Identification of Domains Involved in Tetramerization and Malate Inhibition of Maize C₄-NADP-Malic Enzyme*

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C₄ photosynthetic NADP-malic enzyme (ME) has evolved from non-C₄ isoforms and gained unique kinetic and structural properties during this process. To identify the domains responsible for the structural and kinetic differences between maize C₄ and non-C₄-NADP-ME several chimeras between these isoforms were constructed and analyzed. By using this approach, we found that the region flanked by amino acid residues 102 and 247 is critical for the tetrameric state of C₄-NADP-ME. In this way, the oligomerization strategy of these NADP-ME isoforms differs markedly from the one that present non-plant NADP-ME with known crystal structures. On the other hand, the region from residue 248 to the C-terminal end of the C₄ isoform is involved in the inhibition by high malate concentrations at pH 7.0. The inhibition pattern of the C₄-NADP-ME and some of the chimeras suggested an allosteric site responsible for such behavior. This pH-dependent inhibition could be important for regulation of the C₄ isom in vivo, with the enzyme presenting maximum activity while photosynthesis is in progress.

NAD(P)-malic enzyme (NAD(P)-ME)⁴ (EC 1.1.1.38, 1.1.1.39, and 1.1.1.40) catalyzes the reversible oxidative decarboxylation of L-malate to yield carbon dioxide and pyruvate with the concomitant reduction of NAD(P) (1). The enzyme is found in most living organisms, because the products of the reaction are used as a source of carbon and reductive power in different cell compartments. The amino acid sequences of malic enzymes are highly conserved among many organisms, from bacteria to human. However, malic enzymes do not show recognizable amino acid sequence homology to other proteins such as dehydrogenases, decarboxylases, or other oxidative decarboxylases, comprising a new class of oxidative decarboxylases (2).

In plants, both plastidic and cytosolic NADP-ME (EC 1.1.1.40) playing photosynthetic as well as non-photosynthetic roles have been identified (3). The photosynthetic plastidic isoform, which is expressed in bundle sheath cells of some C₄ plants, represents a unique and specialized form of NADP-ME with particular kinetic, structural, and regulatory properties, and all of these make this enzyme suitable to participate in the CO₂ concentrating mechanism that increases the photosynthetic yield of NADP-ME C₄ plants (3, 4). In contrast, C₄ non-photosynthetic NADP-ME isoforms are expressed in the cytosol (5) and plastids (6–9). Because these non-photosynthetic isoforms represent ancestors of the C₄-specific enzyme they are ideal candidates for studying the evolution process toward the current C₄-NADP-ME.

In maize, the plastidic non-photosynthetic NADP-ME (ZmChlMe2) represents the more recent and direct ancestor of the C₄-NADP-ME (ZmChlMe1, Ref. 9). This enzyme is constitutively expressed in maize and plays housekeeping roles; in contrast to the C₄ isoenzyme (8), 9). By cloning and expressing both the maize plastidic photosynthetic (C₄) and non-photosynthetic NADP-ME (also known as non-C₄-NADP-ME) as functional proteins in Escherichia coli, we identified several structural and kinetic differences between these two proteins (8, 10). Among these structural differences, the most relevant is the oligomeric state: the C₄-NADP-ME assembles as a tetramer and the non-C₄-NADP-ME as a dimer. With regard to the kinetic differences, one of the most outstanding is the inhibition of the C₄-NADP-ME by the substrate malate in a pH-dependent way. The non-C₄-NADP-ME is not inhibited by high concentrations of this substrate at all. This inhibition may be relevant in regulating the C₄ isom in vivo, by allowing the enzyme to increase its activity when photosynthesis is in progress.

The goal of the present work was to identify the segments of the primary structure of the C₄ and non-C₄ isoforms that are responsible for the C₄-specific properties in maize NADP-ME. The high sequence similarity between these two enzymes (85%) allowed us to swap the corresponding segments between the two isoforms, constructing several chimeras. The structural and kinetic properties of the resulting chimerical enzymes were studied identifying segments involved in the tetramerization process and in the inhibition by the substrate malate at low pH.

