after sonication of the mitochondrial suspension and reprecipitation with 10 mm MgCl₂ at 28°C for 30 min. This treatment resulted in the conversion of inactive pyruvate dehydrogenase to the active form (16, 18, 21, 29).

Control experiments were performed in which pyruvate dehydrogenase phosphatase purified up to Step 6 of the procedure described by Beebe and Wieland (29) was added to the mitochondrial suspension together with 10 mm MgCl₂ to ensure that there was a sufficient amount of endogenous phosphatase for the conversion of inactive pyruvate dehydrogenase to the active form. Total activity of pyruvate dehydrogenase obtained after incubation of sonicated mitochondria under described conditions was essentially the same with or without pyruvate dehydrogenase phosphatase (20 μl corresponding to 500 μg of protein of the enzyme).

Maximal activation was obtained after 30 min with endogenous phosphatase and after 15 min with exogenous phosphatase.

**Measurement of Intramitochondrial Adenine Nucleotides—**For assays of intramitochondrial contents of adenine nucleotides, citrate, and isocitrate, the mitochondrial suspension in 0.5 ml containing 8% (w/v) dextran (mol wt 40,000) was centrifuged through a 2-mm silicone oil layer (1.053 specific gravity) into 0.2 ml of 1.5 n perchoric acid. An Eppendorf model 1.2 ml microcentrifuge (Brinkman Instruments) with 1.5-ml test tubes, which developed 8000 × g in less than 10 s, was found satisfactory for this purpose. Transfer of the mitochondria through the silicone oil was complete within this time. The top layer after centrifugation was immediately transferred to perchloric acid (final concentration 1.5 n) and 6 n KOH plus 0.5 ml morpholinopropanesulfonic acid. The contents of ATP, ADP, citrate, isocitrate, Mg²⁺, and Ca²⁺ in the mitochondrial extracts were corrected for the volume of extra-mitochondrial fluid retained with the mitochondria by using 0.1 μCi per ml of [U-¹⁴C]mannitol or [U-¹⁴C]succrose, while the total exchangeable water content of the mitochondria was determined in parallel experiments by addition of 1 μCi per ml of ¹⁴C²O₂ to the mitochondrial incubation medium.

The validity of the procedure used for the rapid separation of
mitochondria from their incubation medium in relation to de-

terminations of the adenine nucleotide content of the mitochon-
drial matrix was ascertained by comparing values obtained from
measurements of the total mitochondrial incubation medium with
the separately determined values obtained from the supernatant
and mitochondrial extracts after correction of the latter for extra
matrix fluid carried down with the mitochondria. Data obtained
for a particular series of experiments, shown in Table I, provide a
good illustration of the over-all reliability both of the separation
procedures and the analytical methods. The greatest deviation
between the sum of the measured contents in the supernatant and
mitochondrial extracts and in the total mitochondrial extract was
obtained with the AMP determinations. This may be caused by
adenylate kinase activity, particularly of the supernatant fluid
prior to its transfer into perchloric acid. Also noteworthy is the
finding that up to 50% of the total content of individual nucleo-
tides may be found in the supernatant fluid, even under State 4
conditions.

### Table I

<table>
<thead>
<tr>
<th>Compartment</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>3.35 ± 0.21</td>
<td>1.01 ± 0.15</td>
<td>0.80 ± 0.11</td>
<td>5.16 ± 0.22</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.77 ± 0.18</td>
<td>1.78 ± 0.07</td>
<td>2.02 ± 0.16</td>
<td>7.57 ± 0.30</td>
</tr>
<tr>
<td>Sum</td>
<td>7.12 ± 0.35</td>
<td>2.79 ± 0.20</td>
<td>2.82 ± 0.18</td>
<td>12.73 ± 0.46</td>
</tr>
<tr>
<td>Total extract</td>
<td>7.26 ± 0.22</td>
<td>2.10 ± 0.07</td>
<td>1.59 ± 0.17</td>
<td>10.95 ± 0.23</td>
</tr>
</tbody>
</table>

