THE ENZYME KINETICS OF THE NADP-MALIC ENZYME FROM TOBACCO LEAVES

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Malic enzyme (L-malate: NADP$^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40, NADP-ME), which was found in chloroplasts, was isolated from tobacco leaves (*Nicotiana tabacum* L.) almost homogenous. The specific enzyme activity was 0.95 µmol min^{-1} mg^{-1}. The enzyme pH optimum was found between pH 7.1 and 7.4. The affinity of NADP-ME to substrates (L-malate and NADP$^+$) was evaluated in the presence of divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}). The value of the apparent Michaelis constant of NADP-ME for L-malate was dependent on the ion cofactor, while no such relationship was found for NADP$^+$. The dependence of the reaction rate on concentration of Mg^{2+} indicates the presence of more than one binding site for these ions in NADP-ME. Likewise, the sigmoidal dependence of the reaction rate on Mn^{2+} concentration and the value of Hill coefficient 7.5 indicate the positive cooperativity of the reaction kinetics in the presence of the ions. The effect of Co^{2+} and Ni^{2+} ions was analogous to that of Mn^{2+} ions; however, the cooperativity was lower (the values of Hill coefficients were 3.0 and 1.3 for Co^{2+} and Ni^{2+}, respectively). Regulation of NADP-ME from tobacco leaves by divalent metal ions is discussed.
Keywords: Oxidoreductases; Enzyme kinetics; NADP-malic enzyme; Divalent metal ions; *Nicotiana tabacum* L.

Malic enzyme (L-malate: NAD(P)$^+$ oxidoreductase (decarboxylating)) catalyzes oxidative decarboxylation of L-malate using NAD$^+$ or NADP$^+$ as coenzymes in the presence of divalent metal ions to produce pyruvate, NAD(P)H and CO$_2$ [1,2]. This is ascribed to three related forms of malic enzymes. The first, EC 1.1.1.38, which uses NAD$^+$ as a coenzyme and can

decarboxylate oxaloacetate in addition to L-malate, is predominantly found in bacteria. The second enzyme, denoted as EC 1.1.1.39, uses $NAD(P)^+$, but it is unable to decarboxylate oxaloacetate. This enzyme is localized in plant mitochondria as well as in animal cells[3]. Finally, NADP-dependent malic enzyme (NADP-ME), EC 1.1.1.40, utilizes $NADP^+$ and catalyzes the decarboxylation of L-malate and other partial reactions including decarboxylation of oxaloacetate and reduction of α-ketocarboxylic acid[1]. This enzyme is found in animal and plant tissues as well as in prokaryotic and eukaryotic microorganisms[1]. NADP-ME is widely distributed among all types of plants and its isoforms are either plastidic or cytosolic[2]. NADP-ME is coded by a small gene family, the expression of which is tissue and ontogenic stage specific. In *Arabidopsis* three isoforms in cytosol and one in chloroplasts are present[4], similar results were obtained in rice[5].

NADP-ME, located in chloroplasts of bundle sheet cells of C_4 plants (a plant, in which the first photosynthetic product is a 4-carbon compound), plays an important role in C_4 photosynthesis. It catalyzes the release of CO_2, which is utilized in the Calvin cycle. In Crasulean acid mechanism (CAM) plants, malic enzyme has an analogous function, but its localization is cytoplasmic[2,6]. NADP-ME is also present in C_3 plants (a plant, in which the first photosynthetic product is a 3-carbon compound), and in non-photosynthetic tissues of C_4 plants[2,7]. It is assumed that its main role is to supply reduced equivalents, NADPH, for synthetic metabolic pathways such as synthesis of fatty acids and pyruvate. Moreover, NADP-ME together with phosphoenolpyruvate carboxylase serves to maintain the intracellular pH. NADP-ME also participates in the mechanism of stomatal closure and can affect the water economy of a plant[8].

The NADP-ME function seems to be associated with metabolic response of plants to stress. The increased activity of NADP-ME in maize roots treated with fungal elicitors, jasmonate, or cellulase was found[9], and in tobacco leaves affected by viral infection[10]. The enhancement of NADP-ME activity was also found in water-stressed tobacco[11] and in transgenic tobacco overproducing endogenous cytokinins[12]. Molecular mechanisms of such responses have not yet been explained. Furthermore, the enzyme of C_3 plants has not been fully characterized until now. Therefore, we have isolated NADP-ME from tobacco leaves and characterized kinetics of the reaction catalyzed by this enzyme. Kinetic parameters of the enzyme in the presence of various divalent metal ions were determined.

