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The regulation and catalytic mechanism of the NADP-malic enzyme from tobacco leaves

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Abstract: The non-photosynthetic NADP-malic enzyme EC 1.1.1.40 (NADP-ME), which catalyzes the oxidative decarboxylation of L-malate and $NADP^+$ to produce pyruvate and NADPH, respectively, and which could be involved in plant defense responses, was isolated from *Nicotiana tabacum* L. leaves. The mechanism of the enzyme reaction was studied by the initial rate method and was found to be an ordered sequential one. Regulation possibilities of purified cytosolic NADP-ME by cell metabolites were tested. Intermediates of the citric acid cycle (α-ketoglutarate, succinate, fumarate), metabolites of glycolysis (pyruvate, phosphoenolpyruvate, glucose-6-phosphate), compounds connected with lipogenesis (coenzyme A, acetyl-CoA, palmitoyl-CoA) and some amino acids (glutamate, glutamine, aspartate) did not significantly affect the NADP-ME activity from tobacco leaves. In contrast, macroergic compounds (GTP, ATP and ADP) were strong inhibitors of NADP-ME; the type of inhibition and the inhibition constants were determined in the presence of the most effective cofactors (Mn^{2+} or Mg^{2+}), required by NADP-ME. Predominantly non-competitive type of inhibitions of NADP-ME with respect to $NADP^+$ and mixed type to L-malate were found.

Keywords: NADP-malic enzyme; macroergic compounds; *Nicotiana tabacum* L.; kinetic mechanism; inhibition.

INTRODUCTION

The NADP-malic enzyme (L-malate: $NADP^+$ oxidoreductase (decarboxylating)), EC 1.1.1.40, NADP-ME) catalyzes the oxidative decarboxylation of L-malate using $NADP^+$ as a coenzyme in the presence of divalent metal ions to produce pyruvate, NADPH and CO_2.[1,2] The presence of a cofactor and the coenzyme is required for the reaction. The most effective cations are $Mg^{2+}$and Mn^{2+}.[3] NADP-MEs have been found in prokaryotic and eukaryotic micro-organisms,

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plants (C_3, C_4 and CAM), animals and humans. Their amino acid sequences are highly conserved among various living organisms, suggesting that NADP-MEs may have an important biological function.[4]

The best-known role of plant NADP-ME is the photosynthetic one; L-malate is decarboxylated and the released CO_2 is fixed into the Calvin Cycle *via* ribulose-1,5-bisphosphate carboxylase/oxygenase. This process occurs in the chloroplasts of bundle sheath cells of some C_4 plants or during the day in the cytosol of CAM plants.[1] The function of the non-photosynthetic NADP-ME isoform, present in C_3 plants and in non-photosynthetic tissues of C_4 plants, is not fully explained. It is assumed that its main role is to supply reduced equivalents (NADPH) for synthetic metabolic pathways, such as the synthesis of fatty acids. NADP-ME together with phosphoenolpyruvate carboxylase serves to maintain the intracellular pH.[1] NADP-ME also participates in the mechanism of stomatal closure and can affect the water economy of a plant.[5] Moreover, NADP-ME function seems to be associated with the metabolic response of plants to stress.[6–17] In animals, the cytosolic isoform (c-NADP-ME) is involved in the generation of NADPH for the biosynthesis of fatty acids and steroids in the liver and adipose tissues. Cytosolic NADP-ME may also have a role in microsomal drug detoxification.[18]

The structure and reaction mechanism of animal malic enzyme has been best studied because the crystal structures of pigeon and rat cytosolic NADP-ME are known.[4,18] The structure of plant and animal NADP-MEs is predominantly tetrameric, with a relative molecular mass of one subunit ranging from 62000 to 67000 Da.[2,4,17,19]

In plants, NADP-MEs are encoded by a small gene family, the expression of which is tissue and ontogenetic stage specific. The best-studied family of malic enzymes is from *Arabidopsis thaliana* (C_3 dicot plant) and rice (C_3 monocot plant), for which the complete genome sequence is known.[12,20] Two recombinant isoforms of *Nicotiana tabacum* L. were characterized (chloroplastic Nt-NADP--ME1 and cytosolic Nt-NADP-ME2) and the transcript of a third putative NADP--ME has also been identified.[17]

The photosynthetic isoform (NADP-ME from maize leaves) is up-regulated by light,[21] or by pH (NADP-ME from sugar cane leaves), which affects the oligomerization state of the enzyme.[1] The enzyme can readily undergo changes between monomer, dimer and tetramer. All three forms of the enzyme possess enzyme activity but the highest specific activity occurs at pH 8 for the tetramer form.[1] Finally, regulation of maize NADP-ME occurs by various compounds: either *via* inhibition by an excess of the substrate (L-malate), or by other effectors. Several organic acids have been found to inhibit the C_4 NADP-ME; the strongest inhibition was observed in the presence of oxaloacetate and α-ketoglutarate.[1] Recombinant non-photosynthetic isoforms of NADP-ME from *Arabidopsis thaliana*

and *Nicotiana tabacum* L. were found to be differently regulated by various intermediates of the citric acid cycle (oxaloacetate, fumarate, and succinate) and ATP.[17,22]

Previously, NADP-ME from tobacco leaves was characterized by kinetic constants and the effect of divalent metal ions on the enzyme activity.[23]

The objective of the present communication was the study of the kinetic mechanism of NADP-ME reaction, which has not been described for C_3 plant enzyme and detailed inhibition studies including the determination of inhibition constants and type of inhibition for important regulators.

