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Comparative Studies of NAD-Malic Enzyme from Leaves of Various C_4 Plants

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Using butyl-TSK-gel chromatography, we purified NAD-malic enzyme (ME) (EC 1.1.1.39), which is involved in C_4 photosynthesis, to electrophoretic homogeneity, from leaves of *Amaranthus tricolor*. Molecular weights of the native and SDS-denatured enzyme from *A. tricolor* were 490 kDa and 61 kDa, respectively. During assay of the enzyme there was a slow reaction transient in the form of a lag before a steady-state rate was reached. The duration of this lag was inversely proportional to the concentration of each substrate and the activator, fructose-1,6-bisphosphate (FBP). The optimal pH of the reaction fell with decreasing concentrations of either malate or FBP. High pH prolonged the lag in reaction.

Double reciprocal plots of the enzymatic activity as a function of the concentration of malate yielded straight lines and did not show any cooperativity for binding of malate. The enzyme from *A. tricolor* was not inhibited by either HCO_3^- or CO_2. At different concentrations of malate, the nature of the activating effect of FBP was compared among the purified enzymes from *A. tricolor* and the C_4 monocots *Eleusine coracana* and *Panicum dichotomiflorum*. At low levels of malate, FBP markedly stimulated the enzyme from each species. In contrast, at saturating levels of malate, the response of enzymes to increasing concentrations of FBP was different and depended on the source of enzyme.

The immunochemical properties of the enzymes from the three species were compared using an enzyme-linked immunoadsorbent assay with antisera raised against the purified enzymes from the three species. Different cross-reactivities were observed among the enzymes from different sources. The N-terminal amino acid sequences of NAD-MEs from the three species were determined and some differences were found among the three enzymes.

Key words: *Amaranthus tricolor* L. — C_4 pathway photosynthesis — Enzyme purification — Fructose-1,6-bisphosphate — NAD-malic enzyme — N-terminal amino acid sequence.

NAD-ME [L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.39] is widespread in nature and catalyzes the oxidative decarboxylation of malate, in the presence of a divalent cation, to produce CO_2 and pyruvate in the mitochondrion (Artus and Edwards 1985). In plants, the enzyme has been found in many tissues of C_3, C_4 and CAM plants and has been purified from various plant tissues and characterized (Grover et al. 1981, Grover and Wedding 1984, Willeford and Wedding 1987). In the leaves of certain C_4 plants, designated NAD-ME type species, NAD-ME plays a key role in photosynthesis by providing CO_2

Abbreviations: ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; ELISA, enzyme-linked immunoadsorbent assay; FBP, fructose-1,6-bisphosphate; ME, malic enzyme; NAD-ME(P) species and NAD-ME(F) species, NAD-ME type C_4 species with centripetal and centrifugal positions of chloroplasts in bundle sheath cells, respectively; PAGE, polyacrylamide gel electrophoresis; PEG, polyethyleneglycol; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase.

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for fixation in the Calvin cycle, in bundle sheath cells (Hatch and Kagawa 1974a, 1974b).

We have recently purified NAD-ME from leaves of *Eleusine coracana* and *Panicum dichotomiflorum* (Murata et al. 1989) which are NAD-ME(P) type species and NAD-ME(F) type species of monocots, respectively (Ohsugi and Murata 1980). The analysis of subunits by SDS-PAGE and the determination of native molecular weight indicate that the enzyme from each species is an octamer of identical subunits, although the molecular weight of subunits was different, depending on the source of enzyme. We have also prepared an antiserum raised in rabbits against the purified NAD-ME from *E. coracana* leaves. Comparative studies with the purified NAD-ME revealed that the kinetic properties and antigenicities of the enzymes were appreciably different for the two monocots. Moreover, the NAD-ME in a crude extract of leaves of Amaranthus *edulis*, a representative of NAD-ME type dicots, was not inhibited by antiserum raised against NAD-ME from *E. coracana*.

It was shown some time ago that the leaf NAD-ME of a C_4 dicot has very different enzymatic properties from that of a C_4 monocot (Hatch et al. 1974, Chapman and Hatch 1977). With the former enzyme, the cooperativity for binding of malate was apparent and the activators, CoA and FBP, and the inhibitor, HCO_3^-, acted as positive and negative allosteric effectors, respectively, with respect to malate. The latter enzyme did not show such allosteric properties. However, these early findings were made with either crude or partially purified enzyme.

The present paper reports the purification of NAD-ME from leaves of a NAD-ME type dicot, *Amaranthus tricolor*, as well as the results of enzymatic and immunochemical studies of the purified enzyme. A comparison is made of these properties with those of the purified NAD-ME from the leaves of two monocots, *E. coracana* and *P. dichotomiflorum*. We also present the partial amino acid sequences from the N-terminus of the NAD-ME from the three C_4 species.

Materials and Methods

Plant materials—A. tricolor L. and *P. dichotomiflorum* Michx. plants were grown in soil under greenhouse conditions to a height of 20–30 cm. The young and fully developed leaves were harvested and stored frozen until use.

