Purification and Characterization of NAD Malic Enzyme from Leaves of Eleusine coracana and Panicum dichotomiflorum

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ABSTRACT

NAD malic enzyme (EC 1.1.1.39), which is involved in C4 photosynthesis, was purified to electrophoretic homogeneity from leaves of Eleusine coracana and to near homogeneity from leaves of Panicum dichotomiflorum. The enzyme from each C4 species was found to have only one type of subunit by SDS polyacrylamide gel electrophoresis. The M, of subunits of the enzyme from E. coracana and P. dichotomiflorum was 63 and 61 kilodaltons, respectively. The native Mr of the enzyme from each species was determined by gel filtration to be about 500 kilodaltons, indicating that the NAD malic enzyme from C4 species is an octamer of identical subunits. The purified NAD malic enzyme from each C4 species showed similar kinetic properties with respect to concentrations of malate and NAD; each had a requirement for Mn2+ and activation by fructose-1,6-bisphosphate (FBP) or CoA. A cooperativity with respect to Mn2+ was apparent with both enzymes. The activator (FBP) did not change the Hill value but greatly decreased K0.5 (the concentration giving half-maximal activity) for Mn2+. The enzyme from E. coracana showed a very low level of activity when NADP was used as substrate, but this activity was also stimulated by FBP. Significant differences between the enzymes from E. coracana and P. dichotomiflorum were observed in their responses to the activators and their immunochromatographic properties. The enzyme from E. coracana was largely dependent on the activators FBP or CoA, regardless of concentration of Mn2+. In contrast, the enzyme from P. dichotomiflorum showed significant activity in the absence of the activator, especially at high concentrations of Mn2+. Both immunodiffusion and immunoprecipitation, using antiserum raised against the purified NAD malic enzyme from E. coracana, revealed partial antigenic differences between the enzymes from E. coracana and P. dichotomiflorum. The activity of the NAD malic enzyme from Amaranthus edulis, a typical NAD malic enzyme type C4 dicot, was not inhibited by the antiserum raised against the NAD malic enzyme from E. coracana.

In the leaves of certain C4 plants, designated NAD-ME4 type species, NAD-ME is the major or sole C4 acid-decarboxylating enzyme (10, 13). The enzyme has been partially purified from the bundle sheath cells of the NAD-ME type species, Atriplex spongiosa, Amaranthus edulis, and Panicum miliaceum (11). The enzyme from each NAD-ME type species was specific for Mn2+ and was significantly activated by either CoA or acetyl-CoA (11) and also by FBP (4). However, the properties of the enzymes from C4 species differ depending on the source of enzyme. The enzyme from dicots (A. spongiosa and A. edulis) showed cooperativity for binding of malate, which was increased by activators and was competitively inhibited by bicarbonate with respect to both malate and activators. The enzyme from a monocot (P. miliaceum) did not show such cooperativity and inhibition (4, 11). Recently, NAD-ME was highly purified from potato tubers (Solanum tuberosum) and leaves of a CAM plant (Crassula argentea) and both enzymes were shown to be composed of two dissimilar 61 and 55 kD subunits. This phenomenon has only been observed in the NAD-ME isolated from these plants (25).

In the Gramineae, most NAD-ME species, such as Eleusine coracana and P. miliaceum, have been characterized by the fact that chloroplasts in the bundle sheath cells are located in a centripetal position [hereafter designated NAD-ME(P) species]. Recently, some NAD-ME species in the dichotomiflorum group of Panicum (17) as well as in Eragositis (9) were found to have centrifugally arranged chloroplasts in their BSC [designated NAD-ME(F) species]. It is noteworthy that NAD-ME(F) species of both genera are found predominantly in relative humid and high-rainfall areas (18, 19), while most NAD-ME(P) species are found in rather arid areas (2, 5).

We, therefore, purified the C4 leaf NAD-ME from two NAD-ME type species, E. coracana, representative of the NAD-ME(P) species and P. dichotomiflorum, representative of the NAD-ME(F) species. The purified enzymes were compared with respect to their enzymic and immunochromatographic properties.

