Functional Characterization of Residues Involved in Redox Modulation of Maize Photosynthetic NADP-Malic Enzyme Activity

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Two highly similar plastidic NADP-malic enzymes (NADP-MEs) are found in the C\(_4\) species maize (\textit{Zea mays}); one exclusively expressed in the bundle sheath cells (BSCs) and involved in \textit{C}\(_4\) photosynthesis (Zm\(_{C4}\)-NADP-ME); and the other (Zm\(_{nonC4}\)-NADP-ME) with housekeeping roles. In the present work, these two NADP-MEs were analyzed regarding their redox-dependent activity modulation. The results clearly show that Zm\(_{C4}\)-NADP-ME is the only one modulated by redox status, and that its oxidation produces a conformational change limiting the catalytic process, although inducing higher affinity binding of the substrates. The reversal of Zm\(_{C4}\)-NADP-ME oxidation by chemical reductants suggests the presence of thiol groups able to form disulfide bonds. In order to identify the cysteine residues involved in the activity modulation, site-directed mutagenesis and MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) analysis of Zm\(_{C4}\)-NADP-ME were performed. The results obtained allowed the identification of Cys\(_{192}\), Cys\(_{246}\) (not conserved in Zm\(_{nonC4}\)-NADP-ME), Cys\(_{270}\) and Cys\(_{410}\) as directly or indirectly implicated in Zm\(_{C4}\)-NADP-ME redox modulation. These residues may be involved in forming disulfide bridge(s) or in the modulation of the oxidation of critical residues. Overall, the results indicate that, besides having acquired a high level of expression and localization in BSCs, Zm\(_{C4}\)-NADP-ME displays a particular redox modulation, which may be required to accomplish the \textit{C}\(_4\) photosynthetic metabolism. Therefore, the present work could provide new insights into the regulatory mechanisms potentially involved in the recruitment of genes for the \textit{C}\(_4\) pathway during evolution.

**Keywords:** \textit{C}\(_4\) photosynthesis \& Maize \& NADP-malic enzyme \& Redox modulation \& Structure–function relationship.

**Abbreviations:** BSC, bundle sheath cell; CD, circular dichroism; DTT, dithiothreitol; IBZ, iodosobenzoate; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; NADP-ME, NADP-malic enzyme; Zm\(_{C4}\)-NADP-ME, maize photosynthetic NADP-ME; Zm\(_{nonC4}\)-NADP-ME, maize non-photosynthetic plastidic NADP-ME.

### Introduction

The decarboxylation of \textit{C}\(_4\) acids in the bundle sheath cells (BSCs) is a key step in the photosynthetic process of \textit{C}\(_4\) plants. Depending on the \textit{C}\(_4\) species, this process can be mediated by NADP-malic enzyme (NADP-ME), NAD-malic enzyme and/or phosphoenolpyruvate carboxykinase (Kanai and Edwards 1999, Drincovich et al. 2011). During the last few years, remarkable advances have been made in the characterization of different isoforms of each \textit{C}\(_4\) decarboxylase. In most cases, non-photosynthetic isoforms of the \textit{C}\(_4\) decarboxylating enzymes involved in primary and/or secondary metabolism were characterized. These isoforms were the starting point for the evolution of the photosynthetic \textit{C}\(_4\)-specific decarboxylases. During this evolutionary process, \textit{C}\(_4\) enzymes have acquired characteristics that make them more suitable to fulfill the requirements of the \textit{C}\(_4\) photosynthetic process (Ku et al. 1996, Drincovich et al. 2001, Drincovich et al. 2011).