Purification of enzymes from the leaves of A. tricolor and P. dichotomiflorum—The initial steps in the purification procedure were as previously described (Murata et al. 1989), and involved preparation of crude extracts, fractionation with 5–11.5% PEG and DEAE-TSK-gel chromatography. Enzymatically active fractions from DEAE-gel chromatography were combined and precipitated with 13% PEG. The precipitate was dissolved in a small volume of 20 mM Tris-acetic acid (pH 7) that contained 2.5 mM $MnCl_2$ and 50 mM 2-mercaptoethanol (hereafter designated as TBM) and then an equal volume of 4 M sodium acetate in TBM was added to give a final concentration of 2 M sodium acetate. After centrifugation at 18,000 rpm for 15 min, the clear supernatant solution was applied to a column of butyl-TSK-gel-650M (Toyo Soda Co. Tokyo, Japan) (2.5 cm × 23 cm) which had been previously equilibrated with 2 M sodium acetate in TBM. After the column was washed with about 130 ml of the same solution, the enzyme was eluted with a 2 to zero M reverse-phase gradient of sodium acetate in 500 ml TBM, and 5 ml fractions were collected at a flow rate of 150 ml·h^{-1}. The peak fractions of NAD-ME (tubes No. 8–16) and PEPC (tubes No. 24–27) were collected separately. The fractions containing NAD-ME were pooled and protein was precipitated by addition of 50% PEG to give a final concentration of 13%. The resulting pellet was then dissociated in a small amount of TBM that contained 10% glycerol (TBMG). The concentrated solution of enzyme was chromatographed on a column of TSK-gel HW-60S (Toyo Soda Co.) (2.5 × 90 cm) which had been previously equibrated with 0.1 M sodium acetate in TBMG. Fractions of 4 ml were collected at a flow rate of 60 ml·h^{-1}. The fractions with maximum enzymatic activity were pooled and concentrated by precipitation with PEG and dissolved in a small amount of TBMG. The final preparation was kept frozen at −35°C. The fractions containing PEPC, obtained by butyl-TSK-gel chromatography, were also further purified by gel filtration as described in the case of NAD-ME, except that ammonium sulfate and 0.1 M phosphate buffer (pH 7) that contained 5 mM $MgCl_2$ were used for concentration and for elution of enzyme, respectively.

Enzyme assay—The NAD-ME was assayed by following the malate-dependent reduction of NAD at 340 nm, as previously described (Murata et al. 1989). Two types of reaction mixture were used. The *ACES system* contained 12.5 mM ACES-NaOH (pH 6.8), 5 mM malate, 2 mM NAD, 2.5 mM $MnCl_2$, 5 mM DTT and 100 μM FBP in 1.0 ml. The *HEPES system* was the same as the ACES system except for the use of 25 mM HEPES-KOH (pH 7) and 500 μM FBP instead of 12.5 mM ACES-NaOH and 100 μM FBP, respectively. The HEPES system was mainly used in the assay of NAD-ME in crude extracts and during purification of enzyme. The enzymes used in the experiments were purified from *P. dichotomiflorum* and *A. tricolor* as described above and from *E. coracana* as described previously (Murata et al. 1989). The reaction was initiated by addition of appropriate amounts of enzyme 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}. Activity is reported as units·(mg protein)$^{-1}$. PEPC was assayed spectrophotometrically in a coupled system with

malate dehydrogenase (Boehringer-Mannheim) as described by Huber et al. (1986).

Production of antibodies—The specific antisera against NAD-ME, purified from *A. tricolor* and from *P. dichotomiflorum*, were obtained by the injection of the purified enzymes into different New Zealand white female rabbits as previously described (Murata et al. 1989).

ELISA—The procedure was performed by the method of Weeden et al. (1982). Duplicate wells of EIA microtitration plates (Flow Lab. Inc.) were used in each experiment. The antisera raised against NAD-ME from *E. coracana*, *P. dichotomiflorum* and *A. tricolor* were used at dilutions of 1 : 400, 1 : 300 and 1 : 400, respectively. Peroxidase-conjugated goat anti-rabbit IgG (Bio Rad Lab Inc.) at a dilution of 1 : 3,000 and 100 μl of assay mixture were used.

N-terminal amino acid sequence—The enzyme protein was separated by SDS-PAGE and electroblotted onto polybrene-coated glass-fiber sheets. Then the N-terminal amino acid sequence was determined directly in a gas-phase protein sequencer, as described by Hirano (1985) and Hirano and Wittman-Liebold (1987).

Other procedures—The native molecular weight of NAD-ME was determined by FPLC on a column of Superose 6 (Phamacia Fine Chem., Uppsala, Sweden) (Murata et al. 1989). SDS-PAGE and determination of molecular weight of subunits were carried out using the Laemmli system (13). Standard proteins used were purchased from Bio-Rad Co. (Pre-stained SDS-PAGE Standard). Protein concentrations were determined by the Bradford method (1976). A standard curve was established using BSA.

