Regulation of Malate Oxidation in Isolated Mung Bean Mitochondria

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ABSTRACT

Effects of ADP and ATP on products of malate oxidation in the presence or absence of respiratory inhibitors and an uncoupler were investigated in mitochondria isolated from mung bean (Phaseolus aureus var. Jumbo) hypocotyls. Changes in levels of products from malate oxidation generally correlated directly with changes in oxygen uptake. Effects of ADP and ATP were indistinguishable from each other when respiratory chain activity was limited. We concluded that adenylates indirectly act on malate oxidation by the oxidation-reduction status of the pyridine nucleotides which are linked to the respiratory chain. The possibility of allosteric action of ADP and ATP on malate dehydrogenase activity was examined in both intact mitochondria and a partially purified enzyme preparation. Although small inhibition, 16% with 500 μM ATP and 8% with 500 μM ADP, was observed at pH 9.5, this effect was abolished by the addition of magnesium ions or by lowering the pH to 7.2. We concluded that these adenylate effects are probably not a significant factor in regulation under physiological conditions. Furthermore, the equilibrium constant of malate dehydrogenase (to 1.5 x 10^-3) in both mitochondria and the partially purified enzyme calculated from the steady state level of NADH formed suggested that the enzyme functions in an equilibrium manner in intact mitochondria.

Studies of citric acid cycle activity in mitochondria isolated from mung bean hypocotyls led to the conclusion that malate oxidation plays an important role in cycle activity (6). Adenylates are implicated in the control of malate oxidation by the following observations in the literature: (a) ADP and ATP drastically altered the levels of OAA produced from oxidation of malate (6); (b) the adenylates have been suggested as regulatory agents for malate dehydrogenase from mammalian mitochondria (18, 22); and (c) ATP was found to be a strong inhibitor of NAD-reducing activity in malate dehydrogenase purified from mung bean cotyledon mitochondria (1). On the other hand, some investigators contend that control of oxidation of cycle intermediates does not reside in the cycle itself but is confined to interaction of adenine nucleotides with the respiratory chain (25, 27). The concept of respiratory control proposed by Chance and Williams (7) can also be viewed as supporting the latter possibility.

This publication is aimed at resolving how adenylates act in the regulation of malate oxidation in isolated mung bean mitochondria, whether they act directly as effectors of malate dehydrogenase, indirectly via their action on the oxidative-phosphorylation system, or both.

MATERIALS AND METHODS

Preparation and Incubation of Mitochondria. Mitochondria were prepared as described previously (6) from mung bean hypocotyls by the method of Ikuma (13). The standard reaction medium contained 500 mM mannitol, 10 mM KCl, 5 mM MgCl₂, and 10 mM K phosphate buffer, pH 7.2. In studies aimed at evaluating the effect of adenylates on malate oxidation products, mitochondria were incubated with 10 mM malate as substrate, and O₂ consumption was followed to a steady state level. During the period of linear O₂ uptake, ADP or ATP was added, and the oxidation products were analyzed at two to three timed intervals thereafter.

Assay Procedures. Oxygen was measured polarographically with a Clark electrode as before (6). Products of malate oxidation were extracted by the perchloric acid method and assayed fluorometrically by enzymic methods as described previously (6). Samples were either collected directly from the O₂ electrode cuvette or, more frequently, prepared in 7-m1 test tubes concurrently with the O₂ electrode runs. The mitochondrial protein content was determined by a biuret procedure (12) after solubilization in deoxycholate. The method of Lowry et al. (20) was used for determination of the partial purification of malate dehydrogenase. Crystalline BSA was used as standard for both methods. Protein distribution in DEAE-cellulose-eluted fractions was estimated by absorbance measurements at 280 nm.

