

Research article

Posttranslational modification of the NADP-malic enzyme involved in C₄ photosynthesis fine-tunes the enzymatic activity during the day

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Short Title: *In vivo* phosphorylation of maize C₄-NADP-ME

One-sentence summary: Phosphorylation of ZmC₄-NADP-ME at S419 decreases the binding affinity to NADP fine-tuning the enzymatic activity during the first hours in the light.

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ABSTRACT

Evolution of the C₄ photosynthetic pathway involved in some cases recruitment of housekeeping proteins through gene duplication and their further neofunctionalization. NADP-malic enzyme (ME), the most widespread C₄ decarboxylase, has increased its catalytic efficiency and acquired regulatory properties that allowed it to participate in the C₄ pathway. Here, we show that regulation of maize C₄-NADP-ME activity is much more elaborated than until now indicated. Using mass spectrometry, we identified phosphorylation of the serine 419 (S419) of C₄-NADP-ME in protein extracts of maize leaves. The phosphorylation event increases after the light turns on, with a peak at ZT2. Phosphorylation of ZmC₄-NADP-ME drastically decreases its activity as shown by the low residual activity of the recombinant phosphomimetic mutant. Analysis of the crystal structure of C₄-NADP-ME indicated that S419 is involved in the binding of NADP at the active site. Molecular dynamics simulations and effective binding energy computations indicate a less favorable binding of the cofactor NADP in the phosphomimetic and the phosphorylated variants. We propose that phosphorylation of ZmC₄-NADP-ME at S419 during the first hours in the light is a cellular mechanism to fine-tune the enzymatic activity to coordinate the carbon concentration mechanism with the CO₂ fixation rate, most probably to avoid CO₂ leakiness from bundle sheath cells.

INTRODUCTION

The world's most productive crops perform C₄ photosynthesis. The ancestors of these plants evolved a biochemical pump to concentrate CO₂ at the site of Rubisco leading to lower photorespiratory fluxes and greater photosynthetic efficiency (Furbank and Hatch, 1987). Compared with the ancestral C₃ photosynthesis, the C₄ pathway allows increased plant productivity in warm habitats due to a more efficient use of nitrogen and water.

The most agronomically important C₄ plants maize, sorghum, and sugar cane belong to the NADP-ME subtype. In these plants, CO₂ is initially fixed in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPC), leading to the formation of oxaloacetate. Oxaloacetate is then predominantly reduced to malate and transported into the bundle sheath cells (BSC), where CO₂ is released by NADP-malic enzyme (NADP-ME) for its refixation

through Rubisco (Hatch, 1987). By this process, CO₂ concentration in the BSC rises to levels up to 1,500 $\mu\text{L L}^{-1}$ (Furbank and Hatch, 1987; Sage et al., 2012).

The transition from C₃ to C₄ metabolism involved complex alterations to leaf anatomy and biochemistry. A crucial step in the evolution of the C₄ photosynthetic pathway was the recruitment of some enzymes through gene duplication of housekeeping isoforms and subsequent neofunctionalization. In the course of its evolution, the C₄-specific NADP-ME isoform increased its catalytic efficiency and acquired regulatory properties that optimized its efficiency in the C₄ pathway (Saigo et al., 2013; Alvarez et al., 2019). During the night, when the delivery of malate from the PEPC reaction is stopped, C₄-NADP-ME activity is inhibited by malate (Saigo et al., 2004; Saigo et al., 2013). This property, exclusive to the C₄ isoform, minimizes the malate consumption to avoid extreme carbon loss during the night period, which would otherwise result in carbon starvation (Fahnenstich et al., 2007; Zell et al., 2010). At the pH of the stroma in darkness, a proportion of C₄-NADP-ME likely loses its quaternary structure and adopts a lower oligomerization state that is less active or even inactive (Iglesias and Andreo, 1990). This likely represents a second level of regulation that ensures that C₄-NADP-ME is less active during the night period.

