Purification and Properties of NAD-Dependent Malate Dehydrogenase from *Mesembryanthemum crystallinum* L. Exhibiting Crassulacean Acid Metabolism

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Abstract: By DEAE-cellulose anion exchange column chromatography, three different forms of NAD-dependent malate dehydrogenase (EC 1.1.1.37) (NAD-MDH) were isolated from leaves of *Mesembryanthemum crystallinum* L. in the crassulacean acid metabolism mode. NAD-MDH that eluted last from an anion exchange column was purified to a specific activity of 1,096 units × (mg protein)^−1 using Blue Sepharose CL-6B chromatography. Citrate and adenosine 5′-triphosphate effectively inhibited the activity of NAD-MDH. The inhibition of the enzymatic activity by citrate was reversed by inorganic phosphate and the degree of reversal increased with increasing the concentration of oxaloacetate, the substrate of the reaction. The optimal pH for NAD-MDH activity was around 7.5. Citrate inhibited the enzymatic activity over a wide range of pH and caused a shift in the optimal pH. The enzymatic activity in the presence of citrate was increased by adding inorganic phosphate below pH 7.5. On the basic side, higher than pH 7.5, however, the inhibition by citrate was enhanced by adding inorganic phosphate.

Key words: Crassulacean acid metabolism, Enzyme purification, *Mesembryanthemum crystallinum* L., NAD-dependent malate dehydrogenase (EC 1.1.1.37).

CAM 型 *Mesembryanthemum crystallinum* L. の NAD-リンゴ酸脱水素酵素の精製と性質 : 斎藤和幸・岡田文武 (九州大学農学部)


Higher plants contain various nicotinamide-adenine dinucleotide (NAD) dependent isozymes of malate dehydrogenase (l-malate: NAD oxidoreductase, EC 1.1.1.37) (NAD-MDH) that catalyze the reduction of oxaloacetate to malate. The NAD-MDH isozymes are involved in different metabolic functions, i.e. the glyoxylate pathway, the tricarboxylic acid cycle and the malate/aspartate shuttle. The individual forms exhibit different molecular properties, and they can be separated by polyacrylamide gel electrophoresis, isoelectric focusing, or other techniques. The association of the isoenzymes with different cellular compartments has been established by electrophoretic analysis of cell organelles which were purified by sucrose density gradient centrifugation.

We are using the halophyte *Mesembryanthemum crystallinum* L. Exposure of the plants to
high soil salinity causes a shift in the mode of carbon assimilation from that typical of C₃ plants to that typical of crassulacean acid metabolism (CAM) plants, which exhibit substantial net carbon gain at night. In CAM plants, β-carboxylation of phosphoenolpyruvate catalyzed by PEPCase is the initiating reaction of CAM, followed by the reduction of oxaloacetate to malate by NAD-MDH. Decarboxylation of malate via malic enzyme prevails in the light and malate synthesis during the light period is inhibited. Feed-back inhibition of PEPCase by malate is assumed to be one of the key factors regulating the day/night rhythm of acidification and decacidification in tissues exhibiting CAM. It has been proposed that inorganic phosphate regulates malate accumulation through the promotion of the activity of PEPCase. However, the relationship between properties of NAD-MDH and the physiology of CAM are poorly understood. The present report describes purification and properties of an NAD-MDH from leaves of *M. crystallinum* in the CAM mode. Special attention has been paid to the regulatory function of NAD-MDH in tissues exhibiting CAM.

**Materials and Methods**

1. **Plant materials**

Plants were grown from seeds of *Mesembryanthemum crystallinum* L. as described previously. All plants were kept in a growth cabinet with a light period of 12 h, a photon flux density (400-700 nm) of 250 μmol m⁻² s⁻¹ on plant level, an air temperature of 25°C and a relative humidity of 70%. Six-week-old plants that had been maintained under hydroponic conditions were used for induction of CAM. CAM was induced by including 400 mM NaCl in the root medium for fourteen days. The fully developed leaves were harvested 4 h before the end of the light period. The leaves were frozen with liquid nitrogen and stored at −55°C until use.