EXPERIMENTAL

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) were grown in a greenhouse under 22/18 °C day/night temperatures. The seeds were sown in pots with sand and the plantlets were transferred to soil after 3 weeks. The leaves of seven-week old plants were collected, frozen immediately in liquid N_2 and stored at –80 °C.

Enzyme purification

A modified procedure described by Ryšlavá *et al*.[23] was used for the purification of NADP--ME enzyme from tobacco leaves. In contrast to the previously used method, the homogenization buffer contained in addition 330 mM sorbitol, which ensures that the chloroplasts remained intact and were removed by centrifugation. Chromatography on DEAE-cellulose, sephacryl S-300 and finally on a 2',5'-ADP-sepharose 4B column yielded a purified enzyme preparation with specific activity 0.95 μmol min^{-1} mg^{-1}. The purified NADP-ME was stored at 4 °C for further studies.

Enzyme activity assays

The NADP-ME activity was determined spectrophotometrically (Hellios α, Thermo Spectronic) at 21 °C by monitoring the NADPH production at 340 nm, as previously described by Ryšlavá *et al*.[9]

Kinetic studies

The initial-rate study of the kinetic mechanism of NADP-ME was performed by varying the concentrations of free $NADP^+$ (0.025–0.166 mmol/l) and varying the concentration of free L-malate (0.879–14.633 mmol/l). The association constants for Me^{2+}–$NADP^+$ and Me^{2+}–L-malate complexes described by Grover *et al*. were used.[24] The concentrations of the other compounds were constant (4 mmol/l $MgCl_2$, 80 mmol/l MOPS–20 mmol/l sodium acetate–NaOH buffer (pH 7.4)). The experimental data were fitted using the equations characterizing a two--substrate mechanism. The sequential initial-rate pattern (Eq. (1)) was found to be the most suitable. Differentiation between ordered sequential and random sequential mechanisms was achieved by scrutinizing the constants K' and constants V'_{lim} (calculated from Eqs. (2)–(4)) *vs*. the $NADP^+$ concentration and *vs*. the L-malate concentration, respectively.

$$v = \frac{V_{lim}[A][B]}{[B]K_{MA} + [A]K_{MB} + [A][B] + K_A K_{MB}} \quad (1)$$

$$K' = \frac{K_A K_{MB}}{[A]} + K_{MB} \quad (2)$$

$$K' = \frac{K_A K_{MB}}{K_{MB} + [B]} \quad (3)$$

$$V'_{lim} = \frac{V_{lim}[B]}{K_{MB} + [B]} \quad (4)$$

where v is the initial reaction rate, V_{lim} is the apparent maximal reaction rate, [A] and [B] are the substrate concentrations; $K_{MA(B)}$ is the Michaelis constant for the particular substrate, K_A the dissociation constant for the complex enzyme–substrate A, B, K' the apparent Michaelis constant.[25]

Effect of various compounds on the NADP-ME reaction rate

The NADP-ME assay mixture contained 80 mmol/l MOPS–20 mmol/l sodium acetate-NaOH buffer (pH 7.4), 16 mmol/l L-malate, 4 mmol/l $MgCl_2$ and 0.2 mmol/l $NADP^+$ in a total volume of 1 cm^3. Alternatively, the reaction mixture contained a subsaturation concentration of some substrate: 2 mmol/l L-malate and 0.05 mmol/l $NADP^+$, respectively. The reaction was started by addition of the enzyme. The tested concentration of the potential modulators (GTP, ATP, ADP, puruvate, α-ketoglutarate, succinate, fumarate, glutamate, glutamine, aspartate, phosphoenolpyruvate, 3-phosphoglycerate and glucose-6-phosphate) in the reaction mixture was 2 or 5 mmol/l. In the case of coenzyme A, acetyl-coenzyme A and palmitoyl-coenzyme A, their concentration in the reaction mixture was 0.01 or 0.1 mmol/l. The reaction rate of NADP-ME without additions of potential regulators was taken as 100 %.