The activity of malate dehydrogenase (EC 1.1.1.37) partially purified from mitochondria was assayed with a Cary model 13 recording spectrophotometer at 340 nm. Initial rates are expressed as μmol NADH produced or oxidized/min·mg protein. The reverse reaction was measured by following NADH oxidation in a reaction medium containing 0.1 mM NADH and 60 mM K phosphate buffer, pH 7.4; addition of 0.2 mM OAA initiated the reaction. Activity in the forward direction was determined by reduction of NAD in a medium of 24 mM malate and 120 mM glycine-NaOH buffer, pH 9.5; the reaction was initiated with 0.6 mM NAD.
Malate dehydrogenase activity in intact mitochondria was measured with the Cary spectrophotometer at 340 nm. Activity in the direction of malate and NAD formation was measured in standard reaction medium supplemented with 0.1 mM NADH and 0.2 mM OAA in substrates and with 3 μg mitochondrial protein/ml. At this very low protein concentration, NADH oxidase activity was not detectable. The assay for malate dehydrogenase in the direction of OAA and NADH formation was carried out in standard reaction medium with Mg omitted in order to prevent activity of NAD-malic enzyme (EC 1.1.1.39) which is present in plant mitochondria (9, 21); the medium was supplemented with 0.6 mM NAD and 30 mM malate as substrates, 1 mM KCN to inhibit NADH oxidase activity, and approximately 35 μg mitochondrial protein/ml. Values of malate-oxidizing activity obtained in complete reaction medium (+Mg++) were assumed to represent the total of malate dehydrogenase and malic enzyme activities. The effects of ADP and ATP on enzyme activities were studied with both low (10 μM) and high (250 or 500 μM) adenylate concentrations.

Partial Purification of Malate Dehydrogenase from Mung Bean Mitochondria. The method was modified from that of Cole and Naron (8). Mitochondrial suspensions were pooled and stored at −20°C prior to extraction of malate dehydrogenase. The suspensions from five mitochondrial preparations containing approximately 150 mg protein were thawed and suspended in 25 ml of 5 mM K phosphate buffer, pH 8, containing 1 mM EDTA and 1 mM β-mercaptoethanol. After stirring at 0°C for 10 min, the suspension was centrifuged for 15 min at 27,000g. The pellet was suspended in the same medium and reextracted twice by freezing in an ethanol-Dry Ice bath, followed by thawing and centrifugation. The three supernatants of 27,000g spins were pooled, yielding 50 ml of enzyme suspension containing approximately 95% of the mitochondrial malate dehydrogenase activity.

The pooled supernatants were subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added slowly with constant stirring at 9°C to give 30% saturation (16.4 g/100 ml). The pH was maintained at 8 by dropwise addition of concentrated NH₄OH. After stirring for 15 min and standing for 15 min, the preparation was centrifuged at 18,000g for 15 min and the pellet discarded. The supernatant was brought to 50% saturation by the addition of ammonium sulfate (an additional 11.7 g/100 ml) and centrifuged; the pellet was discarded. The remaining supernatant was brought to 85% saturation (an additional 23 g/100 ml), stirred 30 min, and allowed to stand for 4 hr. After centrifugation, the pellet was dissolved in a small volume of 5 mM K phosphate, 1 mM EDTA-1 mM β-mercaptoethanol, pH 8, and dialyzed overnight against the same buffer.

The desalted material (1.5 ml) was placed on a DEAE-cellulose column (1.2 × 12 cm), preequilibrated with the dialysis buffer. Elution was carried out with a linear concentration gradient of K phosphate, pH 8, from 5 mM to 200 mM; both 60-ml buffer reservoirs contained 1 mM EDTA and 1 mM β-mercaptoethanol. Two-ml fractions were collected, analyzed for malate dehydrogenase activity, and evaluated for protein content by absorption at 280 nm. Active fractions were pooled and used for studies of adenylate effects on malate dehydrogenase activity, as well as for measurement of kinetic constants. There was no detectable activity of NADH oxidase or NAD-malic enzyme in the malate dehydrogenase preparation.

Chemicals and Enzymes. All chemicals were of analytical reagent grade. Bovine albumin fraction V powder and crystalline BSA were obtained from Nutritional Biochemicals Corporation. ATP, ADP, TTP, and m-Cl-CCP were purchased from Calbiochem. NAD, NADH, oligomycin, and antimycin A were obtained from Sigma Chemical Co. DEAE-cellulose (Cellex-D) was purchased from Bio-Rad Laboratories. Enzymes used in fluorometric assays were obtained from Boehringer-Mannheim.

RESULTS

Effect of Adenylates on Malate Oxidation Products. Effects of ADP and ATP on the oxidation of 10 mM malate in the presence and absence of inhibitors and an uncoupler of oxidative phosphorylation were examined by means of cross-over plots (Figs. 1, 2, and 3). Accompanying O₂ electrode traces indicate the effects of treatment on respiratory activity and also the timing of sampling. Thiamine pyrophosphate, shown to be somewhat depleted from our mitochondrial preparations and to stimulate malate oxidation (5, 6), is included in the reaction media. An intermediate concentration (10 mM) of malate was chosen for these studies in order to approximate our estimated endogenous level of 4 mM (6) rather than the very high concentration required for maximal rates of malate oxidation in the absence of sparkers (5, 6).