### RESULTS

**Distribution of Adenine Nucleotides between Supernatant and Mitochondrial Matrix in Different Metabolic States—Loss of ATP and ADP from the mitochon-
drial matrix during incubation of the mitochondria varied consider-
ably with the metabolic state. Data illustrating the distribution of nucleotides across the mito-
ochondrial membrane in the wide range of experimental conditions
employed in this study are illustrated in Table I. In all cases
no nucleotides were added externally to the mitochondrial incu-
bation medium. Some conditions, notably addition of uncoupler
or ionophore, caused a considerable fall in the sum of total ATP
and ADP, due presumably to an increase of AMP in the super-
natant fluid. Despite wide variations of the ATP:ADP ratio in
the mitochondrial matrix, no large differences were observed in
this ratio between the mitochondria and supernatant in sharp
contrast to the data of Heldt et al. (37). The experimental condi-
tions used by these authors were, however, different from those
reported here in that ADP was routinely added to their incuba-
tion medium. The present demonstration of extensive leakage
of nucleotides from the mitochondrial matrix in the absence
of added nucleotides illustrates the importance of measuring
the ATP and ADP contents of the mitochondria after separation
from the supernatant, rather than in the total mitochondrial incu-
bation medium.

**Effect of Changes of Intramitochondrial ATP:ADP Ratio on Activity of Pyruvate Dehydrogenase—The effect of alterations in
the state of phosphorylation of the intramitochondrial adenine
nucleotides on the activity of pyruvate dehydrogenase in isolated
rat liver mitochondria is shown in Table III. The mitochondria
were incubated with 5 mM glutamate and 1 mM malate as sub-
strates in order to study the interaction between the adenine
nucleotides and pyruvate dehydrogenase directly and to avoid
effects introduced by the presence of pyruvate. All incubations
were of 4-min duration, which was sufficient to achieve steady
state conditions as shown by preliminary kinetic experiments.
Under conditions of State 4 respiration, the nucleotides were pre-
dominantly in the form of ATP, and pyruvate dehydrogenase was
present largely in the inactive phosphorylated form. The endog-
ogenous Ca**2+** content of the mitochondria was about the same
as the ATP content, while Mg**2+** levels were twice as high. It
would be anticipated, therefore, that ATP should be present
mainly as MgATP**2-** and CaATP**2-** chelates rather than as free
ATP (93-95, 38). A high activity of pyruvate dehydrogenase
kinase relative to phosphatase activity presumably accounts for
the small percentage of total pyruvate dehydrogenase present in
the active nonphosphorylated form. Addition of oligomycin
alone resulted in a 30% fall of ATP and 2-fold increase of the
ADP content. The percentage of total pyruvate dehydrogenase
in the active form also doubled. When ADP (4 mM) was added
to the mitochondria together with oligomycin, most of the intra-
mitochondrial ATP exchanged with extramitochondrial ADP,
and the intramitochondrial ATP:ADP ratio fell to 0.5 from a
value of 5.5 observed in State 4. The intramitochondrial ADP
content increased from 1.7 to 6.9 nmoles per mg of protein, and
pyruvate dehydrogenase was almost fully activated. Addition of
uncoupler (0.15 mM P-trifluoromethoxyphenylhydrazone of car-
bonyl cyanide) decreased the ATP:ADP ratio to 0.9, while
pyruvate dehydrogenase activity increased to 75% of the total.
Addition of oligomycin to the uncoupled state increased the
ATP:ADP ratio to 2.2 mainly by lowering the ADP content from
2.9 to 1.2 nmoles per mg of protein, and pyruvate dehydrogenase
Higher activity in the latter state despite a lower ADP content. Plus oligomycin state, where pyruvate dehydrogenase had a noticeable in the comparison between State 4 and the uncoupled importance in determining the steady state percentage of pyruvate dehydrogenase. This is particularly data illustrate that a rough inverse relationship exists between the intramitochondrial ATP:ADP ratio and the activity of nucleotides, but only a small (19 to 27%) loss of Mg++. These without oligomycin resulted in a marked loss of Ca++. The presence of uncoupler either with or activity fell to 51%. The presence of uncoupler either with or without oligomycin resulted in a marked loss of Ca++ and total nucleotides, but only a small (19 to 27%) loss of Mg++. These data illustrate that a rough inverse relationship exists between the intramitochondrial ATP:ADP ratio and the activity of pyruvate dehydrogenase. However, the data suggest that the absolute levels of ATP, ADP, and divalent cations are also of importance in determining the steady state percentage of pyruvate dehydrogenase in the active form. This is particularly noticeable in the comparison between State 4 and the uncoupled plus oligomycin state, where pyruvate dehydrogenase had a higher activity in the latter state despite a lower ADP content.