MATERIALS AND METHODS

Plant Materials

Eleusine coracana (L.) Gaertn, Panicum dichotomiflorum Michx. and Amaranthus edulis Speg, were grown outdoors after germination in a greenhouse. Young leaves were harvested from approximately 3-week-old seedlings and stored at −35°C until use.

Buffers

Buffer A was 50 mM Heps-KOH (pH 7.4) containing 2.5 mM MnCl2, 50 mM 2-mercaptoethanol, and 12.5% glycerol. Buffer B was 20 mM Tris-acetic acid (pH 7) containing 2.5 mM MnCl2, 50 mM 2-mercaptoethanol, and 10% glycerol.

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4 Abbreviations: ME, malic enzyme; FBP, fructose-1,6-bisphosphate; PMSF, phenylmethylsulfonyl fluoride.
Buffer C was 20 mM Tris-acetic acid (pH 7) containing 2.5 mM MnCl₂ and 50 mM 2-mercaptoethanol.

### Preparation of Crude Enzyme from Plant Leaves

About 6 g of young leaves were ground in an equal volume of buffer A and 5% (w/v) leaf tissue Polyclar AT (Sangyo Co, Tokyo, Japan) with a chilled mortar and pestle. After filtration through one layer of Miracloth, the filtrate was centrifuged at 10,000 rpm for 10 min. The supernatant was desalted by passage through a column of Sephadex G-25 equilibrated and eluted with buffer C.

### Purification of NAD-ME

All the following steps were carried out similarly to purify the enzyme from the leaves of *E. coracana* and *P. dichotomiflorum*, at 0 to 4°C, unless otherwise stated.

#### Preparation of Crude Extract

Leaves (about 500 g) were chopped into fine pieces and then ground with a Polytron homogenizer in 3 volumes of buffer A and 25 g of Polyclar AT. After filtration through two layers of cheesecloth, the filtrate was centrifuged at 12,000 rpm for 15 min. The supernatant fraction was used in the next step.

#### PEG Fractionation

PEG (average mol wt 6,000; 50% [w/v] dissolved in H₂O) was slowly added with gentle mixing to the supernatant fraction to bring a final concentration to 5.5%. The solution was allowed to stand for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant fraction, adjusted to 11% (w/v) with 50% PEG, was stirred and allowed to stand for 15 min. After centrifugation, the pellets were resuspended in about 100 mL of buffer B and clarified by centrifugation at 18,000 rpm for 15 min.

#### Ion Exchange Chromatography

The supernatant fraction was adsorbed onto a column (5 × 37 cm) of DEAE-TSK-Gel 650M (Toyo Soda Co, Tokyo, Japan) previously equilibrated with buffer B. The column was washed with 900 mL of the same buffer. The enzyme was eluted with a linear 2-L gradient of sodium acetate (50–250 mM) in buffer B. Fractions of 10 mL were collected at a flow rate of 450 mL·h⁻¹. The peak fractions with enzyme activity were pooled and brought to 12% PEG to precipitate protein. The resulting pellet was dissolved in a small volume of buffer B.

#### Gel Filtration

This step was omitted in the purification of the enzyme from *P. dichotomiflorum*. The concentrated solution was chromatographed on a column of TSK-Gel HW 60S (Toyo Soda Co.) (2.5 × 90 cm) previously equilibrated with 0.1 M sodium acetate in buffer B. Fractions of 3.5 mL were collected at a flow rate of 21 mL·h⁻¹. The fractions with maximum enzyme activity were concentrated by precipitation with PEG as above.