2. **Purification of NAD-MDH**

The following steps were carried out at 0 to 4°C. Leaves (about 300 g) of *M. crystallinum* in the CAM mode were chopped into fine pieces and then ground with a chilled mortar and pestle in 3 volumes of extraction buffer that consisted of 100 mM Tris-acetic acid (pH 8.0), 2.5 mM MgCl₂ and 12.5% (w/v) glycerol. After filtration of the extract through four layers of gauze, the filtrate was centrifuged at 10,000×g for 30 min. To the supernatant fraction, crystalline ammonium sulfate was gradually added up to 55% saturation. After centrifugation at 10,000×g for 30 min, the supernatant was brought to 75% saturation with ammonium sulfate. After centrifugation at 10,000×g for 30 min, the pellet was resuspended in about 18 mL of buffer A that contained 20 mM Tris-acetic acid (pH 7.4), 2.5 mM MgCl₂ and 10% (w/v) glycerol, and centrifuged at 10,000×g for 10 min. The supernatant fraction was passed through a NAP column (Pharmacia Biotech, Uppsala, Sweden; 1.5 cm inside diameter × 5 cm) which had previously been equilibrated with buffer A. The eluate was then applied to a column of DE 52 (Whatman, Maidstone, England; 1.6 cm i.d. × 10 cm) that had previously been equilibrated with buffer A. The column was washed with 200 mL of the same buffer. The enzyme was eluted with a linear 200-mL gradient of KCl (0-200 mM) in buffer A. Fractions of 2 mL were collected at a flow rate of 180 mL h⁻¹. The peak fractions with enzymatic activity were pooled and were passed through a NAP column which had previously been equilibrated with buffer A. The eluate was chromatographed on a column of Blue Sepharose CL-6B (Pharmacia Biotech; 1.6 cm i.d. × 12 cm) that had previously been equilibrated with buffer A. The enzyme was eluted with a linear 200-mL gradient of NADH (0-0.4 mM) in buffer A. The most active fractions were pooled and stored at 4°C.

3. **Assay of NAD-MDH and units of enzymatic activity**

The enzyme was assayed by following the oxaloacetate-dependent oxidation of NADH at 340 nm. The assay condition was modified from the referred source to give optimum activities in extracts from *M. crystallinum*. The standard reaction mixture (1.0 mL) contained 100 mM TES-Tris (pH 7.5), 0.12 mM NADH and 0.5 mM oxaloacetate. The reaction was initiated by the addition of oxaloacetate and carried out at 30°C. One unit of enzyme was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH per min under these conditions.

4. **Quantitation of protein**

Protein concentrations were determined
Table 1. Purification of NAD-MDH from leaves of *M. crystallinum* in the CAM mode.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (mL)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Recovery (%)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>540</td>
<td>297</td>
<td>5,296</td>
<td>100,0</td>
<td>17.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate, 55-75% fraction</td>
<td>72</td>
<td>104</td>
<td>4,937</td>
<td>93.2</td>
<td>47.5</td>
<td>2.7</td>
</tr>
<tr>
<td>DE 52</td>
<td>42</td>
<td>6.48</td>
<td>1,851</td>
<td>35.0</td>
<td>285.6</td>
<td>16.0</td>
</tr>
<tr>
<td>Blue Sepharose CL-6B</td>
<td>36</td>
<td>0.24</td>
<td>263</td>
<td>5.0</td>
<td>1,095.8</td>
<td>61.6</td>
</tr>
</tbody>
</table>

Fig. 1. Elution profile of ion exchange chromatography on DE 52 of NAD-MDH from leaves of *M. crystallinum* in the CAM mode. Circles, activity of NAD-MDH; solid line, concentration of KCl. Note that enzymatic activity is expressed in arbitrary units.

spectrophotometrically at 595 nm by the method of Bradford with Coomassie brilliant blue G-250 (Bio-Rad Co., Richmond, CA). A standard curve was established using bovine serum albumin.

**Results**

The purification of NAD-MDH from leaves of *Mesembryanthemum crystallinum* in the CAM mode is summarized in Table 1. The enzyme was predominantly recovered in the 55-75% ammonium sulfate precipitate. The enzyme was then applied to a column of DE 52. Peak I shown in Fig. 1 was not adsorbed onto the column of DE 52. The remaining adsorbed activity could be eluted with a linear gradient of KCl (0-200 mM) in buffer A (Fig. 1). Two distinct NAD-MDH protein peaks were obtained. The peak III fractions were pooled and applied to a column of Blue Sepharose CL-6B. A single peak was eluted with a linear gradient of NADH (0-0.4 mM) (data not shown). The enzyme was purified 62-fold to a final specific activity of 1,096 units mg⁻¹.