Control results for the analysis of oxidation products are shown in Figures 1B, 2B, and 3B. Although considerable variation in absolute values for intermediate levels was obtained in this series of experiments, the following dramatic trend was consistently observed: OAA levels are elevated with ADP treatment and lowered with ATP treatment as compared with no adenylate addition. A second consistent trend is that citrate levels are higher with ADP treatment than with ATP treatment or in the sample with no adenylate addition. Pyruvate levels are most variable in the controls, but they are usually elevated by both adenylates above the levels in the absence of adenylates and are sometimes elevated even more by ADP than by ATP. The contents of citrate and α-ketoglutarate were also measured in these studies, but their concentrations were too small to assess meaningful differences between ADP and ATP treatments.

In the presence of antimycin A, which inhibits electron transport between b- and c-type Cyt, O₂ uptake patterns (Fig. 1C) are essentially the same for both ADP and ATP treatments (15); the ADP rate is inhibited nearly to that of ATP. Similarly, the levels of intermediates following ADP and ATP treatment are practically identical (Fig. 1D); both resemble ATP treatment in the control (Fig. 1B).

Oligomycin, an inhibitor of energy transduction or ATPase activity, acted to lower the rate of O₂ uptake with ATP down to that with ATP (Fig. 2C, ref. 14). Respiratory rates with no adenylate addition and of the ATP-treated sample are very slightly inhibited (Fig. 2C). As predicted from the O₂ electrode traces, the levels of oxidation products are essentially the same with both ADP and ATP treatments (Fig. 2D), approximating the ATP control changes.

In the presence of the uncoupler, m-Cl-CCP, the O₂ electrode traces show an initially slower rate of malate oxidation with no addition or with addition of both adenylates (Fig. 3D) than the control traces without m-Cl-CCP (Fig. 3A).* The respiratory rate increases with time, and at 12 min after adenylate addition, the traces with ADP and ATP treatments show a linear rate of O₂ uptake, signifying the attainment of an uncoupled condition. The sample without ADP or ATP treatment shows no stimulation by uncoupler; this is probably due to the low level of adenylate in our mitochondria and the need for ADP as a potential in uncoupling by m-Cl-CCP (6, 19). In the presence of uncoupler, intermediate levels 6 min after addition of ADP and ATP are essentially similar for the two treatments (Fig. 3E). Twelve min after treatment, OAA and pyruvate levels remain the same, but citrate levels are elevated with ADP (Fig. 3F). Large variations in OAA levels are not observed.

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* Although the O₂ uptake rates in Figure 3, A and D are low. effects of m-Cl-CCP on oxidation rates and the changes in intermediate levels are comparable to results found in a similar experiment where the O₂ uptake rates were 2 times higher than those of Figure 3A.
Fig. 1. Effect of ADP and ATP on O_2 uptake and levels of intermediates produced during oxidation of 10 mM malate in the presence of antimycin A. Mitochondria (0.4 mg protein/ml) were added to standard reaction medium supplemented with 10 mM malate, 0.25 mM TPP, and either 0.5% ethanol (A and B) or ethanol containing 1 mM antimycin A (C and D). Mitochondria were incubated in this medium until a steady state level of O_2 uptake was attained. At this time, adenylate treatment (750 mM ADP or ATP) was administered as indicated in A and C. Numbers on traces represent O_2 uptake rates in nmol O_2/min/mg protein. Samples for intermediate analyses were taken at times indicated by arrows in A and C. The cross-over plots in B and D show levels of intermediates 4 min after adenylate addition in the presence of ADP or ATP compared with values with no additions as control (= 100%). Absolute values in nmol/2 ml reaction medium for samples with no adenylate treatment (= 100% level) are as follows: B: malate, 20,000; OAA, 11.6; pyruvate, 45.3; citrate, 43. D: malate, 20,000; OAA, 25.4; pyruvate, 49.7; citrate, 25.1.