EXPERIMENTAL

Plant Material

Tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) were grown in a greenhouse under 22/18 °C day/night temperatures. Seeds were sown in pots with sand and plantlets were transferred to soil after 3 weeks. Leaves of seven-week old plants were collected for isolation, frozen immediately in liquid N_2 and stored at –75 °C.

In vivo Localization of NADP-ME

Malic enzyme was localized by staining in fresh hand-cut sections of leaf midribs. The sections were incubated immediately in 10 ml of staining solution consisting of 100 mM Tris–HCl buffer (pH 7.4), 10 mM L-malate, 10 mM $MgCl_2$, 7.6 mg $NADP^+$, 12 μM Nitroblue Tetrazolium chloride, and 0.16 μM phenazine methosulfate at room temperature for 30 min in the dark. The control staining was done without L-malate or $NADP^+$. The blue color indicated NADP-ME activity, which was not present in the control staining. The sections were examined in light microscope Nicon Eclipse E600 equipped with CCD camera.

Enzyme Purification

Tobacco leaves (50 g) were homogenized in 100 mM Tris–HCl (pH 7.8) containing 1 mM dithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$ (buffer A) using a disperser Turrax DI-18. The homogenate was filtered through cheesecloth, 1 g of poly(vinylpyrrolidone) was added, and the mixture was centrifuged at 9400g for 30 min. The extract was precipitated with ammonium sulfate. The fraction with NADP-ME activity precipitated between 30 and 55% saturation of $(NH_4)_2SO_4$. The precipitated protein was dissolved in 6 ml of 25 mM Tris–HCl (pH 7.8) containing 0.5 mM dithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$ (buffer B) and dialyzed overnight against 1 l of buffer B. The solution was applied onto a DEAE-cellulose column (1.5 × 10 cm) equilibrated with buffer B. After washing with buffer B, elution was performed with a linear NaCl gradient (0–300 mM NaCl in buffer B) at flow rate 1 ml min^{-1}. NADP-ME activity was eluted as a single peak at 250 mM NaCl. Fractions containing the highest activity were pooled, precipitated with 80% saturation of ammonium sulfate and centrifuged at 9400g for 10 min. The pellet was dissolved in a minimal volume of buffer B and applied onto a column of Sephadex G-200 (1.5 × 30 cm) previously equilibrated with buffer B. Elution with buffer B was performed and fractions with NADP-ME activity were pooled.

2′,5′-ADP-Sepharose 4B was swollen and washed with buffer B, packed into a column (1 × 8 cm), equilibrated by washing with buffer B containing 5 mM L-malate and 5 mM $MgCl_2$, and then used for the separation of NADP-ME. Elution was performed with buffer C (25 mM Tris–HCl pH 7.8, 0.5 mM dithiothreitol, 1 mM EDTA).

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard[13].

The Enzyme Activity Assays

The NADP-ME activity was determined spectrophotometrically (Hellios α, Thermo Spectronic) at 21 °C by monitoring NADPH production at 340 nm. The NADP-ME assay mixture contained 80 mM 3-morpholinopropane-1-sulfonic acid (MOPS)–20 mM sodium ace-

tate–NaOH buffer (pH 7.4), 16 mM L-malate, 2 mM MgCl$_2$ and 0.2 mM NADP$^+$ in total volume of 1 cm^3. The reaction was started by addition of the enzyme. Apparent Michaelis constants ($S_{0.5}$ constants, respectively) of NADP-ME for the substrates and cofactors were determined by varying its concentration and maintaining the other substrates and cofactors concentrations saturating (16 mM L-malate, 0.2 mM NADP$^+$ and 2 mM cofactor). Kinetic parameters were obtained by fitting the data from a substrate saturation curve to the equation: Michaelis–Menten equation (*A*); Hill equation (*B*); expression, which describes two binding sites for Mg^{2+} ions[14] (*C*)

$$v = \frac{V[\text{S}]}{K_\text{m}[\text{S}]} \tag{A}$$

$$v = \frac{V[\text{S}]^n}{[S_{0.5}]^n + [\text{S}]^n} \tag{B}$$

$$v = \frac{V_1[\text{S}]^{n1}}{[S_{0.5_1}]^{n1} + [\text{S}]^{n1}} + \frac{V_2[\text{S}]^{n2}}{[S_{0.5_2}]^{n2} + [\text{S}]^{n2}} \tag{C}$$

where *V* is apparent maximal velocity of enzyme reaction (μmol min^{-1} mg^{-1}), [S] substrate concentration (mol l^{-1}), K_m Michaelis constant (mol l^{-1}), $S_{0.5}$ is substrate concentration when the reaction rate is half of the maximal velocity in non-Michaelis–Menten kinetics, and *n* is Hill coefficient under the given experimental conditions. The data were processed by non-linear regression with MS Excel program.