Inhibition studies

The inhibition constants and type of inhibition for GTP, ATP and ADP were established with 3 concentrations of inhibitor towards 5 various concentrations of L-malate (free concentrations result from particular graphs in Figs. 2–4A and 4B) or 5 various concentrations of $NADP^+$ (free concentrations result from particular graphs in Figs. 2–4C and 4D) and in the presence of Mg^{2+} (Figs. 2–4A and 4C) or Mn^{2+} (Figs. 2–4B and 4D). The NADP-ME assay mixture for the inhibition studies together with varying substrates and inhibitors contained 80 mmol/l MOPS, 20 mmol/l sodium acetate–NaOH buffer (pH 7.4), 2.0 mmol/l $MgCl_2$ or 0.10 mmol/l $MnCl_2$ in a total volume of 1 cm^3. The reaction was started by addition of the enzyme (50 μl). The concentrations of the free inhibitors ATP, ADP and GTP are listed in the Figure legends (Figs. 2, 3 and 4, respectively). The free ATP (GTP, ADP) was calculated using the association constants of the complexes ATP–Mg^{2+} (log β = 4.29), ATP–Mn^{2+} (log β = = 5.01), GTP–Mg^{2+} (log β = 4.31), GTP–Mn^{2+} (log β = 5.36), ADP–Mg^{2+} (log β = 3.36) and ADP–Mn^{2+} (log β = 4.22).[26-28] In the presence of ATP (GTP and ADP), the chelations by the substrate or cofactors are negligible.[29] The inhibition constants were obtained by fitting the experimental data to Eq. (5) (equation characterizing non-competitive inhibition), Eq. (6) (equation characterizing competitive inhibition) and Eq. (7) (equation characterizing mixed inhibition):[30]

$$v = \frac{V_{lim}[A]}{(K_M + [A])\left(1 + \frac{[I]}{K_i}\right)} \quad (5)$$

$$v = \frac{V_{lim}[A]}{K_M\left(1 + \frac{[I]}{K_{ic}}\right) + [A]} \quad (6)$$

$$v = \frac{V_{\text{lim}}[\text{A}]}{K_{\text{M}}\left(1+\frac{[\text{I}]}{K_{\text{ic}}}\right)+\left(1+\frac{[\text{I}]}{K_{\text{iu}}}\right)[\text{A}]} \quad (7)$$

where V_{lim} is the apparent maximal reaction rate, [A] the substrate concentration; [I] the inhibitor concentration; K_{M} the Michaelis constant for the substrate, K_{ic} and K_{iu} the inhibition constants for the inhibitor (free ATP, ADP or free GTP) derived from the slope and intercept, respectively in a Lineweaver-Burk plot. The data were processed by non-linear regression with the MS Excel program.

RESULTS

Mechanism of reaction catalyzed by NADP-ME

The kinetic mechanism of the reaction catalyzed by the isoform of NADP-ME present in the cytosol of the leaves of *Nicotiana tabacum* L., cv. Petit Havana, SR1 was analyzed by initial rate studies with five various concentrations of $NADP^+$ at five fixed concentrations of L-malate as sequential (Figs. 1A and 1B). This type

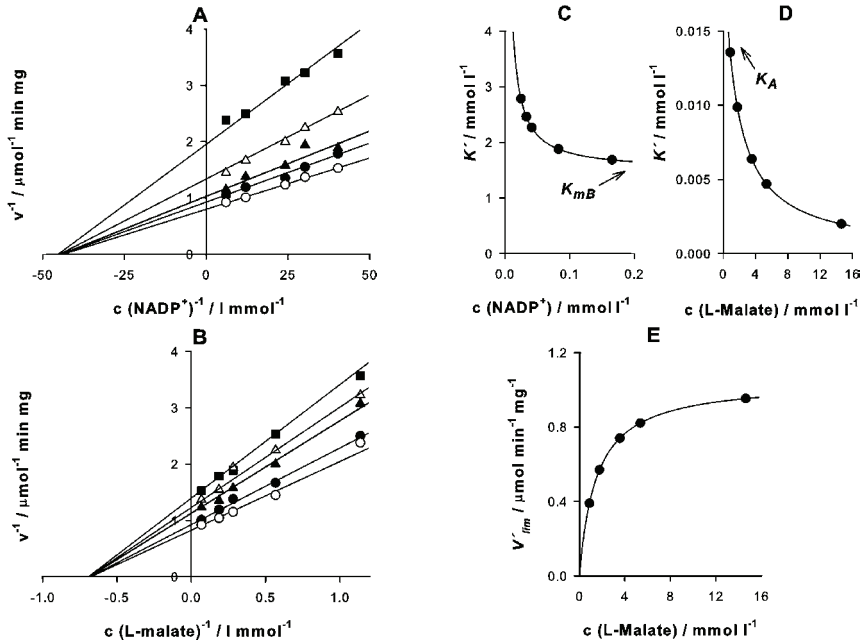

Fig. 1. Double reciprocal plots of the dependence of the reaction rate on the concentration of $NADP^+$ (A) and L-malate (B) fitted to Eq. (1) and the dependences of the apparent Michaelis constants (K') and constants V'_{lim} calculated from Eqs. (2), (3) and (4), respectively, on the concentration of $NADP^+$ (C) and L-malate (D and E) specified sequential mechanism as ordered. Plot C also indicated the Michaelis constant for substrate B (K_{mB}) and plot D dissociation constant for complex enzyme–substrate A, B (K_{A}) (both labeled with arrows). Concentrations of the free (chelation-corrected) $NADP^+$: 0.025–0.166 mmol/l; concentration of free L-malate: 0.879–14.633 mmol/l.

of two-substrate reaction is characterized by all lines intercepting in one point at (or above) the *x*-axis in a double reciprocal plot (Figs. 1A and 1B). The ping-pong mechanism, characterized by a set of parallel lines in a double reciprocal plot, was excluded. The dependence of the apparent Michaelis constants (*K*') and V'_{lim}, calculated from Eqs. (2), (3) and (4), respectively, on the concentration of the fixed substrate, was used for an additional specification of the sequential mechanism, which was determined as ordered (Figs. 1C–1E). Fig. 1E (the dependence of the concentration of the on V'_{lim}) indicates that the second substrate binding to the enzyme is L-malate. From determinations of *K*' at different concentrations of substrate, it is possible to obtain estimations of K_{MB} and K_A (Figs. 1C and 1D).