**EXPERIMENTAL PROCEDURES**

Construction of Chimerical NADP-ME Sequences—Maize C₄ (pET-ME, Ref. 10) and non-C₄-NADP-ME (pPET-RME, Ref. 8) sequences were inserted into the pET32 expression vector (Novagen). The sequences correspond to the mature proteins (without transit peptides) in both cases. The plasmids pET-ME and pET-RME were treated with the corresponding restriction
endonucleases (Fig. 1) and the fragments obtained were purified and recombined to obtain the chimerical NADP-ME sequences into the expression vector pET-32. The plasmids obtained were named as follows: pET-LR-NADP-ME, pET-RL-NADP-ME, pET-RpL-NADP-ME, and pET-LpR-NADP-ME (see Fig. 1). Two more plasmids (pET-LRL-NADP-ME and pET-RLR-NADP-ME) were constructed by changing only the segment between the cleavage sites for the restriction enzymes BamHI and EcoRI (Fig. 1). The inserts of all the chimerical constructs were sequenced to verify correct swapping of the corresponding fragments and that no mistakes were made during subcloning procedures. BL21(DE3) E. coli was then transformed with the pET plasmid containing the chimerical NADP-MEs.

Expression and Purification of Recombinant Chimerical NADP-ME—The pET-chimeric ME expression vectors contained a His tag sequence and the resulting fusion proteins were purified using a His-Bind column (Novagen). Induction and purification of the fusion proteins were done as previously described for the photosynthetic NADP-ME (10) and non-photosynthetic NADP-ME (8). Fusion enzymes were then concentrated on Centricon YM-30 (Amicon) and desalted using Buffer B. Purified fusion NADP-ME proteins were then incubated with enterokinase (1:100, w/w) in buffer B at 10 °C for 2 h to remove the N terminus encoded by the expression vector. Proteins were further purified by affinity chromatography (Affi-Gel Blue), and eluted with buffer C (100 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 500 mM NaCl, 0.1 mM EDTA, 20% (v/v) glycerol, and 20 mM β-mercaptoethanol). Purified fusion NADP-ME proteins were then further purified by affinity chromatography (Affi-Gel Blue), and eluted with buffer C (100 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 500 mM NaCl, 0.1 mM EDTA, 20% (v/v) glycerol, and 20 mM β-mercaptoethanol). Eluted enzymes were then concentrated and desalted on Centricron YM-30 (Amicon) using Buffer B. Purified enzymes were stored at −80 °C in Buffer B (with 50% glycerol) for further studies.

Circular Dichroism (CD) Measurements—CD spectra were obtained on a Jasco J-810 spectropolarimeter using a 1.0-cm path length cell and averaging five repetitive scans between 250 and 200 nm. Typically, 50 μg of C₄, non-C₄, or chimerical NADP-MEs in phosphate buffer (20 mM NaPi, pH 8.0, 5 mM MgCl₂) were used for each assay.

NADP-ME Activity Assays—NADP-ME activity was determined spectrophotometrically as previously described (10). Initial velocity studies were performed by varying the concentration of one of the substrates around its Km while keeping the concentration of the other substrates at saturating levels. All kinetic parameters were calculated at least by triplicate determinations and subjected to non-linear regression. All substrate concentrations reported refer to the free, uncomplexed, reactant concentrations and were calculated considering the dissociation constant of the different complexes formed (Mg²⁺-NADP or Mg²⁺-malate). When testing different compounds as possible inhibitors or activators of the enzymatic activity, NADP-ME activity was measured in the presence of 2 or 5 mM of each effector (fumarate, succinate, citrate, α-ketoglutarate, glucose-6-P, fructose-1,6-bisphosphate, or fructose-6-P), and saturating or non-saturating concentrations of malate (typically, 1/5 or 1/10 of the Km, malate value). When analyzing the inhibition of NADP-ME by high malate concentration at pH 7.0, the model that fitted best the kinetic data was

\[
\frac{v}{V_{max}} = \frac{\left(\frac{[M]}{K_{c} + \phi M^{2}/K_{r}}\right)}{1 + \frac{[M]}{K_{c}} + \frac{[M]}{K_{r}} + \frac{[M^{2}]}{K_{r} K_{c}}} \quad (Eq. 1)
\]