The pH optimum of NADP-ME was measured utilizing 80 mM MOPS–20 mM sodium acetate–NaOH buffer (pH 6.5–7.7) and 100 mM Tris–HCl buffer (pH 7.1–9.0).

Electrophoretic Separations

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out according to Laemmli[15] using a Biometra apparatus. Gels (10%) were stained for protein with Coomassie Brilliant Blue. Native gel electrophoresis was performed according to Lee and Lee[16]. The gels (6%) were assayed for the NADP-ME activity by incubating in a solution of 100 mM Tris–HCl (pH 7.4) containing 10 mM L-malate, 10 mM MgCl$_2$, 2 mM NADP$^+$, 0.1 mg ml^{-1} Nitroblue Tetrazolium chloride and 5 μg ml^{-1} phenazine methosulfate at room temperature[9].

Isoelectric focusation was performed according to the Pharmacia manual except the prolongation of the procedure to 6000 V h instead of 3500 V h. The standard protein mixture obtained from Serva was used for calibration.

RESULTS

Localization of NADP-ME in Tobacco Plants

The localization of NADP-ME was studied in tobacco leaf midribs by staining for its activity (Figs 1A–1C). The dark blue colour, which is formed by
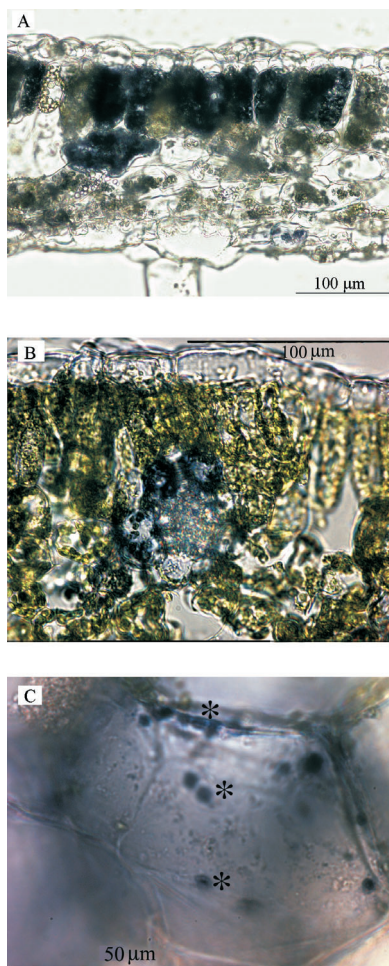

FIG. 1

Histochemical localization of the NADP-ME activity on fresh hand-cut section of tobacco leaf. Blue staining indicates the enzyme activity. Cross-section of the tobacco leaf (A), more detailed view of leaf cross-section (B), detailed view of mesophyll cell with stained chloroplast (C). Chloroplasts marked by ✻ showed the NADP-ME activity

reduction of Nitroblue Tetrazolium chloride and phenazine methosulfate, corresponds to activity of NADP-ME. Figures 1A and 1B document higher activity of NADP-ME in the cells surrounding the bundle sheets than in other cells of tobacco leaf, i.e. L-malate transported in veins can be decarboxylated in these surrounding cells. Within the cell, NADP-ME is localized particularly in the chloroplasts. In Fig. 1C the chloroplasts are marked with asterisk. However, the presence of this enzyme in other cell compartments, such as vacuole or cytosol, cannot be excluded.

Purification of NADP-ME

The enzyme was isolated from seven-week old tobacco plants and purified to a final specific activity of 0.95 μmol min^{-1} mg^{-1}, using chromatography on DEAE-cellulose followed by gel and affinity chromatography. The purification procedure is summarized in Table I. Purified NADP-ME was analyzed by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). The band with molecular weight of 67 000 corresponded to NADP-ME. Another band with lower molecular weight was also present, but it was probably a contaminant protein (Fig. 2A). This was proved by non-denaturing electrophoresis tested for NADP-ME activity, which showed only the presence of one protein band with the NADP-ME activity (Fig. 2B). Native isoelectric focusation assayed for activity indicated also only one form of NADP-ME, with the estimated isoelectric point pI 5.5 (Fig. 2C).