The enzymatic activities of some enzymes of C₄ metabolism and also of the CAM carbon concentrating pathway are known to be regulated through post-translational modifications (PTMs). PTMs provide mechanisms for rapid and reversible control of activity of these proteins in a coordinated fashion during the day and night and in response to light intensity (Walker et al., 2002; Bailey et al., 2007; Chen et al., 2014). The activity of C₄-PEPC is regulated through phosphorylation of a serine residue located at the N-terminal region by a small PEPC kinase (PEPC-PK) (Nimmo, 2003). C₄-PEPC is mainly phosphorylated in the light, where it has a higher catalytic activity and is less sensitive to the allosteric inhibition by malate than in darkness (Jiao and Chollet, 1988; 1989; 1990). Transgenic lines of *Flaveria bidentis* with antisense or RNA interference constructs targeted at the mRNA of the PEPC-PK demonstrated that the kinase is essential for the phosphorylation of C₄-PEPC in vivo (Furumoto et al., 2007). In the transgenic lines C₄-PEPC was not phosphorylated in the light, nevertheless no differences in the CO₂ and light response of CO₂ assimilation rates between them and the wild type (Furumoto et al., 2007). These results suggest that phosphorylation of PEPC in the light is not essential for efficient C₄ photosynthesis in plants grown under standard glasshouse conditions but it remains to be determined if photosynthetic rates are affected under stress conditions.

PEPC of the obligate CAM species *Kalanchoë fedtschenkoi* is also regulated by phosphorylation (Hartwell et al., 1999; Nimmo, 2000). A specific PEPC kinase phosphorylates CAM-PEPC during the dark period, reducing its sensitivity to feedback inhibition by malate, enabling nocturnal fixation of CO₂ into malate (Boxall et al., 2017). C₄-pyruvate orthophosphate dikinase (C₄-PPDK) activity, in contrast, is not controlled by the light/dark transition *per se* but rather by the light intensity. In this case, increased phosphorylation takes place with waning light intensity in the evening leading to a decrease in activity (Chen et al., 2014). C₄-PPDK is regulated by reversible phosphorylation of a threonine residue of the active site (Burnell and Hatch, 1985; Chastain et al., 2000) catalyzed by the PPDK regulatory protein (PDRP) (Chen et al., 2014). PDRP is bifunctional; it catalyzes both PPDK activation/dephosphorylation and PPDK inactivation/phosphorylation (Burnell and Chastain, 2006; Chastain et al., 2008; Astley et al., 2011). The regulatory phosphorylation of C₄-PPDK is a very ancient mechanism as genes coding for PDRP are present in C₃ plants and prokaryotes (Agarie et al., 1997; Imaizumi et al., 1997; Wei et al., 2000; Burnell, 2010). In some C₄ plants such as Guinea grass (*Panicum maximum*) and maize, PEP carboxykinase (C₄-PEPCK) is phosphorylated in the dark when the enzyme is less active, and dephosphorylated under illumination (Walker and Leegood, 1996; Walker et al., 2002; Chao et al., 2014). Modifications in the phosphorylation state of PEPCK lead to changes in its sensitivity to adenylates, which would be involved in the activation of the enzyme in the light (Walker et al., 2002). Recently, it was shown that phosphorylation of a serine residue in maize C₄-PEPCK is also dependent on the light regime (Chao et al., 2014).

Little is known about the participation of PTMs in the regulation of the C₄-NADP-ME isoform *in vivo*. Until now, only the effects of redox modulation on the recombinant ZmC₄-NADP-ME activity were reported (Alvarez et al., 2012). Oxidation of ZmC₄-NADP-ME decreases the catalytic activity and increases the affinity for malate and the cofactor; in combination, these changes produce no significant changes of the catalytic efficiency. The oxidation of C192, C246, C270 and C410 may contribute to the changes observed *in vitro* (Alvarez et al., 2012).