Results

Purification of NAD-ME from leaves of A. tricolor—The DEAE-TSK-gel chromatography was a very efficient method for purifying the enzyme from leaves of *E. coracana* (Murata et al. 1989). However, this procedure was less efficient for purification of the enzyme from *A. tricolor*, since in the latter case the NAD-ME was eluted from the column together with the large amounts of contaminating proteins, the major contaminant being PEPC. NAD-ME and PEPC could not be separated from each other by affinity chromatography on 5'AMP-Sepharose, which was used in the purification of the enzyme from *E. coracana*. As shown in Figure 1, the efficient resolution of the NAD-ME from PEPC was achieved by reverse-phase chromatography on butyl-TSK-gel. When the same procedure was applied for the purification of enzyme from *P. dichotomiflorum* leaves, NAD-ME was successfully resolved from PEPC, although the final preparation of the enzyme obtained in earlier experiments was still contaminated with a small amount of PEPC. The overall purification of the enzyme from *A. tricolor* is shown in Table 1. The enzyme was purified about 380-fold to a

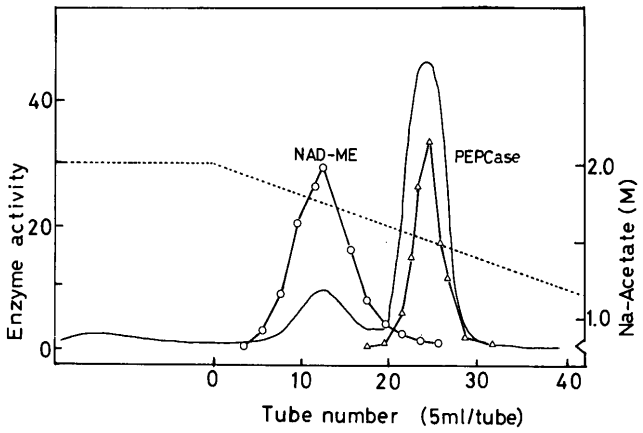

Fig. 1 Elution profile of reverse-phase chromatography on butyl-TSK-gel of NAD-ME from leaves of *A. tricolor*. Active fractions from DEAE ion-exchange chromatography were applied and eluted as described in "Materials and Methods". Solid line, concentration of protein expressed as A_{280}; dotted line, concentration of sodium acetate; circles, activity of NAD-ME; triangles, activity of PEPC. Note that enzyme activity is expressed in arbitrary units and the scale for activity of PEPC is 5-fold larger than that for NAD-ME.

Table 1 Purification of NAD-ME from *A. tricolor* leaves

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity	Purification
Crude extract	1,500	16,184	2,412	100	0.15	1
PEG fraction	128	6,507	2,529	104.9	0.39	2.6
DEAE-TSK-Gel chromatography	50	736	2,142	88.8	2.91	19.4
Butyl-TSK-Gel chromatography	1.5	29.8	1,089	45.1	36.54	243.6
TSK-Gel filtration	1.3	11.4	654	27.1	57.37	382.5

specific activity of 57 units·(mg protein)$^{-1}$ with a yield of 27%. These values are similar to those for the enzyme from leaves of *E. coracana* and *P. dichotomiflorum*. (Murata et al. 1989)

Figure 2 shows the electrophoretic pattern of SDS-treated preparations of NAD-ME from different C_4 species

