The Effect of Adenosine Triphosphate on the Tricarboxylate Transporting System of Rat Liver Mitochondria*

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SUMMARY

ATP has two significant effects on the mitochondrial tricarboxylate transporting system.

First, it alters the concentration gradients at equilibrium for the substrates of this transporter. ATP (2 mM) caused the uptake of 10 nmol of citrate into the mitochondria coincident with the output of a similar amount of L-malate. This redistribution was dependent on ATP transport, the effect being inhibited by atractyloside and mimicked by the nonmetabolizable derivative adenylyl imidodiphosphate. A mechanism to account for these observations is proposed.

Secondly, preincubation of mitochondria with ATP resulted in a 2- to 3-fold increase in the \( K_m \) of the mitochondrial citrate transporter. This effect of ATP was not produced by ADP and \( P_i \), nor by \( N,N,N',N'-\text{tetramethyl-p-phenylenediamine} \) and ascorbate. It was prevented by the addition of rotenone and antimycin A. This effect of ATP was observed in the presence of oligomycin and could not be attributed to a change in the content of the known tricarboxylate carrier inhibitor, palmityl-CoA, nor to the ATP concentration. The origin of possible regulatory factor (or factors) is discussed.

It has been demonstrated that the tricarboxylate transporting system of rat liver mitochondria has affinity for a number of anionic species, the major ones being citrate, isocitrate, L-malate, and phosphoenolpyruvate (1-3). A relationship between this transporting system and the adenine nucleotide transporting system has been suggested because both of these transport systems are inhibited by long chain acyl-CoA esters (4-6), both transport phosphoenolpyruvate (7), and both are inhibited by carboxyatractyloside (8).

High rates of fatty acid oxidation in the intact liver are associated with a fall in the total tissue content of ATP with no change in total adenine nucleotide content (9), while high rates of fatty acid oxidation in isolated mitochondria are associated with higher rates of adenine nucleotide translocation, a lowering of affinity of the adenine nucleotide transporting system for ADP, and an increase in mitochondrial ATP content (10). Hypoxia on the other hand leads to conditions of reduced ATP levels both in intact liver and isolated mitochondria (11).

Transport of citrate from mitochondria to cytosol in liver may be important from a control standpoint since it provides acetyl-CoA for fatty acid synthesis (12), it activates acetyl-CoA carboxylase (13), and inhibits phosphofructokinase (14). Thus, any effect of ATP on the transport of citrate might result in modulation either of the glycolytic or lipogenic pathways. In the course of evaluating the influence of ATP on the tricarboxylate carrier, two separate effects of ATP have been established. In this communication, we shall present data indicating that ATP influences the kinetics of the tricarboxylate carrier, and it also appears to alter the concentration gradients at equilibrium for the substrates of this transporter.

METHODS AND MATERIALS

Rat liver mitochondria were loaded with [\(^{14}C\)]citrate as described previously (15). "Inhibitor stop" experiments for the tricarboxylate carrier were carried out as previously described (15). Further experimental details are given either in the text or in the legends to figures. L-Malate (16), citrate (17), ATP, ADP, AMP, and CoA-SH were determined by enzymic fluorimetric methods (18).

Loading of Mitochondria with [\(^{32}P\)]ATP—Rat liver mitochondria (50 mg of protein) were added to 5 ml of a medium containing 125 mm KCl, 20 mm Tris-HCl, 2 \( \mu \)M rotenone, 1 \( \mu \)M of antimycin A, 2 \( \mu \)M of oligomycin, 0.5 mm ATP, 2.5 mm [\(^{32}P\)]ATP, pH 7.4, at 10°. The suspension was then made up to 100 ml with ice-cold buffer containing 0.25 m sucrose, 5 mm Tris-HCl, pH 7.4, and the mitochondria separated by centrifugation at 18,000 \( \times g \) for 10 min. The adhering supernatant was decanted off as much as possible and the mitochondria resuspended in 3 to 4 ml of the sucrose-Tris buffer for use in isotopic exchange experiments. The adenine nucleotide content of these loaded mitochondria were 8 nmol of ATP, 0.6 nmol of ADP, and 1.4 nmol of AMP per mg of protein.