Here, we show a novel PTM, phosphorylation at S419, of ZmC₄-NADP-ME, which takes place *in vivo* after the light is turned on, with a peak at 2 h after the onset of light. The results of the characterization of the recombinant phosphomimetic mutant S419E together with the analysis of the crystal structure of C₄-NADP-ME, and molecular modeling and dynamic simulations of wild-type and mutant enzymes indicate that phosphorylation at S419 inactivates ZmC₄-NADP-ME because S419 is involved in NADP binding at the active site.

We postulate that the novel PTM of ZmC₄-NADP-ME described here is a mechanism to fine-tune the enzymatic activity, most probably to coordinate malate decarboxylation with carbon fixation in BSC.

RESULTS

Identification of a phosphorylation site in ZmC₄-NADP-ME

Mass spectrometric identification of phosphorylation sites in complex protein extracts faces several challenges. Phosphopeptides are often of low abundance and often exhibit low MS signals and inadequate fragmentation patterns. To overcome these issues and to be able to identify phosphorylated peptides of ZmC₄-NADP-ME in cell lysates obtained from maize leaf macerates, we loaded maize protein extracts onto 4-12% NuPAGE gels and electrophoresed them for a short time. Upon in-gel digestion, samples were subjected to a phosphopeptide enrichment step and analyzed by LC-MS using an AB Sciex TripleTOF 6600 mass spectrometer. The acquired data was analyzed with Protein Pilot 5.0 and PeakView 2.2. This approach was successful in unambiguously detecting the phosphorylation of ZmC₄-NADP-ME at a single amino acid residue, S419. Figure 1 shows a tandem MS spectrum of the obtained peptide acVWLVDpSK. The quality of the MS/MS data for this peptide enabled the correct assignment of the phosphorylated residue. In fact, with the exception for the y₁-ion, the entire y-ion series of the peptide was assigned, and the loss of the phosphate group (H₃PO₄), corresponding to a mass-to-charge ratio of 98, was detected for the whole y-ion series.

The MS analysis did not identify any phosphorylation event in the other maize NADP-ME isoforms, as the V414 from the identified phosphorylated peptide is unique to the ZmC₄NADP-ME (Alvarez et al., 2013). We conclude that a functional role for this phosphorylation site most probably represents a C₄-trait. We next compared the protein sequences of plastidic photosynthetic and non-photosynthetic NADP-ME isoforms within the Panicoideae subfamily of the Poaceae. This sequence alignment indicated that the serine corresponding to amino acid position 419 in the C₄-NADP-ME protein sequence is conserved among all NADP-ME sequences analyzed (Supplemental Figure 1).

Production of the phosphomimetic mutant and characterization of the biochemical properties

In order to analyse the effect of phosphorylation of S419 on the biochemical properties of ZmC₄-NADP-ME, we generated the phosphomimetic mutant S419E by changing the S419 to E by site-directed mutagenesis. Phosphorylation adds negative charge to amino acids, and thus the negatively charged amino acid E can be used to mimic the phosphorylated state of a protein (Dissmeyer and Schnittger, 2011). In order to assess the importance of the amino acid position for the enzymatic activity, we also created the S419A variant by changing the Ser419 for A, a small non-polar amino acid.

ZmC₄-NADP-ME wild-type (WT), S419E, and S419A were heterologously expressed and purified to homogeneity by affinity chromatography. The recombinant proteins, showing in all cases the expected molecular masses (Supplemental Figure 2), were subjected to a comparative analysis of their enzymatic properties. Analysis of the dependence of the enzymatic activity on the pH of the reaction media indicated that while the recombinant WT ZmC₄-NADP-ME has a pH optimum of 8.0, the S419E variant presents a shift of the pH optimum towards more acidic pHs between 6.5 and 7.0 (Figure 2A). The S419A variant showed a pH optimum of 7.0 - 7.5 (Figure 2A). At the physiologically relevant pH 8.0 of the photosynthetically active stroma, the S419E variant presents only 8.7% of the WT activity, while the S419A variant retains a 72.3% of the WT activity (Figure 2A).