Inhibition of NADP-ME by macroergic compounds

NADP-ME from tobacco leaves was inhibited by ATP, ADP and GTP. ATP and GTP were stronger inhibitors than ADP (Table I). Detailed inhibition studies were performed with ATP, ADP and GTP as inhibitors with respect to L-malate and $NADP^+$ in the presence of Mg^{2+} or Mn^{2+} as NADP-ME cofactors (Figs. 2–4). The obtained results based on various diagnostic plots of experimental sets of data (double reciprocal plot (Figs. 2–4), the Dixon plot, the Hanes-Woolf plot and the Woolf-Augustinsson-Hoffstee plot (data not shown)) showed that the ATP (GTP, ADP) concentration had to be corrected for Me^{2+}–ATP (Me^{2+}–GTP,

TABLE I. Inhibition constants (mmol l^{-1}) and types of inhibition of NADP-ME from tobacco leaves by ATP, ADP or GTP with respect to $NADP^+$ or L-malate in the presence of Mg^{2+} or Mn^{2+} as cofactors. Standard deviations from 3 independent measurements are shown. K_{ic} and K_{iu} inhibition constants for the inhibitor: free ATP, ADP or free GTP, derived from slope and intercept, respectively, of Lineweaver-Burk plots, calculated from Eqs. (5)–(7)

Inhibitor with respect to	Constant	Mg^{2+}	Mn^{2+}
		Mixed	
ATP/L-malate	K_{ic}	0.19±0.06	0.053±0.013
	K_{iu}	0.94±0.13	0.39±0.06
ATP/$NADP^+$		Non-competitive	
	K_i	0.54±0.20	0.40±0.13
ADP/L-malate		Competitive	Mixed
	K_{ic}	0.68±0.15	0.45±0.02
	K_{iu}	–	2.76±0.64
ADP/$NADP^+$		Non-competitive	
	K_i	5.08±0.43	3.58±0.39
GTP/L-malate		Mixed	
	K_{ic}	0.23±0.08	0.047±0.025
	K_{iu}	1.65±0.32	0.35±0.10
GTP/$NADP^+$		Non-competitive	
	K_i	0.70±0.20	0.23±0.03

Me^{2+}–ADP) complex, because only free ATP (GTP, ADP) was assumed to be the inhibitory species. These results are also in agreement with those of Hsu *et al.*[29]

ATP, ADP and GTP are non-competitive inhibitors with respect to $NADP^+$

The dependences of the enzyme reaction rate on the $NADP^+$ concentration were measured in the presence of Mg^{2+} or Mn^{2+} and three concentrations of inhibitors (ATP, GTP or ADP). The lines in the double reciprocal plots of these dependences in all cases intercepted the *x*-axis (Figs. 2C and 2D, 3C and 3D and 4C and 4D), indicating a non-competitive type of inhibition.

This means that macroergic inhibitors (ATP, GTP or ADP) are bound to another site than the binding site for the coenzyme $NADP^+$. The relevant inhibi-