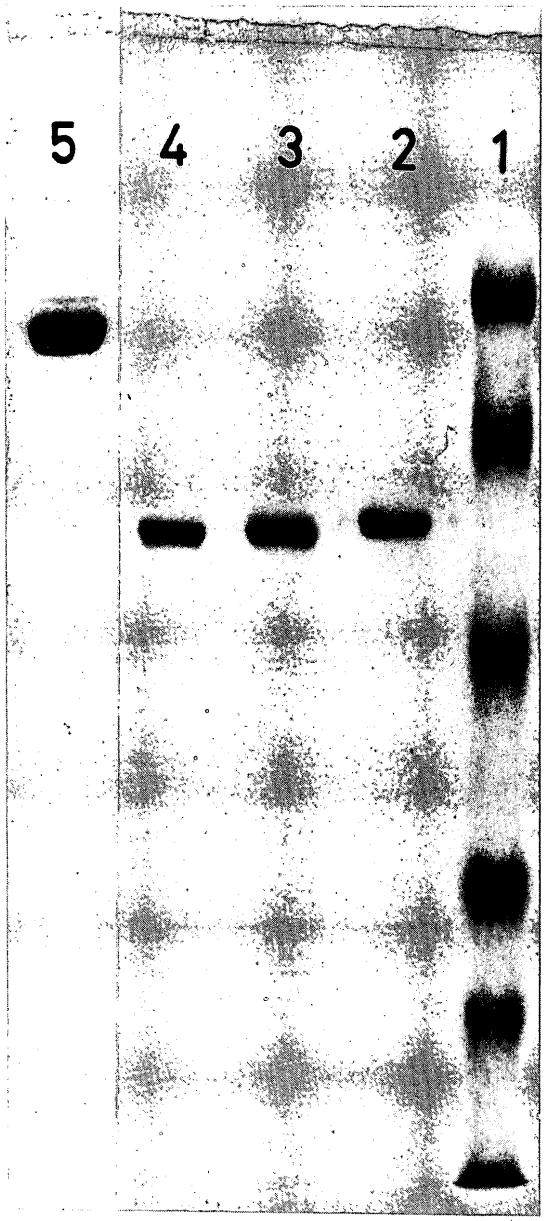

Fig. 2 SDS-PAGE of the purified NAD-ME from leaves of three C_4 species and PEPC from leaves of *A. tricolor*. Lane 1, standard proteins, from upper band: phosphorylase b (mol wt 130 kDa), BSA (75 kDa), ovalbumin (50 kDa), carbonic anhydrase (39 kDa), soybean trypsin inhibitor (27 kDa) and lysozyme (17 kDa). Lane 2: the purified NAD-ME from *E. coracana* which was obtained in previous experiments (Murata et al. 1989). Lane 3: NAD-ME from *P. dichotomiflorum*. Lane 4: NAD-ME from *A. tricolor*. Lane 5: PEPCase from *A. tricolor*.

and the preparation of PEPC from *A. tricolor*. Each preparation exhibited only a single major band of protein. The NAD-ME from *A. tricolor* migrated slightly more rapidly than the enzyme from *P. dichotomiflorum* and clearly more rapidly than the enzyme from *E. coracana*. The molecular weight of subunits of the enzyme from *A. tricolor* was determined to be 60 kDa (data not shown). Gel filtration on a column of Superose 6 of the native enzyme gave a molecular weight of 490 kDa, which is similar to that of the enzyme from monocots.

The lag in the enzymatic reaction—In the course of kinetic analysis in 25 mM HEPES-KOH buffer (pH 7.2), it was observed that a substantial time was required for attainment of a steady-state rate in the assay. In particular, the lag was remarkable at low concentrations of either malate or activator FBP and was prolonged at high concentrations of buffer and at higher pH than the optimum. The range of the lag encountered is shown in Figure 3. Line A, determined in the standard assay mixture, shows little lag, while line B, determined without FBP, represents a reaction with a substantial lag. The lag was almost entirely eliminated when the enzyme was assayed after preincubation in practically complete reaction mixture (line C). The effect of preincubation was probably due to an activation of the enzyme by the reaction product(s) produced during preincubation. In fact, the addition of 10 μM NADH (line E), but not 2 μM (line D), to the reaction mixture eliminated the lag.

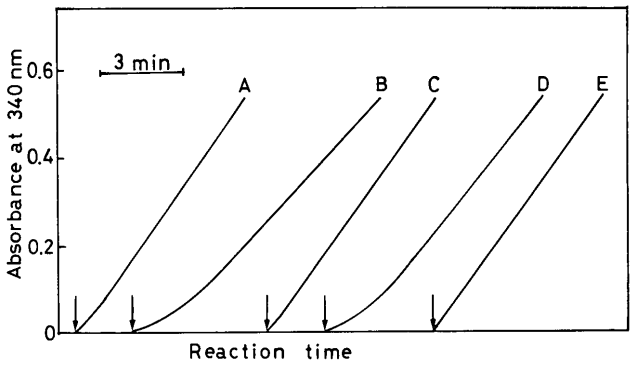

Fig. 3 Effect of substrates on the lag in the reaction exhibited by the purified NAD-ME from *A. tricolor*. Time courses of enzymatic reaction were determined using 1-ml reaction mixtures that contained 25 mM HEPES-KOH (pH 7), 5 mM malate, 2 mM NAD, 2.5 mM $MnCl_2$, 2.5 mM DTT and the compound specified. A: enzyme was assayed with 100 μM FBP in the reaction mixture. B and C: enzyme was assayed after incubation in the mixture that contained 25 mM HEPES-KOH (pH 7), 1 mM NAD, 0.5 mM $MnCl_2$, 5 mM DTT, in the absence (B) or in the presence (C) of 5 mM malate at room temperature for 30 min. D and E: enzyme was assayed in the presence of NADH at 2 μM (D) or 10 μM (E) in the reaction mixture. Arrows indicate the start of the respective reaction.

The effect of pH—As shown in Figure 4-A, the activity of NAD-ME from both *E. coracana* and *P. dichotomiflorum* showed similar dependence on pH, with a sharp decline on the acidic side of the optimum. The optimal pH was between 6.5 to 7 for both enzymes. The lag in the reaction was not observed at any pH, even though the assay was carried out at a low concentration of malate (1 mM, data not shown). In contrast, the pH-activity profile of the NAD-ME from *A. tricolor* showed considerable variation with respect to both optimal pH and the lag in the reaction, depending upon the concentration of either malate or FBP (Fig. 4-B). The optimal pH of between 6.8 to 7.3, with the standard assay mixture, was reduced either by decreasing the concentration of malate or by omitting FBP. The optimal pH was found to be about 6 with an assay mixture that contained 1.0 mM malate and without FBP. The lag in the reaction was also dependent on pH, being remarkable on the alkaline side of the optimal pH, but not so evident on the acidic side.