Analysis of the affinity to NADP indicated that the exchange of the S residue for E in the S419E variant dramatically decreases the affinity of the mutant for the cofactor, as saturation was not observed even at NADP concentrations as high as 5 mM. The S419A variant presented a 13-fold lower affinity (K_m 159.5 μ M) for the cofactor in comparison to the WT (K_m 12.3 μ M) (Figure 2B), indicating that S419 is essential for the normal operation of the enzyme.

The affinity for the substrate malate at pH 8.0 of the WT and S419A was measured at saturating NADP concentrations. As we found that S419E is not saturated by NADP even at very high concentrations, the K_{mapp} was determined at a sub-saturating concentration of the cofactor. In comparison to the WT (K_m 0.19 mM), the apparent affinity for malate decreased by approximately 14-fold in the S419E variant (K_{mapp} 2.68 mM) and only 2-fold in the S419A variant (K_m 0.47 mM). The turnover number (k_{cat}) was measured at pH 8.0, at saturating levels of malate, and at 0.5 mM NADP. Under these conditions, the S419E variant displayed a 6-fold lower catalytic activity (k_{catapp} 2.1 s⁻¹) with respect to the WT (k_{cat} 12.2 s⁻¹) (Figure 2B). In contrast, the k_{cat} of the S419A variant (k_{cat} 8.3 s⁻¹) was in the same range as that of the WT (Figure 2B).

Structural organization of the mutant variants

As the phosphomimetic mutant showed dramatic changes of its kinetic properties in comparison to the WT enzyme, we analysed if these could be the result of alterations in the structural organisation of the engineered protein. We used circular dichroism (CD) to explore the secondary structure organization and analytical ultracentrifugation (AUC) to explore the quaternary composition.

CD measurements at both pH 7.0 and 8.0 indicated no significant changes in the secondary structure organization of the mutated variants in comparison to the WT (Figure 3A and B). The data obtained indicated that ZmC₄-NADP-ME WT and the S419E and S419A variants presented similar fractions of different types of secondary structures depending on the pH. At pH 8.0, all enzymes possess almost twice the amount of helical structures observed at pH 7.0, an approximately 1.5-fold lower amount of β -strand structures, an almost unchanged amount of turns, and an only slightly decreased amount of unordered secondary structures (Supplemental Figure 4 and Supplemental Table 1). As shown in Supplemental Figure 4 for the case of the WT, the higher signal at 195 nm together with the more pronounced minimum at 208 nm is indicative of an overall increase in α -helical structure at pH 8. The decrease of beta-strand structure is hardly visible directly in the spectra, since the CD-effect of β -strand structure is lower than that of α -helix. This is why slight changes are less visible.

A comparison of the analysis presented in Supplemental Table 1 with the secondary structure determined from the crystal structure of ZmC₄-NADP-ME (pdb-code: 5OU5; (Alvarez et al., 2019)) indicates that the total helix content measured by CD increases with the pH from 24% at pH 7.0 to 37% at pH 8.0. The increase in helical structure is achieved mainly at the expense of total β -strand conformation, which decreases from 28% at pH 7.0 to 20% at pH 8.0. The change in the fractions of turn and unordered conformations is less pronounced. In comparison the crystal structure of ZmC₄-NADP-ME has 46% β -helical structure and about 11% β -strand conformation. While obviously the change in pH has a significant impact on the conformation of the protein, the structure measured by CD at pH 8.0 is more similar to the crystal structure than the measured at pH 7.0. That the structure of a protein in solution differs from its crystal structure is expected; in solution different conformational ensembles exist, while in a crystal a single conformation prevails. Additionally, different environments in solution and in the crystal and the presence or absence of the cofactor might favor different conformations. Either an ordering of the protein as the pH decreases or alterations due to

changes in domain interactions are possible causes of the deviation in secondary structure composition obtained by the different methods.