Fig. 2. Effect of ADP and ATP on O_2 uptake and levels of intermediates produced during oxidation of 10 mM malate in the presence of oligomycin. Conditions as in Figure 1 except 2.5 mM oligomycin were used in place of antimycin A, the adenylate concentrations added were 500 mM, and cross-over data are from 5 min after adenylate addition. Absolute values for 100% intermediate levels: B: malate, 20,000; OAA, 18.4; pyruvate, 127; citrate 43.5. D: malate, 20,000; OAA, 11.2; pyruvate, 132; citrate, 29.6.
A summary of the results of this series of experiments is presented in Table 1. Average relative values from several experiments including Figures 1, 2, and 3 are given in order to compensate for the variability in individual experiments. Absolute values for 100% intermediate levels: B, malate, 20,000; OAA, 17.2; pyruvate, 33; citrate, 28.6. C: malate, 20,000; OAA, 8.6; pyruvate, 55.4; citrate, 52.2. E: malate, 20,000; OAA, 50.6; pyruvate, 22; citrate, 13.8. F: malate, 20,000; OAA, 40.4; pyruvate, 27.0; citrate, 20.7.

Fig. 3. Effect of ADP and ATP on O₂ uptake and levels of intermediates produced during oxidation of 10 mM malate in the presence of m-Cl-CCP. Conditions as in Figure 1 except 0.5 μM m-Cl-CCP was used in place of antimycin A, the adenylate concentrations added were 500 μM, and crossover data are from 6 and 12 min after adenylate addition. Absolute values for 100% intermediate levels: B: malate, 20,000; OAA, 17.2; pyruvate, 33; citrate, 28.6. C: malate, 20,000; OAA, 8.6; pyruvate, 55.4; citrate, 52.2. E: malate, 20,000; OAA, 50.6; pyruvate, 22; citrate, 13.8. F: malate, 20,000; OAA, 40.4; pyruvate, 27.0; citrate, 20.7.

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Fig. 3. Effect of ADP and ATP on O₂ uptake and levels of intermediates produced during oxidation of 10 mM malate in the presence of m-Cl-CCP. Conditions as in Figure 1 except 0.5 μM m-Cl-CCP was used in place of antimycin A, the adenylate concentrations added were 500 μM, and crossover data are from 6 and 12 min after adenylate addition. Absolute values for 100% intermediate levels: B: malate, 20,000; OAA, 17.2; pyruvate, 33; citrate, 28.6. C: malate, 20,000; OAA, 8.6; pyruvate, 55.4; citrate, 52.2. E: malate, 20,000; OAA, 50.6; pyruvate, 22; citrate, 13.8. F: malate, 20,000; OAA, 40.4; pyruvate, 27.0; citrate, 20.7.

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Table I. Summary of Effects of Adenylates on Products of 10 mM
Malate Oxidation in the Absence or Presence of Antimycin A,
Oligomycin, and m-C1-CPP

<table>
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<tr>
<th>Treatment</th>
<th>Oxidation Products</th>
<th>O₂ Utilized</th>
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<tr>
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<td>OAA</td>
<td>PYR</td>
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<td>Control</td>
<td>No Add</td>
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</tr>
<tr>
<td></td>
<td>ADP</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>50</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>No Add</td>
<td>100</td>
</tr>
<tr>
<td>(1 μM)</td>
<td>ADP</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>30</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>No Add</td>
<td>100</td>
</tr>
<tr>
<td>(2.5 μM)</td>
<td>ADP</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>90</td>
</tr>
<tr>
<td>m-C1-CPP</td>
<td>No Add</td>
<td>100</td>
</tr>
<tr>
<td>(0.5 μM)</td>
<td>ADP</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>90</td>
</tr>
</tbody>
</table>

Table II. Effect of Adenylates on Activity of Malate Dehydrogenase in
the Direction of OAA Formation in Mung Bean Mitochondria

Increase in absorbance at 340 nm was measured in a mixture of
standard reaction medium (± Mg²⁺), pH 7.2, 30 mM malate, 0.6 mM
NAD, 1 mM KCN, and 67 μg mitochondrial protein. Volume was 2 ml.