**Effect of Ionophore A23187 on Pyruvate Dehydrogenase Activity**

Rat liver mitochondria were incubated for 4 min in different metabolic states with 5 mM glutamate and 1 mM malate as substrates. The oligomycin-inhibited states included oligomycin (1 μg per mg of mitochondrial protein). The uncoupled states contained 0.2 mM p-trifluoromethoxyphenylhydrazone carbonyl cyanide (FCCP). Ionophore was used in concentrations of 0.4 to 1.2 nmoles per mg of protein. The final concentration of EGTA was 0.2 mM; Mg++ and Ca++ were 3 mM and 0.8 mM, respectively. Mitochondria were centrifuged into perchloric acid through silicone oil. Values shown are the mean ± S.E.M. of three to six determinations.

### Table II

**Distribution of adenine nucleotides between mitochondrial matrix and supernatant in mitochondria incubated in different metabolic states**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Mitochondria</th>
<th>Supernatant</th>
<th>Sum of ATP and ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
<td>ATP:ADP</td>
</tr>
<tr>
<td>None (State 4)</td>
<td>9.3 ± 0.7</td>
<td>1.7 ± 0.2</td>
<td>5.5</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>6.5 ± 0.5</td>
<td>3.6 ± 0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>FCCP</td>
<td>2.7 ± 0.3</td>
<td>3.0 ± 1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>A23187</td>
<td>1.0 ± 0.1</td>
<td>6.0 ± 0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>A23187 + EGTA</td>
<td>6.8 ± 0.7</td>
<td>2.3 ± 0.2</td>
<td>2.9</td>
</tr>
<tr>
<td>FCCP + oligomycin</td>
<td>2.8 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>2.4</td>
</tr>
<tr>
<td>FCCP + A23187</td>
<td>2.1 ± 0.1</td>
<td>4.0 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>FCCP + oligomycin + A23187</td>
<td>3.1 ± 1.0</td>
<td>2.0 ± 0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>FCCP + A23187 + EGTA</td>
<td>2.3 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>FCCP + A23187 + oligomycin + EGTA</td>
<td>3.7 ± 1.0</td>
<td>4.3 ± 1.7</td>
<td>0.9</td>
</tr>
<tr>
<td>A23187 + EGTA + Mg++ + Ca++</td>
<td>1.8 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>FCCP + A23187 + EGTA + Mg++ + Ca++</td>
<td>1.9 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

### Table III

**Pyruvate dehydrogenase activity of mitochondria in different metabolic states**

All incubations included buffer (130 mM KCl, 20 mM Tris chloride, and 5 mM KH₂PO₄, pH 7.2), 5 mM glutamate plus 1 mM malate as substrates, and 6 to 8 mg per ml of mitochondrial protein. State 4 contained no further additions. The oligomycin-inhibited state included oligomycin (1 μg per mg of mitochondrial protein). The oligomycin-ADP state also included 4 mM ADP. The uncoupled state contained p-trifluoromethoxyphenylhydrazone carbonyl cyanide (FCCP) (~0.2 mM). All reactions were started by addition of mitochondria except the oligomycin-inhibited states, in which case mitochondria were preincubated with oligomycin for 1 min. Incubations were continued for 4 min. Values shown are the means of three to six separate determinations ± S.E.M. Absolute activity of pyruvate dehydrogenase accounts for 14.4 milliunits ± 2.8 when 1 milliunit is defined by the formation of 1 nmole of p-nitroacetanilide per mg of protein per min at 30°. The value shown represents a mean of 13 experiments ± standard error of the mean.

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP:ADP</th>
<th>ATP</th>
<th>ADP</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>Pyruvate dehydrogenase activity of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (State 4)</td>
<td>5.5</td>
<td>9.3 ± 0.7</td>
<td>1.7 ± 0.2</td>
<td>21.3 ± 1.4</td>
<td>10.1 ± 0.8</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>1.8</td>
<td>6.5 ± 0.5</td>
<td>3.6 ± 0.9</td>
<td>17.7 ± 3.2</td>
<td>8.1 ± 2.2</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>Oligomycin + ADP</td>
<td>0.5</td>
<td>3.4 ± 0.5</td>
<td>6.9 ± 1.5</td>
<td>17.3 ± 2.4</td>
<td>7.0 ± 0.4</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>FCCP</td>
<td>0.9</td>
<td>2.5 ± 0.3</td>
<td>2.9 ± 0.8</td>
<td>17.0 ± 2.5</td>
<td>3.2 ± 0.7</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>FCCP + oligomycin</td>
<td>2.2</td>
<td>2.6 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>15.5 ± 2.2</td>
<td>2.6 ± 0.4</td>
<td>51 ± 6</td>
</tr>
</tbody>
</table>