TABLE I
Procedures used for purification of NADP-ME from tobacco leaves

Step	Total protein mg	Total activity μmol min^{-1}	Specific activity μmol min^{-1} mg^{-1}	Purification fold	Yield %
Crude extract	67.62	0.99	0.015	1	100
$(NH_4)_2SO_4$	29.61	0.73	0.025	2	74
Dialysis	25.83	0.65	0.025	2	65
DEAE-cellulose	3.84	0.34	0.088	6	34
Gel chromatography	0.36	0.21	0.591	41	22
Affinity chromatography	0.04	0.04	0.950	65	4

Kinetic Properties of NADP-ME

The activity of NADP-ME was pH-dependent, having pH optimum between 7.1 and 7.4. A significant decrease in the enzyme activity was found below pH 6.8 and above pH 7.6 (Fig. 3).

NADP-ME is known[2] to catalyze oxidative decarboxylation of L-malate in the presence of divalent metal ions, particularly Mg^{2+}. Therefore, in addition to Mg^{2+} ions, other ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} were also analyzed (Table II). While Mg^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} ions acted as the NADP-ME cofactors stimulating the enzyme activity (Table II), Ca^{2+} and Cu^{2+} were ineffective (not shown). The presence of a divalent metal ion is essential, no NADP-ME activity was observed without this cofactor. Zero activity without addition of divalent metal ions to the reaction mixture also confirms, that the enzyme extract is free of divalent metal ions after all isolation procedures. The Mn^{2+} ions served as the most efficient cofactor of NADP-ME, being 1.4-fold more efficient than Mg^{2+} ions (Table II). Ni^{2+} and Co^{2+} ions were less effective to stimulate the NADP-ME activity, exhibiting
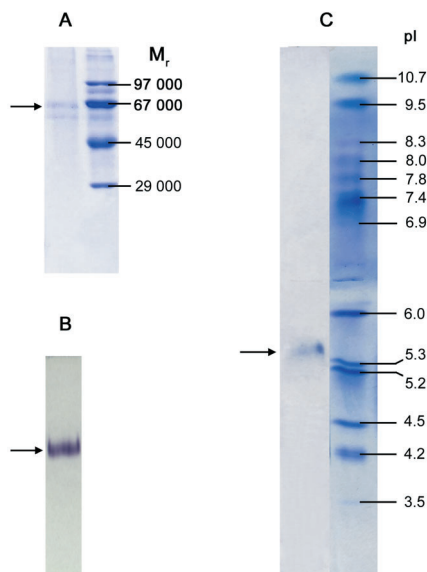

FIG. 2
NADP-ME from tobacco leaves separated by SDS-PAGE stain-tested for proteins (A), by native electrophoresis stain-tested for enzyme activity (B), by isolectric focusation stain-tested for enzyme activity (C). Positions of molecular weight markers (A) and isolectric focusation markers are indicated on the right-hand side

only 54 and 59% of the value of the reaction rate in the presence of Mg^{2+} ions, respectively. The activity of NADP-ME in the presence of Zn^{2+} was only 20% compared with Mg^{2+} ions (not shown).

The experimental data obtained from the dependence of the reaction rate on the concentration of $MgCl_2$ corresponded to the equation that describes two binding sites for Mg^{2+} ions (see Eq. (*C*) in Experimental). The particu-

TABLE II
Kinetic parameters of NADP-ME from tobacco leaves characterizing the dependence of the reaction rate on the concentration of Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} ions

Cofactor	Mg^{2+}		Mn^{2+}	Co^{2+}	Ni^{2+}
	1	2			
$S_{0.5}$ (Me^{2+}), mM	0.21 ± 0.03	1.15 ± 0.55	0.04 ± 0.01	0.06 ± 0.01	0.16 ± 0.02
n	1.6 ± 0.4	1.2 ± 0.5	7.5 ± 1.3	3.0 ± 0.3	1.3 ± 0.1
V, µmol min^{-1} mg^{-1}	0.42 ± 0.07	0.65 ± 0.11	1.49 ± 0.25	0.63 ± 0.11	0.58 ± 0.10