<table>
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<tr>
<th>Treatment</th>
<th>Activity</th>
<th>- Mg²⁺</th>
<th>%</th>
<th>+ Mg²⁺</th>
<th>%</th>
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<tr>
<td></td>
<td>Specific</td>
<td>Relative</td>
<td>Specific</td>
<td>Relative</td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>0.15</td>
<td>100</td>
<td>0.23</td>
<td>100</td>
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</tr>
<tr>
<td>10 μM ADP</td>
<td>0.16</td>
<td>107</td>
<td>0.25</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>250 μM ADP</td>
<td>0.15</td>
<td>100</td>
<td>0.23</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>10 μM ATP</td>
<td>0.14</td>
<td>93</td>
<td>0.25</td>
<td>109</td>
<td></td>
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<tr>
<td>250 μM ATP</td>
<td>0.13</td>
<td>87</td>
<td>0.23</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

accumulation of pyruvate and to lower citrate as compared to O₂
uptake is consistent with the possibility that ATP inhibits citrate
synthase activity in these mitochondria.

Effect of Adenylates on Malate Dehydrogenase Activity in
Intact Mitochondria. The preceding studies clearly indicated that
major adenylate effects are found on the accumulation or disappear-
ance of OAA during oxidation of malate. Therefore, it was
of interest to examine the effects of ADP and ATP directly on
malate dehydrogenase activity under conditions similar to those of
the malate oxidation studies with isolated mitochondria.

Our assays showed no effect of adenylates on the malate
dehydrogenase reaction in the direction of malate and NAD
formation. The data in Table II suggest that there is no effect of
adenylates on malate dehydrogenase activity in the direction of
OAA and NADH formation in the presence or absence of 5 mM magnesium. However, rates in the presence of
Mg are about 50% greater than in its absence and probably
include the activity of NAD-malic enzyme, which has a divalent
metal cation requirement (9, 21). NAD-reducing activity in the
absence of Mg is presumably due mainly to malate dehydro-

ase. Although the data rule out large adenylate effects, ATP at a
high concentration may inhibit malate dehydrogenase activity.
Furthermore, strong inhibition of malate dehydrogenase activity
by ATP has been reported for the partially purified enzyme from
mung bean cotyledon mitochondria (1).

The effects of adenylates on steady state levels of NADH
formed under conditions favoring malate utilization (high malate
and NAD concentrations) were next examined with intact mito-
chondria. Figure 4 shows an example of the spectrophotometric
traces for the four conditions used in these assays. In the absence of both Mg²⁺ and KCN, a steady state level of NADH is reached
after an initial rapid rise followed by a slow leveling off—within
2 min. This is interpreted as due to malate dehydrogenase and
NADH oxidase activities. Generally, Mg slightly raises the
NADH concentration at which a steady state condition is
attained (A and B versus C and D in Fig. 4). The magnitude of
enhancement by Mg varies with mitochondrial preparations. The
Mg effect is likely due to addition of NAD-malic enzyme in
utilizing added malate and NAD. KCN further raises the steady
state level of NADH and also abolishes the "peak" seen in the
control trace (B and D versus A and C in Fig. 4). This effect can
be attributed to inhibition of NADH oxidase. If we assume equal
amounts of OAA and NADH are present at the steady state
level, we can calculate the equilibrium constant from these data.
Using 0.100 as the approximate value for A₄₅₀, we calculate the
NADH steady state level as 1.6 × 10⁻⁵ M. Then,

\[
K_{eq} = \frac{[NADH][OAA]}{[Mal][NAD]} = \frac{(1.6 \times 10^{-5} \text{ M})^2}{(3.0 \times 10^{-3} \text{ M})(1.0 \times 10^{-3} \text{ M})} = 1.0 \times 10^{-4}.
\]

Since the equilibrium constant of isolated malate dehydrogenase
is 2.3 × 10⁻⁵ at 22°C and pH 7.2 (4), the value obtained here
suggests that the steady state level reached during oxidation of
malate by intact mitochondria is determined by the malate dehy-

![FIG. 4. Conditions for study of NADH steady state levels during malate oxidation. Mitochondria (0.4 mg protein) were added to standard reaction medium (±5 mM MgCl₂), pH 7.2, supplemented with 1 mM NAD and ±1 mM KCN in a total volume of 2 ml. Reaction was initiated with 30 mM malate (A₄₅₀ = 0.020). NADH levels were determined by absorption at 340 nm. Numbers indicate maximal A₄₅₀ values.](https://example.com/fig4.png)
The effect of adenylates on NADH steady state levels is shown in Table III. Since the experimental error in these experiments is rather large, especially when maximal absorbance values are small, data from three different preparations of mitochondria are given. On the basis of average per cent differences, slight inhibition by high levels of ATP is consistently obtained. The presence of Mg appears not to alter the values for average per cent differences.