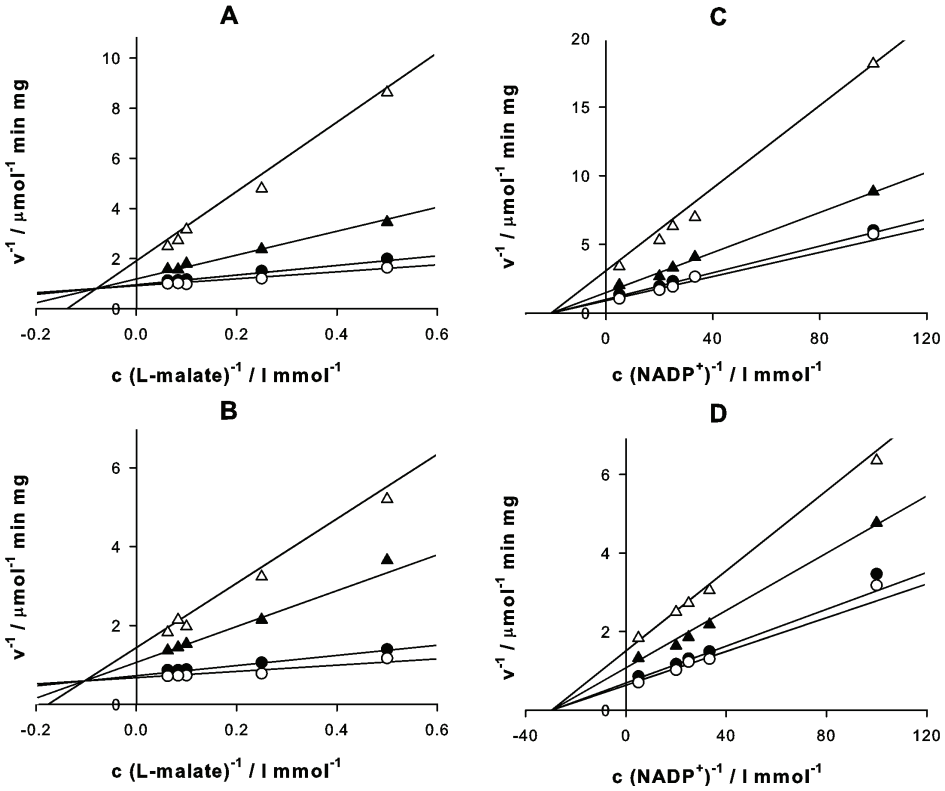

Fig. 2. Mixed and non-competitive type of inhibition of NADP-ME from tobacco leaves by ATP with respect to L-malate and $NADP^+$ in the presence of Mg^{2+} (A and C) or Mn^{2+} (B and D). The NADP-ME activity was measured at different concentrations of L-malate (A and B) or $NADP^+$ (C and D) at various concentrations of free ATP (from top to bottom, the chelation-corrected free ATP concentrations were 1.090, 0.296, 0.047 and 0 mmol/l in A and C, and 0.402, 0.200, 0.027 and 0 mmol/l in B and D. The experimental data were fitted to Eqs. (5) and (7).

tion constants are summarized in Table I. $K_{i,ADP(free)}$ in the presence of Mg^{2+} is approximately 10-times higher than $K_{i,ATP(free)}$ and $K_{i,GTP(free)}$, and in the presence of Mn^{2+}, the value of $K_{i,ADP\ (free)}$ was the highest (7-times higher than $K_{i,ATP\ (free)}$ and 12-times higher than $K_{i,GTP(free)}$).

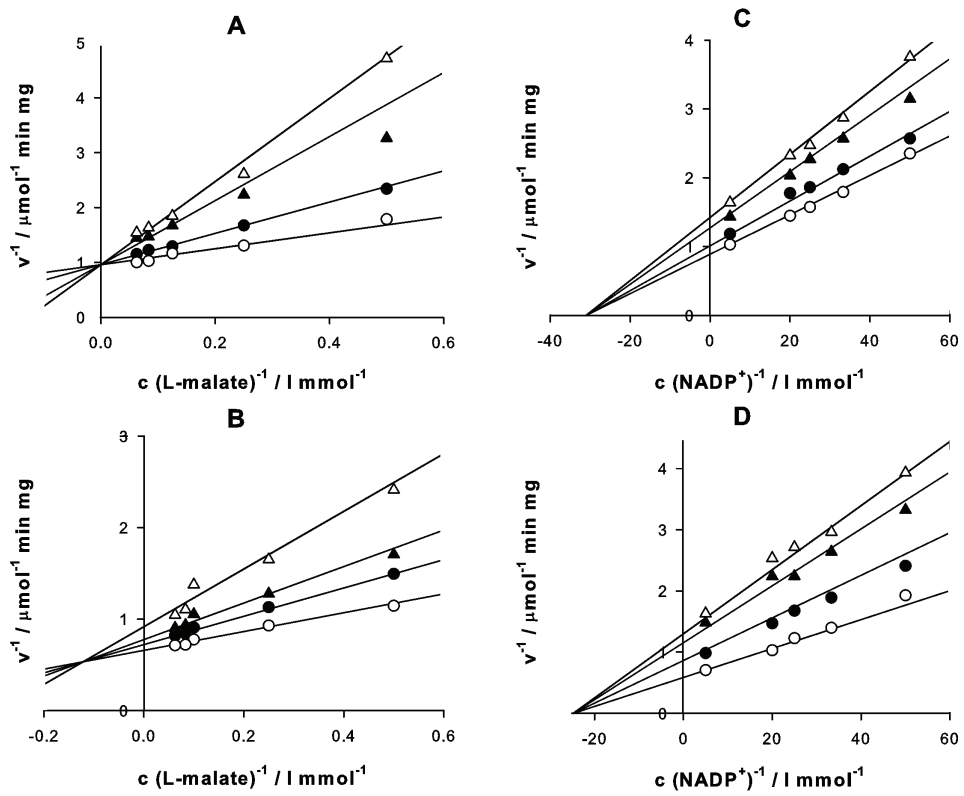

Fig. 3. Competitive, mixed and non-competitive type of inhibition of NADP-ME from tobacco leaves by ADP with respect to L-malate and $NADP^+$ in the presence of Mg^{2+} (A and C) or Mn^{2+} (B and D). The NADP-ME activity was measured at different concentrations of L-malate (A and B) or $NADP^+$ (C and D) at various concentrations of free ADP (from top to bottom, the chelation-corrected free ADP concentrations were 3.238, 2.317, 0.741 and 0 mmol/l in A and C, 1.903, 0.906, 0.510 and 0 in B and 4.901, 3.902, 1.903 and 0 mmol/l in D. The experimental data were fitted to Eqs. (5)–(7).