AUC measurements conducted at both pH 7.0 and 8.0 (Figure 3C and D) confirmed a stable tetrameric state of WT ZmC₄-NADP-ME at pH 8.0, with a sedimentation coefficient of 11.7 S (Figure 3D). Both mutated variants also assemble as tetramers at pH 8.0, showing sedimentation coefficients of 11.6 S and 11.1 S (Figure 3D). The sedimentation coefficient distribution of all proteins at pH 7.0 showed two peaks, with sedimentation coefficients of approximately 11.5 S and 5.4 S (Figure 3C), indicating that at this pH the enzymes exist as a mixture of monomers/dimers and tetramers with predominance of the latter. The results also indicate that the generated mutant variants have no altered quaternary structure at any pH in comparison to the WT. It can be speculated that the changes in amount of helical and turn components observed in the CD measurements at the different pHs might be related to the occurrence of a dimeric fraction at pH 7.0, as these changes may contribute to the alterations in the interface contacts between the monomers in the tetramer.

Taken together, the results of CD and AUC analyses indicate that the observed changes in the kinetic properties of the mutant variants are not due to changes in the structural organization of the proteins with respect to the WT at each pH analysed.

Diurnal profiles of ZmC₄-NADP-ME phosphorylation state, NADP-ME activity and malate concentration in maize leaves

We quantified ZmC₄-NADP-ME total protein amount in leaves of maize at different time points during a day using SWATH-MS (sequential window acquisition of all theoretical fragment ion spectra-mass spectrometry). We found that during the day, there are no major fluctuations of the ZmC₄-NADP-ME total protein amount (Figure 4A). Following the light-to-dark transition, there is an increase in total ZmC₄-NADP-ME protein level (Figure 4A). Despite a decreasing trend over the dark period from ZT16.5 to ZT23.5, the relative levels of ZmC₄-NADP-ME to the total protein levels (as measured by SWATH-MS) are higher during the whole dark period when compared to the light period.

Quantification of phosphorylation in S419 throughout the day indicated a significant increase of phosphorylation events after the light is turned on, with a peak at ZT2 that accounts for about 13% of the total ZmC₄-NADP-ME protein. This peak is followed by a substantial

decrease to phosphorylation levels as low as 3% at ZT4 in the light. These low levels are maintained for the remainder of the day and also during the night (Figure 4B).

Measurements of total NADP-ME activity in extracts of maize leaves indicated a slight but no significant increment of the activity between ZT2 and ZT4 in the light, followed by a constant decline during the rest of the light period (Figure 4C). The activity measured in total leaf extracts is the sum of the activity of all maize NADP-ME isoforms. As the C₄ isoform is the most highly expressed isoform in photosynthetic tissues with respect to the other isoforms (Detarsio et al., 2008; Alvarez et al., 2013), we conclude that the tendencies observed in the profile of measured NADP-ME activity represents the activity of the C₄ isoform.

We next investigated the profile of malate concentration in whole maize leaf extracts during a diurnal cycle (Figure 4D). Similar high malate concentrations were observed during the light period and at ZT16.5 (530 to 670 nmol g⁻¹ fresh weight). These values decreased during the dark period, reaching a value of approx. 340 nmol g⁻¹ fresh weight by the end of the night (Figure 4D).

Localization of Ser419 in the C₄-NADP-ME structure

We recently solved the crystal structures of C₄-NADP-ME of maize (ZmC₄-NADP-ME; pdb-code 5OU5) and sorghum (SbC₄-NADP-ME; pdb-code 6C7N) which both display a homotetrameric assembly composed of two dimers (monomers A and B and monomers C and D, respectively (Figure 5A) (Alvarez et al., 2019). The overall structure of SbC₄-NADP-ME is highly similar to that of ZmC₄-NADP-ME (rmsd 0.81 Å over 1584 Cα atoms), although parts of monomer C and D (residues 360 to 530) are not unambiguously traceable in the electron density. However, SbC₄-NADP-ME monomer A and B are of high quality and clearly show a NADP molecule bound in the active site in each monomer (Figures 5A,B and Supplemental Figure 3A).