Preincubation Procedure—Rat liver mitochondrial preincubations were carried out in a final volume of 5 ml of 20 mm Tris-HCl and 125 mm KCl, pH 7.4, for 10 min at 23°. The mitochondrial protein concentration ranged from 30 to 90 mg/ml. The final concentrations where indicated were: L-malate, ADP, and inorganic phosphate 1 mm, ATP 10 mm, oligomycin 10 to 15 \( \mu \)g/ml, rotenone 2.0 \( \mu \)M, antimycin 0.6 \( \mu \)M, ascorbate 1 mm, and \( N,N,N',N'-\text{tetramethyl-p-phenylenediamine} \), 100 \( \mu \)M. In experiments with hexokinase, 4 units of dialyzed enzyme, 25 mm glucose, 7 mm inorganic phosphate, and 5 mm ATP were present. After each preincubation, the mitochondrial suspension was washed with sucrose-Tris buffer and centrifuged for 5 min at 13,000 \( \times g \). The mitochondrial pellet was then suspended in the KCl, Tris-HCl buffer above and loaded with \([^{14}C]\)citrate as described (12).

Chemicals—ADP, ATP, adenylyl imidodiphosphate, and hexo-

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from rat liver mitochondria in the presence and absence of K & K Laboratories, Plainview, N. Y. Sodium [14C]bicarbonate N,N,N',N'-tetramethyl-p-phenylenediamine were from British Glucose, ascorbate, and were from Sigma Chemical Co., St. Louis, MO. glutamate anion [14C]citrate (1 mM) was the substrate for the provision of energy, TMPD and ascorbate were employed. As expected, L-malate caused an efflux of citrate from the mitochondria, while ATP caused an uptake of citrate into the mitochondria separated by centrifugation. The presence of TMPD and ascorbate did not cause [14C]citrate movement as did ATP, but in contrast did alter the equilibrium position after an L-malate citrate exchange (Fig. 3). In contrast there was little or no effect of ATP when the nonmetabolizable ATP analogue, adenylyl imidodiphosphate was added to mitochondria loaded with [α-32P]ATP from New England Nuclear, Boston, Mass. We thank Dr. Henry Lardy (Wisconsin) for a gift of atractyloside.

**RESULTS**

Rat liver mitochondria loaded with [14C]citrate were added to 1-ml incubations of a buffer containing 125 mM KCl, 20 mM Tris-HCl, pH 7.4, at 10°C in the presence of either L-malate, ATP, or L-malate plus ATP and the movement of labeled citrate was followed with time using the inhibitor stop technique (Fig. 1). As expected, L-malate caused an efflux of citrate from the mitochondria, while ATP caused an uptake of citrate into the mitochondria. When present together, ATP and L-malate had a complex effect on citrate movement resulting in inward movement followed by an efflux. When no external anions were added to the medium (not shown) little citrate movement occurred over the 2-min incubation. ATP added after 1 min of exchange with 1 mM L-malate resulted in a prompt uptake of [14C]citrate followed by the establishment of a new equilibrium position (Fig. 2). In contrast there was little or no effect of ATP when the tricarboxylate anion [14C]citrate (1 μM) was the substrate for the exchange.

In order to determine whether this effect of ATP was caused by the provision of energy, TMPD3 and ascorbate were employed. The presence of TMPD and ascorbate did not cause [14C]citrate uptake as did ATP, but in contrast did alter the equilibrium position after an L-malate citrate exchange (Fig. 3). In order to delineate the mode of action of ATP on the citrate transporting system, 2-min inhibitor stop incubations were performed and the results reported in Tables I and II. The intramitochondrial citrate rose from 37.5 to 47.5 nmol and the extramitochondrial citrate fell by a similar quantity (20.8 to 11.1 nmol) when ATP was added. This confirms the radioactive exchange data of Figs. 1 and 3. In contrast, the mitochondrial L-malate fell from 46.2 to 36.2 nmol coincident with a similar rise in the extramitochondrial L-malate (36.1 to 45.2 nmol). This suggests a stoichiometric 1:1 relationship between the reciprocal movements of these anions. This effect of ATP was not influenced by oligomycin or mersalyl but was inhibited by atractylloside, indicating possible involvement of the adenine nucleotide transporting system (19). Atractylloside also prevented ATP from reducing the extent of L-malate exchange for citrate while mersalyl (20) and 2-pentylmalonate (21) were without effect.

When the nonmetabolizable ATP analogue, adenylyl imidodiphosphate was added to mitochondria loaded with [α-32P]ATP and the time course of ATP efflux followed by stopping the reaction at various times with atractylloside (Fig. 4), it was found to give an extensive exchange with the labeled compound although not as extensive as when ATP itself was added as the

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1 The abbreviations used are: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; AMP-PNP, adenylyl imidodiphosphate.
**TABLE I**

Effect of oligomycin, mersalyl, and atractyloside on ATP-induced citrate uptake in \(^{14}C\)citrate-loaded rat liver mitochondria