#### Affinity Chromatography

The solution of NAD-ME was applied to the column (1.2 × 7 cm) of 5'AMP-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with buffer B. The column was washed with 30 mL of 0.2 mM sodium acetate in buffer B and then eluted with 0.6 mM sodium acetate in the same buffer at a flow rate of 43 mL·h⁻¹. Fractions of 2.8 mL were collected and those containing enzyme activity were pooled. The solution was concentrated as above and passed through a small column of Sephadex G-50 to remove salt and PEG, with buffer B used both for equilibration and for elution of enzyme. The purified preparations were stored at −35°C.

#### Assay of NAD-ME and Unit of Activity

The enzyme was assayed by following the malate-dependent reduction of either NAD or NADP at 340 nm (11). The standard reaction mixture (1.0 mL) contained 25 mM Hepes-KOH (pH 7.2), 5 mM malate, 2 mM NAD, 2.5 mM MnCl₂, 5 mM DTT, and 50 μM FBP. The reaction was initiated by the addition of appropriate amounts of enzyme (0.5–2 μg protein) and carried out at 31°C. One unit of activity is defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD·min⁻¹. Specific activity is expressed as units·mg⁻¹ protein.

#### SDS-PAGE

The Laemmli (14) system for SDS-PAGE was used. Analytical polyacrylamide gels containing SDS were either stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes using an electroblotting apparatus (HORIZON AE-6670, ATTAXO Co, Tokyo, Japan) for Western blotting.

#### M, Determination

The mol wt of the subunit of NAD-ME was determined by SDS-PAGE (10% slab gel). The proteins used as SDS mol wt standards were lysozyme (*M.14.4 kDa*), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66.2 kDa), and phosphorylase B (92.5 kDa). The native mol wt of the enzyme was determined by gel filtration chromatography using the fast protein liquid chromatographic system (Pharmacia Fine Chemicals) with a Superose 6 column (1 × 30 cm) at a flow rate of 30 mL·h⁻¹. The column was equilibrated with 0.1 mM sodium acetate in buffer B and precalibrated with 100 to 300 μg of Cyt c (12.4 kDa), chymotrypsinogen (24 kDa), ovalbumin (45 kDa), BSA (66.2 kDa), aldolase (156 kDa), catalase (208 kDa), and apoferritin (475 kDa).

#### Immunochemical Procedures

Antiserum against purified NAD-ME from *E. coracana* leaves was raised by inoculating a New Zealand white rabbit with a homogeneous preparation of the enzyme. Four intradermal injections of the purified enzyme (about 600 μg) were given at 15-d intervals; the first injection with complete
Freund's adjuvant and the three others with incomplete adjuvant. Ten d after the final injection, blood was taken from the ears and centrifuged at 5,000 rpm. The plasma was stored at -35°C until use. The double immunodiffusion was carried out using the procedure described by Ouchterlony. Agar (0.8% [w/v]) plates were incubated for 48 h at 4°C and the protein precipitates were photographed. Immunoprecipitation of NAD-ME with antiserum raised against NAD-ME from *E. coracana* was tested (15). A solution of crude enzyme (about 0.05 unit of activity), extracted from plant leaves, was incubated for 15 min at room temperature with 15 mM Hepes-KOH (pH 7.2) that contained antiserum (0.5 µL), 12.5% glycerol, 3.75 mM MnCl_2, and 75 mM 2-mercaptoethanol in a total volume of 55 µL. The incubation of the mixture was continued for another 15 min following the addition of 30 µL Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) suspended in 10 mM Hepes-KOH (pH 7.2). After centrifugation at 10,000 rpm for 10 min, the 20-µL supernatant was subjected to the assay for NAD-ME in the standard reaction mixture. Western blotting was performed, after SDS-PAGE and electrophoretic separation, as described by Campbell and Remmler (3) using an assay kit (Immun-Blot [Goat Anti-Rabbit IgG Horseradish Peroxidase], Bio-Rad Co., Richmond, CA).

**Protein Determination**

Protein concentrations were determined spectrophotometrically by the Bradford method at 595 nm, utilizing the Coomassie dye-binding reagent (Bio-Rad Co.). A standard curve was established using BSA.