in which the free enzyme is capable of binding malate at two different sites, one catalytic and the other allosteric (regulatory). In this kinetic scheme, E symbolizes the enzyme, M, the malate bound to the catalytic binding site (with dissociation constant Kc), Mr malate bound to the regulatory allosteric site (with dissociation constant Kr), and P the products of the enzymatic reaction. Thus, two different catalytic constants are postulated, namely k₁ and k₂, to indicate product formation when the allosteric site is empty or occupied, respectively. Considering k₂ = φ × k₁, the following expression for the initial rate of malate oxidative decarboxylation obtained,

\[
v = \frac{V_{max} [M/K_{c} + \phi M^{2}/K_{r}]}{1 + [M/K_{c} + [M/K_{r} + M^{2}/K_{r} K_{c}]} \quad (Eq. 1)
\]

where v is the activity measured at a specific malate concentration ([M]); Vmax is the maximum activity; Kc and Kr are the dissociation constants of the catalytic and regulatory site, respectively; and φ is the ratio between k₂ and k₁. This last value (φ) should be less than 1, as the activity when the allosteric site is occupied is less than when it is empty. When no inhibition by high substrate concentration was found, the kinetic data obtained were fit to typical Michaelis-Menten equations.

Gel Electrophoresis—SDS-PAGE was performed in 8 or 10% (w/v) polyacrylamide gels according to Laemmli (11). Proteins were visualized with Coomassie Blue or electroblotted onto a nitrocellulose membrane for immunoblotting. The membrane was incubated with affinity purified anti-maize recombinant photosynthetic NADP-ME antibodies (8). Bound antibodies were located with alkaline phosphatase-conjugated goat antirabbit IgG according to the manufacturer’s instructions (Sigma).

Native PAGE was performed employing a 6% (w/v) polyacrylamide separating gel. Gels were analyzed by Western blotting or assayed for malic enzyme activity as described in Ref. 8.

RESULTS

Metabolites Tested as Effectors of Maize C₄ and Non-C₄ NADP-ME—To detect other regulatory differences between maize C₄ and non-C₄-NADP-ME, in addition to the previously reported malate inhibition at pH 7.0 of the C₄ isofrom, we analyzed several metabolites as effectors of the activity of these two enzymes. No definitive activation by fumarate or succinate was found for both maize NADP-ME isofroms, although these metabolites were characterized as activators of other MEs. On the other hand, partial inhibition of both isofroms (up to 40%) was found in the case of citrate and α-ketoglutarate,
probably due to competition with the substrate malate. Other metabolites tested (glucose-6-P, fructose 1,6-bisphosphate, and fructose-6-P) did not show any significant effect on NADP-ME activity for either of the two isoforms. In this way, the pH-dependent inhibition of C4-NADP-ME by malate represents the major metabolite regulation difference found between maize photosynthetic and non-photosynthetic NADP-ME isoforms.

Construction and Expression of Chimerical Maize NADP-ME—To examine the sequence determinants responsible for the structural and kinetic differences between both maize NADP-MEs, we constructed six chimeras of these proteins by interchanging different sequence segments using two conserved restriction sites (BamHI at position 306 and EcoRI at position 749 of the cDNA of maize C4-NADP-ME) (Fig. 1). We will refer to the C4 and non-C4-NADP-ME as L (leaf) and R (root), respectively, considering the tissues where the isoforms are highly expressed. The chimerical proteins were named: LR-NADP-ME, LpR-NADP-ME, RL-NADP-ME, RpL-NADP-ME, LRL-NADP-ME, and RLR-NADP-ME (Fig. 1). All of these chimerical proteins were successfully expressed in E. coli, and purified to homogeneity. They presented molecular masses between 62 and 66 kDa determined by SDS-PAGE and reacted with antibodies against maize recombinant C4-NADP-ME (not shown). They also presented NADP-ME activity, so they were kinetically and structurally characterized in the same way as the parental enzymes.