Incubations were carried out with 9.1-mg aliquots of mitochondria as described in Fig. 1 for 2 min stopping the reaction with 50 mM 1,2,3-tricarboxybenzene. After separation samples of supernatant and pellet were taken for counting and assay of citrate. The above figures are the mean values obtained upon duplicate incubations. The reference point for calculating exchanges was an incubation with 50 mM 1,2,3-tricarboxybenzene added at zero time. Oligomycin, mersalyl, and atractyloside added alone gave no exchange with \(^{14}C\)citrate.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Percentage exchange with (^{14}C)citrate</th>
<th>Citrate</th>
<th>L-Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol</td>
<td>mmol</td>
<td>mmol</td>
</tr>
<tr>
<td>None</td>
<td>0.8</td>
<td>20.8</td>
<td>37.5</td>
</tr>
<tr>
<td>2 mM ATP</td>
<td>-25.4</td>
<td>11.1</td>
<td>47.3</td>
</tr>
<tr>
<td>2 mM ATP + 1 µg/ml oligomycin</td>
<td>-22.0</td>
<td>12.2</td>
<td>46.1</td>
</tr>
<tr>
<td>2 mM ATP + 0.1 mM mersalyl</td>
<td>-19.4</td>
<td>14.2</td>
<td>44.3</td>
</tr>
<tr>
<td>2 mM ATP + 10 µM atractyloside</td>
<td>0.4</td>
<td>19.8</td>
<td>38.2</td>
</tr>
</tbody>
</table>

**TABLE II**

Exchanges given by L-malate in 8-min incubations in presence of ATP with \(^{14}C\)citrate-loaded mitochondria

See Table I for details.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Percentage exchange with (^{14}C)citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol</td>
</tr>
<tr>
<td>None</td>
<td>1.2</td>
</tr>
<tr>
<td>1 mM L-malate</td>
<td>28.8</td>
</tr>
<tr>
<td>1 mM L-malate + 2 mM ATP</td>
<td>12.3</td>
</tr>
<tr>
<td>1 mM L-malate + 2 mM ATP + 10 µM atractyloside</td>
<td>25.8</td>
</tr>
<tr>
<td>1 mM L-malate + 2 mM ATP + oligomycin 0.1 µg/ml</td>
<td>13.8</td>
</tr>
<tr>
<td>1 mM L-malate + 2 mM ATP + 0.1 mM mersalyl</td>
<td>14.1</td>
</tr>
<tr>
<td>1 mM L-malate + 2 mM ATP + 10 mM pentyvlmalonate</td>
<td>12.1</td>
</tr>
<tr>
<td>1 mM L-malate + 10 mM pentyvlmalonate</td>
<td>24.1</td>
</tr>
</tbody>
</table>

When citrate, L-malate, and phosphoenolpyruvate were tested for their ability to exchange for \([\alpha-P]ATP\) only phosphoenolpyruvate caused an exchange (Fig. 5), the extent of this exchange being very small. When AMP-PNP was added to mitochondria loaded with \(^{14}C\)citrate it had a similar effect to ATP in that it caused an uptake of labeled citrate into the mitochondria (Fig. 6).

When the K_m of the citrate transporter of rat liver mitochondria was measured using the inhibitor stop technique to evaluate rates of citrate transport at varying concentrations of citrate the values varied from 250 to 350 µM (23 and Table III). If these mitochondria are preincubated with ATP and the ATP removed by washing, the K_m for the citrate transporter increased from 261 ± 49 µM to 407 ± 69 µM \((n = 6, p < 0.01\) by paired analysis). Since similar results were seen with oligomycin present, this agent was employed to minimize ATP breakdown (Table III). When ATP was replaced by its metabolites, ADP and inorganic phosphate, the citrate transporter was not inhibited. Furthermore, intramitochondrial generation of energy with TMPD and ascorbate did not result in alterations of the citrate transporter. The mitochondrial content of long chain fatty acyl-CoA, a known inhibitor of the tricarboxylate carrier \((4,6,8)\), did not increase when ATP was present in the preincubation period (Table III). When rotenone and antimycin were also
the driving force for the following reasons. Any mechanism boxylate carrier, we suggest that an ionic gradient may provide inhibition by pentylmalonate and mersalyl. And dicarboxylate transporting systems as shown by the lack of oligomycin. The ATP effect is independent of both the phosphate exchanges and the atractyloside sensitivity of the effect dictates citrate for L-malate appear to be involved in these ATP-induced metabolism, the ATP added no longer resulted in inhibition of its short term effect on citrate-L-malate equilibrium since the inhibition is not prevented by ATP (Tables I and II and Fig. 2). Therefore, the following mechanism is suggested to account for the observed fluxes (Scheme 1). When ATP is added to mitochondria a rapid exchange of ATP\(^{4-}\) for ADP\(^{-}\) ensures (24) which leaves an electrical gradient, positive outside and negative inside. Since the citrate transporter is not electrogenic (1, 2, 15) and at physiological pH, citrate exists predominantly in the triply charged form, citrate\(^{3-}\) would be protonated to citrate\(^{2-}\) and enter for malate\(^{2-}\) (Tables I and II, Figs. 1 to 3). The ATP transport-induced movement of citrate into these mitochondria could then be explained in terms of a concentration gradient of citrate\(^{2-}\), citrate\(^{3-}\) being formed outside and dissociating to give a proton inside the mitochondrion in order to minimize the electrical gradient. This mechanism is compatible with the lack of effect of ATP on a citrate-citrate exchange (Fig. 2) an exchange which does not produce pH disequilibrium (15). Since phosphate is not added in these experiments we are assuming that electroneutral or electrogenic phosphate-hydroxyl exchange (25) is an extremely poor substrate for the adenine nucleotide carrier (Fig. 5). Mechanical linkage between the two carriers such that movement on one of them obligates movement on the other is also unlikely, because the citrate-citrate exchange was virtually unaffected by ATP (Tables I and II). Therefore, the following mechanism is suggested to account for the observed effect of ATP on a citrate-citrate exchange (Fig. 2).