**RESULTS**

**Purification of NAD-ME**

The purification of NAD-ME from leaves of *Eleusine coracana* and *Panicum dichotomiflorum* is summarized in Tables I and II, respectively. The enzymes were purified about 400-fold to a final specific activity of 57 to 58 units·mg^{-1} protein. In the case of *E. coracana*, the purification was particularly successful after chromatography on DEAE gel, with specific activity being 74-fold greater than in the PEG fraction. This efficient separation was due to the elution of enzyme activity at approximately 80 mM sodium acetate, far ahead of most of the other proteins. Thus, the subsequent gel filtration step yielded one peak of enzyme activity which coincided with the peak of protein. SDS-PAGE of the final preparation obtained by affinity chromatography on 5'-AMP-Sepharose revealed a single band without any contaminating bands (Fig. 1). In the case of the enzyme from *P. dichotomiflorum*, however, the gel filtration procedure was omitted since this procedure caused remarkable decrease in the yield of the enzyme without any significant enrichment of the enzyme activity. As judged from the specific activity (Table II), the final chromatographic step on 5'-AMP-Sepharose was quite efficient, but it did not remove a small amount of contaminating protein from the preparation. The minor band on SDS-PAGE gel corresponded to a mol wt of about 100 kD, and was possibly due to PEP-carboxylase (Fig. 1).

**M, and Subunit Structures**

The mol wt of native NAD-ME purified from *E. coracana* and *P. dichotomiflorum* leaves were estimated to be about 500 kD; we did not find an appreciable difference in the mol wt of the enzymes from the two species (Fig. 2). The mol wt of the subunit of the NAD-ME were clearly different between the two plant sources being 63 and 61 kD for NAD-ME from *E. coracana* and *P. dichotomiflorum*, respectively (Fig. 3). However, there was the possibility that the difference in mol wt of the enzymes from the two plant sources was due to an artifact; for example, the degradation of the enzyme by proteolytic activity contained in the extract of plant material during purification. In order to test the possibility, crude preparations of enzyme were extracted from each source with buffer A that contained inhibitors of proteases (1 mM PMSF and 0.1 mg·mL^{-1} soybean trypsin inhibitor). The extracts were subjected to SDS-PAGE and analyzed by Western blotting using the rabbit antiserum raised against NAD-ME from leaves of *E. coracana*. Only a single immunoreactive polypeptide of 63 kD in the case of the *E. coracana* extract and of 61 kD in the case of *P. dichotomiflorum* extract were observed. This result indicates that the mol wt of the subunits was truly different between the two plant sources (data not shown). The native NAD-ME from *C. pec* plant source appears to be an octamer composed of identical subunits.

**Cataytic Properties of NAD-ME from Leaves**

The NAD-ME in crude extracts from various *C. pec* (20), *C. dica* (12), and CAM plants (22, 23) demonstrated a slow reaction transient in the form of a lag before reaching a steady state rate in assay. This lag was not observed with the purified enzymes from *E. coracana* and *P. dichotomiflorum*, even when the amount of each substrate and cofactor was lowered below the optimal concentration. The optimal pH for NAD-ME activity was similar for enzymes from both *E. coracana* and *P. dichotomiflorum*, being between 6.5 and 7.0 (data not shown). This value is consistent with the values reported for the analogous enzyme from various plants (7, 11, 24). The

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**Table I. Purification of NAD-ME from Leaves of *E. coracana***