ATP, ADP and GTP are predominantly mixed inhibitors with respect to L-malate

Detailed kinetic studies of NADP-ME inhibition by ATP (GTP) with respect to L-malate in the presence of Mg^{2+} or Mn^{2+} showed a mixed type of inhibition. In double reciprocal plot, all lines met at a joint intercept left of the ordinate, *i.e.*, they differed in slope and ordinate intercept, because this type of inhibition influences both the apparent Michaelis constant and the maximum rate, Figs. 2A

and 2B; 3A and 3B; 4Aand 4B. The two constants, K_{ic} and K_{iu}, characterizing this type of inhibition are summarized in Table I for both inhibitors (ATP and GTP) and cofactors (Mg^{2+} and Mn^{2+}). ADP with respect to L-malate was a milder inhibitor than ATP. Inhibition of NADP-ME by ADP toward L-malate was evaluated as competitive in the presence of $Mg^{2+}$and mixed in the presence of Mn^{2+}, analogous to the inhibitors ATP and GTP.

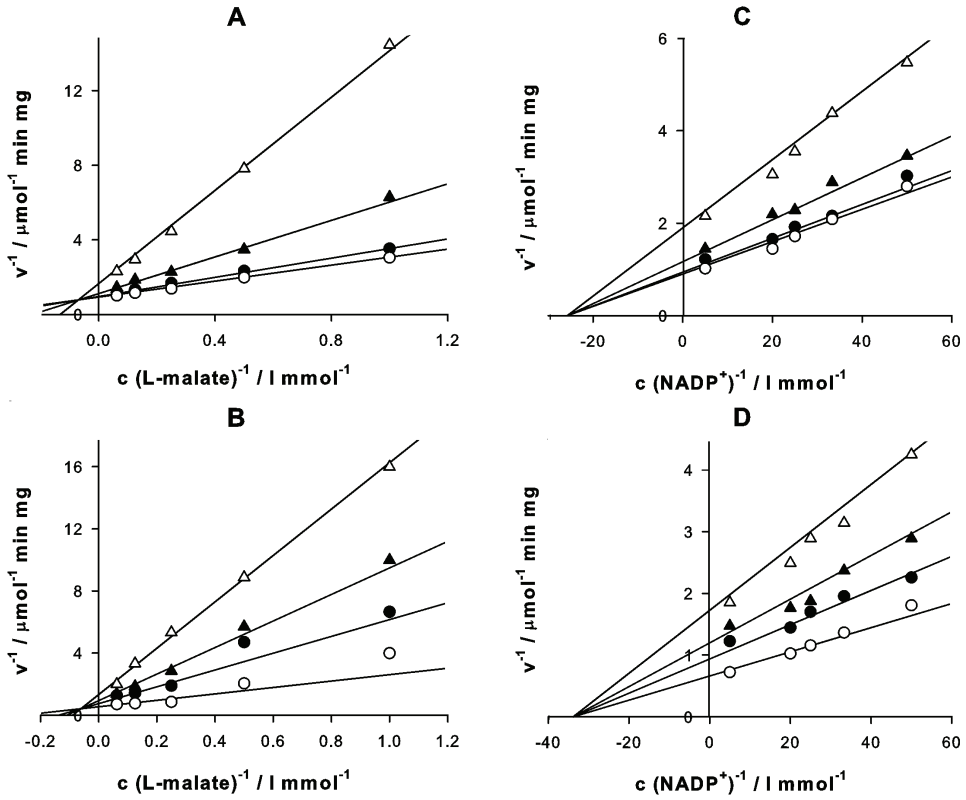

Fig. 4. Mixed and non-competitive type of inhibition of NADP-ME from tobacco leaves by GTP with respect to L-malate and $NADP^+$ in the presence of Mg^{2+} (A and C) or Mn^{2+} (B and D). The NADP-ME activity was measured at different concentrations of L-malate (A and B) or $NADP^+$ (from top to bottom, the chelation-corrected free GTP concentrations were 1.086, 0.289, 0.049 and 0 mmol/l in A and C, and 0.401, 0.202, 0.104 and 0 mmol/l in B and D. The experimental data were fitted to Eqs. (5) and (7).

Effect of other compounds on the NADP-ME reaction rate

Five groups of compounds important in metabolism were tested as inhibitors or modulators of NADP-ME activity: macroergic compounds (ATP, ADP, and GTP), intermediates of the citric acid cycle (α-ketoglutarate, succinate and fumarate), metabolites of glycolysis (glucose-6-phosphate, 3-phosphoglycerate, phos-

phoenolpyruvate and pyruvate), compounds related to lipogenesis (coenzyme A, acetyl-CoA, palmitoyl-CoA) and some amino acids (glutamate, glutamine, aspartate). The influences of these compounds were tested as possible modulators with respect to L-malate and to $NADP^+$ not only at saturation concentrations, but also at subsaturation concentrations to indicate *e.g.* competition between the inhibitor and the NADP-ME substrate. Only the macroergic compounds (ATP, ADP and GTP) significantly affected (inhibited) the reaction rate of NADP-ME. The other compounds, with exception of a slight inhibition effect of intermediates of glycolysis, did not influence the reaction rate of NADP-ME within the frame of standard deviations (Table II).