In Mitochondria—In an attempt to study the effect of alterations of the mitochondrial energy state on pyruvate dehydrogenase activity at lower levels of endogenous Mg²⁺ and Ca²⁺, use was made of the divalent cation ionophore A23187. Respiration was stimulated and oxidative phosphorylation was uncoupled after addition of A23187 to rat liver mitochondria, as previously observed by Reed and Lardy (32). The uncoupling effect of A23187 was reversed by EGTA as shown in Fig. 2. Respiration could be stimulated again by subsequent addition of Ca²⁺ but not Mg²⁺ (Fig. 2A). Ruthenium red, which is known to inhibit

The abbreviation used is: EGTA, ethylene glycol bis(α-amino-ethyl ether)-N,N'-tetraacetic acid.
Mitochondria (0.9 mg of protein) were incubated with 5 mM glutamate plus and concentration of EGTA was 0.2 mM. Mg*+ and Ca*+ were 3 mM and 1 mM, respectively. B, mitochondria were incubated as described in the legend for Trace A except that ruthenium red was added at the concentration of 10 nM of protein before addition of both cations.

In further experiments (Table IV), mitochondria were incubated for 4 min in the presence of A23187, and residual Ca*+ and Mg*+ in the mitochondrial pellet were measured by the atomic absorption method. The amount of intramitochondrial magnesium decreased from 2.1 to 5 nmoles per mg of protein, and calcium decreased from 10 to 2.8 nmoles per mg of protein, relative to State 4 conditions, after addition of A23187 (see also Table III). The intramitochondrial ADP level increased to 6 nmoles per mg of protein, and the ATP:ADP ratio decreased to 0.2. Under these conditions pyruvate dehydrogenase was about 80% active. It is apparent that inhibition of pyruvate dehydrogenase kinase activity by ADP prevented conversion of pyruvate dehydrogenase to the inactive phosphorylated form. When EGTA was added to the incubation medium to bind calcium released by A23187, the calcium level in the mitochondrial matrix decreased further to 1.4 nmoles per mg of protein. The intramitochondrial ADP content decreased to 2.3 nmoles per mg of protein, and the ATP:ADP ratio increased to 2.9, while pyruvate dehydrogenase activity fell to 16% of total. Presumably pyruvate dehydrogenase kinase activity increased with a fall of the intramitochondrial ADP content, and a condition resembling State 4 was achieved except for the lowered Mg*+ and Ca*+ contents.

It may be assumed that the intramitochondrial concentration of ATP-Mg*+ is sufficient for maximal activation of pyruvate dehydrogenase kinase under conditions described in Table IV, since apparent K_m values for ATP-Mg*+ are very low, i.e. 20 μM (17). Furthermore, ADP levels remained approximately the same under the different conditions shown in Table IV except when A23187 was added alone. These results clearly demonstrate that pyruvate dehydrogenase activity may be regulated by the intramitochondrial divalent cation content, presumably via changes of pyruvate dehydrogenase phosphatase activity. The best condition for revealing these effects is when pyruvate transport through mitochondrial membranes (30-41), prevented this effect of Ca*+ (Fig. 2B).

Fig. 3 shows that addition of A23187 caused a rapid release of endogenous magnesium and calcium from the mitochondria. In these experiments, kinetics of Mg*+ release was measured by following the changes in absorbance (575 to 540 nm) of the indicator 3-[5-(chloro-2-hydroxyphenyl)-azol]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid as indicator (Fig. 3C, b) showed good agreement with the amount released as measured with atomic absorption spectroscopy (Fig. 3C,a). However, the amount of Ca*+ released calculated from the murexide method (Fig. 3D, b) was slightly greater than that measured by atomic absorption (Fig. 3D, a). These data confirm the original observations of Reed and Lardy (32) that the ionophore A23187 causes a rapid release of both Ca*+ and Mg*+ from mitochondria, and show in addition that release of Mg*+ is faster than that of Ca*+.

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hydrogenase phosphatase can also be demonstrated by addition of oligomycin together with uncoupler and A23187 caused a 350%, i.e. slightly less active than the 75% observed with uncoupler activity as a result of a fall of the ADP content and partly to a decreased phosphatase activity since Mg2+ levels fell 3-fold. A Mg2+ content of 5.6 nmoles per mg of protein and a Ca2+ content of 1.4 nmoles per mg of protein was insufficient to activate the phosphatase, while the fact that supplements of both Mg2+ and Ca2+ are required to produce conversion of pyruvate dehydrogenase to the active form confirms the requirements of the phosphatase for both cations as first observed with the iso-}