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Recovery</th>
<th>Specific Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>mg</td>
<td>units</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>1,580</td>
<td>17,380</td>
<td>2,233</td>
<td>100</td>
<td>0.13</td>
<td>1</td>
</tr>
<tr>
<td>PEG fraction</td>
<td>115</td>
<td>2,473</td>
<td>1,851</td>
<td>82.9</td>
<td>0.75</td>
<td>5.7</td>
</tr>
<tr>
<td>DEAE-TSK-gel chromatography</td>
<td>4.6</td>
<td>33.1</td>
<td>1,824</td>
<td>81.7</td>
<td>55.11</td>
<td>423.9</td>
</tr>
<tr>
<td>TSK-gel filtration</td>
<td>2.5</td>
<td>17.5</td>
<td>965</td>
<td>43.2</td>
<td>55.14</td>
<td>424.2</td>
</tr>
<tr>
<td>5'-AMP-Sepharose chromatography</td>
<td>0.8</td>
<td>7.0</td>
<td>401</td>
<td>18.0</td>
<td>57.29</td>
<td>440.7</td>
</tr>
</tbody>
</table>
optimal temperature of reaction was found to be about 35 and 45°C for the enzyme from E. coracana and from P. dichotomiflorum, respectively (Fig. 4). Arrhenius plots of the activities of both enzymes gave straight lines without any breaking point (data not shown).

Specificity for Activators of NAD-ME

The NAD-ME isolated from the bundle sheath cells of different C₄ species is reported to be activated by metabolites such as CoA, acetyl-CoA, and FBP, as well as by sulfate (12). The enzyme from E. coracana and from P. dichotomiflorum was shown in preliminary assays to be activated by CoA and FBP in a similar manner and by sulfate in a different manner. Therefore, the results with FBP and sulfate are described in this report. Figure 5 shows the effect of the concentration of FBP on the activity of NAD-ME at different concentrations of MnCl₂. The NAD-ME activity from E. coracana was largely dependent on the presence of FBP, regardless of the concentration of MnCl₂, activation being more than 10-fold at both 0.4 and 2.5 mM MnCl₂ (Fig. 5A). The response of the enzyme activity to increasing concentrations of FBP was hyperbolic.

The $K_a$ (the concentration of activator giving half-maximal activity) for FBP and the $\Delta V_{max}$ ($V_{max}$ with FBP-$V_{max}$ without FBP) were significantly affected by the concentration of MnCl₂, the $K_a$ being 71 and 280 $\mu$M, and the $\Delta V_{max}$ being 62.7 and 26.0 units with 2.5 and 0.4 mM MnCl₂, respectively. With the enzyme from P. dichotomiflorum (Fig. 5B), the
Figure 4. Effect of temperature on the activity of NAD-ME. a. Enzyme from *E. coracana* assayed in the standard reaction mixture; b. enzyme from *P. dichotomiflorum* assayed in the standard reaction mixture; c. enzyme from *P. dichotomiflorum* assayed without FBP. The standard reaction mixture is described in "Materials and Methods."

Activity without FBP was markedly increased with increasing concentrations of MnCl₂. The activating effect of FBP (activity with FBP versus activity without FBP) was decreased with increasing concentrations of MnCl₂. The activation was found to be 13.7-fold, 3.8-fold, and 1.9-fold at 0.2, 0.4, and 2.5 mM MnCl₂, respectively. At any concentration of MnCl₂ tested, the $K_a$ for FBP and the $\Delta V_{\text{max}}$ were not very different, being 15 to 16 μM and 28.7 to 30.6 units mg⁻¹ protein, respectively. It is noteworthy that the $K_a$ was significantly lower than that of the enzyme from *E. coracana*.

The effects of sulfate on NAD-ME activity are shown in Figure 6. When the assays were carried out with the standard reaction mixture that contained 500 μM FBP, both enzyme activities were inhibited irreversibly but the degree of inhibition was larger with the enzyme from *E. coracana*. By contrast, when FBP was omitted, the enzyme activity for each species was stimulated by sulfate, but the extent of activation was very different between them. The enzyme from *P. dichotomiflorum* was activated only by 20 to 30% of its initial activity within a limited concentration of ammonium sulfate (<20 mM) and was inhibited at higher concentrations, while the enzyme from *E. coracana* was activated over a broad range of sulfate concentrations up to 200 mM and was not inhibited. The maximum activation was about threefold. The plots of activity as a function of the concentration of ammonium sulfate were sigmoidal, giving an $n$ value of 2.06, a $K_a$ of 50 mM, and a $V_{\text{max}}$ of 8.3 units mg⁻¹ protein. The effects of Cl⁻ on NAD-ME activity were found to be inhibitory: concentrations of Cl⁻ needed for 50% inhibition with the standard reaction mixture being 23 and 51 mM for the enzyme from *E. coracana* and *P. dichotomiflorum*, respectively. In contrast, acetate showed a little stimulation of both enzyme activities at concentrations up to 100 mM (data not shown).