To determine whether the construction of the chimerical proteins resulted in a loss of overall structural integrity, CD spectra for all chimerical and parental proteins were compared. In all cases, the CD spectra obtained were superimposable after corrections for protein concentration (not shown). In this way, the structural changes should be minimal, without causing a severe loss of protein secondary structure.

Kinetic Characterization of Chimerical NADP-MEs at pH 8.0—Kinetic characterization of chimerical NADP-MEs, L-NADP-ME and R-NADP-ME was performed at pH 8.0 (optimum for the L and R isoforms, Table 1) and pH 7.0 (required for malate inhibition of the C4 isoform, Table 2).

The chimerical NADP-MEs analyzed (RL, LR, LpR, RpL, LRL, and RLR) showed less specific activity (kcat) than the L and R isoforms at pH 8.0 (Table 1). The kcat values obtained for RL, LpR, and RpL were between 39 and 58% of the value obtained for the L isoform; whereas LRL and RLR presented considerably lower specific activities, between 2 and 12% of the value for the L isoform (Table 1). However, chimerical protein LR-NADP-ME displayed the lowest kcat value of all the recombinant proteins analyzed (more than 2 orders of magnitude, Table 1). Nevertheless, the Kcat of this chimerical enzyme was more than 2 times lower than that for the non-C4 counterpart (Table 1), indicating that, at least, the binding site for NADP is integral in this chimerical NADP-ME. The catalytic efficiency for the non-C4 and chimerical NADP-MEs when using NADP as substrate ranged from nearly 1 to 3 orders of magnitude lower than for the C4 counterpart (Table 1).

On the other hand, the Kmalate value at pH 8.0 for all the NADP-ME isoforms analyzed was very similar, with the L isoform showing the lower value followed by RpL-NADP-ME (Table 1). No inhibition by high malate concentration (up to 30 mM) was observed for any of the NADP-ME isoforms at pH 8.0 (Fig. 2).

Kinetic Characterization of Chimerical NADP-MEs at pH 7.0—Purified NADP-ME from maize green leaves is inhibited by high concentrations of the substrate malate in a pH-dependent way, that is, the inhibition is observed only at pH lower than
Tetramerization and Inhibition of Maize $C_4$-NADP-Malic Enzyme

**TABLE 2**

Kinetic parameters assayed at pH 7.0 for the recombinant maize $C_4$-NADP-ME (10), the non-$C_4$-NADP-ME (8), and the chimerical NADP-MEs obtained in the present work.

Values are given as average ± S.D. Each value is the average for at least two different preparations of each enzyme. When inhibition by high malate concentration was observed, $K_{c,malate}$ and $K_{r,malate}$ values were obtained using the equation indicated under “Experimental Procedures.”

| Parameters | $k_{cat}$ s$^{-1}$ | $K_{m,malate}$ mM | $k_{cat}/K_{m,malate}$ | $K_{c,malate}$ mM | $K_{r,malate}$ mM | $\varphi$ |
|------------|----------------|----------------|----------------|----------------|----------------|-------|
| C$_4$-NADP-ME (L isoform) | 104.4 ± 5.8 | 0.040 ± 0.007 | 2610 | 1.580 ± 0.004 | 0.27 ± 0.02 | 0.57 |
| Non-$C_4$-NADP-ME (R isoform) | 151.7 ± 1.5 | 0.51 ± 0.06 | 297 | 0.3 | NI | NI |
| LpR-NADP-ME | 55.4 ± 0.6 | 0.42 ± 0.02 | 131 | NI | NI | NI |
| RL-NADP-ME | 51.7 ± 8.8 | 0.21 ± 0.06 | 271 | 2.23 ± 1.09 | 0.32 ± 0.05 | 0.57 |
| Rpl-NADP-ME | 73.7 ± 3.4 | 0.040 ± 0.005 | 1842 | 1.21 ± 0.24 | 0.38 ± 0.02 | 0.57 |
| LRL-NADP-ME | 2.8 ± 0.2 | 0.130 ± 0.007 | 21 | 1.29 ± 0.25 | 0.44 ± 0.03 | 0.57 |
| RLRL-NADP-ME | 12.9 ± 0.7 | 0.55 ± 0.02 | 23 | NI | NI | NI |

* No inhibition was observed. Data were fit to a typical Michaelis–Menten equation.