\begin{table}[h]
\centering
\caption{Effect of ionophore A23187 and EGTA on activity of pyruvate dehydrogenase in uncoupled mitochondria}
\begin{tabular}{|c|c|c|c|c|}
\hline
Additions & ATP:ADP & ATP & ADP & Mg2+ & Ca2+ \\
\hline
FCCP + A23187 & 0.5 & 2.1 ± 0.1 & 4.0 ± 0.1 & 6.0 ± 0.1 & 2.8 ± 0.3 & 78.3 ± 4.3 \\
FCCP + oligomycin + A23187 & 1.2 & 3.1 ± 1.0 & 2.6 ± 0.9 & 6.2 ± 1.6 & 2.1 ± 0.7 & 28.7 ± 6.3 \\
FCCP + A23187 & 0.9 & 3.7 ± 1.0 & 4.5 ± 1.7 & 8.3 ± 1.6 & 1.0 ± 0.4 & 20.4 ± 4.1 \\
FCCP + A23187 + EGTA & 0.5 & 2.8 ± 0.1 & 5.1 ± 0.4 & 9.7 ± 0.6 & 1.0 ± 0.3 & 26.8 ± 3.3 \\
FCCP + A23187 + EGTA + Ca2+ & 0.7 & 1.2 ± 0.2 & 1.7 ± 0.1 & 3.2 ± 0.5 & 6.2 ± 1.2 & 17.8 ± 3.6 \\
FCCP + A23187 + EGTA + Ca2+ + Mg2+ & 1.1 & 1.9 ± 0.3 & 1.6 ± 0.2 & 10.6 ± 1.7 & 5.0 ± 0.5 & 106.2 ± 0.4 \\
\hline
\end{tabular}
\end{table}

dehydrogenase kinase is not fully inhibited by a high intramitochondrial ADP content (e.g. Lines 2 to 5 of Table IV). Hence an inactive phosphatase causes pyruvate dehydrogenase to remain in the inactive phosphorylated form. The present data show that a Mg2+ content of 5.6 nmoles per mg of protein and a Ca2+ content of 1.4 nmoles per mg of protein was insufficient to activate the phosphatase, while the fact that supplements of both Mg2+ and Ca2+ are required to produce conversion of pyruvate dehydrogenase to the active form confirms the requirements of the phosphatase for both cations as first observed with the isolated components.

\section*{Effect of ionophore A23187 and EGTA on Pyruvate Dehydrogenase Activity in Uncoupled Mitochondria}

Under these conditions the intramitochondrial ATP:ADP ratio was 1 or below (Table V). With A23187 and uncoupler present, pyruvate dehydrogenase was 60% in the active form, i.e. slightly less active than the 75% observed with uncoupler alone added (Table III). This difference may be accounted for by a fall of the Mg2+ content from 17 to 6.6 nmoles per mg of protein. However, regulation of pyruvate dehydrogenase phosphor-ylation under these conditions is probably controlled mainly by the activity of pyruvate dehydrogenase kinase since addition of oligomycin together with uncoupler and A23187 caused a 35% fall of ADP content and a similar fall of pyruvate dehydrogenase activity (Table V). Nonetheless, regulation via pyruvate dehydrogenase phosphatase can also be demonstrated by addition of EGTA together with uncoupler and A23187 since this did not affect the ADP content but decreased the Ca2+ content to 1.0 nmoles per mg of protein and decreased pyruvate dehydrogenase activity from 59% to 26% of total (cf. Lines 1 and 4 of Table V). Hence, a lowered pyruvate dehydrogenase phosphatase activity at a constant kinase activity shifted the pyruvate dehydrogenase equilibrium over toward the inactive phosphorylated form. Subsequent addition of Ca2+ alone caused a further fall of pyruvate dehydrogenase activity, due partly to an increased kinase activity as a result of a fall of the ADP content and partly to a decreased phosphatase activity since Mg2+ levels fell 3-fold. A Mg2+ limitation of pyruvate dehydrogenase phosphatase under these conditions can be deduced from the fact that addition of Mg2+ together with Ca2+ fully restored pyruvate dehydrogenase activity without any further change of the ADP content.