In many \textit{C}\(_4\) species, such as maize, sugarcane and sorghum, NADP-ME (EC 1.1.1.40) is the unique or major decarboxylase and malate is the predominant \textit{C}\(_4\) acid formed during photosynthesis. This enzyme catalyzes the oxidative decarboxylation of l-malate to yield carbon dioxide and pyruvate with the concomitant reduction of NADP (Edwards and Andreo 1992, Drincovich et al. 2001). In maize, the photosynthetic NADP-ME (Zm\(_{C4}\)-NADP-ME) is exclusively expressed in the BSC chloroplasts (Maurino et al. 1997, Maurino et al. 2001, Saigo et al. 2004, Detarsio et al. 2008), and displays particular kinetic, structural and regulatory properties (Detarsio et al. 2003, Detarsio et al. 2007). Apart from Zm\(_{C4}\)-NADP-ME, other NADP-ME isoforms not involved in the photosynthetic process are expressed in maize (Drincovich et al. 2001, Saigo et al. 2004, Detarsio et al. 2008). One of these isoforms, maize...
non-photosynthetic NADP-ME (ZmnonC\textsubscript{4}-NADP-ME), is constitutively expressed in maize plastids and plays housekeeping roles, representing the more recent and direct ancestor of ZmC\textsubscript{4}-NADP-ME (Tausta et al. 2002, Saigo et al. 2004). Although the two maize plastidic NADP-MEs share 85% identity, they show distinctive differences (Detarsio et al. 2007). The most relevant structural difference is the oligomeric state; ZmC\textsubscript{4}-NADP-ME assembles as a tetramer and ZmnonC\textsubscript{4}-NADP-ME as a dimer. Regarding kinetic properties, one of the most outstanding peculiarities is ZmC\textsubscript{4}-NADP-ME inhibition by the substrate malate in a pH-dependent way (Drincovich and Andreo 1992); while ZmnonC\textsubscript{4}-NADP-ME is not inhibited at all (Detarsio et al. 2007). Thus, high malate levels found in C\textsubscript{4} plant tissues, along with the pH decrease in the chloroplast stroma when photosynthesis is off, would both produce a reduction in the C\textsubscript{4} NADP-ME activity.

Redox regulation plays a key role in many plastid functions. Chloroplasts undergo changes in redox potential during the day/night cycle as well as during variations in metabolic demands. In this regard, NADP-ME purified from maize leaves is inactivated by cysteine oxidants (Drincovich et al. 1992, Drincovich and Andreo 1994). Moreover, NADP-ME activity increases in maize leaves due to illumination, which has been suggested to be due to a change in the redox state of cysteine residues (Murmu et al. 2003). However, the analysis of the redox modulation of the various NADP-ME isoforms, which are co-expressed in maize leaves (Saigo et al. 2004, Detarsio et al. 2007), has not been performed yet. Thus, in the present work, redox activity modulation of the two plastidic ZmNADP-ME isoforms from leaves was analyzed using recombinant purified proteins. The results obtained indicate a specific redox regulation of ZmC\textsubscript{4}-NADP-ME activity, modulation that is not observed in the case of ZmnonC\textsubscript{4}-NADP-ME. Moreover, the specific amino acids responsible for ZmC\textsubscript{4}-NADP-ME redox regulation were mapped, and the location of cysteine residues in NADP-MEs from other species were in silico analyzed. Overall, the results indicate that, besides acquiring a high level of expression and localization in BSCs, ZmC\textsubscript{4}-NADP-ME displays a particular redox modulation of its activity, which may be required to accomplish the C\textsubscript{4} photosynthetic metabolism successfully.

**Results**

**Redox modulation of recombinant ZmC\textsubscript{4}- and ZmnonC\textsubscript{4}-NADP-ME activity**

Purified recombinant ZmC\textsubscript{4}- and ZmnonC\textsubscript{4}-NADP-ME were incubated with chemical oxidants in order to assess the redox modulation of their activities (Fig. 1A). Three different oxidants that differ in redox potentials were used: iodosobenzoate (IBZ) and CuCl\textsubscript{2}, which are highly oxidative agents; and diamide, which has been widely used to induce the formation of disulfides (Kosower and Kosower 1987). The results show a time-dependent decrease of ZmC\textsubscript{4}-NADP-ME activity in the presence of the three oxidants, reaching nearly 40% of activity at 60 min of incubation in all cases (Fig. 1A). In contrast, ZmnonC\textsubscript{4}-NADP-ME activity is not significantly modified by any chemical oxidant, at least until 60 min of incubation (Fig. 1A).

In order to assess the reversal of ZmC\textsubscript{4}-NADP-ME inactivation, the enzyme was incubated with diamide for 20 min (60% of initial activity), desalted and further incubated with 10 mM DTT (open circles). For each time course incubation experiment, typical results are shown from at least three different incubation experiments using different preparations, in which similar results were obtained. In each incubation experiment, the activity was measured once at the indicated time intervals. In each experiment the initial activity of ZmC\textsubscript{4}-NADP-ME was 200 ± 15 U mg\textsuperscript{-1} and that of ZmnonC\textsubscript{4}-NADP-ME was 180 ± 10 U mg\textsuperscript{-1}.
DTT, a complete recovery of the initial NADP-ME activity is observed after 90 min of incubation (Fig. 1B).