Measured with 16 mM L-malate and 0.2 mM $NADP^+$ at 21 °C, SEMs were calculated from at least 3 sets of data. V is apparent maximal velocity, $S_{0.5}$ substrate concentration when the reaction rate is half of maximal velocity in non-Michaelis–Menten kinetics, n Hill coefficient under the given experimental conditions. Column 1: the first binding site for Mg^{2+} ions, column 2: second binding site for Mg^{2+} ions.
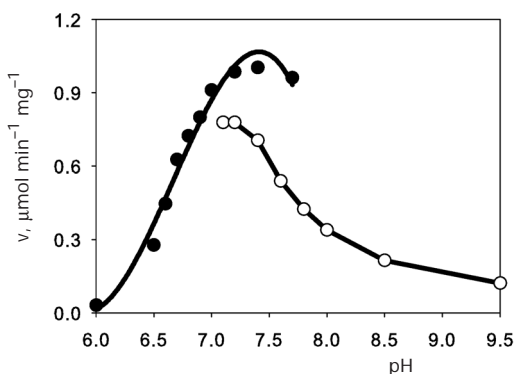

FIG. 3
Effect of pH on activity of NADP-ME from tobacco leaves. ● 80 mM MOPS–20 mM sodium acetate–NaOH buffer (pH 6.5–7.7), ○ 100 mM Tris–HCl buffer (pH 7.1–9.0)
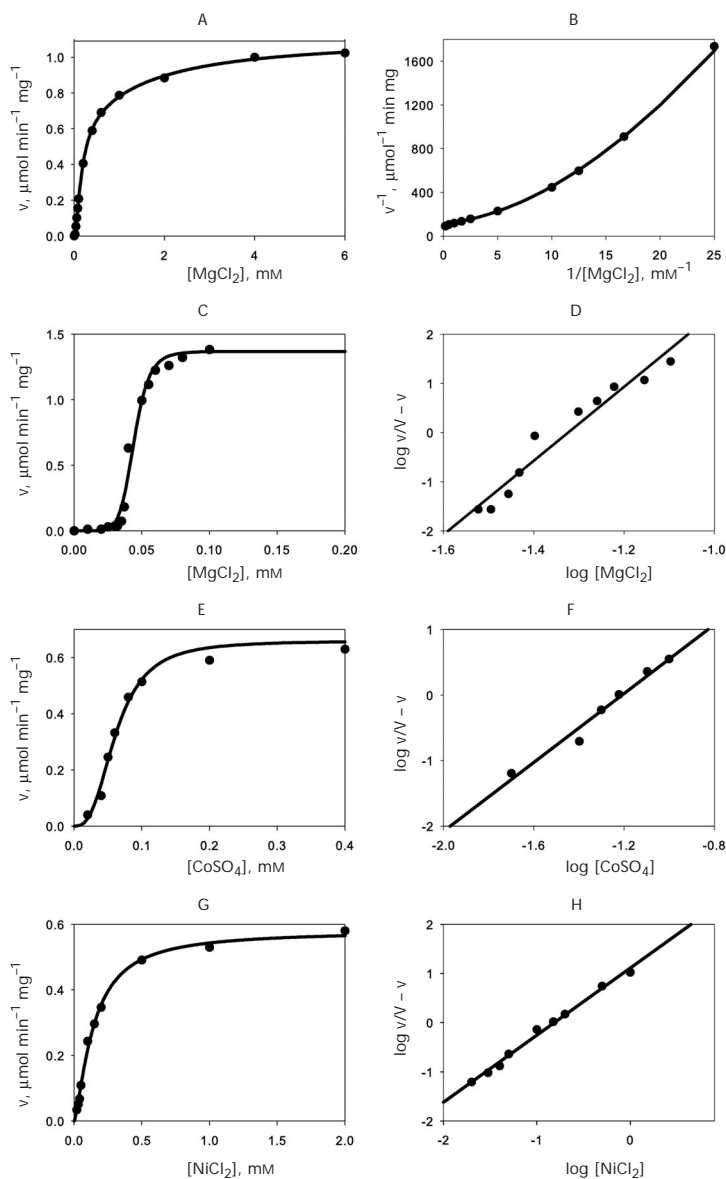
FIG. 4