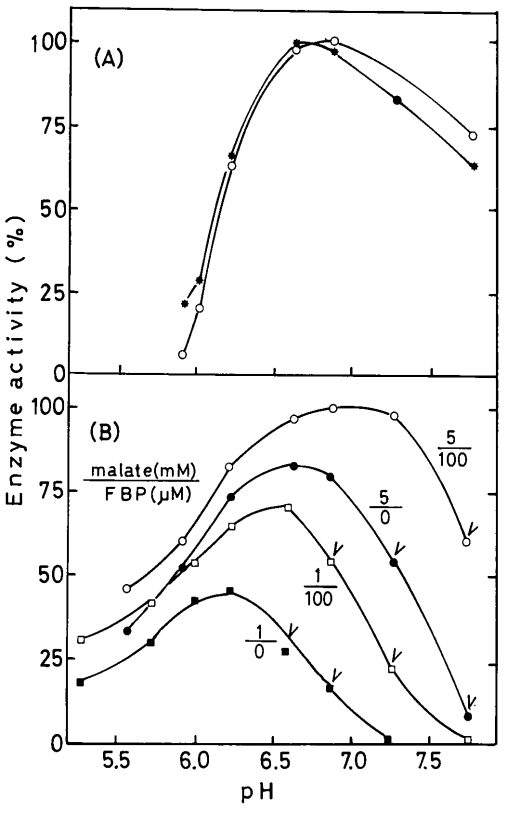

Fig. 4 Effect of pH on the activity of NAD-ME from leaves of the three C_4 species. (A): NAD-ME from *E. coracana* (open circles) and from *P. dichotomiflorum* (closed stars). Enzyme was assayed using the reaction mixture of the HEPES system except for changes in pH. (B): NAD-ME from *A. tricolor*. Reaction mixture was the ACES system except that the pH and concentrations of malate and FBP were changed as shown in the Figure. A symbol (v) represents the activity that showed a lag in the reaction.

Effect of the concentration of malate—The activity of NAD-ME from *A. tricolor* was determined as a function of the concentration of malate at different concentrations of FBP (0, 10 and 100 μM). At a high concentration of FBP (100 μM), the plots for activity were hyperbolic and Lineweaver-Burk plots revealed a straight line that gave a K_m of 0.38 mM (Fig. 5). Either with limiting FBP (10 μM) or without FBP, the Lineweaver-Burk plots of activity against the concentration of malate were not linear. Since the lag in the reaction was increased with decreasing concentrations of malate and FBP, as described above, sigmoidal activity curves may possibly have been due to the fact that the enzyme was not fully activated at low concentrations of malate. Therefore, except for that activity that showed a lag in the reaction, the activity was plotted against the concentration of malate according to the Lineweaver-Burk equation (Fig. 5). Straight lines were obtained and gave K_m values of 0.87 and 1.42 mM in the presence of 10 μM and in the absence of FBP, respectively. All these lines intersected the 1/v axis at the same point, indicating the same Vmax at any concentration of FBP. Similar results were obtained by plotting the activity assayed at pH 6, where no lag was observed (Fig. 4-B), as a function of the concentration of malate with 100 μM FBP and without FBP (Fig. 6). The straight lines in the double reciplocal plots gave K_m values for malate of 0.71 mM and 2.0 mM with 100 μM FBP and without FBP, respectively. These values were somewhat higher than those found at the optimal pH. The intersections with the 1/v axis were at the same point. Since the Vmax was the same at any concentration of FBP

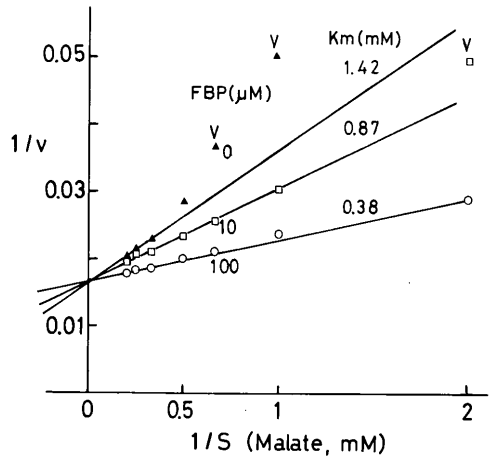

Fig. 5 Double reciprocal plots of activity of NAD-ME from *A. tricolor* as a function of the concentration of malate in the presence of 100 μM (open circle) and 10 μM (open squares) or in the absence (closed triangles) of FBP. The reaction mixture was the ACES system except for the use of various concentrations of malate and FBP as shown in the Figure. A symbol (v) indicates the activity that showed a lag in the reaction. K_m values are shown above the respective lines in the Figure.