**Kinetic and structural properties of oxidized and reduced ZmC₄-NADP-ME**

Kinetic parameters of reduced and oxidized ZmC₄-NADP-ME were estimated at two pH values, i.e. 7.0 and 8.0. ZmC₄-NADP-ME was incubated with 10 mM DTT for 2 h (redZmC₄-NADP-ME) or with 2 mM diamide for 20 min (oxZmC₄-NADP-ME) and desalted. The results indicate that ZmC₄-NADP-ME oxidation decreases the catalytic activity at both pH values tested (Table 1). On the other hand, a decrease in $K_m$ values for NADP of nearly three or four times is detected after oxidation, while $k_{cat}$ values for malate measured at both pH values decreased nearly 2-fold after oxidation (Table 1). In the case of ZmnonC₄-NADP-ME, kinetic parameters were not significantly modified after incubation with DTT or diamide (not shown).

The oligomeric state of red- and oxZmC₄-NADP-MEs was also analyzed by native gel electrophoresis (Detarsio et al. 2007). The enzyme treated with either DTT or diamide shows the same mobility in native electrophoresis, with a molecular mass consistent with a tetramer in all cases (Supplementary Fig. S1). In addition, circular dichroism (CD) spectra were determined for red- and oxZmC₄-NADP-MEs. The CD spectra obtained were superimposable, indicating that no severe loss of protein secondary structure takes place after ZmC₄-NADP-ME oxidation (Supplementary Fig. S2A).

**Functional characterization of ZmC₄-NADP-ME cysteine residues**

ZmC₄-NADP-ME and ZmnonC₄-NADP-ME share nearly 85% protein identity (Fig. 2). From the seven cysteine residues found in ZmC₄-NADP-ME (Cys192, Cys231, Cys246, Cys270, Cys410, Cys499 and Cys625), only one (Cys246) is not conserved in ZmnonC₄-NADP-ME (Fig. 2). A prediction of the three-dimensional model of ZmC₄-NADP-ME based on the crystallographic data for the human mitochondrial NAD(P)-ME complexed with its substrates (1EFL from the Protein Data Bank; http://www.rcsb.org/pdb/; Detarsio et al. 2004) was analyzed regarding the position of the seven cysteine residues (Fig. 3A). The relative position among the seven residues as well as the spatial relationship with respect to the substrate-binding site were taken into account to select residues that could be involved in the redox modulation of the enzyme. The predicted model shows that Cys192, Cys231, Cys246 and Cys270 are close enough to participate in a disulfide bridge (Fig. 3A). Thus, four cysteine residues, i.e. Cys192, Cys231, Cys246 and Cys270, were selected for site-directed mutagenesis analysis, replacing them with alanine residues.

**Purification and structural characterization of ZmC₄-NADP-ME mutants**

Four different ZmC₄-NADP-ME mutants were constructed by replacing cysteine residues with alanine: C192A, C231A, C246A and C270A. Although expressed to lower levels than the wild type, the mutated NADP-MEs were successfully expressed in *Escherichia coli* BL21(DE3) and purified to homogeneity using the same procedure previously described for the mature wild-type NADP-ME (not shown; Detarsio et al. 2003). The oligomeric state of ZmC₄-NADP-ME mutants was analyzed by native gel electrophoresis (Detarsio et al. 2007). The four mutants show the same mobility in native electrophoresis as the wild type, with a molecular mass consistent with a tetramer (not shown). The mobility in native gel electrophoresis of C231A in comparison with the wild type is shown in Supplementary Fig. S1.

To determine whether the introduced mutations resulted in a loss of overall structural integrity, CD spectra were obtained for all mutant proteins and compared with the one obtained for the wild-type NADP-ME. In all cases, the CD spectra were superimposable after corrections were made for protein concentration (Supplementary Fig. S2B). In this way, structural changes, if any, should be limited to the active site without generating a severe loss of protein structure.

**Kinetic characterization of C192A, C231A, C246 and C270A**

The apparent kinetic parameters of the purified recombinant ZmC₄-NADP-ME mutants C192A, C231A, C246A and C270A were estimated and compared with those of the wild type (Table 2). All mutants display a marked decrease in $k_{cat}$, presenting values from 8.6% to 0.4% compared with the wild type.