Figure 5. Effect of the concentration of FBP on the activity of NAD-ME from *E. coracana* (A) and *P. dichotomiflorum* (B) at different concentrations of MnCl₂. Note the different scales used on the abscissa.

**Effects of the Concentration of MnCl₂ on NAD-ME Activity**

The NAD-ME from *E. coracana* and *P. dichotomiflorum* had a requirement for Mn²⁺, which could not be replaced by any other metal ions, such as Mg²⁺ or Co²⁺ at concentrations up to 10 mM. The response of the enzyme activity to increasing concentrations of MnCl₂ with different concentrations of FBP was also sigmoidal (Fig. 7). The Hill plots of the activity against varying concentrations of MnCl₂ showed that the $n$ value was not affected by the concentration of FBP added, but the $n$ value differed between the enzymes from *E. coracana* and *P. dichotomiflorum*, being 2.0 and 1.65, respectively. The interaction between MnCl₂ and FBP was qualitatively similar for the enzymes from both *E. coracana* and *P. dichotomiflorum*, except that the latter enzyme had significant activity in the absence of FBP. FBP affected both the $K_{0.5}$ (the concentration giving half-maximal activity) for MnCl₂ and the $V_{\text{max}}$. The addition of 500 μM FBP decreased the $K_{0.5}$ from 2.3 to 0.63 mM, and from 0.66 to 0.16 mM, and increased the $V_{\text{max}}$ from 5.2 to 62.4 units, and from 27.7 to 59.1 units, for the enzyme from *E. coracana* and from *P. dichotomiflorum*, respectively.
Substrate Specificity and Kinetic Parameters

The purified enzyme from *E. coracana* had very much lower activity in the presence of NADP than in the presence of NAD. The activity with NADP also had a requirement for Mn$^{2+}$, which could not be replaced by Mg$^{2+}$, and was activated by FBP, as was the activity with NAD (Fig. 8). The maximal activity with FBP was observed at about 0.5 mM NADP.

Table III compares the kinetic parameters determined for substrates with and without activator. Compared to the NAD-ME from several other C$_4$ plants, a somewhat higher $K_m$ for NAD was obtained for both enzymes in this study: 2.2 and 2.5 mM for the enzyme from *E. coracana* and from *P. dichotomiflorum*, respectively. The $K_m$ for NADP was smaller than that for NAD by one order of magnitude. The $K_m$ for both NAD and NADP was increased by omitting FBP from the reaction. The $\Delta V_{\text{max}}$ was decreased for the enzyme from *E. coracana* and unchanged for that from *P. dichotomiflorum* by the omission of FBP. In the presence of a saturating concentration of FBP (500 $\mu$M), the $K_m$ for malate and the $V_{\text{max}}$ were very similar for both enzymes. In the absence of FBP, however, the $K_m$ for malate was increased ninefold and the $V_{\text{max}}$ was significantly decreased for the enzyme from *E. coracana* in the absence of FBP, while the $K_m$ was not changed and the $V_{\text{max}}$ fell by about 40% for the enzyme from *P. dichotomiflorum*.

Immunochemical Properties of NAD-ME

Antiserum was raised in a rabbit against purified NAD-ME from *E. coracana*. The specificity of the antibody and the relative antigenicity of NAD-ME from different plants were determined by double immunodiffusion and immunoprecipitation. Figure 9 shows the precipitin lines produced by crude extract and the PEG fraction from *E. coracana*, and by the crude extract from *P. dichotomiflorum* with the antibody. The antibody formed a single precipitin line with the extract from *E. coracana*, which spurs over the faint line produced by the extract of *P. dichotomiflorum*.