We conclude from this series of experiments that ATP and ADP probably exert no direct effect on the activity of malate dehydrogenase or on NAD-malic enzyme in mitochondria at pH 7.2. Possibly, ATP at a high concentration inhibits NAD-reducing activity of malate dehydrogenase approximately 10%. Furthermore, we infer from the experiments on the steady state levels of NAD that the malate dehydrogenase equilibrium, coupled with NADH oxidase activity, plays a major role in controlling malate utilization in intact organelles under the conditions used here. Evidence for competition between NADH oxidase and malate dehydrogenase in intact mitochondria is contained in Figure 5, which shows that increasing concentrations of OAA cause increasing inhibition of NADH oxidation. Also, it is of interest to note that no state 4 increase in fluorescence can be seen during oxidation of 30 mM malate unless TPP (and, better, pyruvate too) is added to prevent accumulation of OAA, which can remove available NADH via the malate dehydrogenase reaction (Fig. 6).

Effects of Adenylates on Activity of Partially Purified Malate Dehydrogenase. Results of a typical purification show a 70-fold increase in specific activity from the mitochondrial suspension to the DEAE-pooled active fractions (Table IV). For partial char-

**Table III. Effect of Adenylates on Steady State Levels of NADH Produced by Malate Oxidation by Mung Bean Mitochondria**

Increase in absorbance at 340 nm was measured in a mixture of standard reaction medium (± Mg2+), pH 7.2, 30 mM malate, 0.6 mM NAD, 1 mM KCN. Preps 1, 2, and 3 contained 270, 400, and 260 μg protein, respectively. Volume was 2 ml. Values for Prep 1 represent a single assay; for preps 2 and 3, the average of two replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prep 1</th>
<th>Prep 2</th>
<th>Prep 3</th>
<th>Average % Difference</th>
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<tbody>
<tr>
<td>- Mg2+ No addition</td>
<td>50 100</td>
<td>48 100</td>
<td>29 100</td>
<td>100 100</td>
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<tr>
<td>- KCN 10 μM ADP</td>
<td>63 126</td>
<td>57 119</td>
<td>26 90</td>
<td>112</td>
</tr>
<tr>
<td>250 μM ADP</td>
<td>67 134</td>
<td>50 103</td>
<td>24 83</td>
<td>101</td>
</tr>
<tr>
<td>10 μM ATP</td>
<td>58 116</td>
<td>50 103</td>
<td>24 83</td>
<td>101</td>
</tr>
<tr>
<td>250 μM ATP</td>
<td>60 120</td>
<td>44 92</td>
<td>19 66</td>
<td>93</td>
</tr>
<tr>
<td>- Mg2+ No addition</td>
<td>104 100</td>
<td>67 100</td>
<td>45 100</td>
<td>100</td>
</tr>
<tr>
<td>+ KCN 10 μM ADP</td>
<td>96 92</td>
<td>62 93</td>
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<tr>
<td>250 μM ADP</td>
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<td>62 93</td>
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<td>91</td>
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<td>10 μM ATP</td>
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<tr>
<td>250 μM ATP</td>
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<td>56 84</td>
<td>46 102</td>
<td>91</td>
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<td>57 100</td>
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</tr>
<tr>
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<td>61 107</td>
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<td>250 μM ADP</td>
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<tr>
<td>10 μM ATP</td>
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<tr>
<td>+ Mg2+ No addition</td>
<td>103 100</td>
<td>96 100</td>
<td>88 100</td>
<td>100</td>
</tr>
<tr>
<td>+ KCN 10 μM ADP</td>
<td>101 102</td>
<td>100 104</td>
<td>80 91</td>
<td>99</td>
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<tr>
<td>250 μM ATP</td>
<td>95 92</td>
<td>95 99</td>
<td>74 84</td>
<td>92</td>
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<tr>
<td>10 μM ATP</td>
<td>109 106</td>
<td>91 95</td>
<td>77 87</td>
<td>96</td>
</tr>
<tr>
<td>250 μM ATP</td>
<td>85 83</td>
<td>80 83</td>
<td>73 83</td>
<td>83</td>
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FIG. 6. Fluorescence trace (A) of malate oxidation ± 0.25 mM TPP. Oxygen electrode trace (B) is included for comparison (+ TPP). Assays were made in standard reaction medium with 0.3 mg mitochondrial protein/ml.