**Table 1** Kinetic parameters of recombinant oxidized and reduced ZmC₄-NADP-ME at pH 7.0 or 8.0

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ NADP ($\mu$M)</th>
<th>$K_m$ malate (mM)</th>
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<tbody>
<tr>
<td>8.0</td>
<td>redZmC₄-NADP-ME</td>
<td>269.0 ± 0.5</td>
<td>27.6 ± 1.2</td>
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<tr>
<td></td>
<td>oxZmC₄-NADP-ME</td>
<td>91.0 ± 8.3</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>7.0</td>
<td>redZmC₄-NADP-ME</td>
<td>8.35 ± 0.89</td>
<td>14.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>oxZmC₄-NADP-ME</td>
<td>3.55 ± 0.30</td>
<td>3.6 ± 0.2</td>
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</table>

Purified recombinant ZmC₄-NADP-ME was treated with either 10 mM DTT (redZmC₄-NADP-ME) for 2 h or 2 mM diamide (oxZmC₄-NADP-ME) for 20 min. After the treatment, the enzyme was desalted and kinetic parameters were estimated at pH 8.0 or 7.0. Values are given as average ± SD. Each value is the average obtained using at least three different preparations of each enzyme.
With regard to the $K_m$ values for NADP, all mutants show higher affinity for this substrate (Table 2). Notably, C246A exhibits a nearly 5-fold increase in its affinity towards NADP. Considering malate, C246A also shows a decrease (nearly 3-fold) in the $K_m$ for this substrate (Table 2). However, the other mutants display lower malate affinity than the wild type, with increases between four and 11 times in the $K_m$ for malate (Table 2). Thus, all the ZmC4-NADP-ME cysteine mutants constructed and analyzed show significantly lower catalytic efficiencies for both NADP and malate than the wild type (Table 2).

### Redox modulation of ZmC4-NADP-ME mutants activity

Purified recombinant ZmC4-NADP-ME mutants C192A, C231A, C246A and C270A were incubated with 1 mM IBZ (Fig. 3B). The results indicate that while the wild-type ZmC4-NADP-ME and C246A also shows a decrease (nearly 3-fold) in the $K_m$ for this substrate (Table 2). However, the other mutants display lower malate affinity than the wild type, with increases between four and 11 times in the $K_m$ for malate (Table 2). Thus, all the ZmC4-NADP-ME cysteine mutants constructed and analyzed show significantly lower catalytic efficiencies for both NADP and malate than the wild type (Table 2).

### MALDI-TOF-MS analysis of oxidized and reduced ZmC4-NADP-ME

In order to identify further the cysteine residues involved in the modulation of ZmC4-NADP-ME activity, oxZmC4-NADP-ME (treated with 2 mM diamide) and redZmC4-NADP-ME (treated with 10 mM DTT) were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Fig. 4). The pattern of peptides obtained for both enzymes after trypsin digestion were carefully analyzed in order to detect differences. In silico estimation of the m/z ratio of the peptides after ZmC4-NADP-ME trypsin digestion was considered in order to identify the peptides containing cysteine residues (Supplementary Table S1).
Signals for peptides containing Cys231 and Cys625 were clearly detected in both red- and oxZmC4-NADP-ME spectra (Fig. 4). Signals for the peptide containing Cys499 were also detected in both spectra, although that in the oxidized form was very small. In contrast, the signal for the peptide containing Cys410 was detected only in the case of redZmC4-NADP-ME (Fig. 4). In the case of the oxZmC4-NADP-ME spectrum, no peak was detected with the m/z ratio corresponding to this peptide (Fig. 4). The identity of peptides containing Cys231, Cys410 (from redZmC4-NADP-ME), Cys499 and Cys625 was corroborated by MS/MS analysis. Finally, no signals with the predicted m/z ratio could be detected for peptides containing Cys192, Cys246 or Cys270 either in the redZmC4-NADP-ME or in the oxZmC4-NADP-ME spectrum (not shown), which may be due, for example, to high m/z ratios, the presence of more than one peptide with similar m/z ratios, or improper trypsinization of the protein near these residues.

Discussion

The two maize plastidic NADP-MEs display distinct susceptibility to redox modulation

Redox regulatory mechanisms are essential in chloroplasts, where the activity of numerous enzymes is linked to the redox status of the photosynthetic electron transport chain (Buchanan and Balmer 2005). In the present work, the two maize plastidic NADP-MEs were analyzed regarding the redox modulation of their activities. The results obtained clearly show a distinct redox modulation of the two plastidic ZmNADP-MEs, the photosynthetic enzyme (ZmC4-NADP-ME) being the only one modulated by the redox status (Figs. 1A, 5). Loss of activity in ZmC4-NADP-ME occurs concomitantly with the oxidation of thiol groups to disulfide bonds, as the oxidation is reversed by incubation with reductant agents (Fig. 1B). ZmC4-NADP-ME oxidation produces a decrease in the maximum catalytic activity of the enzyme, which is associated with an increase in the affinity for both malate and NADP (Table 1, Fig. 5). Thus, enzyme oxidation produces a conformational change that limits the catalytic process although inducing a higher affinity binding of the substrate.