The effects of antisera on the activity of NAD-ME from each C$_4$ species are shown in Figure 10. The antisera inhibited NAD-ME activity in extracts from *E. coracana* and *P. dichotomiflorum*. The amounts of antisera required for 50% inhibition were about 15 and 115 $\mu$L of antisera per unit of enzyme from *E. coracana* and *P. dichotomiflorum*, respectively. By contrast, the NAD-ME activity in an extract from *A. edulis* was not inhibited by the antisera at any concentrations tested.

DISCUSSION

NAD malic enzyme is widespread in nature and has been purified from various organisms (1). In plants, it has been purified from the nonphotosynthetic organs of C$_3$ plants, i.e. from cauliflower buds (6) and from potato tubers (7) and from the leaves of a CAM plant (21). However, NAD-ME has never, to our knowledge, been fully purified from leaves of a C$_4$ plant, in spite of its higher levels in certain species of C$_4$ plant than in C$_3$ and CAM plants. Difficulties encountered in purifying the enzyme were mainly due to the instability of the enzyme during fractionation and storage (11). In the
Figure 8. Effect of the concentration of NAD and NADP on the activity of NAD-ME from *E. coracana*. The enzyme was assayed with various concentrations of NAD (circles) and NADP (squares) in the presence (open symbols) and absence (closed symbols) of 500 µM FBP.

**Table III. Kinetic Parameters of NAD-ME from *E. coracana* and *P. dichotomiflorum* Determined for the Substrates shown, with (+) and without (−) FBP (500 µM)**

<table>
<thead>
<tr>
<th>Substrate (FBP)</th>
<th><em>E. coracana</em></th>
<th><em>P. dichotomiflorum</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>NAD (+)</td>
<td>2.2</td>
<td>120.5</td>
</tr>
<tr>
<td>NAD (−)</td>
<td>14.3</td>
<td>34.9</td>
</tr>
<tr>
<td>NADP (+)</td>
<td>0.25</td>
<td>13.6</td>
</tr>
<tr>
<td>NADP (−)</td>
<td>0.35</td>
<td>0.8</td>
</tr>
<tr>
<td>Malate (+)</td>
<td>0.63</td>
<td>62.4</td>
</tr>
<tr>
<td>Malate (−)</td>
<td>5.5</td>
<td>9.4</td>
</tr>
</tbody>
</table>

In the present study, by use of relatively high concentrations of 2-mercaptoethanol and glycerol, NAD-ME was stabilized and successfully purified from leaves of *E. coracana* and *P. dichotomiflorum*. Our success in purification of the enzyme was also due in large part to the use of PEG fractionation, as used in the case of the CAM enzyme, instead of ammonium sulfate fractionation which causes loss of most of the enzyme activity (21).

A difference in mol wt of the subunits was observed between NAD-MEs from *E. coracana* (63 kD) and *P. dichotomiflorum* (61 kD). The possibility that the smaller subunit of the latter enzyme may be a proteolytic artifact was eliminated by experiments using protease inhibitors and by the observations that the mol wt of the subunits did not change during the fractionation and purification of the enzymes. Recently (25), the purified NAD-MEs from potato tubers and CAM plant leaves were shown to be composed of two dissimilar 61 and 55 kD subunits, that occur in the enzyme at a molar ratio 1:1. However, the SDS-PAGE of the purified NAD-ME from leaves of *E. coracana* and *P. dichotomiflorum* revealed a single major band of protein. In addition, the analysis of crude extracts from the leaves of each C₄ species by Western blotting, using the antiserum raised against NAD-ME from leaves of *E. coracana* showed the same result as the SDS-PAGE of the purified enzymes. These results indicate that the NAD-ME of *E. coracana* plant leaves is composed of identical subunits. However, our results do not exclude the possibility that a small amount of NAD-ME composed of two dissimilar subunits, like in

Figure 9. Double immunodiffusion of crude extracts from *E. coracana* and *P. dichotomiflorum* leaves. Central well, antiserum raised against NAD-ME from *E. coracana* leaves; wells 1 and 3, crude extract from *E. coracana*; wells 2 and 4, PEG fraction from *E. coracana*; wells 5 and 6, crude extract from *P. dichotomiflorum*.