Effect of increasing concentrations of divalent metal ions on the reaction rate of NADP-ME from tobacco leaves. The concentrations of L-malate and NADP⁺ were at saturating levels. Mg^{2+} saturation curve of NADP-ME (A), double reciprocal plots (B), Mn^{2+} saturation curve of NADP-ME (C), Hill plots (D), Co^{2+} saturation curve of NADP-ME (E), Hill plots (F), Ni^{2+} saturation curve of NADP-ME (G), Hill plots (H)

lar constants for both binding sites $S_{0.5}$ (concentration of $MgCl_2$ at half-saturation; for first binding site (subscript 1) and for second binding site (subscript 2), $S_{0.5_1}$, $S_{0.5_2}$) and V (maximal reaction rate, V_1, V_2) were calculated (Table II). The mild positive cooperativity in the presence of variable concentrations of Mg^{2+} characterized by Hill coefficients of 1.6 and 1.2 for these two binding sites were found (Figs 4A and 4B, Table II).

A typical sigmoidal kinetic curve, indicating a positive cooperativity, was detectable in the presence of different concentrations of $MnCl_2$. The value of the $S_{0.5}$ was equal to 0.04 mM, the value of Hill coefficient was 7.5 (Figs 4C and 4D, Table II). The enzyme kinetics in the presence of variable concentrations of Co^{2+} and Ni^{2+} ions was analogous to that in the presence of Mn^{2+} ions, but the positive cooperativity characterized by values of Hill coefficients and maximal reaction rate was much lower (Figs 4E–4H, Table II).

The divalent metal ions affected the affinity of NADP-ME to L-malate (Table III). Hyperbolic kinetic curves were found for the NADP-ME reactions in the presence of different concentrations of L-malate and all the ions tested in the study (Fig. 5A). The values of apparent K_m for L-malate at saturation level of $NADP^+$ and relevant metal ions are summarized in Table III. For comparison, we calculated values of Michaelis constants in the presence of Mg^{2+} using association constants Mg–malate (Table IV). The highest K_m value for L-malate was found in the presence of $MgCl_2$, whereas K_m was twice lower with $MnCl_2$. None of the tested ions affected the affinity of $NADP^+$ to the NADP-ME (Fig. 5B, Table III). No inhibition of NADP-ME by L-malate was found (Fig. 5A).
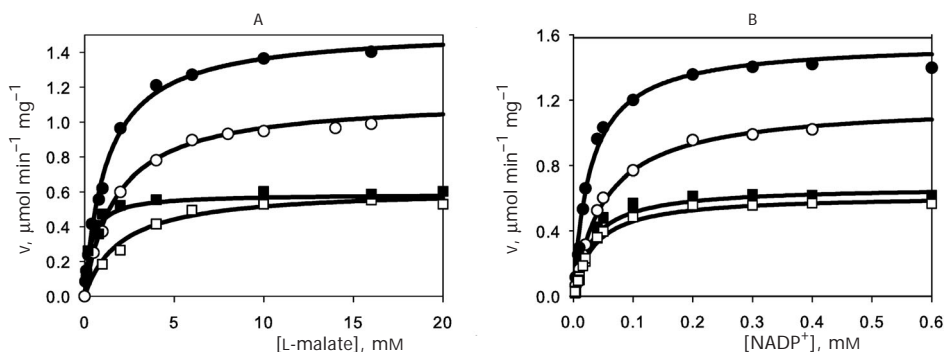

FIG. 5

Effect of L-malate cocentration (A) and $NADP^+$ concentration (B) on the reaction rate of NADP-ME from tobacco leaves in the presence of divalent metal ions. ● 2 mM Mn^{2+}, ○ 2 mM Mg^{2+}, ■ 2 mM Co^{2+}, □ 2 mM Ni^{2+}

Apparent Michaelis constants of NADP-ME for L-malate and NADP$^+$ at saturating concentrations (2 mM) of Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} ions

Cofactor	Mg^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}
K_m (L-malate), mM	2.0 ± 0.4	1.4 ± 0.4	0.4 ± 0.1	2.2 ± 1.1
K_m (NADP$^+$), mM	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

SEMs were calculated from at least 3 sets of data.

