Characterization of the Phosphoenolpyruvate Inhibition of Mitochondrial Protein Synthesis*

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The interaction of phosphoenolpyruvate with isolated rat liver mitochondria has been further investigated. The uptake of P-enolpyruvate is accompanied by the stoichiometric release of intramitochondrial adenine nucleotides resulting in the inhibition of mitochondrial protein synthesis. Addition of specific inhibitors of either the adenine nucleotide carrier or the tricarboxylic acid carrier blocks the P-enolpyruvate-stimulated loss of adenine nucleotides and thereby prevents the resultant inhibition of mitochondrial protein synthesis.

These data suggest that there is a specific interaction between the mitochondrial adenine nucleotide translocase and the tricarboxylic acid carrier resulting in the control of intramitochondrial adenine nucleotide levels by phosphoenolpyruvate.

In recent years, increased attention has been focused on the mechanisms by which substrate anions penetrate the selectively permeable inner mitochondrial membrane. In contrast to the well characterized systems for cation transport, the transport of anions appears to be energy independent and to proceed by a carrier mediated diffusion. To date, seven anion carriers have been ascribed to the inner mitochondrial membrane. These include transport systems for the adenine nucleotides (1-3), inorganic phosphate (4-6), dicarboxylic acids (7-10), L-ketoglutarate (7), tricarboxylic acids (11-13), aspartate (14), and glutamate (14, 15). In addition to catalyzing exchanges with endogenous anions of the same molecular species, several of the anion carriers participate in heterologous exchange reactions. Heterologous exchanges have been shown to occur between phosphate and dicarboxylic acids, dicarboxylic acids and tricarboxylic acids, and L-ketoglutarate and dicarboxylic acids (16, 17).

Harris has shown that phosphoenolpyruvate, long thought to be a nonpermeating species because of its hydrophilic nature, can influence the net accumulation of citrate within the mitochondrial matrix (18). This observation is consistent with the earlier observation by Gamble and Mazur (19) that citrate facilitated the release of endogenous P-enolpyruvate from rabbit liver mitochondria. Furthermore, Robinson (20) demonstrated that mitochondria preloaded with either citrate or malate rapidly exchange endogenous anion for exogenously added P-enolpyruvate. The exchange of exogenous P-enolpyruvate for both endogenous citrate and malate was inhibited by benzene 1,2,3-tricarboxylic acid, an inhibitor of the tricarboxylic acid carrier (13). We have previously reported (21) that P-enolpyruvate is capable of inhibiting protein synthesis in isolated rat liver mitochondria. We showed that P-enolpyruvate inhibition could be overcome by either increasing the concentration of externally added ADP or by the addition of atracyloside, an inhibitor of the adenine nucleotide translocase system, and that addition of P-enolpyruvate caused the loss of radioactivity from mitochondria that had been prelabeled with [14C]ADP. We concluded that the ability of P-enolpyruvate to inhibit mitochondrial protein synthesis was due to its ability to cause release of intramitochondrial adenine nucleotides.

This report further documents the inhibitory effect of P-enolpyruvate on mitochondrial protein synthesis and presents evidence that the P-enolpyruvate inhibition of mitochondrial protein synthesis can be directly correlated with the loss of adenine nucleotides from mitochondria. In addition, we will show that the exchange of exogenous P-enolpyruvate for endogenous adenine nucleotide requires the participation of at least two different carrier systems, namely the adenine nucleotide carrier and the tricarboxylic acid carrier.