Figure 10. Immunoprecipitation curves of NAD-ME in crude extracts from leaves of *E. coracana* (E.c), *P. dichotomiflorum* (P.d) and *A. edulis* (A.e). Immunoprecipitations were performed with the same antiserum as mentioned in the legend to Figure 9 and as described in "Materials and Methods."
potato tubers and a CAM plant leaves, is present in the C4 plant leaves, since our experiment did not allow us to estimate levels of a minor species of NAD-ME. We are interested in investigating this possibility with respect to the genetic diversity of NAD-ME in plants. In this respect, it is of interest that light induced the synthesis and increased the activity of NAD-ME in E. coracana leaves, in parallel with an increase in PEP carboxylase activity and Chl content after the illumination of dark-grown seedlings (16), while light can not increase the activity of NAD-ME in nonphotosynthetic tissues, such as potato tubers.

NAD-ME from potato tubers (8, 9) and CAM plant leaves (21, 23) was shown by gel filtration to exist in a dimeric, tetrameric, or octameric form, and appeared to interconvert readily between these three states. The gel filtration of native purified NAD-ME from the leaves of E. coracana and P. dichotomiflorum gave a single symmetric peak in each case, corresponding to a mol wt of about 500 kD. These results indicate that NAD-ME from each C4 species is present in one state under our experimental conditions, namely, as an octamer of identical subunits.

The purified NAD-ME from E. coracana and P. dichotomiflorum gave a hyperbolic activity curve for the concentration of malate; it had a requirement for Mn2+, a low level of activity with NADP and was activated by FBP as well as by CoA. These properties are similar to those of the enzyme from P. miliaceum (4, 11) which is a typical NAD-ME type C4 species of monocot. A cooperativity with respect to Mn2+ was apparent with the enzyme from E. coracana and from P. dichotomiflorum. With the activator, FBP, the n value was not changed but K0.5 for Mn2+ was significantly decreased. The physiological rationale for this allosteric regulation is not yet apparent.

A significant difference between the enzymes from E. coracana and P. dichotomiflorum was observed in their responses to the activators FBP and ammonium sulfate. The enzyme from E. coracana was significantly activated by these activators, regardless of the concentration of Mn2+ and was not inhibited at any concentration of each activator tested. In contrast, the enzyme from P. dichotomiflorum showed significant activity without any activator, especially at high concentrations of Mn2+. The enzyme was very slightly activated by ammonium sulfate at low concentrations and was inhibited by high concentrations. Both double immunodiffusion and immunoprecipitation revealed partial antigenic differences between NAD-MEs from E. coracana and P. dichotomiflorum. These results were expected from the experiments that showed some differences in the enzymatic properties of the enzymes from the two monocots, as described above.

The NAD-ME of leaves of C4 dicots, such as A. edulis, was reported to have very different enzymatic properties from those of NAD-ME from C3 monocot leaves. In particular, the former enzyme showed marked allosteric effects while the latter lacked them (4, 11). These findings indicate that significant differences in antigenicities should be present between the enzymes from monocots and dicots. In fact, NAD-ME activity from A. edulis was not inhibited by the antisera raised against NAD-ME from E. coracana in our experiments. These species-dependent differences in the enzymatic properties and antigenicities of NAD-ME from leaves of C4 plants must reflect a considerable divergence between species among the genes that encode the enzyme. It remained to be determined what specific differences in amino acid residues in the protein are responsible for the differences in catalytic and allosteric properties observed between these C4 species.

LITERATURE CITED
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