The effects of various organic acids on the activity of the purified NAD-MDH were investigated in the presence of 0.5 mM oxaloacetate at pH 7.0 (Table 2). Minimal effects were observed upon inclusion, at 2 mM, of the following compounds in the standard assay mixture: glucose-6-phosphate, fructose-6-phosphate, fructose-1, 6-bisphosphate, fructose-2, 6-bisphosphate, dihydroxyacetone phosphate, glycerate-3-phosphate, glycerate-2-phosphate, succinate, fumarate, adenosine 5'-monophosphate, inorganic phosphate, l-alanine and glutarate. Phosphoenolpyruvate, glyceraldehyde-2, 3-diphosphate, pyruvate, 2-ketoglutarate, glyoxylate, l-malate, acetyl-CoA and adenosine 5'-diphosphate caused 20-40% inhibition of the enzymatic activity. In the presence of citrate, the enzymatic activity was inhibited by 43%. ATP caused a 54% reduction of the enzymatic activity.

Because citrate and ATP inhibited the activity of NAD-MDH more than the other organic acids tested, a more detailed study of the inhibition by citrate and ATP was performed. The effect of the concentration of citrate or ATP on the activity of NAD-MDH from *M. crystallinum* are shown in Fig. 2. When the assays were carried out at pH 7.0 in the presence of 0.25 mM oxaloacetate, the inhibitory effects of citrate and ATP were increased with increasing concentrations of citrate and ATP.

The optimal pH for the activity of NAD-MDH was around 7.5 (Fig. 3). This value was
Table 2. Effect of various solutes on the relative activity of NAD-MDH from _M. crystallinum_ in the CAM mode.

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Relative activity (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate</td>
<td>90.4</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>90.4</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate</td>
<td>87.6</td>
</tr>
<tr>
<td>Fructose-2,6-bisphosphate</td>
<td>93.2</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>82.3</td>
</tr>
<tr>
<td>Glycerate-2,3-diphosphate</td>
<td>63.5</td>
</tr>
<tr>
<td>Glycerate-3-phosphate</td>
<td>87.6</td>
</tr>
<tr>
<td>Glycerate-2-phosphate</td>
<td>82.3</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>66.6</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>62.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>57.4</td>
</tr>
<tr>
<td>2-Ketoglutarate</td>
<td>74.6</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>78.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>90.4</td>
</tr>
<tr>
<td>Fumarate</td>
<td>100.0</td>
</tr>
<tr>
<td>L.-Malate</td>
<td>78.1</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>67.3</td>
</tr>
<tr>
<td>Adenosine 5'-' triphosphate</td>
<td>45.5</td>
</tr>
<tr>
<td>Adenosine 5'-diphosphate</td>
<td>64.5</td>
</tr>
<tr>
<td>Adenosine 5'-monophosphate</td>
<td>91.1</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>100.0</td>
</tr>
<tr>
<td>L.-Alanine</td>
<td>90.4</td>
</tr>
<tr>
<td>L.-Glutamate</td>
<td>96.6</td>
</tr>
</tbody>
</table>

*The concentration of various solutes was 2 mM.
**The activity in the absence of various solutes was taken as 100%.

consistent with the value reported for the analogous enzyme from pea seeds\(^{26}\) and French bean.\(^{29}\) Over a wide range of pH, citrate inhibited the activity of NAD-MDH. Citrate caused a shift in the optimal pH. The enzymatic activity in the presence of 3 mM citrate culminated at pH values around 8. The same result was obtained in the presence of 3 mM ATP (data not shown).

It was observed that the inhibition of the enzymatic activity by 3 mM citrate was completely reversed by 40 mM inorganic phosphate (Fig. 4 and see also Fig. 6). The inhibition of the enzymatic activity by 3 mM ATP was also completely reversed by 80 mM inorganic phosphate. When citrate and ATP were not included in the reaction mixture, however, inorganic phosphate did not affect the enzymatic activity at pH 7.0 in the presence of 0.25 mM oxaloacetate (Fig. 5). The enzymatic activity in the presence of citrate was increased by adding inorganic phosphate below pH 7.5 (Fig. 3). On the basic side, higher than pH 7.5, however, the enzymatic activity in the presence of citrate was reduced by adding inorganic phosphate. The enzymatic activity in the presence of 3 mM citrate and 40 mM inorganic phosphate culminated at pH values around 7.5. These
Fig. 4. Effect of the concentration of inorganic phosphate on the activity of NAD-MDH in the presence of 3 mM citrate (●) or 3 mM ATP (■). Enzyme was assayed at pH 7.0 in the presence of 0.25 mM oxaloacetate.

Fig. 5. Effect of the concentration of inorganic phosphate on the activity of NAD-MDH. Enzyme was assayed at pH 7.0 in the presence of 0.25 mM oxaloacetate.

values were consistent with the optimal pH for the enzymatic activity.