8.0 (12). This kinetic behavior was also observed when maize photosynthetic NADP-ME was expressed as a recombinant protein in E. coli (10), but was not observed in the case of maize recombinant non-photosynthetic NADP-ME (8). To locate the differences in the primary structure of these two enzymes that could account for malate inhibition, the activity of the chimerical NADP-MEs as a function of malate concentration was studied (Fig. 2 and Table 2).

First of all, a detailed study of the activity of recombinant L and R isoforms as a function of malate concentration at both pH 7.0 or 8.0 was performed. Fig. 2 clearly shows that inhibition by malate concentrations higher than 0.5 mM is observed for the L isoform at pH 7.0 but not at pH 8.0 (Fig. 2). Data obtained for the L isoform (pH 8.0, L8) and the R isoform (pH 7.0 and 8.0; R7 and R8; Fig. 2) were fit to a typical Michaelis–Menten equation, yielding parameter values indicated in Tables 1 and 2. On the other hand, in the case of the L isoform at pH 7.0 (L7, Fig. 2), the data obtained fitted very well to an equation in which two different binding sites for malate are considered, one catalytic ($K_{c,malate}$) and the other allosteric ($K_{r,malate}$) (see Model 1 and Equation 1 under “Experimental Procedures”). This second allosteric site decreases the activity of the enzyme when occupied, rendering a partial inhibition of the activity. It is worth mentioning that lower regression fitting values were obtained when kinetic data were fit to other models and equations for inhibition by high substrate concentrations (13).

Malic enzyme activity as a function of malate concentration at pH 7.0 was assessed for the chimerical NADP-ME LR, LpR, RL, Rpl, LRL, and RLR (Fig. 2C) to identify segments in the primary structure implicated in malate inhibition. RL-NADP-ME, Rpl-NADP-ME, and LRL-NADP-ME were the only chimerical NADP-MEs analyzed showing inhibition by malate concentrations higher than 0.5 mM at pH 7.0 (Fig. 2C). Data obtained fitted very well to the same equation used in the case of the L isoform at pH 7.0 and the kinetic parameters obtained after data fitting were compared with the values obtained for the C$_4$ isoform (Table 2). On the other hand, LR-NADP-ME, LpR-NADP-ME, and RLR-NADP-ME did not show inhibition by high malate concentrations at pH 7.0 (as the R isoform), and the Michaelis-Menten
**Tetramerization and Inhibition of Maize C_{4}-NADP-Malic Enzyme**

The quaternary structure of chimerical NADP-ME from maize was investigated to understand the factors associated with NADP-ME oligomerization. The data obtained by native electrophoresis indicated that whereas the active form of LRL-NADP-ME migrates as a dimer, the chimera RLR-NADP-ME present an equilibrium between tetrameric and dimeric states (Fig. 3).

**DISCUSSION**

Because the C_{4}-NADP-ME isozyme (L isoform) has evolved from the non-C_{4}-NADP-ME (R isoform), we searched for changes in the primary structure of maize C_{4}-NADP-ME responsible for its C_{4} properties, allowing it to participate in a highly active metabolism such as photosynthesis. The results obtained analyzing different recombinant chimeras of the maize L and R isoforms allowed us to find key sequence elements associated with the more relevant kinetic and structural differences between these isoforms: inhibition by malate and oligomeric state.

All the chimerical NADP-MEs obtained in the present work were functional. Nevertheless, the $k_{cat}$ obtained for LR-NADP-ME at both pH 7.0 and 8.0 was significantly lower than the value obtained for the other chimerical NADP-MEs, including the reciprocal RL-NADP-ME (Tables 1 and 2). However, all the $K_m$ values estimated for LR-NADP-ME are in the same range as those of others chimeras (Tables 1 and 2), suggesting that one or more residues are not appropriately located for catalysis in this particular enzyme, although the binding of substrates is not seriously affected.