Kinetic parameters NADP-ME calculated using association constants (Me^{2+}–L-malate): Michaelis constants NADP-ME for L-malate in the presence of Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} ions and $S_{0.5}$ for Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} ions

Cofactor	Mg^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}
log β (Me^{2+}-L-malate)[28]	1.71	2.24	2.82	3.17
K_m (L-malate), mM	1.8 ± 0.4	1.1 ± 0.3	0.17 ± 0.02	0.72 ± 0.25
$S_{0.5}$ (Me^{2+}), mM	0.11 ± 0.03	0.67 ± 0.37	0.03 ± 0.01	0.010 ± 0.006	0.006 ± 0.001
n	1.6 ± 0.4	1.2 ± 0.5	7.5 ± 1.3	2.8 ± 0.3	1.3 ± 0.1

DISCUSSION

The results shown in this paper demonstrate that in young tobacco leaves only one form of NADP-ME is present. It follows from our data: (i) using native gel electrophoresis, only one band detecting the enzyme activity was found (Fig. 2); (ii) likewise, using native focusation, only one band detecting the enzyme activity was observed (Fig. 2); (iii) symmetric peaks of NADP-ME activity were evident after all chromatographic separations, especially after DEAE-cellulose chromatography, when the enzyme is eluted by a very mild gradient of ionic strength; (iv) localization of NADP-ME activity in chloroplasts (Fig. 1). Even though these data strongly suggest that the NADP-ME in green tobacco leaves is present as one isoform, other isoforms localized in other parts of tobacco plant or taking part in the metabolism of other developmental stages of the plant cannot be excluded. Indeed, in other C$_3$ plants, *Arabidopsis thaliana* and *Oryza sativa*, the genes for four NADP-MEs were found[4,5]. However, in these plants the proteins of individual isoforms were not investigated.

Figure 1C demonstrates that most enzyme activity is localized in chloroplasts and the contribution of possible cytosolic isoform is negligible. NADP-ME was detected via enzyme activity (see Experimental), not immunochemically, which could interfere with heat shock protein 70 [17].

A higher activity of NADP-ME was found in cells surrounding the veins (Fig. 1B) although NADP-ME was detected also in other mesophyll cells (Fig. 1A). Hibberd and Quick reported similar results, decarboxylation activity of NADP-ME in stems and petioles of tobacco and celery plants can cover a part of CO_2 supply required for photosynthesis under higher energy needs[18].

In order to characterize the properties of non-photosynthetic C_3 NADP-ME, we isolated the enzyme from leaves of tobacco. The isolated enzyme with the specific activity 0.95 µmol min^{-1} mg^{-1} was obtained during the isolation procedure. The activity detected for the isolated enzyme is much lower than that of the enzyme isolated from maize leaves (30.9 µmol min^{-1} mg^{-1}), but it is comparable with the activity of the enzyme isolated from etiolated maize leaves (leaves developed without chlorophyll by being deprived of light) (1.4 µmol min^{-1} mg^{-1}), maize roots (1.4 µmol min^{-1} mg^{-1}), and wheat (0.98 µmol min^{-1} mg^{-1})[2,19,20]. The specific activity of NADP-ME from tobacco leaves is comparable with the enzyme activities of other C_3 plants and with those of non-photosynthesizing parts of C_4 plants. Moreover, the values of pH optimum of NADP-ME from tobacco leaves correspond to pH optimum of the NADP-ME of other C_3 plants such as wheat[20]. In contrast, the pH optimum of photosynthetic form (C_4) of this enzyme lies in the alkaline region, at pH 8.0 [2].

There are different values of the isoelectric point (pI) of NADP-ME from green leaves and from non-photosynthetic parts of maize[2]. The pI value of NADP-ME from tobacco leaves, 5.5 (see Fig. 2C), is analogous to that of NADP-ME from maize roots and etiolated leaves[9].

Malic enzymes isolated from various sources require Mg^{2+} or Mn^{2+} ions for their activity. Generally, NADP-MEs from plants are known to prefer Mg^{2+} to Mn^{2+} ions[2,21,22], but the activity of the enzyme from tobacco leaves was stimulated more efficiently (by 140%) in the presence of Mn^{2+} compared with Mg^{2+} ions (Table II). This effect was observed also for malic enzymes of animal origin[23,24]. We have also tested other ions for their ability to substitute Mg^{2+} and Mn^{2+} and proved that Co^{2+}, Ni^{2+} and Zn^{2+} could act as additional cofactors of tobacco NADP-ME. However, they were less effective than Mg^{2+} and Mn^{2+} ions. Co^{2+} was found also by other authors to stimulate NADP-ME from maize leaves[22].