### DISCUSSION

ATP at physiological concentrations is able to bring about a rapid change in the equilibrium position of the citrate transporting system as judged by its effect on the distribution of L-malate and citrate between the mitochondria and suspending medium. The uptake of [\(^{14}\)C]citrate in the presence of ATP cannot be mimicked by substitution of ascorbate-TMPD as an energy source instead of ATP, nor does ascorbate-TMPD appear to nullify the exchange reaction of L-malate for [\(^{14}\)C]citrate in the manner shown by ATP in Fig. 2. Stoichiometric 1:1 exchanges of citrate for L-malate appear to be involved in those ATP induced exchanges and the atractyloside sensitivity of the effect dictates that ATP must enter the mitochondria to bring about its effect. Any involvement of the oligomycin-sensitive ATPase in this process is unlikely since the induced changes are unaffected by oligomycin. The ATP effect is independent of both the phosphate and dicarboxylate transporting systems as shown by the lack of inhibition by pentylmalonate and mersalyl.

In order to account for these effects of ATP on the tricarboxylate carrier, we suggest that an ionic gradient may provide the driving force for the following reasons. Any mechanism involving a common substrate for the citrate and adenine nucleotide transporting systems can be discarded under "Methods and Materials." Citrate was added at seven concentrations for each incubation period (1 min). Incubations were terminated by the addition of 50 mM 1,2,3-tricarboxybenzene. Results are shown as mean ± S.E. Each individual assay was performed in triplicate.

<table>
<thead>
<tr>
<th>Additions to preincubation</th>
<th>No. of experiments</th>
<th>Mitochondrial parameters</th>
<th>K(_m) (µM)</th>
<th>Acid-insoluble CoA (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
<td></td>
<td>288 ± 13</td>
<td>512 ± 30</td>
</tr>
<tr>
<td>ADP, P(_i), malate</td>
<td>5</td>
<td></td>
<td>357 ± 36</td>
<td>459 ± 80</td>
</tr>
<tr>
<td>ATP, P(_i), glucose, hexokinase</td>
<td>14</td>
<td></td>
<td>320 ± 26</td>
<td>278 ± 24*</td>
</tr>
<tr>
<td>Oligomycin experiments</td>
<td></td>
<td></td>
<td>309 ± 22</td>
<td>444 ± 34</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td></td>
<td>341 ± 58</td>
<td>630 ± 56</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
<td></td>
<td>296 ± 108*</td>
<td>618 ± 44</td>
</tr>
<tr>
<td>ATP, rotenone, antimycin A</td>
<td>7</td>
<td></td>
<td>402 ± 59</td>
<td>605 ± 23</td>
</tr>
<tr>
<td>Rotenone, antimycin A</td>
<td>5</td>
<td></td>
<td>373 ± 58</td>
<td>725 ± 60</td>
</tr>
<tr>
<td>TMPD, ascorbate</td>
<td>5</td>
<td></td>
<td>256 ± 28</td>
<td>719 ± 90</td>
</tr>
<tr>
<td>TMPD, ascorbate, oligomycin</td>
<td>5</td>
<td></td>
<td>357 ± 74</td>
<td>722 ± 103</td>
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</table>

* P < 0.01 as compared to none.
by oligomycin. We postulate therefore that some event in oxidative metabolism, triggered in conditions of elevated mitochondrial ATP, by raising the \( K_m \) for citrate transport may alter the rate of export of mitochondrially generated citrate. The identification of this regulatory factor (or factors) should provide insight into the co-ordinated regulation of the lipogenic pathway.

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