**The Allosteric Binding Site for Fumarate and Malate as Activators of Non-plant MEs Are Not Found in Maize NADP-ME**—Human mitochondrial NAD(P)-ME can be regulated (activated) allosterically by fumarate, which binds at the dimer interface of this protein (14). By site-directed mutagenesis, 4 amino acid residues have been suggested to be involved in binding fumarate at this allosteric site: Arg-67, Arg-91, Glu-59, and Asp-102 (14, 16) (Fig. 4A). With regard to maize C_{4}-NADP-ME, among the 4 residues found to be involved in fumarate activation of human NAD(P)-ME, only Asp-102 is conserved, which is in agreement with the fact that this enzyme is not activated by fumarate at all (Fig. 4A).

On the other hand, **Ascaris suum** NAD-ME is activated by both malate and fumarate, which bind to different binding sites that act synergically (17). Site-directed mutagenesis studies indicated that 2 amino acid residues were found to be implicated in this activation: Arg-105 (homologous to R91 of human NAD(P)-ME) and Lys-143 (Fig. 4A) (17). The effect of malate on **A. suum** NAD-ME is opposite to the effect of this substrate on C_{4}-NADP-ME (Fig. 2) and, in correlation with this, the residue Arg-105 involved in malate activation in **A. suum** NAD-ME (17) is not conserved in C_{4}-NADP-ME (Fig. 4A). On the other hand, these residues present homologues in non-C_{4}-NADP-ME (Fig. 4A), which is not activated nor inhibited by

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**Figure 3.** NADP-ME activity gels for the recombinant chimerical NADP-MEs. Between 1 and 3 milliunits of the different recombinant NADP-MEs were loaded in each lane: C_{4}-NADP-ME (L isoform), non-C_{4}-NADP-ME (R isoform), LR-NADP-ME (LR), RpL-NADP-ME (LpR), RL-NADP-ME (RL), RpL-NADP-ME (Rpl), LpR-NADP-ME (LpR), and RLR-NADP-ME (RLR). Native molecular mass markers were run in parallel and stained with Coomassie Blue (MWM, left).

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**Table 2.** A summary of the kinetic parameters obtained at pH 7.0 for the chimerical NADP-MEs, in comparison to the L and R isoforms. When inhibition by high malate concentrations is observed, the data fitted correctly the equation indicated under “Experimental Procedures,” obtaining two different binding constants for malate, one catalytic ($K_{cat,malate}$) and the other regulatory ($K_{r,malate}$). When comparing the $k_{cat}$ lower values are observed at pH 7.0 versus 8.0 for all the recombinant NADP-MEs, with the exception of RpL-NADP-ME and LRL-NADP-ME (Table 1 versus 2). Nevertheless, the decrease in $k_{cat}$ at pH 7.0 range from 47% (L isoform) to only 16%, as is the case for the R isoform. The $K_{cat,malate}$ at pH 7.0 for the L isoform, RpL-NADP-ME, and LRL-NADP-ME is nearly 1 order of magnitude lower at pH 7.0 than at 8.0, and for the case of RL-NADP-ME, is nearly 3 times lower. However, in the case of the R isoform and all the other chimerical NADP-MEs that are not inhibited by malate, this value is almost invariant when comparing measurements at pH 7.0 versus 8.0. In this way, a correlation between lower $K_{cat,malate}$ values at pH 7.0 than at pH 8.0 and inhibition by high malate concentration is observed (Table 2).

With regard to the inhibition by high malate concentrations, the $K_{r,malate}$ is very similar among the four NADP-MEs that showed inhibition (L isoform, RL-NADP-ME, RpL-NADP-ME, and LRL-NADP-ME). The decrease ratio in the $k_{cat}$ obtained for LR-NADP-ME are in the same range as those of others chimeras (Tables 1 and 2), suggesting that one or more residues are not appropriately located for catalysis in this particular enzyme, although the binding of substrates is not seriously affected.

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malate at all, suggesting that other amino acid residues are necessary for activation of ME by malate.