In order to elucidate the influence of S419 phosphorylation on the biochemical changes observed in C₄-NADP-ME, we analyzed the cofactor binding site on the crystal structure of SbC₄-NADP-ME in a complex with NADP (pdb-code 6C7N; (Alvarez et al., 2019)). The nicotine amide moiety of NADP interacts the O-atoms of S535, whereas both oxygen atoms of the ribose are hydrogen bonded by the N-atoms of N492 and the side chain O of S491. The phosphate O-atoms show several interactions to the side and main chain atoms of E386, A387 and N331, while the ribose 3'-OH-group is hydrogen bonded to the backbone nitrogen of

A384. Most interactions are present between the 2'-phosphate of the adenosine moiety and the protein, i.e. to main and side chain atoms of K435 and S419 (Figure 5C, Supplemental Table 2). These analyses clearly demonstrate that S419 plays an important role in NADP binding.

Using the structural information, we also found that Ser419 is located on the solvent-exposed surface of the enzyme. It might, therefore, be spatially available for the interaction with a kinase/phosphatase (Supplemental Figure 3B,C). Analysis of known kinase/phosphatase binding motifs associated with the identified phosphorylation site in ZmC₄-NADP-ME indicated the presence of a β -adrenergic receptor kinase (BARK) substrate motif DSKGL (Amanchy et al., 2007) (Supplemental Figure 1).

NADP binding is less favorable to the S419E and phosphorylated variants than to wild type ZmC₄-NADP-ME

In order to assess differences in the binding affinity of NADP to WT ZmC₄-NADP-ME, the variant carrying the phosphomimetic substitution S419E, and the phosphorylated enzyme pS419, we performed ten independent molecular dynamics (MD) simulations of 100 ns length each on the different complexes. As to the protein systems, only moderate structural changes were observed during the MD simulations, in particular in the second halves of the trajectories (Supplemental Figure 5). However, preliminary simulation results showed that, especially for the variant systems, NADP has the tendency to unbind (Supplemental Figure 6). This finding already indicates that the affinity of NADP to the S419E and pS419 variants is lower than to the WT. Furthermore, the higher mobility of the cofactor in the variant's binding site may explain the ~6-fold lower turnover rate (k_{cat}) of the S419E variant compared to WT ZmC₄-ME (Figure 2), as then obtaining a reactive configuration with malate becomes less likely.

As the NADP unbinding would compromise the comparability of results of effective binding energy calculations (Homeyer et al., 2014), the MD simulations were repeated restraining NADP to the respective binding sites (Supplemental Figure 7). Effective binding energies were then computed by the MM-PBSA method (Gohlke and Case, 2004; Miller et al., 2012; Wang et al., 2016), employing the single-trajectory approach on the second halves of the trajectories and considering contributions due to gas-phase energies and solvation free energies but neglecting contributions due to changes in the configurational entropy (see Methods part for a justification). In line with the preliminary simulation results, the effective

energy of binding of NADP towards the S419E and pS419 variants is significantly larger compared to that of NADP binding to the WT (Figure 6A), indicating that binding to the two variants is much less favorable than to the WT. This finding agrees with the experimental observation that no saturation concentration of NADP could be reached during biochemical characterization of the S419E variant (Figure 2).

Furthermore, a structural decomposition on a per-residue level of the terms contributing to the effective binding energy revealed only minor differences between the variants and the WT in the van der Waals interactions and non-polar contributions of the solvation free energy (Figure 6B). By contrast, the variants' lower binding affinity to NADP is caused by an increase in the electrostatic energy, which is only partially compensated for by a more favorable polar solvation (Figure 6B). These energetic changes coincide on the structural level with a loss of the hydrogen bond between S419 and the 2'-phosphate of NADP in WT and charge repulsion of the negatively charged opposing groups in the variants (Figure 6C).