**EXPERIMENTAL PROCEDURE**

Materials—Firefly lantern extract (FLE-50); adenylate kinase; pyruvate kinase (type II); adenosine 5'-monophosphate, sodium salt; adenosine 5'-diphosphate, sodium and barium salt; adenosine 5'-triphosphate, sodium and barium salt; phosphoenolpyruvate, tricyclohexylammonium and barium salt; L-ketoglutaric acid; malic acid; and atracyloside, potassium salt, were obtained from Sigma Chemical Co. Citric acid, succinic acid, and ethylenediaminetetraacetic acid were purchased from Fisher Chemical Co. N-Tri(hydroxymethyl)methylglycine was obtained from Calbiochem. Sucrose, special enzyme grade, was obtained from Schwarz/Mann Research Corp. Benzene 1,2,3-tricarboxylic acid was obtained from Aldrich Chemical Co.
8-14C]Adenosine-5'-diphosphate (17.7 mCi/mmol) and L-14C]valine (210 to 248 mCi/mmol) were obtained from New England Nuclear Corporation. Polyelectrolyneine (PEI) chromatogram sheets were obtained from Brinkmann Instruments Inc., Westbury, N.Y.

All solutions were prepared with double distilled water and brought to desired pH (usually 7.4) by the addition of dilute potassium hydroxide or hydrochloric acid. Final solutions were sterilized either by autoclaving or by filtering through 0.45-μm Millipore filters. Incubation mixtures for all experiments were filtered through 0.45-μm Millipore filters immediately before use. Barium salts of adenine nucleotides and phosphoeno pyruvate were converted to the potassium salts by following the procedures suggested by the manufacturer. Norit A was prepared as described by Zimmerman (23). Toluene-fluors scintillation cocktail was made from a 25-fold dilution of Nuclear Chicago Lique fluor in toluene.

Mitochondria were prepared from the livers of adult male CD rats (descended from Sprague-Dawley) purchased from Charles River Breeding Laboratories, Inc. as described previously (21).

**Assay of Protein Synthesis**—The rate of in vitro protein synthesis was measured in an incubation medium consisting of 100 mM sucrose; 2 μmol of EDTA; 1 μmol of MgCl2; 10 μmol of KH2PO4, pH 7.4; 33 μmol of Tricine buffer, pH 7.5; 750 μg of complete equimolar amino acid mixture minus valine; 0.5 μCi of 14C]valine; 1 to 3 mg of mitochondrial protein. Final volume was 1.0 ml. Other additions were as described in figures and tables. Mitochondria were added to the complete incubation mixture minus radioactive valine and were incubated for 5 min at 30°C in a Dubnoff metabolic shaking incubator. Following the addition of labeled amino acid, tubes were incubated for 45 min at 30°C. At the end of the incubation period, 0.1-ml aliquots of the incubation media were removed, placed on 2.5-cm paper filter discs (Whatman No. 3MM), and prepared for liquid scintillation counting by the procedure of Mano and Novelli (23). Discs were placed in scintillation vials containing 6.0 ml of toluene-fluor mixture and counted in a Beckman LS-100 liquid scintillation system using the procedure described in figures and tables. Bacterial contamination was checked by plating 0.1-ml aliquots of mitochondrial preparations and incubation media immediately following the end of each experiment on either trypticase soy agar or nutrient agar plates. Plates were incubated at room temperature and 37°C because it was observed that a frequent contaminant (Pseudomonas) did not grow at 37°C. The number of colonies per ml of inoculation mixture was consistently less than 100 colonies in all experiments reported here.

**Labeling of Mitochondrial Adenine Nucleotide Pool**—Mitochondria were incubated for 10 min at 4°C in a medium consisting of 200 μmol of sucrose; 4 μmol of EDTA; 20 μmol of KH2PO4; 2 μmol of MgCl2; 66 μmol of Tricine buffer, pH 7.4; 80 nmol of ADP; 0.4 μCi of 14C]ADP; 4 to 5 mg of mitochondrial protein in a final volume of 2.0 ml. The mitochondria were resuspended by centrifugation at 12,000 × g for 5 min, washed once, and resuspended to give a final protein concentration of approximately 20 mg of protein/ml.