The results obtained suggest that, although the two ZmNADP-MEs share the same subcellular compartment, they display divergent properties that may be linked to a particular metabolic function in vivo. Thus, under oxidative conditions, ZmnonC4-NADP-ME could be fully active, while ZmC4-NADP-ME would be partially inactivated. It may be proposed that under conditions linked to oxidative processes, such as senescence or stress, ZmnonC4-NADP-ME would be responsible for malate oxidative decarboxylation in plastids. Besides, an enzyme for malate oxidative decarboxylation not susceptible to oxidation may be required in non-photosynthetic plastids where ZmnonC4-NADP-ME is the only ME expressed (Saigo et al. 2004; Fig. 5). Moreover, the reversible modulation of ZmC4-NADP-ME may be important for fine-tuning of the C4 metabolic pathway, as discussed below.

Fig. 3 (A) Three-dimensional model view of ZmC4-NADP-ME. The predicted model was obtained using human mitochondrial NAD(P)ME complexed with NAD, and tartronate used as a malate analog (1EFL from the Protein Data Bank: http://www.rcsb.org/pdb/). Relative distances among the seven cysteine residues are indicated in Ångstroms. Cys192, Cys231, Cys246 and Cys270 are highlighted as they display the lowest distances between each other and were further selected for mutagenesis analysis. (B) Redox modulation of ZmC4-NADP-ME mutant activity. Purified recombinant ZmC4-NADP-ME and C192A, C231A, C246A and C270A were incubated with 1 mM IBZ. At the indicated times, NADP-ME activity was measured and expressed as a percentage of the initial activity. For each time course incubation experiment, typical results are shown from at least three different incubation experiments using different purification batches, in which similar results were obtained. In each incubation experiment, the activity was measured once at the indicated time intervals. The initial activity of ZmC4-NADP-ME was 200 ± 15 U mg⁻¹; 15 ± 0.6 U mg⁻¹ for C231A; 4.0 ± 0.2 U mg⁻¹ for C192A; and 1.0 ± 0.06 U mg⁻¹ for C270A and C246A.
Identification of cysteine residues involved in the redox modulation of ZmC₄-NADP-ME

In the present work, two approaches were applied in order to identify the residues involved in the redox modulation of ZmC₄-NADP-ME activity: site-directed mutagenesis and MALDI-TOF-MS analysis of red- and oxZmC₄-NADP-ME (Figs. 3, 4).

The predicted three-dimensional model of ZmC₄-NADP-ME indicated that only four cysteine residues (Cys192, Cys231, Cys246 and Cys270) out of the seven residues would be close enough to be involved in a disulfide bridge (Fig. 3A). Thus, these four cysteine residues were replaced by alanine and the corresponding mutated NADP-MEs were characterized. The four mutants display significantly lower catalytic efficiency than the wild type (Table 2), indicating the relevance of the cysteine residues for the maximum catalytic performance of the enzyme. When analyzing the redox modulation of activity of the four cysteine mutants, the results indicate that mutation of either Cys192, Cys246 or Cys270 to alanine prevents the loss of activity upon exposure to oxidative agents (Fig. 3B). However,

Table 2 Kinetic parameters of the recombinant wild-type NADP-ME and site-directed mutants

<table>
<thead>
<tr>
<th></th>
<th>kcat (s⁻¹)</th>
<th>Km NADP (μM)</th>
<th>kcat/Km NADP</th>
<th>Km malate (mM)</th>
<th>kcat/Km malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>redZmC₄-NADP-ME</td>
<td>269.0 ± 0.5</td>
<td>27.6 ± 1.2</td>
<td>9.7</td>
<td>0.39 ± 0.03</td>
<td>690</td>
</tr>
<tr>
<td>C192A</td>
<td>5.6 ± 0.3</td>
<td>15.4 ± 1.2</td>
<td>0.36</td>
<td>3.0 ± 0.12</td>
<td>1.9</td>
</tr>
<tr>
<td>C231A</td>
<td>23.2 ± 1.6</td>
<td>15 ± 0.9</td>
<td>1.5</td>
<td>1.70 ± 0.08</td>
<td>13.6</td>
</tr>
<tr>
<td>C246A</td>
<td>1.1 ± 0.06</td>
<td>5.3 ± 0.51</td>
<td>0.21</td>
<td>0.14 ± 0.01</td>
<td>7.8</td>
</tr>
<tr>
<td>C270A</td>
<td>1.6 ± 0.08</td>
<td>13.5 ± 0.8</td>
<td>0.12</td>
<td>4.30 ± 0.2</td>
<td>0.37</td>
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</table>