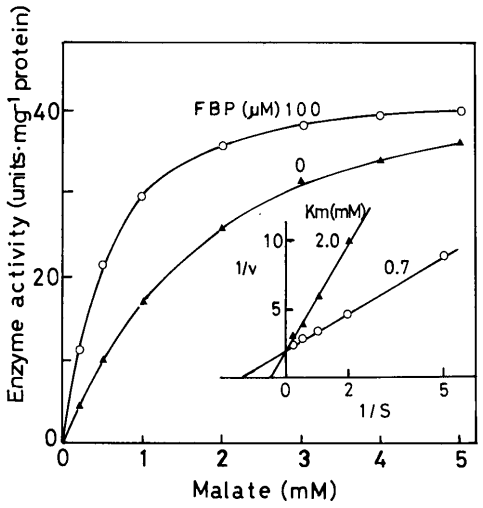

Fig. 6 Effect of the concentration of malate on the activity of NAD-ME from *A. tricolor* at pH 6 in the presence (open circles) or absence (closed circles) of 100 μM FBP. The reaction mixture was the ACES system at pH 6. K_m values are shown above the respective lines in the Figure.

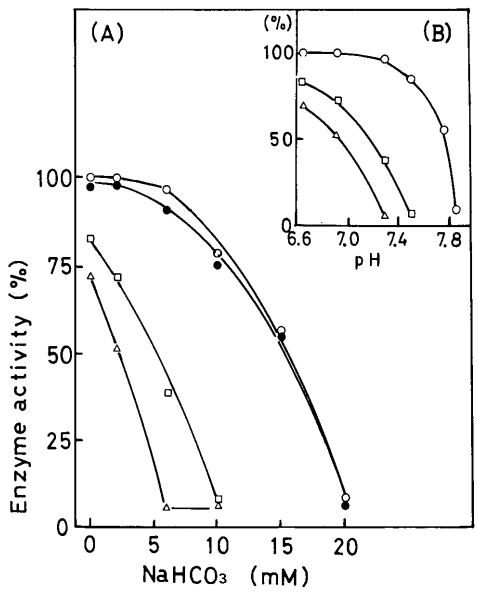

Fig. 7 Effect of the concentration of $NaHCO_3$ on the activity of NAD-ME from *A. tricolor* at various concentrations of FBP. The reaction mixture was the ACES system except that malate was present at 2 mM and the concentration of FBP was changed as follows; 100 μM (open and closed circles), 5 μM (open squares) and zero (triangles). To monitor the effect of CO_2, carbonic anhydrase (2 unit) was added (closed circles). The inset shows replots of the activity against the pH of the reaction mixture, as determined immediately after the reaction.

at any given pH, it is possible that FBP functions by increasing the affinity for malate rather than by increasing the maximum velocity. These results relating to the effect of FBP on the NAD-ME from *A. tricolor* are quite different from those obtained with the enzymes from *E. coracana* and *P. dichotomiflorum*. With the enzyme from *E. coracana*, both the affinity for malate and Vmax were greatly increased in the presence of FBP, while with the enzyme from *P. dichotomiflorum*, the K_m for malate was not changed but Vmax was increased about 1.5-fold in the presence of FBP (Murata et al. 1989).

Effect of HCO_3^-—The partially purified NAD-ME from leaves of *Atriplex spongiosa* and *Am. edulis* has been reported to be inhibited competitively by bicarbonate with respect to both malate and the activators, CoA (Hatch et al. 1974) and FBP (Chapman and Hatch 1977). In our preliminary experiments, the elimination of CO_2 and bicarbonate dissolved in the reaction mixture by boiling and by gassing with N_2 did not affect the activity of NAD-ME from *A. tricolor*. Therefore, the effect of the concentration of bicarbonate on the purified NAD-ME from leaves of *A. tricolor* was tested at different concentrations of FBP with 2 mM malate. The addition of $NaHCO_3$ to the reaction mixtures resulted in the reduction of the NAD-ME activity from *A. tricolor* as shown in Figure 7. The reduction in activity was more remarkable at low concentrations of either malate or FBP. However, the activity was not affected by the addition of carbonic anhydrase to the assay mixture that contained HCO_3^-. The concentration of buffer (12.5 mM ACES-NaOH) was not enough to maintain a constant pH when the relatively concentrated $NaHCO_3$ was added. The pH of the assay mixture was determined after each reaction and the activity was reploted against the actual pH (inset of Fig. 7). The shapes of these pH-activity curves are very similar to those in Figure 4-B, indicating that the reduction in activity by the addition of $NaHCO_3$ was due to the increase in pH rather than any direct effect of HCO_3^- or CO_2.

Effect of FBP on the purified NAD-ME from the three different C_4 species—Some effects of FBP on the activity of NAD-ME were dependent on the source of enzyme, namely *E. coracana, P. dichotomiflorum* and *A. tricolor*, as shown in Figure 8. With the enzyme from *E. coracana*, the response to increasing concentrations of FBP was hyperbolic and was remarkable at any of the tested concentrations of malate. The concentrations of FBP giving half of maximum activity (K_A) were 160 μM and 65 μM at 0.5 mM and 5 mM malate, respectively. Similar results were obtained for the enzymes from *P. dichotomiflorum* and *A. tricolor* at low concentrations of malate (less than 0.5 mM). The K_A for FBP was 12.5 μM and 5.5 μM with the enzyme from *P. dichotomiflorum*, and was 47.6 μM and 12.7 μM with the enzyme from *A. tricolor*, at 0.1 mM and 0.5 mM malate, respectively. In contrast, at a saturating level of malate (5 mM), the activity without FBP was comparable to that with FBP for the enzyme from *A. tricolor*