**Measurement of P-enolpyruvate Uptake and Adenine Nucleotide Release**—Prelabeled mitochondria (2 mg/ml) were added to media consisting of 100 μmol of sucrose; 2 μmol of EDTA; 1 μmol of MgCl2; 10 μmol of KH2PO4; 33 μmol of Tricine buffer, pH 7.4, in a final volume of 1.0 ml. Assay tubes were incubated for 5 min at 30°C in a Dubnoff metabolic shaking incubator prior to the addition of substrates. Following the addition of substrate, tubes were incubated for 10 min at 30°C. Experiments were terminated by centrifugation at 12,000 × g for 0 min. Supernatants were saved for adenine nucleotide analysis. Mitochondrial pellets were resuspended and reisolated by centrifugation and then extracted as described by Strehler (24) for P-enolpyruvate and adenine nucleotide analysis.

Total adenine nucleotides were determined by conversion of AMP and ADP to ATP and by measurement of the resultant ATP with luciferin-luciferase as described by Adams (25). The adenine nucleotide content of mitochondrial preparations assayed in this manner varied from 8 to 10 nmol/mg of protein which is in excellent agreement with published results obtained with other assay systems (26, 27).

**Separation of Adenine Nucleotides**—Polyelectrolyneine chromatograms were developed in 0.75% KH2PO4, pH 3.4, until the solvent front migrated 10 cm from the origin. Chromatograms were then oven dried and adenine nucleotides were visualized by ultraviolet light. Visualized spots as well as a representative portion of the origin were scraped from the chromatogram, placed in scintillation vials, and counted as described previously for protein synthesis.

**RESULTS**

The effect of atracyloside concentration on preventing the inhibition of mitochondrial protein synthesis by 5 mM P-enolpyruvate is shown in Fig. 1. As little as 0.5 μM atracyloside was sufficient to give 50% prevention and complete prevention was obtained with 6 μM atracyloside. These values are in excellent agreement with those reported by others for the interaction of atracyloside with whole mitochondria (2, 29), isolated inner mitochondrial membranes (30), and reconstituted vesicles (31).

Since P-enolpyruvate was an effective inhibitor of the endogenous rate of in vitro mitochondrial protein synthesis (21), we investigated the effect of P-enolpyruvate on the respiratory substrate-supported rate of protein synthesis. Table I shows that 5 mM P-enolpyruvate produced greater than 90% inhibition of the rate of mitochondrial protein synthesis supported by either succinate, malate, or citrate as well as the endogenous rate. Table I also shows that the P-enolpyruvate inhibition could be substantially blocked by the addition of 6 μM atracyloside.

The effect of varying the P-enolpyruvate concentration on the endogenous rate of mitochondrial protein synthesis as well as}

![Fig. 1](https://example.com/fig1.png)
as release of mitochondrial adenine nucleotides is shown in Fig. 2, A and B. The amount of P-enolpyruvate required for either 50% inhibition of protein synthesis or 50% release of mitochondrial adenine nucleotides varied from 0.4 to 0.7 mM with maximum effect occurring at P-enolpyruvate concentrations greater than 3 mM.

Enzymatic analysis of the released adenine nucleotide indicated that while ATP was the major nucleotide released, substantial amounts of ADP and AMP were also found (Table II). Chromatographic analysis of the radioactivity released from mitochondria that had been prelabeled with [14C]ADP showed that essentially all of the radioactivity co-migrated with adenine nucleotide standards. Since the same specific radioactivity was obtained for each of the three adenine nucleotides, it appears that either ATP and/or ADP was transported across the inner mitochondrial membrane and acted upon by adenylate kinase. Adenylate kinase activity in the intermembrane space would cause the appearance of ATP, ADP, and AMP of the same specific radioactivity.

Since P-enolpyruvate and adenine nucleotides exist as anions at physiological pH, it was of interest to see if other anions were capable of causing the release of mitochondrial adenine nucleotides. As can be seen in Table III, none of the anions other than P-enolpyruvate was able to release significant amounts of adenine nucleotides from mitochondria.