Purified recombinant ZmC₄-NADP-ME was treated with either 10 mM DTT (redZmC₄-NADP-ME) for 2 h or 2 mM diamide (oxZmC₄-NADP-ME) for 20 min. After the treatment, the enzyme was desalted and kinetic parameters were estimated at pH 8.0 or 7.0. Values are given as average ± SD. Each value is the average obtained using at least three different preparations of each enzyme.

Fig. 4 Portions of MALDI mass spectra of the trypsinized redZmC₄-NADP-ME and oxZmC₄-NADP-ME. Each panel shows the portion of the spectrum with the signals for peptides containing cysteine residues for oxZmC₄-NADP-ME (treated with 2 mM diamide, top) and redZmC₄-NADP-ME (treated with 10 mM DTT, bottom). The arrow in each panel indicates signals for peptides containing Cys231, Cys410, Cys499 or Cys625.
mutation of Cys231 to alanine does not prevent oxidation at all (Fig. 3B). These results clearly indicate that Cys231 is not involved in ZmC4-NADP-ME redox modulation, although it is implicated in the kinetic mechanism and/or binding of substrates (Table 2). Furthermore, these results indicate that Cys192, Cys246 and Cys270 contribute to the changes leading to ZmC4-NADP-ME inactivation by oxidation, and may be directly involved in forming a disulfide bridge. Additionally, it may also be possible that mutation of one of these residues may induce a conformational change in the enzyme that prevents oxidation, for example increasing the distance between the cysteine residues involved in the disulfide bridge, and thus impeding bridge formation.

Regarding the MALDI-TOF-MS analysis of red- and oxZmC4-NADP-ME, the results indicate that Cys231, Cys499 and Cys625 are not involved in the redox modulation of the enzyme, as the peptides containing these residues are found in both red- and oxZmC4-NADP-ME spectra (Fig. 4). Moreover, the presence of the peak corresponding to the peptide containing Cys410 only in redZmC4-NADP-ME, and not in oxZmC4-NADP-ME, indicates the participation of this residue in the formation of a disulfide bridge (Fig. 4). Unfortunately, the peaks for peptides containing Cys192, Cys246 and Cys270 could not be detected under any condition, which could be due, for example, to the high m/z ratio of the peptide containing Cys270, the presence of a lysine at the end of the peptide containing Cys192 or the presence of more than one peptide with similar m/z ratios (this was the case for the peptide containing Cys246).

Overall, considering the two approaches used, it can be postulated that Cys192, Cys246, Cys270 and Cys410 are directly or indirectly involved in the redox modulation of ZmC4-NADP-ME. Several hypotheses agree with the results obtained. First, a disulfide bridge between Cys410 and one of the other three cysteine residues partially inactivates the enzyme, and the mutation of the two remaining cysteine residues induces a conformational change that avoids the oxidation of the enzyme to form the bridge. Secondly, the four cysteine residues contribute to the formation of disulfide bridge(s), maybe by producing alternative disulfide bonds or by additive effects. In this regard, it is also worth mentioning that the replacement of Cys246 with serine in ZmnonC4-NADP-ME (Fig. 2) may be responsible for the absence of redox modulation (Fig. 1A). In this sense, when analyzing the two putative plastidic NADP-MEs (SbME1 and -2) from Sorghum bicolor, another C4 species that uses NADP-ME as the main decarboxylase in BSCs, it is also notable that the residue homologous to Cys246 is found in only one of the two isoforms (Supplementary Fig. 53). Notably, the isoform with a cysteine at position 246 is the one exhibiting the highest degree of identity with the maize photosynthetic enzyme (Supplementary Fig. 53). In this way, it is possible that the redox modulation of NADP-MEs is a feature needed for the fine-tuning of the C4 flux in the photosynthetic process.