\section*{Effect of Citrate Accumulation on Activity of Pyruvate Dehydrogenase in Mitochondria}

It is well known that citrate combines with magnesium to form a citrate-magnesium complex (44, 45). It was expected that accumulation of a high amount of citrate in mitochondria depleted of endogenous magnesium should shift the equilibrium of the two molecular forms of pyruvate dehydrogenase toward the inactive form of the enzyme due to a Mg2+ limitation of pyruvate dehydrogenase phosphatase activity. In order to investigate these interrelationships in more detail, mitochondria were incubated with 1 mM malate and 1 mM pyruvate as substrates (Table VI). It may be noted that the relatively high level of pyruvate dehydrogenase activity found in State 4 is
due to the protective effect of pyruvate against inactivation of pyruvate dehydrogenase by its kinase. The concentration of pyruvate in the incubation medium of each sample at the end of the incubation period was about 0.3 to 0.4 mM, which according to Portenhauser and Wieland (31) is sufficient for half-maximal activation of the pyruvate dehydrogenase complex in liver mitochondria. ADP (4 mM) plus oligomycin were added to the incubation mixture to provide maximal activation of pyruvate dehydrogenase by inhibition of the kinase (Table VI). Fluorocitrate was used to inhibit the aconitase reaction (46, 47) and benzene-1,2,3-tricarboxylate to block citrate transport from mitochondria (48). Under these conditions the intramitochondrial citrate content increased to 5 nmoles per mg of protein, but there was no effect on pyruvate dehydrogenase activity (Table VI). Addition of A23187 to the incubation mixture caused release of intramitochondrial calcium and magnesium and conversion of pyruvate dehydrogenase into the inactive form. Subsequent addition of Mg++, caused full activation, presumably by restoring activity of pyruvate dehydrogenase phosphatase. Addition of Ca++ alone produced only partial activation indicating that the phosphatase was still limited by the low intramitochondrial free Mg++ concentration.

**DISCUSSION**

An inverse relationship between ATP contents and the activity of pyruvate dehydrogenase has been shown by Martin et al. (16) using isolated rat epidymal fat cell mitochondria incubated under State 4 conditions and in the presence of oligomycin or uncoupler. However, the authors determined only the total amount of ATP in the incubation mixture without separation of mitochondria. Activation of pyruvate dehydrogenase by addition of ADP to rat liver mitochondria incubated in the absence of substrate has also been reported by Portenhauser and Wieland (31). These results are consistent with competitive inhibition of pyruvate dehydrogenase kinase by ADP, as observed with the isolated enzyme (5, 7, 16, 31). The data presented in this paper permit a more detailed evaluation of the various factors contributing to the regulation of pyruvate dehydrogenase in intact mitochondria respiring in different metabolic states. Of importance is the demonstration of substantial loss of both ATP and ADP from the mitochondria to the medium during even brief incubations at 28°. Greatest depletions of intramitochondrial ATP and ADP were observed after addition of p-trifluoromethoxyphenylhydrazone of carbonyl cyanide or Ca++, agents which uncouple oxidative phosphorylation and stimulate ATPase activity. Consequently, the influence of the phosphorylation state of the adenine nucleotides on over-all pyruvate dehydrogenase activity can most reliably be ascertained only after separation of the mitochondria from the medium. Furthermore, the method of separation must be sufficiently fast to maintain aerobic conditions and avoid phosphate transfer between nucleotides prior to enzyme inactivation by the perchloric acid. Likewise, the supernatant fluid after separation of the mitochondria must be added to perchloric acid as rapidly as possible to minimize nucleotide interconversions resulting from adenylate kinase activity.

Over-all activity of pyruvate dehydrogenase as measured by the percentage of total enzyme in the active nonphosphorylated form is determined by the relative activities of the kinase and the phosphatase. Available evidence indicates that there is not a significant difference in the maximum activities of the pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase (17). With both enzymes active, phosphorylation and dephosphorylation of pyruvate dehydrogenase will proceed in a cyclic manner with expenditure of ATP, while pyruvate dehydrogenase will remain partially active. Low activity of the kinase should result in a shift of the pyruvate dehydrogenase equilibrium toward the active form, while an inhibited phosphatase should result in a shift of the equilibrium in the opposite direction toward the inactive form. A key factor in the regulation of the kinase is the ADP concentration. The increased activity of pyruvate dehydrogenase observed after addition of oligomycin or uncoupler to mitochondria incubated with glutamate plus malate was found to correlate directly with the intramitochondrial ATP content and inversely with the ATP:ADP ratio (Table III). Under these conditions, the Mg++ and Ca++ contents of the mitochondrial matrix were high relative to the ATP content so that an active phosphatase may be postulated. Consequently, inhibition of the kinase by a rise of the ADP content (e.g. after addition of oligomycin and ADP) allows dephosphorylation and complete activation of pyruvate dehydrogenase.