When the initial velocity of the reaction was studied as a function of the concentration of oxaloacetate, in the presence of saturating concentration of NADH (0.12 mM), a hyperbolic response was obtained (Fig. 6). There was no apparent oxaloacetate substrate inhibition below the concentration of 0.5 mM at pH 7.5. The inhibition of the activity of NAD-MDH by citrate was not influenced by the concentration of oxaloacetate. The inhibition of the enzymatic activity by citrate was reversed by inorganic phosphate and the degree of reversal increased with increasing in the concentration of oxaloacetate; it was observed that NAD-MDH recovered about 100% of the enzymatic activity in the absence of citrate above the concentration of 0.25 mM oxaloacetate. The enzymatic activity was inhibited by inorganic phosphate below the concentration of 0.25 mM oxaloacetate, while slight stimulation of the enzymatic activity by inorganic phosphate was observed above the concentration of 0.25 mM oxaloacetate.

Discussion

Multiple forms of NAD-MDH have been reported in numerous plant tissues\(^1,2,14,15,16,18,30,31,32\). It has been reported that spinach\(^32\) and pea leaf tissue\(^31\) and green stem tissue of Opuntia ficus-indica\(^14\) contain at least three unique forms of the enzyme, one associated with the leaf microbodies, second form associated with the mitochondria, and third nonparticulate form was detected as the soluble or cytosol isoenzyme. The three NAD-MDH proteins were separated on a DEAE-cellulose column, and the cytoplasmic form was eluted from DEAE-cellulose column later than the microbody form and the mitochondrial form\(^14,32\). Three different NAD-MDH
isoenzymes were isolated from green leaf tissue of *M. crystallinum* in the CAM mode by DEAE-cellulose anion exchange column chromatography (Fig. 1). The present report describes the properties of NAD-MDH that was most strongly retained by DEAE-cellulose. Although the total activity of NAD-MDH in extracts from leaves in the C₃ mode was a third of that in extracts from leaves in the CAM mode, extracts from leaves in the C₃ mode exhibited almost identical behaviour to those from leaves in the CAM mode when chromatographed on a DEAE-cellulose column (data not shown).

NAD-MDH from leaves in the CAM mode was purified about 62-fold to a specific activity of 1,096 units · (mg protein)⁻¹. It has been reported the specific activities of purified mitochondrial and soluble isoenzymes of NAD-MDH from *Zea mays* to be 800 and 700 units · (mg protein)⁻¹, respectively. NAD-MDH purified to apparent electrophoretic homogeneity from animal sources exhibited a range of specific activities from 300 to 1,400. This purified NAD-MDH in this report appears to have a higher specific activity by comparison.

Cennamo et al. reported that commercially obtained pig heart mitochondrial NAD-MDH was specifically inhibited by 3.3 mM fumarate and citrate, but the soluble form was not inhibited. Mukerji and Ting reported that no significant differences were apparent in the inhibition patterns among NAD-MDH isoenzymes in green stem tissue of *Opuntia ficus-indica* and metabolites such as 2-ketoglutarate, cis-aconitate, citrate, α-isocitrate, succinate, fumarate, aspartate, glutamate and maleate partially inhibited the isoenzymes. The isoenzymes were inhibited by 2-ketoglutarate more than the other metabolites tested. In the present study, the activity of NAD-MDH from leaves of *M. crystallinum* in the CAM mode was inhibited by citrate more than 2-ketoglutarate. It is likely that the properties of the enzymes differ depending on the source of enzyme, and that the difference in the properties is relevant for the difference in the regulation of enzymatic activity in vivo. The importance of such physiological effect remains to be determined.

In the present study, we found that the inhibition of the activity of NAD-MDH by citrate was completely reversed by 40 mM inorganic phosphate (Figs. 4 and 6). *M. crystallinum* in the CAM mode accumulates substantial amounts of citrate and isocitrate in the mesophyll cells. Accordingly, the activity of NAD-MDH might be inhibited in the mesophyll cells of *M. crystallinum* in the CAM mode. It has been reported that inorganic phosphate (appropriate concentration of 30 mM) restores maleate-caused inhibition of the activity of PEPCase isolated from *M. crystallinum*, and that inorganic phosphate regulates malate accumulation through the promotion of the activity of PEPCase. It is likely that inorganic phosphate regulates malate accumulation through the promotion of the activity of NAD-MDH as well as of PEPCase. Further experimentation is required to substantiate this hypothesis.

It was reported that NAD-MDH of spinach and pea showed oxaloacetate substrate inhibition at pH 6.0 and 7.5, and that these phenomena were absent in the purified enzyme. NAD-MDH purified from leaves of *M. crystallinum* in the CAM mode did not show oxaloacetate substrate inhibition (Fig. 6). This difference could be mainly adjudged to the purification state of the enzyme as well as to the different experimental conditions.

### References