Finally, the participation of Cys410 in the disulfide bond of ZmC4-NADP-ME (Fig. 4) was rather surprising when considering the three-dimensional model of this enzyme (Fig. 3A). However, it is worth noting that this model was constructed using non-plant NADP-MEs, which show several and relevant sequence differences with plant NADP-MEs (Detarsio et al. 2004, Detarsio et al. 2007). Moreover, from the seven cysteine residues of ZmC4-NADP-ME, only three (Cys192, Cys270 and Cys499) are conserved in human mitochondrial NAD(P)-ME (not shown). Therefore, the three-dimensional model of ZmC4-NADP-ME used in the present work may not precisely reflect the real tertiary structure of the enzyme, making it necessary to collect crystallographic data for plant NADP-MEs and refine the modelling analysis.

Redox modulation in BSC chloroplasts for C4-NADP-malic enzymes?

In the present work, the redox modulation for an enzyme exclusively localized in maize BSC chloroplasts and involved in C4 photosynthesis is presented. Previous work described an increase in NADP-ME activity due to illumination of leaves with the participation of redox processes (Drincovich and Andreo 1994, Murmu et al. 2003). When using maize leaf discs, the increase in NADP-ME activity after illumination was due, at least in part, to the reduction of dithiols, indicating a change in the activation state of the enzyme (Murmu et al. 2003; Fig. 5). However, when considering the relevance of such regulation in vivo, it must be taken into account that maize BSC chloroplasts show low levels of active PSII complexes and only limited amounts of NADPH production from linear photosynthetic electron transport, NADP-ME being the main source of reducing power for BSC chloroplasts (Majeran and van Wijk 2009). It is interesting to note that recent proteomic studies comparing maize mesophyll and BSC chloroplasts have indicated the compartmentalization of several redox regulators and antioxidative defense systems, quantifying higher expression levels of several redox regulators in the mesophyll, with the exception of some BSC-specific components (Majeran et al. 2005, Majeran and van Wijk 2009). These results raise the question of whether the bundle sheath and mesophyll redox activation systems are specific for some particular enzymes, and also if some of the BSC-specific components are involved in maintaining ZmC4-NADP-ME in its reduced state.

The operation of the C4 photosynthetic pathway requires a deep synchronization between mesophyll and BSCs (Bailey et al. 2007). For C4 photosynthesis to concentrate CO2 around RubisCO in BSC chloroplasts, the NADP-ME decarboxylation and RubisCO carboxylation rates must be coordinated to avoid leakage of the CO2 produced by NADP-ME. Moreover, the carboxylation (in mesophyll cells) and decarboxylation (in BSCs) reactions must be also regulated to accommodate the large and rapid flux changes that occur during photosynthesis. In this regard, the relevance of the light activation of NADP-malate dehydrogenase from S. bicolor, mediated by thioredoxin, which occurs in mesophyll cells of this C4 species is well known (Goyer et al. 2001). Another example is the redox
regulation by thioredoxin of glycerate 3-kinase in maize mesophyll cells, an enzyme participating in photorespiration (Bartsch et al. 2010). Thus, modulation of ZmC4-NADP-ME activity by the redox state appears to be a suggestive regulatory mechanism for enzyme adaptation to the changing functional status of the BSC chloroplasts. It may be possible that activation of ZmC4-NADP-ME by reduction of thiol groups occurs under light or reductive conditions in BSC chloroplasts to allow a fine-tuning of the flux through the C4 pathway (Fig. 5). In this way, when photosynthesis is operating, ZmC4-NADP-ME displays its maximum catalytic activity. Although more C4-NADP-ME family members should be analyzed, it is probable that the redox modulation of this enzyme was a critical step towards the evolution to C4 NADP-MEs. Therefore, the present work provides new insights towards the understanding of the regulation of C4 enzymes, which is crucial for fully exploiting the potential of this type of metabolism (Hibberd et al. 2008, Hibberd and Covshoff 2010).