TABLE II. Effect of different compounds on the reaction rate of NADP-ME from tobacco leaves. The activity of the enzyme is expressed in percentage, control experiment without inhibitors or modulators were taken as 100 %. Standard deviations from 3 independent measurements are shown

Compounds		Composition of the reaction mixture		
		16 mmol/l L-malate 0.2 mmol/l $NADP^+$ 4 mmol/l $MgCl_2$	2 mmol/l L-malate 0.2 mmol/l $NADP^+$ 4 mmol/l $MgCl_2$	16 mmol/l L-malate 0.05 mmol/l $NADP^+$ 4 mmol/l $MgCl_2$
Macroergic compounds[a]	GTP	59±2	50±2	62±2
	ATP	54±4	46±8	51±2
	ADP	71±8	70±5	78±4
Intermediates of glycolysis[a]	Glucose-6-phosphate	98±4	97±6	100±3
	3-Phosphoglycerate	92±5	84±9	90±5
	Phosphoenolpyruvate	94±6	100±17	85±5
	Pyruvate	97±4	91±2	98±3
Metabolites of citric acid cycle[a]	α-Ketoglutarate	97±2	91±6	101±1
	Succinate	100±4	109±14	104±6
	Fumarate	102±4	103±9	100±4
Compounds related to lipogenesis[b]	Coenzyme A	103±10	103±4	96±5
	Acetyl-CoA	106±4	99±6	97±3
	Palmitoyl-CoA	101±2	97±18	95±5
Amino acids[a]	Glutamate	105±10	104±4	107±17
	Glutamine	102±3	102±6	99±4
	Aspartate	100±2	102±5	101±4

Concentration of tested compounds: [a]5.0 mmol/l; [b]0.10 mmol/l

DISCUSSION

In this study, the mechanism of the reaction catalyzed by the non-photosynthetic NADP-ME from tobacco leaves and its regulation possibilities were investigated.

Although the enzyme is present in tobacco both in the chloroplasts and the cytosol, the enzyme used in this study was most probably the cytosolic isoform, because the chloroplasts were removed during the enzyme isolation (see Experimental, *Enzyme purification*).

It was previously found that the reaction mechanism of cytosolic NADP-MEs is ordered sequential for NADP-ME from pigeon liver and from human breast cancer cell lines[31,32] but random sequential for mitochondrial (NAD(P)-ME from *Ascaris suum* and from hepatoma tumor cells).[33,34] The reaction mechanism catalyzed by NADP-ME from tobacco leaves was found to be ordered sequential (Fig. 1). This means that the enzyme binds only one substrate first (substrate A), followed by the binding of substrate B, to form a ternary enzyme–substrate A–substrate B complex.[25] With respect to Fig. 1E, which can be used as an indicator of substrate-binding order (a fixed dependence of V'_{lim} on a substrate concentration is associated with the second substrate to bind the enzyme)[25] and from results shown in Figs. 1C and 1D, it is supposed that $NADP^+$ is the leading substrate, followed by L-malate. This mechanism is in agreement with the results published for animal cytosolic NADP-ME.[31,32] The kinetic mechanism for plant NADP-ME was studied only for photosynthetic chloroplastic NADP-ME from maize leaves and an ordered sequential mechanism was also determined.[35]

In the present study, the regulation of cytosolic NADP-ME from tobacco leaves by cell metabolites, the most important of which were ATP, GTP and ADP (Table II), was also investigated. ATP was previously found to be an inhibitor for all NADP-ME isoenzymes of *Arabidopsis thaliana* and both chloroplastic and cytosolic ones of *Nicotiana tabacum* L. plants.[17,22] The inhibition was characterized in detail by the type of inhibition and the inhibition constants (Figs. 2––4, Table I). A non-competitive inhibition of NADP-ME by ATP, GTP and the ADP with respect to $NADP^+$ and a predominantly mixed inhibition by ATP, GTP and ADP with respect to L-malate in the presence of Mg^{2+} or Mn^{2+} were established. The type of inhibition of human m-NAD-ME by ATP toward NAD^+ and L-malate was reported to be competitive in both cases.[29] Free ATP (GTP, ADP, respectively) was assumed to be the inhibiting species of both tobacco NADP-ME (by evaluating the various diagnostic plots) and of human m-NAD-ME (from structural studies).[29] Divalent metal ions (cofactors) significantly influenced the inhibition constants. The values of $K_{i,ATP(free)}$, $K_{i,GTP(free)}$ and $K_{i,ADP(free)}$ for non-competitive inhibition by ATP (GTP, ADP) toward $NADP^+$ were lower in the presence of Mn^{2+} than in the presence of Mg^{2+}; this means that ATP (GTP, ADP) is a stronger inhibitor in the presence of Mn^{2+} as the cofactor. Also, the $K_{ic,ATP(GTP)free}$ and $K_{iu,ATP(GTP)free}$ constants of the mixed type of inhibition by ATP (GTP) to L-malate were lower in the presence of Mn^{2+} than in the presence of Mg^{2+}. The only exception was the inhibition by ADP with respect to L-malate,

which is, however, competitive in the presence of Mg^{2+} and mixed in the presence of Mn^{2+}.