Recent work (13, 20) has suggested that P-enolpyruvate is transported through the inner mitochondrial membrane by the tricarboxylic acid carrier system. Since this system is inhibited by benzene 1,2,3-tricarboxylic acid, it was expected that this inhibitor would counteract the inhibition of mitochondrial protein synthesis if transport of P-enolpyruvate was required for the observed inhibition. Fig. 3 shows the concentration-dependent prevention of the inhibition caused by 5 mM P-enolpyruvate by increasing concentrations of benzene 1,2,3-tricarboxylic acid. Maximal prevention occurred with 15 to 25 mM benzene1,2,3-tricarboxylic acid. Increasing benzene1,2,3-tricarboxylic acid concentration beyond 25 mM greatly decreased its effectiveness.

**TABLE II**

Distribution of adenine nucleotides released from mitochondria in presence of 5 mM P-enolpyruvate

<table>
<thead>
<tr>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
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</thead>
<tbody>
<tr>
<td>3.08</td>
<td>1.69</td>
<td>2.68</td>
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</table>

Reported values are averages of triplicate determinations done in one experiment. Absolute values varied from experiment to experiment but the same relative values were consistently obtained.

**TABLE III**

Effect of Krebs cycle intermediates on adenine nucleotide release from mitochondria

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenine nucleotide released</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.10</td>
</tr>
<tr>
<td>5.0 mM α-ketoglutarate</td>
<td>0.51</td>
</tr>
<tr>
<td>10.0 mM succinate</td>
<td>0.20</td>
</tr>
<tr>
<td>5.0 mM citrate</td>
<td>0.14</td>
</tr>
<tr>
<td>5.0 mM P-enolpyruvate</td>
<td>7.59</td>
</tr>
</tbody>
</table>

**Fig. 2.** The effect of increasing concentrations of phosphoenolpyruvate (PEP) on (A) mitochondrial protein synthesis and (B) release of intramitochondrial adenine nucleotides (AdN).

**Fig. 3.** Concentration-dependent protection of phosphoenolpyruvate inhibition of mitochondrial protein synthesis by benzene 1,2,3-tricarboxylic acid (BTA). Mitochondria were incubated for 5 min at 30° prior to the addition of label. The rate of label incorporation in the uninhibited controls was 960 cpm/45 min/mg of protein.
The data presented in Table IV presents data from experiments that were carried out to determine the stoichiometry of P-enolpyruvate uptake and adenine nucleotide release. Mitochondria were incubated at 30° for 10 min in the presence of 5 mM P-enolpyruvate. Mitochondria were reisolated by centrifugation, washed once, extracted, and assayed for P-enolpyruvate. The incubation media was assayed directly for adenine nucleotides. It was found that the ratio of P-enolpyruvate taken up to adenine nucleotide released was 1:1.

The effect of atractyloside and benzene tricarboxylic acid on the uptake of P-enolpyruvate and the release of adenine nucleotides from mitochondria is presented in Table V. It can be seen that concentrations of the two inhibitors which prevented the inhibition of mitochondrial protein synthesis by P-enolpyruvate were equally effective in inhibiting the release of mitochondrial adenine nucleotides. In contrast, while benzene tricarboxylic acid greatly diminished the uptake of P-enolpyruvate, atractyloside did not.

**DISCUSSION**

Our previous observations that the inhibition of mitochondrial protein synthesis by P-enolpyruvate could be overcome either by the addition of atractyloside or by the addition of ADP suggested that the observed inhibition was due to the stimulation of adenine nucleotide release via the mitochondrial adenine nucleotide carrier (21). It was not known if P-enolpyruvate was itself a substrate for the adenine nucleotide translocase or if entry of P-enolpyruvate was mediated by another carrier. The data presented in this paper suggest that the interaction of P-enolpyruvate with intramitochondrial adenine nucleotides requires the operation of at least two distinct mitochondrial transport systems, namely, the atractyloside-sensitive adenine nucleotide carrier system for the transport of adenine nucleotides out of the mitochondria, and the benzene tricarboxylic acid-sensitive tricarboxylic acid carrier system for the entry of P-enolpyruvate into the mitochondria. Such heterologous exchange reactions have been previously described for competing transport between mitochondrial substrate anions, but the adenine nucleotide carrier has not been implicated previously in heterologous exchange reactions. The stoichiometry of the exchange appears to be 1:1, that is, for each molecule of P-enolpyruvate taken in, a molecule of adenine nucleotide is released.