**Maize C4-NADP-ME Presents a Regulatory Region Associated to Malate Inhibition**—When analyzing maize C4-NADP-ME inhibition by malate, we first examined if the oligomeric state of this isoform (tetrameric) was associated to the inhibition by high malate concentration. This would have been the case if the malate binding site responsible for malate inhibition was located in the interface between the dimers, as is the case of the allosteric site for fumarate in the human NADP-ME (14), and, possibly, the allosteric activating site for malate in A. suum NAD-ME (17). Alternatively, if the inhibition was related to heterogeneity of the malate active binding sites in the tetramer, as was suggested for the pigeon liver NADP-ME (18, 19) (see below), it was reasonable that the tetramer (C4-NADP-ME) was inhibited by malate, whereas the dimer (non-C4-NADP-ME) was not. Nevertheless, analysis of the chimera obtained in the present work indicates that no correlation is observed between the oligomeric state and malate inhibition at all (Fig. 1), so these two first working hypotheses were discarded. Thus, the mechanism of maize C4-NADP-ME inhibition by malate was not in accordance with other kinetic mechanisms previously reported for non-plant MEs.

When considering substrate inhibition, three general mechanisms appear (13): pseudo-substrate inhibition, where the substrate affects some assay component rather than the enzyme directly; kinetic substrate inhibition, in which the substrate interferes or alters the catalytic mechanism; and allosteric substrate inhibition, in which the substrate binds a site different from the active site causing inhibition by a conformational change. In the present work, when assaying malate inhibition, free metal concentration was maintained constant, and therefore this could not be the cause of malate inhibition. Moreover, inhibition studies were performed in identical conditions for all isoforms tested, ruling out the possibility of a pseudo-inhibition mechanism (Fig. 2). In this way, the inhibition by high malate
The Mechanism of Oligomerization of Maize NADP-ME Differently from Other Malic Enzymes—By analyzing the maize NADP-ME chimeras constructed in the present work, we identified a region between amino acid residues 102 and 247 of the C₄ isoform and its homologue region in the non-C₄ isoform, which is strongly associated with the particular active oligomeric state of each maize NADP-ME (Figs. 1 and 4B). It is interesting to note that the isoform LRL-NADP-ME, which contained the mentioned region from the non-C₄ isoform (Fig. 3), does not tetramerize, indicating that some residues from this region are essential for tetramerization. On the other hand, the isoform RLR-NADP-ME exhibits an equilibrium between tetrameric and dimeric states (Fig. 3), indicating that the intermediate segment originated from the C₄ isoform induces tetramerization, but not in a complete way. The isoforms LR-NADP-ME and RpL-NADP-ME can completely tetramerize (Fig. 3), indicating that other interactions of the mentioned region contribute also to the oligomerization process but are not strong enough to drive complete tetramerization by their own. In this way, it seems that, for complete tetramerization, the region between amino acid residues 102 and 247 is indispensable, along with some residues from the N or, alternatively, C terminus of the L-NADP-ME. Although this oligomerization-related segment presents a high degree of conservation between these two NADP-ME isoforms, several non-conservative amino acid residue substitutions can be identified (Fig. 4B) that could be responsible, at least partially, for the different oligomeric states of these isoforms. The fact that the plastidic A. thaliana isoform presents similar homology to both the tetrameric and dimeric maize MEs in the region postulated to be involved in tetramerization (Fig. 4B), and that it also has an intermediate oligomeric state (equilibrium between tetramer and dimer, Ref. 29) reinforces the conclusion that this region has an essential role in tetramerization. On the other hand, the functional significance of tetrameric or dimeric states is not obvious from the present work, as both dimeric and tetrameric chimeric isoforms presented, in several cases, almost the same catalytic efficiency and substrate affinities.