Possible regulation of pyruvate dehydrogenase phosphatase activity by Mg++ or Ca++ limitation was investigated by using the divalent cation ionophore A23187 to release these ions from the mitochondria (32). However, interpretation of these data is complicated by changes in the phosphorylation state of the intramitochondrial adenine nucleotides as well as by loss of total nucleotides from the matrix space. Limitation of phosphatase activity should be revealed by conditions of low ADP, which allows pyruvate dehydrogenase kinase to be active. A relatively

**TABLE VI**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Mg++</th>
<th>Ca++</th>
<th>Citrate</th>
<th>Isocitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (State 4)</td>
<td>23.8</td>
<td>8.5</td>
<td>2.8 ± 0.3</td>
<td>0.056 ± 0.006</td>
</tr>
<tr>
<td>ADP + oligomycin</td>
<td>19.4</td>
<td>7.3</td>
<td>1.5 ± 0.1</td>
<td>0.007 ± 0.005</td>
</tr>
<tr>
<td>ADP + oligomycin + BTCA + F-Cit + A23187</td>
<td>11.4</td>
<td>6.8</td>
<td>5.0 ± 0.1</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>ADP + oligomycin + BTCA + F-Cit + A23187 + Mg++</td>
<td>1.8</td>
<td>1.3</td>
<td>6.2 ± 0.6</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>ADP + oligomycin + BTCA + F-Cit + A23187 + Ca++</td>
<td>12.6</td>
<td>1.8</td>
<td>0.003 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>ADP + oligomycin + BTCA + F-Cit + A23187 + Ca++</td>
<td>1.9</td>
<td>6.3</td>
<td>0.003 ± 0.001</td>
<td></td>
</tr>
</tbody>
</table>

%  

10.8 ± 10.6

0.7 ± 0.7

25.5 ± 2.7

103.7 ± 3.5

40.8 ± 10.6
inactive phosphatase will then be associated with pyruvate dehydrogenase being present predominantly in the inactive form, while activation of the phosphatase should cause a shift in the equilibrium toward the active form. Since pyruvate dehydrogenase is predominantly inactive under State 4 conditions, it is apparent that in the absence of pyruvate and with normal intramitochondrial cation contents, the activity of the kinase is greater than that of the phosphatase. Interconversion between the phosphorylated and nonphosphorylated forms of pyruvate dehydrogenase acts as a potential ATPase. This could well be mistaken for respiratory chain ATPase activity since addition of oligomycin, particularly in the presence of ADP, may cause an inhibition of respiration as a result of inhibition of pyruvate dehydrogenase kinase. The degree to which State 4 respiration is caused by pyruvate dehydrogenase phosphorylation and dephosphorylation, therefore, is likely to depend on the activity of pyruvate dehydrogenase phosphatase. A most attractive form of regulation in terms of energy conservation would be for the phosphatase to be fully inhibited due to deficiency of cations when the energy state of the mitochondria is high, and for Mg$^{2+}$ or Ca$^{2+}$, or both, to be released from MgATP$^{2-}$ and CaATP$^{2-}$ complexes under conditions of active respiration. At the same time, inhibition of pyruvate dehydrogenase by ADP when respiration is stimulated minimizes energy wastage due to a cyclic interconversion between the two forms of pyruvate dehydrogenase. The present data provide clear evidence in favor of an ADP control of pyruvate dehydrogenase kinase activity in the intact mitochondria over a range of ADP concentrations associated with maximal inhibition and maximal activation of respiratory chain phosphorylation. However, the data are less readily interpretable in relation to Mg$^{2+}$ and Ca$^{2+}$ effects on pyruvate dehydrogenase phosphatase. The principal reason for this difficulty lies in the lack of knowledge of the free cation concentration for a given measured content of Mg$^{2+}$ or Ca$^{2+}$, together with the possibility that mitochondria as isolated have an atypically high content of Ca$^{2+}$ as a result of an active uptake during the tissue homogenization procedure. Perfusion of livers with Ca$^{2+}$-free medium prior to homogenization for instance has been found to diminish the mitochondrial Ca$^{2+}$ content to 1 to 2 nmoles per mg of protein.