Numerous investigators have been able to show that anionic mitochondrial substrates are capable of competing with one another for occupancy in the sucrose-impermeable space of the mitochondrial (i.e. that space which is enclosed by the inner mitochondrial membrane) and that the nature of this competition appears to be a result of the net charge of the competing anions (18). It was therefore important for us to determine whether the release of adenine nucleotides in the presence of P-enolpyruvate could be duplicated by mitochondrial substrate anions. The data in Table III show quite clearly that none of the other anions tested was able to cause observable release of adenine nucleotides. Similar experiments by Shug and Shrago (32) have shown that citrate, malate, and pyruvate are unable to release mitochondrial adenine nucleotides. These experiments suggest that the interaction of P-enolpyruvate with endogenous adenine nucleotides is highly specific and cannot be explained on the basis of a nonspecific charge displacement hypothesis.

It seems clear that both the inhibition of mitochondrial protein synthesis which we observe, as well as the release of preloaded calcium from mitochondria that was first observed by Chudapongse and Haugaard (33), are the result of the decrease in mitochondrial adenine nucleotides caused by the translocation of P-enolpyruvate. The factors which coordinate the interaction of the two carrier systems are as yet undefined. It is of interest that Peng et al. have shown that the P-enolpyruvate-stimulated release of calcium from preloaded mitochondria requires phosphate, and can be blocked by sulfhydryl reagents which are known to inhibit the phosphate-hydroxyl exchange system (34). It is not yet clear whether phosphate is required for the uptake of P-enolpyruvate or the release of calcium or both. Further insight into the nature of the interaction of P-enolpyruvate with mitochondrial adenine nucleotides has been provided by the work of Shug and Shrago who have shown that low concentrations (5 μM) of oleoyl-CoA which are capable of reversibly inhibiting both the uptake of ADP as well as P-ATP exchange in isolated rat liver mitochondria (35), are equally effective in inhibiting the uptake of P-enolpyruvate (32).

The fact that intramitochondrial adenine nucleotides are capable of being mobilized and translocated from their sequestered compartment within the inner mitochondrial membrane by a key intermediate of the glycolytic pathway has potential regulatory significance. It is possible that P-enolpyruvate functions as a readily reversible cytoplasmic regulator of intramitochondrial adenine nucleotide levels.

**REFERENCES**


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**TABLE IV**

*Relation of P-enolpyruvate taken up and adenine nucleotide released by mitochondria*

<table>
<thead>
<tr>
<th>P-enolpyruvate uptake</th>
<th>Adenine nucleotide released</th>
<th>Ratio of adenine nucleotide released to P-enolpyruvate uptake</th>
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</thead>
<tbody>
<tr>
<td>4.59 ± 0.11 (5)</td>
<td>4.98 ± 0.17 (5)</td>
<td>1.08</td>
</tr>
<tr>
<td>5.03 ± 0.31 (5)</td>
<td>5.19 ± 0.26 (5)</td>
<td>1.03</td>
</tr>
</tbody>
</table>

**TABLE V**

*Effect of mitochondrial transport inhibitors on P-enolpyruvate uptake and adenine nucleotide release*

<table>
<thead>
<tr>
<th>Additions</th>
<th>P-enolpyruvate uptake</th>
<th>Adenine nucleotide release</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>0.16</td>
</tr>
<tr>
<td>+ 5 mM P-enolpyruvate</td>
<td>6.13</td>
<td>6.59</td>
</tr>
<tr>
<td>+ 5 mM P-enolpyruvate + 20 mM benzene tricarboxylic acid</td>
<td>2.14</td>
<td>1.29</td>
</tr>
<tr>
<td>+ 5 mM P-enolpyruvate + 6 μM atractyloside</td>
<td>6.03</td>
<td>1.34</td>
</tr>
</tbody>
</table>

*Not applicable.*
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