The structural derivate of $NADP^+$, β-nicotinamide mononucleotide had no effect on the activity of tobacco NADP-ME (data not shown), suggesting that the adenosine diphosphate part of the molecule is important for $NADP^+$ binding.

Regulation of NADP-ME from tobacco leaves by its cofactors was studied previously.[23] Not only Mg^{2+} and Mn^{2+}, but also Co^{2+} and Ni^{2+} were found to be cofactors of NADP-ME. The dependence of the reaction rates on the Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} concentrations does not correspond to Michaelis-Menten kinetics. Two binding sites were determined for Mg^{2+} and binding of Mn^{2+} caused a strong positive cooperation.[23] NADP-ME from tobacco leaves was not regulated *via* inhibition by L-malate, which is a characteristic trait for C_3 plants.[2]

Other metabolites, such as intermediates of glycolysis, intermediates of the citric acid cycle, compounds related to lipogenesis and amino acids, did not affect significantly the activity of cytosolic NADP-ME from tobacco leaves, neither at saturation nor subsaturation concentrations of the substrate or coenzyme (Table II). Mild inhibition was observed with 3-phosphoglycerate (Table II). Müller *et al.*[17] obtained slightly different results, *i.e.*, pyruvate, glucose-6-phosphate, fumarate, succinate and oxaloacetate inhibited recombinant cytosolic tobacco NADP--ME. The reason lies in the different experimental conditions (especially the substrate and coenzyme concentrations, the employed buffer and its concentration, pH). Furthermore, the recombinant protein could be differently folded compared with protein isolated from plant material. Oxaloacetate was not tested in this study because this compound could also be a substrate and could be decarboxylated in a reaction catalyzed by NADP-ME. The regulation of NADP-ME by cell metabolites depended on the particular isoform; chloroplastic NADP-ME was activated by aspartate, while the cytosolic one was not.[17] The regulation of NADP-ME isoenzymes in *Arabidopsis thaliana* was different but all isoenzymes were inhibited by ATP.[22]

NADP-ME from germinating *Ricinus communis* cotyledons was activated by coenzyme A, acetyl-CoA, palmitoyl-CoA and succinate. Therefore, cotyledon NADP-ME was suggested to play a role in the metabolism of fatty acids.[36]

The regulation of NADP-ME by macroergic compounds (ATP, ADP and GTP) (Figs. 2–4) and the slight inhibition by intermediates of glycolysis (Table II) indicate that the enzyme could participate in maintaining the energy balance in the plant. It could be more important under stress than under physiological conditions. Higher activity of NADP-ME caused by viral infection[9] and by abiotic stress factors in plants were found.[6–8,10–17]

In regard to the significance of NADP-ME in plant defense response, and the quite different regulation of NADP-ME in respective organism, cells and cell compartments, it is important to bring new information into this field.

CONCLUSIONS

The kinetic mechanism of reactions catalyzed by cytosolic NADP-ME from tobacco leaves was determined as ordered sequential. This enzyme was inhibited by ATP, GTP and ADP; the type of inhibition was non-competitive toward $NADP^+$ and predominantly mixed toward L-malate.

Other cell metabolites, such as intermediates of the citric acid cycle, amino acids and compounds related to fatty acids metabolism did not significantly affect the activity of cytosolic NADP-ME from tobacco leaves, neither in saturation nor in subsaturation concentrations of L-malate and $NADP^+$.

ABBREVIATIONS

CAM – Crassulacean acids metabolism
C_3 plant – A plant that produces the 3-carbon compound 3-phosphoglyceric acid as the first photosynthetic product
C_4 plant – A plant that produces the 4-carbon compound oxaloacetic acid as the first photosynthetic product
MOPS – 3-Morpholinopropanesulfonic acid
NADP-ME – NADP-dependent malic enzyme EC 1.1.1.40

ИЗВОД

РЕГУЛАЦИЈА И МЕХАНИЗАМ КАТАЛИЗЕ NADP-МАЛАТНОГ ЕНЗИМА ИЗ ЛИСТА ДУВАНА

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Из листа *Nicotiana tabacum* L. је изолован нефотосинтетишући NADP-малатни ензим EC 1.1.1.40 (NADP-ME), који катализује оксидативну декарбоксилацију L-малата и стварање пирувата и NADPH, укључених у одбрамбени одговор биљке. Механизам ензимске реакције је проучаван методом почетне брзине, за коју је нађено да је првог реда. Испитане су могућности регулације пречишћеног цитосолног NADP-ME ћелијским метаболитима. На активност NADP-ME из листа дувана нису значајно утицали интермедијери циклуса лимунске киселине (*α*-кетоглутарат, сукцинат, фумарат), метаболити гликолизе (пируват, фосфоенолпируват, глукоза-6-фосфат), једињења која учествују у липогенези (коензим А, ацетил-CoA, палмитоил-CoA), и неке амино-киселине (глутамат, глутамин, аспартат). Супротно томе, једињења GTP, ATP и ADP су јаки инхибитори NADP-ME; тип и константа инхибиције су одређени у присуству најефикаснијих кофактора NADP-ME (Mn^{2+} и Mg^{2+}). Констатован је претежно некомпетитивни тип инхибиције NADP-ME у односу на $NADP^+$ и мешовити тип у односу на L-малат.

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