**Materials and Methods**

**Site-directed mutagenesis of ZmC4-NADP-ME**

Site-directed mutagenesis of maize mature NADP-ME was carried out according to the procedure of Mikaelian and Seargeant (1992). The primers that introduced mutations in the wild-type protein were as follows: C192A, 5'-AGGTGAGGCAGCAGAAAGTAT-3'; C232A, 5'-TCAAGTTATCGCTGTACTGATGGTGAG-3'; C246A, 5'-GGAGATTTGGGTGCTCAGGGAATG-3'; and C290A, 5'-GACCCATCAGTTGCCTATC-3'. The mutated positions are in bold in the oligonucleotide sequence. These mutagenic primers were used in combination with the following primers in order to obtain the complete sequence of NADP-ME: SEMMut, 5'-TAGCATGACATGGCGGATC-3'; and 3EMMut, 5'-CAACGCTGACGGAACATTCACT-3'. PCR was carried out using a mixture of Taq and Pfu polymerases (10:1) in order to minimize possible errors introduced by Taq polymerase. The resulting sequences obtained by PCR were purified from agarose gels. These fragments were used as megaprimers, in combination with the 5EMMut and 3EMMut primers, to amplify the complete sequences. The resulting fragments were separated on agarose gels, purified by QIAprep columns (Qiagen) and ligated into pGEMT-Easy vectors (Promega). The introduced sequences were checked to verify the presence of the desired mutations and that no errors were added due to PCR and subcloning procedures. The different sequences obtained were cloned into pET32a vectors (Novagen).

**Expression and purification of recombinant NADP-MEs**

*Escherichia coli* BL21(DE3) cells were transformed with the pET32 plasmids containing the mutated NADP-MEs. The resulting fusion proteins were purified using a His-Bind column (Novagen). Induction and purification of the mutated proteins were performed as previously described for the photosynthetic NADP-ME (Detarsio et al. 2003). Fusion enzymes were then concentrated on Millipore MW-30 (Amicon) and desalted using a buffer containing 100 mM Tris pH 8.0 and 10 mM MgCl2. Purified enzymes were stored at −80°C with the addition of 50% glycerol. ZmC4-NADP-ME and ZmnonC4-NADP-ME were purified as previously described (Detarsio et al. 2003, Saigo et al. 2004, Detarsio et al. 2007).

**Redox modulation of the activity of recombinant NADP-MEs**

The different purified recombinant NADP-MEs (approximately 50 μg) were incubated at 0°C in buffer containing 100 mM Tris–HCl (pH 8.0) and 10 mM MgCl2 with different redox compounds (1 mM IBZ, 25 μM CuCl2, 2 mM diamide or 10 mM DTT). At different incubation times, aliquots were withdrawn and assayed for NADP-ME activity. Modification of the
recombinant enzymes was stopped by dilution (at least 100-fold) of redox compounds in the assay media. No significant decrease in activity was found when the recombinant NADP-MEs were incubated in the absence of redox compounds. Addition of these compounds (100-fold diluted) to the NADP-ME assay medium had no significant effect on the enzyme activity assay.

NADP-ME activity assay and estimation of kinetic parameters

NADP-ME activity was measured spectrophotometrically at 30°C by monitoring NADPH production at 340 nm. The standard reaction mixture contained 100 mM Tris–HCl pH 8.0 (or 7.0 when stated), 10 mM MgCl₂, 0.5 mM NADP and 4 mM l-malate. The reaction was started by the addition of l-malate. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADPH min⁻¹ under the specified conditions. Initial velocity studies were performed by varying the concentration of one of the substrates around its Kₘ while keeping the remaining substrate concentrations at saturating levels. All kinetic parameters were calculated at least in triplicate and subjected to non-linear regression, using free concentrations of all substrates (Detarsio et al. 2003). Kinetic parameters of NADP-ME were also obtained after treatment with either 10 mM DTT (redZmC₄-NADP-ME) for 2 h or 2 mM dithiobis(succinimidyl propionate) (oxZmC₄-NADP-ME) for 20 min at 0°C. After both treatments, the enzyme was desalted using Microcon MW-30 (Amicon) with 100 mM Tris–HCl pH 8.0 and 10 mM MgCl₂.

Gel electrophoresis

SDS–PAGE was performed in 8% or 10% (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized by Coomassie Blue staining. Native PAGE was performed in the absence of any reductant. Coomassie-stained bands were excised from the gel and treated with 10 mM iodoacetamide to block thiol groups by carbamidomethylation. MS data from tryptic digestion of SDS–polyacrylamide gel bands were obtained using a MALDI-TOF spectrometer, Ultraflex II (Bruker), in the MS facility CEQUIBIEM (Centro de Estudios Químicos y Biológicos por Espectrometría de Masa; Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). Spectra obtained from redZmC₄-NADP-ME or oxZmC₄-NADP-ME were manually evaluated and compared with the in silico digestion predicted peptides of redZmC₄-NADP-ME (Supplementary Table S1). Peaks of putative peptides containing cysteine residues were sequenced for verification. At least two different preparations of redZmC₄-NADP-ME or oxZmC₄-NADP-ME were used for MS analysis.

Supplementary data

Supplementary data are available at PCP online.

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