Acetylation and oxidation of the malate dehydrogenase, we measured kinetic constants (Fig. 7) and obtained the following apparent Km values: 2.5 x 10⁻³ M for malate, 1.0 x 10⁻³ M for NAD, 7.0 x 10⁻³ M for OAA, and 2.5 x 10⁻³ M for NADH. The apparent Km for NAD could not be determined at pH 9.5. At this pH, the measured activity was still increasing at an NAD concentration of 5 mM. Similar anomalous behavior was observed by Englard and Siegel (10) during purification of malate dehydrogenase from beef heart mitochondria. No such difficulties were met with tris buffer at pH 8.4 was used. The Km values for OAA, NADH, and malate agree well with data on malate dehydrogenase preparations from mitochondria of other plants (23, 26) and animals (10). Values reported in the literature for the Km for NAD vary considerably, from 10⁻³ to 10⁻⁷ M. Asahi and Nishimura (1), working with a malate dehydrogenase from mung bean cotyledon mitochondria, found a Km of 10⁻⁴ for both NAD and NADH. Figure 7C also contains data showing that 500 μM ATP increases the Km of malate 2-fold from 2.5 mM to 5 mM, as
compared with 5-fold reported by Asahi and Nishimura (1). Product inhibition by OAA was not observed in the concentration range (0–1.5 mM) studied.

The effect of adenylates on activity of partially purified malate dehydrogenase was examined similarly to the studies with intact mitochondria. However, in these studies, conditions optimal for the enzyme reaction under study were used in place of mitochondrial reaction medium. Effects of ADP and ATP at low (10 μM) and high (500 μM) concentrations were studied.

The assays showed no effect of adenylates on activity of malate dehydrogenase in the direction of malate formation. There was also no detectable effect of adenylates on activity in the direction of OAA formation when a high malate concentration (24 mM) was used. However, with 2 mM malate as substrate, a small inhibitory effect of adenylates on malate dehydrogenase becomes evident. Table VA shows 16% inhibition by 500 μM ATP and 8% inhibition by 500 μM ADP. These effects are statistically significant. Since adenylates have a very high affinity for Mg ion, which is present in the standard reaction medium, and are known to function as an Mg2+-adenylate complex in many reactions, the effect of Mg on inhibition of malate dehydrogenase by adenylates was investigated. Addition of Mg abolishes any inhibition of malate dehydrogenase activity by adenylates (Table VB).

When effects of adenylates on malate dehydrogenase activity were examined under conditions approaching physiological

<table>
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<tr>
<th>Table IV. Partial Purification of Malate Dehydrogenase from Mung Bean Hypocotyl Mitochondria</th>
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<td>One unit of enzyme activity is that amount of enzyme required</td>
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<tr>
<td>to convert 1 μmol NADH into NAD/min.</td>
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<td>Procedure</td>
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<td>(NH4)2SO4 fraction</td>
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<td>DEAE fractions</td>
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Fig. 7. Apparent kinetic constants of partially purified mung bean malate dehydrogenase. A: OAA titration; assay medium consisted of 60 mM K phosphate buffer, pH 7.2, 0.1 mM NADH, and OAA of variable concentration. Reaction was initiated with addition of malate dehydrogenase (0.1 μg protein). B: NADH titration; same buffer as in (A) with 0.2 mM OAA and NADH of variable concentration. Reaction starter was 0.12 μg enzyme protein. C: Malate titration; assay medium contained 120 mM glycine-NaOH buffer, pH 9.5, 0.6 mM NAD, and variable concentrations of malate. Reaction was initiated with malate dehydrogenase (0.7 μg protein). Titration was also conducted in the presence of 0.5 mM ATP (O). D: NADH titration; assay medium contained 60 mM tris-HCl buffer, pH 8.4, 24 mM malate, and variable concentrations of NAD. Reaction was initiated with malate dehydrogenase (0.7 μg protein). Rates were calculated as change in absorbance at 340 nm/min.
ones, i.e. at pH 7.2 rather than 9.5 and with 2 mM malate (Table VC), a high concentration of ADP or ATP did not cause detectable inhibition. From the steady state level of NADH formed (4.2 μM) under these conditions, the equilibrium constant was calculated to be 1.5 × 10^{-5}.