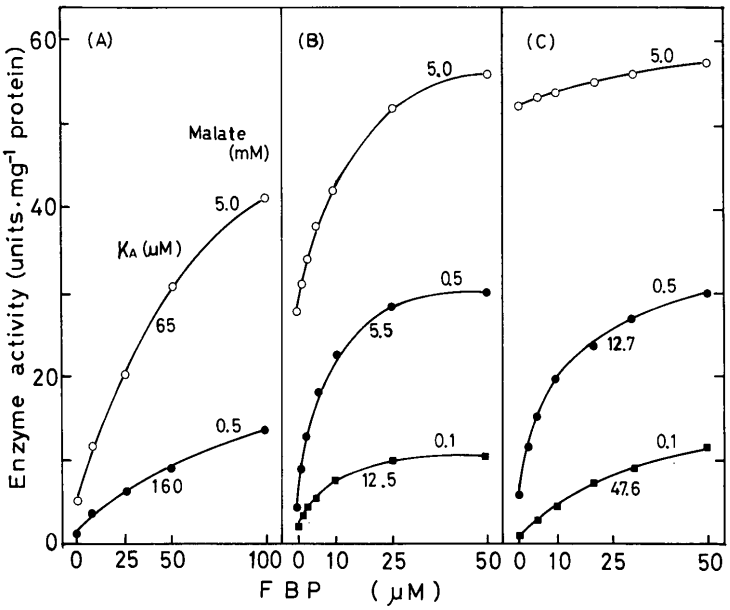

Fig. 8 Effect of the concentration of FBP on the NAD-ME from the leaves of *E. coracana* (A), *P. dichotomiflorum* (B) and *A. tricolor* (C). The reaction mixture was the ACES system except that the concentration of malate was chaned as follows; 5 mM (open circles), 0.5 mM (closed circles) and 0.1 mM (closed squares). K_A values are shown under the respective curves in the Figure.

and was about half of the activity with FBP for the enzyme from *P. dichotomiflorum*. That is, the stimulatory effect of FBP was very small and only doubled the activity in the cases of the enzymes from *A. tricolor* and *P. dichotomiflorum*, respectively.

Immunological analysis of the purified NAD-ME from different C_4 species (ELISA)—The cross-reactivities of the purified NAD-ME from *E. coracana*, *P. dichotomiflorum* and *A. tricolor* with the antisera raised against the enzymes from the three species were determined by the ELISA procedure. With the antiserum raised against NAD-ME from *E. coracana* (Fig. 9-A), the enzymes from *P. dichotomiflorum* and *A. trilcolor* were about 49% and 37% as reactive as the enzyme from *E. coracana*, respectively. Similar results were obtained using antiserum raised against NAD-ME from *A. tricolor* (Fig. 9-C). The cross-reactivity of the enzymes from *E. coracana* and *P. dichotomiflorum* was 43% and 38% of the antigen enzyme, respectively. In contrast, the antiserum raised against NAD-ME from *P. dichotomiflorum* showed similar cross-reactivities against the enzymes from each of the three different species, the enzymes from *E. coracana* and *A. tricolor* showing 93% and 84% cross-reactivity compared to that of the enzyme from *P. dichotomiflorum*, respectively (Fig. 9-B).

N-terminal amino acid sequence—The first 29, 14 and 28 amino acid residues from the N-terminus of the purified NAD-MEs from the three C_4 species, *E. coracana*, *P. dichotomiflorum* and *A. tricolor*, were determined (Table 2). Eleven out of 20 and 7 out of 12 amino acids in the enzyme from *A. tricolor* were identical to the corresponding amino acids in the enzyme from *E. coracana* and the enzyme from *P. dichotomiflorum*, respectively (but a deletion was introduced to maximize homology).

Table 2 Comparison of N-terminal amino acid sequences of NAD-ME from leaves of three C_4 species, *E. coracana*, *P. dichotomiflorum* and *A. tricolor*. (One-letter symbols)

Position from the N-terminus	1	5	10	15	20	25	30
E. coracana	S S A A P A A G A A V P G P I I V H K × G N D I L N D P ×						
P. dichotomiflorum	- - - - - P - - - L A V - P						
A. tricolor	- T - E - - - L A F - N - × - × × F - E - - V F N K G T						

A dash (–) represents a residue identical to that in the sequence of the enzyme from *E. coracana*. A blank represents a deletion introduced in order to maximize homology. A symbol (×) indicates an unidentified residue.