Another target of our study was the investigation of the affinity of NADP-ME to various substrates (L-malate, NADP^{+}) in the presence of divalent metal ions. Divalent metal ions form complexes with organic acid anions such as L-malate, oxaloacetate, pyruvate and coenzyme NADP(H), which are all components of the reaction mixture for measurement of NADP-ME activity. Therefore, it is very complicated to consider the incidence of all these factors because of equilibrium between all complexes, which is influenced not only by association constants but also by the actual concentrations of substrates and products in the course of the reaction. A further problem is to determine the accurate values of these constants. Even though the values of the constants were partly estimated by more authors about 30 years ago[25–27], some of the constants are not available. This is a reason why we used in our calculations analytical concentrations of metal ions and other compounds.

For comparison, we calculated values of Michaelis constants in the presence of Mg^{2+} using association constants Mg–malate, which are available not only for Mg^{2+}, Mn^{2+}, but also for Co^{2+} and Ni^{2+} ions[28]. The values are summarized in Table IV.

The values of apparent Michaelis constants for L-malate and NADP^{+} of enzymes from C$_4$, CAM and C$_3$ plants in the presence of Mg^{2+} were reviewed by Drincovich et al.[2]. The value of Michaelis constant of NADP-ME for L-malate is lower for the enzyme of C$_4$ plants (i.e. 0.08–0.46 mM) than for that of wheat (0.96 mM)[2,29]. We found higher values of this constant for NADP-ME from tobacco leaves (see Table III). Nevertheless, we detected that in the presence of Mn^{2+} and predominately Co^{2+} the K_m value of NADP-ME for L-malate is lower and thus comparable with those reported for other plants[2]. In C$_4$ plants, inhibition of NADP-ME by excess of L-malate was described[2]. No such inhibition was observed in our results (Fig. 5A); this is in agreement with other C$_3$ plants[2]. The K_m value of NADP-ME for NADP^{+} in C$_4$ plants is 3.15–12 μM and that of wheat (37 μM) is the same as that of NADP-ME from tobacco leaves (see Table III). The affinity of tobacco NADP-ME to NADP^{+} was not affected by the presence of other metal ions. Variations in the affinity of plant NAD-ME to L-malate and also to NAD^{+} in the presence of Mg^{2+}, Mn^{2+} and Co^{2+} were found[3].

As demonstrated in this work, more than one binding site (probably two binding sites) for Mg^{2+} ions might be situated in one subunit of NADP-ME from tobacco leaves with different affinity to Mg^{2+}. This is in agreement with properties of malic enzyme from maize[2] and sugar-cane[29]. Moreover, we found the sigmoidal kinetics for the reaction catalyzed with NADP-ME from tobacco in the presence of variable concentrations of Mn^{2+}, with pro-

nounced positive cooperativity. The value of the Hill coefficient was 7.5, which might indicate the presence of at least two binding sites also for Mn^{2+} in one subunit of the enzyme. The binding of Co^{2+} and Ni^{2+} showed also positive cooperativity, although the value of the Hill coefficient was lower.

Our results showed that the regulation of NADP-ME from tobacco is affected not only by the substrate accessibility, but also by the presence of different concentrations of divalent metal ions such as Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}. They differ in some properties; of those the ionic radius is probably the most important. Only ions with the radius 72–80 pm could bind to the NADP-ME molecule, which becomes able to catalyze enzyme reactions. Larger ions (i.e. Ca^{2+} and Cu^{2+}) are not able to play this role. The different character of Mg^{2+} and other ions, such as Mn^{2+}, Co^{2+}, Ni^{2+}, can be explained by their different ability to deform electron cloud and their different polarization potentials. Mg^{2+} ion is a "hard" Lewis acid, while Mn^{2+}, Co^{2+} and Ni^{2+} are on the borderline[30].

The physiological role of these metal ions in regulation of NADP-ME is unclear. One of the reasons is that the exact concentration of those ions is not known in relevant cell compartments. Nevertheless, we suggest that in spite of its low content present in cells, Mn^{2+} may affect the activity of NADP-ME by smaller changes in its concentration even more efficiently than Mg^{2+} ions. This suggestion is supported by results found in our previous work[10]. We have found an increase in the NADP-ME activity in tobacco plants infected by *Potato virus* Y. Although the virus presence was not proved inside the chloroplasts, the reduction of pigment content and the signs of plastid degradation were found[31]. An increase in Mn^{2+} concentration, which might result from decomposition of the pigment–protein complexes in chloroplasts, could be responsible for the stimulation of the enzymes obtained in these experiments.

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