DISCUSSION

ZmC₄-NADP-ME is phosphorylated *in vivo* at a serine that is directly involved in NADP binding

Through mass spectrometry, we identified a single phosphorylation event of ZmC₄-NADP-ME at S419 in maize leaves. Phosphorylation levels increase after the light is turned on, with a peak at ZT2. Through mutational analysis and biochemical characterization of ZmC₄-NADP-ME WT and enzyme variants, we demonstrate that the phosphomimetic mutant S419E has highly reduced affinity to the cofactor NADP as well as lower catalytic activity compared to the non-phosphorylated enzyme. S419E presented a low residual activity at the stromal pH in the light, indicating that a negative charge at this site of ZmC₄-NADP-ME drastically decreases its activity. Analysis of the crystal structures of Zm- and SbC₄-NADP-ME indicated that S419 is located on the surface of the enzyme, which makes it spatially available for the interaction with a kinase/phosphatase. Moreover, analysis of the crystal structure indicates that S419 is directly involved in NADP binding. Molecular dynamics simulations and free energy calculations revealed that the effective energy for NADP binding is less favorable in the phosphomimetic (S419E) and the phosphorylated variants than in the WT. Additionally, the higher mobility of the cofactor in the variants' binding sites likely lead to binding positions of NADP that are not suitable for a reaction with the substrate. This explains the highly reduced affinity of S419E for NADP and suggests that phosphorylation of S419

modulates the activity of ZmC₄-NADP-ME by creating unfavorable binding conditions for its cofactor.

Furthermore, we observed conservation of the S419, as well as of a β -adrenergic receptor kinase (BARK) substrate motif (DSKGL), which includes the S419, among plastidic photosynthetic and non-photosynthetic NADP-ME isoforms within the Poaceae (Supplemental Figure 1). This pattern of sequences conservation and the fact that we found that only the C₄-NADP-ME isoform is phosphorylated in leaves suggests that the use of reversible phosphorylation of S419 to control the activity of NADP-ME in photosynthetic tissues appeared with the evolution of the C₄ pathway and should involve a BSC chloroplastic kinase, which might have been co-opted for this specific task.

Phosphorylation of ZmC₄-NADP-ME at S419 is a mechanism to fine-tune the enzymatic activity

The operation of the C₄ pathway requires a deep synchronization between mesophyll cells and BSCs during a day-night cycle (Bailey et al., 2007). To efficiently concentrate CO₂ in BSC chloroplasts, coordination of C₄-NADP-ME decarboxylation and Rubisco carboxylation rates is imperative as this will prevent loss of the CO₂ released by C₄-NADP-ME. During the night, when the photosynthetic pathway is inactive, C₄-NADP-ME activity is regulated through two processes: on the one hand, the enzyme is allosterically inhibited by malate, on the other hand, it partially loses its active quaternary oligomerization state (Iglesias and Andreo, 1990; Saigo et al., 2004; Saigo et al., 2013; Alvarez et al., 2019). Production of malate in mesophyll cells and its decarboxylation in BSCs must also be coordinated to accommodate the large and rapid flux changes that occur during the day. We show that malate concentrations in total maize leaf extracts tend to decrease during the night and increase after the onset of light, quickly reaching values that are maintained during the whole light period. This profile of malate concentrations in maize leaves is different from that described in some C₃ plants such as *Arabidopsis*, where malate functions as a transient carbon storage molecule; in these C₃ plants, malate concentrations increase continuously during the day and decrease during the night (Fahnenstich et al., 2007; Zell et al., 2010). The differences of malate levels between the end of the night and the day in maize leaves is likely mostly due to the activity of the C₄ concentrating mechanism.