With regard to the tetramer interaction of human NAD(P)-ME, it has been determined that it is mediated by a segment of ~20 residues at the C terminus of the enzyme monomer (1). Nevertheless, a multiple segment alignment of maize NADP-ME isoforms, and the NAD(P)-MEs for which crystal structures are available (Fig. 4C), clearly shows that this part of the protein is not present in maize NADP-ME nor in other plant-sequenced NADP-MEs (not shown). In this way, plant NADP-MEs have a different strategy for tetramerization and different amino acid residues involved in the tetramer interface. Moreover, it has been shown that mutation of Trp-548 of pigeon liver NADP-ME, which is completely buried at the tetramer interface, has a tremendous effect in the quaternary structure of the enzyme (23). This residue, which is conserved among crystallized NAD(P)-MEs, is absent from maize NADP-ME (Fig. 4C). Besides this particular residue, the region between residues 541 and 544 of human NAD(P)-ME also contributes to the formation of the tetramer interface (1). Specifically, mutation of Arg-542 of human NAD(P)-ME at the tetrameric interfacial exo site that binds ATP, resulted in dimeric mutants (25). Nevertheless, this residue is conserved in both dimeric and tetrameric maize isoforms (Fig. 4C). These observations indicate that the oligomerization interactions of plant NADP-MEs clearly differ from those of animal NADP-MEs.

Evolution of C₄-NADP-ME from a Non-C₄ Isoform: Regulation by Malate of Key C₄ Isoforms—The results obtained herein give new insights into the special requirements of C₄-NADP-ME, in relation to C₄ isoforms, to be well suitable for C₄ photosynthesis. In this regard, C₄-NADP-ME presents notably more affinity for the substrates, nearly 10 times lower $k_{\text{NADP}}$ and 2 times lower $k_{\text{malate}}$ than the C₄ enzyme (Table 1). However, the molecular determinants for the higher substrate affinity of the C₄-NADP-ME could not be mapped by the approach used in the present work, as the affinity for the substrates of all the chimerical NADP-ME analyzed was between the values obtained for the parent enzymes (Table 1). Thus, the molecular determinants involved in the higher substrate affinity of the C₄-NADP-ME may be distributed in several segments of the protein.
Tetramerization and Inhibition of Maize C4-NADP-Malic Enzyme

On the contrary, the other relevant kinetic difference, the inhibition by malate in a pH-dependent way, could be mapped associated to the C terminus of the C4-NADP-ME. In this regard, the C4-NADP-ME from *Flaveria bidentis* (20) and sugarcane (21) are also inhibited by high malate concentrations. Although more C4-NADP-ME family members should be analyzed, it is probable that this inhibition may be important for the C4-pathway regulation *in vivo*. In this way, the high level of malate concentration found in plant tissues (22) and the decrease in pH observed in chloroplasts in darkness, would both produce a decrease in NADP-ME activity when carbon fixation is not active in C4 plants.

Another C4 enzyme that evolved from C3 counterparts that were analyzed is P-enolpyruvate carboxylase (PEPC) (26, 27). The C4 and C3 PEPCs presented, as the case of NADP-ME, nearly the same $k_{cat}$ but differed drastically in the affinity for PEP, which was opposite to NADP-ME, lower for the C4 isoform. An increase in the inhibition constant for malate is also observed in the C4 isoform. With regards to regulation, the C4 PEPC activity is regulated by diurnal phosphorylation, which decreases the degree of inhibition by malate of the C4 enzyme. In this way, in the case of PEPC, the phosphorylation/dephosphorylation of the enzyme is involved in the activation/inhibition of the enzyme by malate. This was also the case for the PEPC from C4 single cell-type photosynthesis (28).

In summary, it seems that during the evolution to C4 enzymes, apart from a higher level and selective expression (PEPC in mesophyll and NADP-ME in bundle sheath cells), changes in kinetic and regulatory properties were also necessary to adapt the enzymes to new metabolic contexts. It is not surprising that, in both C4 PEPC and NADP-ME evolution, the inhibition of the high enzyme activity found in C4 leaves is controlled by the same metabolite (malate), which mediates activity inhibition when C4 photosynthesis is off.

Concluding Remarks—In the present work, we demonstrate that maize NADP-ME and crystallized non-plant NAD(P)-MEs present markedly different mechanisms for oligomerization and allosteric regulation. The active site of these enzymes present significant conservation, whereas their corresponding regulatory mechanism and oligomerization interactions are more divergent. These observations suggest that the emergence of different types of allosteric sites during the evolution of NAD(P)-MEs, led to the creation of enzymes with unique regulatory mechanisms and best suited to fulfill different metabolic roles.

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