In summary, the studies on partially purified malate dehydrogenase indicate that small inhibition by ATP and even less by ADP is obtained in media optimized for measuring enzyme activity in vitro. However, this inhibition is abolished by Mg ions and is not detectable under more nearly physiological conditions.

**DISCUSSION**

To summarize: (a) ADP and ATP treatments yield very different OAA levels, as well as smaller changes in pyruvate and citrate, from oxidation of 10 mM malate (Figs. 1, 2, 3, and Table I); (b) application of respiratory inhibitors eliminates adenylate-associated differences in OAA, pyruvate, and citrate levels (Figs. 1 and Table I); (c) addition of uncoupler eliminates the differences in OAA and pyruvate levels, but not in citrate levels (Fig. 3 and Table I); (d) adenylates possibly inhibit activity of malate dehydrogenase in the direction of OAA formation in intact mitochondria to the extent of a 10% level (Tables II and III); (e) adenylates effect on NAD-malic enzyme activity in intact mitochondria cannot be detected (Tables II and III); (f) although small inhibition by high concentrations of ADP and ATP is seen on activity of partially purified malate dehydrogenase at pH 9.5, lowering of the pH to 7.2 and/or addition of Mg ions eliminates adenylate inhibition (Table V); (g) the steady state level of NADH during malate oxidation by intact mitochondria appears to be determined by the activity of malate dehydrogenase + NADH oxidase (Fig. 4 and Table III); (h) addition of OAA to mitochondria-oxidizing NADH shows that malate dehydrogenase competes effectively with NADH for exogenous NADH (Fig. 5); (i) no state 4 increases in fluorescence can be seen during oxidation of 30 mM malate unless TPP is added to prevent OAA accumulation (Fig. 6).

We concluded that adenylates play a significant role in regulation of malate oxidation. The role of adenylates is mediated primarily from the level of the electron transfer system affecting malate dehydrogenase activity. The results suggest that the electron transfer system exerts its effect via NADH levels. NADH that is not used by the respiratory chain reacts with OAA to produce malate and NAD.

Asahi and Nishimura (1) reported that ATP strongly inhibits the NAD-reducing activity of partially purified malate dehydrogenase from mung bean cotyledon mitochondria. Results in the present study with mung bean hypocotyl mitochondria differ from this report in that malate dehydrogenase extracted from mitochondria and possibly malate dehydrogenase in intact organelles are subject to only small inhibition by ATP. Furthermore, since this small inhibition of the isolated enzyme is abolished by addition of Mg ions or adjustment of the pH to a more nearly physiological level, inhibition in vivo seems unlikely. Therefore, we conclude that regulation of malate dehydrogenase by ATP in intact mitochondria probably does not play a major role in the regulation of malate oxidation. An analogous observation was made by Williamson et al. (28) who reported relief of ATP inhibition of purified citrate synthase by Mg ions and concluded that inhibition of this enzyme by ATP is not significant in vivo.

The results indicate that malate dehydrogenase in these mitochondria functions essentially in an equilibrium manner (11). NADH has been reported to inhibit NAD-reducing activity of malate dehydrogenase with Ki values of 10^{-3} M (1). Since equilibrium is reached with NADH and OAA at a concentration of 5 × 10^{-6} M when malate and NAD are supplied at very high levels, the question of NADH inhibition is perhaps a moot point. Instead, regulation of malate dehydrogenase activity can be readily accounted for by equilibrium effects alone.

The possibility that adenylates indirectly affect malate oxidation through direct regulation of another cycle or cycle-related enzyme has not been ruled out. Enzymes which remove OAA may indirectly regulate malate oxidation. Since aspartate could not be detected as a product of malate oxidation (6), effects of adenylates on glutamate-OAA transaminase activity were not investigated. ATP inhibits citrate synthase activity in a number of organisms (2, 3, 16, 24). Results of studies with m-Cl-CCP can be interpreted as implicating adenylate effects on citrate synthase in mung bean mitochondria. The studies with intact mitochondria revealed no effect of adenylates on NAD-malic enzyme activity. However, the repeated observation in cross-over analyses that pyruvate tends to accumulate with ATP as opposed to ADP might suggest a stimulating effect of ATP on this enzyme. Therefore, direct examination of adenylate effects on malic enzyme activity in both mitochondria and a partially purified enzyme preparation is of interest for future work.

**LITERATURE CITED**