A free Mg$^{2+}$ concentration in the mitochondrial matrix of at least 2.5 mM under State 4 conditions is indicated from the high ratio of citrate to isocitrate (Table VI), according to the Mg$^{2+}$ dependence of the aconitase equilibrium reported by England et al. (44). It thus appears unlikely that with a total magnesium plus calcium content three times higher than the ATP content (Table III), a conversion of ATP to ADP would cause activation of pyruvate dehydrogenase phosphatase by release of free Mg$^{2+}$ due to the different stability constants of the MgATP$^{2-}$ and MgADP$^{2-}$ complexes. The data obtained by addition of A23187 to mitochondria in State 4 indicate that up to 90% of the total Ca$^{2+}$ and 75 to 85% of the total Mg$^{2+}$ is readily exchangeable, in accordance with suggestions from the aconitase equilibrium that the free Mg$^{2+}$ is in the millimolar concentration range even in the presence of high ATP levels. The free Ca$^{2+}$ concentration cannot readily be estimated but is probably considerably lower than that of Mg$^{2+}$, because of the relative insolubility of calcium phosphate at the slightly alkaline pH of the mitochondrial matrix.

In the present experiments, regulation of pyruvate dehydrogenase interconversion by changes of phosphatase activity could only be demonstrated under extreme conditions of depleted divalent cation content. Since the ionophore A23187 lacked specificity between Ca$^{2+}$ and Mg$^{2+}$, and since Ca$^{2+}$ transport is associated with ATPase activity, it was not possible to investigate the effects of Mg$^{2+}$ depletion in the absence of changes of Ca$^{2+}$ and ADP contents. However, when EGTA was added to the mitochondria in addition to A23187, the ADP content remained low enough to allow activity of the kinase, but pyruvate dehydrogenase itself was not converted to the active form unless both Mg$^{2+}$ and Ca$^{2+}$ were added to the medium in excess of the EGTA present (Table IV). With Ca$^{2+}$ alone added, the magnesium content of the mitochondria was only 3.3 nmoles per mg of protein relative to an ATP content of 2.1 nmoles per mg of protein, indicating that the maximum possible concentration of free Mg$^{2+}$ was about 1 mM, although the actual concentration was probably lower than this because of chelation with negatively charged species other than ATP$^{4-}$. The fact that a 3-fold increase of Mg$^{2+}$ content (to 9.2 nmoles per mg of protein) produced full activation of pyruvate dehydrogenase is consistent with the reported $K_a$ for Mg$^{2+}$ of 2 to 3 mM with isolated pyruvate dehydrogenase phosphatase (10, 11, 22). The tricarboxylate carrier is very active in liver mitochondria so that it is unlikely that citrate normally accumulates in the mitochondrial matrix to a sufficient extent to complex much of the available Mg$^{2+}$. In the present experiments only a limited success was achieved in raising the matrix citrate content by inhibiting its utilization with fluorocitrate and its transport with benzene tricarboxylate, possibly because the citrate distribution is limited by the ΔpH across the mitochondrial membrane (49). However, the data of Table VI show that a 3-fold increase of citrate content using mitochondria with a relatively undepleted Mg$^{2+}$ content failed to cause an inactivation of the phosphatase. This was achieved only by subsequent addition of A23187 which produced a severe depletion of both Mg$^{2+}$ and Ca$^{2+}$. The fact that addition of Mg$^{2+}$ alone under these conditions caused full activation of pyruvate dehydrogenase, presumably by increasing the phosphatase activity, despite a low Ca$^{2+}$ content of 1.8 nmoles per mg of protein suggests an additional compartmentation of calcium within the matrix which may affect the availability of Ca$^{2+}$ to the phosphatase.

The $K_a$ of pyruvate dehydrogenase phosphatase for calcium is not yet known. Pettit et al. (20) described stimulation of bovine kidney and heart pyruvate dehydrogenase phosphatase activity in the presence of dihydroxyacetone phosphate and pyruvate dehydrogenase by 1.25 mM Ca$^{2+}$ with or without 1 mM EGTA. Denton et al. (21) found that the activity of partially purified pyruvate dehydrogenase from pig heart, pig kidney cortex, and rat epididymal fat cells in the presence of Mg$^{2+}$ was stimulated by 9 μM Ca$^{2+}$, but enzyme from rat liver mitochondria appeared to have a lower sensitivity to Ca$^{2+}$. The present experiments confirm that both Ca$^{2+}$ and Mg$^{2+}$ are required for activity of pyruvate dehydrogenase phosphatase in intact rat liver mitochondria, and suggest that an intramitochondrial content of 1 to 2 nmoles per mg of protein of total calcium is sufficient when the ATP content is low. However, the sensitivity of pyruvate dehydrogenase phosphatase to calcium could not be ascertained with intact mitochondria since addition of Ca$^{2+}$ to mitochondria caused alterations of both the phosphorylation state and total content of the intramitochondrial adenine nucleotides.

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Regulation of Pyruvate Dehydrogenase in Rat Liver Mitochondria by Phosphorylation-Dephosphorylation
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