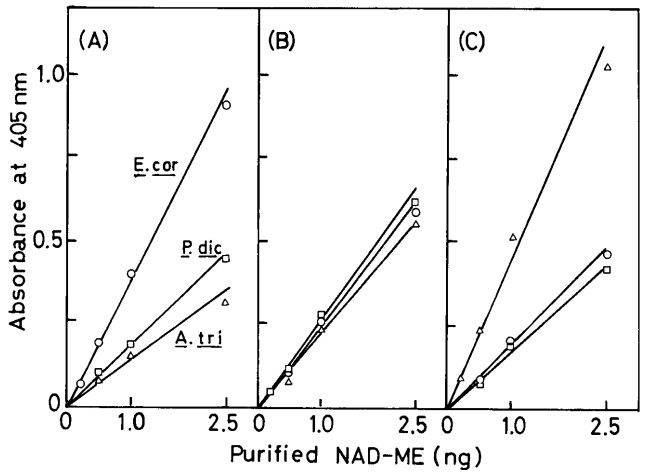

Fig. 9 Cross-reactivities of NAD-MEs from leaves of *E. coracana* (circles), *P. dichotomiflorum* (squares) and *A. tricolor* (triangles) with antiserum raised against purified NAD-ME from *E. coracana* (A), *P. dichotomiflorum* (B) and *A. tricolor* (C), as determined by the ELISA procedure.

Discussion

NAD-ME is widely distributed in plant tissues and has been purified from mitochondria of non-photosynthetic tissues of a C_3 plant (Grover et al. 1981) and from leaves of a CAM plant (Wedding and Black 1983). We recently purified the NAD-ME from leaves of C_4 monocots (Murata et al. 1989), but the enzyme from leaves of a C_4 dicot had not yet been fully purified. In this series of experiments, NAD-ME was purified from leaves of *A. tricolor*, a NAD-ME type C_4 dicot, to an electrophoretically homogeneous preparation with about 27% recovery. The procedures used were similar in principle to those described in our previous paper, except the butyl-TSK-gel chromatography was used instead of 5'AMP-Sepharose chromatography. The purified enzyme had a specific activity of 57 units·(mg protein)$^{-1}$ and purification was 380-fold. These values are very similar to those obtained for the enzymes from leaves of *E. coracana* and *P. dichotomiflorum* (Murata et al. 1989). These similarities indicate that both the content and the activity of NAD-ME in vivo are not very different among the NAD-ME type species.

It was of interest to ascertain the subunit composition of the enzyme from *A. tricolor*, since a difference in the molecular weight of subunits was observed between the enzymes from *E. coracana* (63 kDa) and *P. dichotomiflorum* (61 kDa) (Murata et al. 1989) and a subunit composition with two dissimilar subunits has been reported for the enzymes from dicots, potato tubers and *Crassula* leaves (Willeford and Wedding 1987). SDS-PAGE of the enzyme from *A. tricolor* revealed a single major band of protein which migrated slightly more rapidly than the denatured enzyme from *P. dichotomiflorum*, indicating a molecular weight of subunits of 60 kDa. This result together with the native molecular weight of 490 kDa indicates that the enzyme from *A. tricolor* is an octamer of identical subunits.

A lag in the reaction was observed with NAD-ME from *A. tricolor* as with that from cauliflower buds (Valenti and Pupillo 1981) and *Crassula* leaves (Wedding et al. 1981). The lag was marked when malate was limiting and activator FBP was omitted from the assay mixture. However, the lag was almost completely eliminated by the addition of 10 μM NADH to the assay mixture. This elimination of the lag may occur via the formation of the quarternary complex, Enzyme-Mn^{2+}-NADH-Malate (Wedding et al. 1981).

The leaf NAD-ME from C_4 dicots, such as *Am. edulis* and *At. spongiosa*, exhibited an allosteric property (Chapman and Hatch 1977, Hatch et al. 1974). The purified enzyme from *A. tricolor* showed a hyperbolic activity curve when activity was plotted against the concentration of malate, either in the presence of saturating activator or at low pH in reactions where no lag was observed. In the absence of FBP, the double reciprocal plots of activity, when the reactions that showed a lag were excluded, *versus* the concentration of malate also gave straight lines. Neither CO_2 nor HCO_3^- was found to inhibit the enzyme regardless of the presence or absence of the activator. The reasons for the apparent discrepancies between the results obtained here and those reported by Hatch's group are not clear. The most likely possibilities are that our enzyme was fully purified and that special regard was paid to the lag in the kinetic studies of the enzyme. In this connection, it is of interest to note that the purified NAD-ME from dicots, such as cauliflower buds (Canellas and Wedding 1984, Valenti and Pupillo 1981), potato tubers (Grover and Wedding 1984) and *Crassula* leaves (Wedding and Black 1983) exhibited no cooperativity for binding of malate, with or without activator, when the fully activated enzyme was used to analyze the reaction of NAD-ME. The stimulatory effect of FBP on the enzyme was quite different among the enzymes from the leaves of the three C_4 species when malate was present at saturating concentrations. Similar results were obtained in the presence of high concentrations of Mn^{2+} (Murata et al. 1989).

ELISA studies using antisera raised against the purified enzymes from three C_4 species revealed different antigenicities of the enzyme that depended on the source of enzyme. Some differences in N-terminal amino acid sequences were also found among the enzymes from the three species, although the homology between sequences was relatively high. These results indicate the genetic diversity of NAD-ME among species. Further studies are in progress to elucidate the molecular basis for the diversity of the enzyme by comparative analysis of the genes that encode the enzyme in various species.

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