Apart from the contribution of malate decarboxylation, respiration of CO₂ in BSC also contributes to the carbon concentrating mechanism (Bellasio and Griffiths, 2014a;b). It is

possible that under our conditions of growth, at ZT2, respiration in BSC is high enough to make an important contribution to CO₂ fixation by Rubisco. In such conditions, decarboxylation of malate by a fully active C₄-NADP-ME could lead to high CO₂ levels in BSC resulting in increased wasteful leakage of CO₂ to mesophyll cells (Kromdijk et al., 2014). The naturally evolved efficiency of C₄ photosynthesis requires the tight regulation of CO₂ supply to Rubisco within the BSCs in order to minimize leakiness and associated energy costs (Furbank et al., 1990). As a consequence, a decrease in C₄-NADP-ME activity would avoid extra waste of ATP for the operation of the C₄ carbon concentration mechanism. The cooperation of the PEPC-driven carbon concentration and respiration-driven mechanisms require plasticity, as the extent in which each of these mechanisms is involved may depend on changes of environmental conditions. Phosphorylation of ZmC₄-NADP-ME at S419 during the first hours in the light might thus be a mechanism to fine-tune the enzymatic activity, helping to adjust the use of malate in the BSC to avoid CO₂ leakiness and energy waste. The functional importance of this molecular switch is probably linked to environmental changes such as the photosynthetically active radiation, shade by canopy, and water availability environmental conditions.

Regulation of C₄-NADP-ME activity via phosphorylation may also be important in the context of biochemical flexibility of the C₄ pathway. Aspartate movement to maize BSCs carries approximately 4% of the CO₂ for the C₃ cycle. Approximately 40% of this C₄-acid is decarboxylated by PEPCCK (Arrivault et al., 2017); the remainder aspartate is converted to malate and decarboxylated through NAD(P)-ME. There exists a flexible partitioning of C₄ decarboxylation activity between NADP-ME and PEPCCK in response to environmental conditions in maize (Furbank, 2011; Sharwood et al., 2014). In this context, we hypothesize that phosphorylation of ZmC₄-NADP-ME at Ser419 might be a mechanism to rapidly decrease the activity of C₄-NADP-ME and with this to switch the use of malate to aspartate according to the metabolic demands imposed by a constantly changing environment.

A currently highly popular goal within the plant scientific community is the improvement of photosynthesis by introducing the C₄ concentrating mechanism into C₃ plants. Our results are of great importance in this context, as they describe a novel regulation of a C₄ enzyme critical for an efficient C₄ cycle. Further work should be now directed towards elucidating the environmental conditions in which this regulatory mechanism is required, and if it effectively contributes to the coordination of the C₄ and C₃ cycles and hence to the metabolic flexibility of C₄ plants in response to fluctuating environments.

METHODS

Plant growth conditions

For the mass spectrometry analysis, *Zea mays* (B73) seeds were germinated for 48h in the dark at 28°C and then transferred to a soil:turf (1:1) mixture. Plants were grown under the following conditions: light/dark cycles of 16/8 h at 28°C/26°C; light intensity of 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For metabolite analysis and determination of total NADP-ME enzyme activity, plants were grown in Floraton I soil in a Conviron® E15 plant growth chamber under following conditions: light/dark cycles of 16/8 h at 25°C/20°C; light intensity of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Sample preparation for LC-MS analysis

One-third (from leaf tip) of maize leaves (3rd leaf) from 12-day-old seedlings were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Maize leaves were collected at ZT (Zeitgeber time) 0.5, 2, 4, 15.5, 16.5, 20, and 23.5; where ZT0 = light on and ZT16 = light off. At each time point, 4 pools of 5 maize leaves were collected. Each pool constitutes one biological replicate. Frozen maize leaves were ground with mortar and pestle in liquid nitrogen until a fine powder was obtained. Protein extraction was performed directly in Lysis Buffer as previously described (Luis et al., 2016). For the total protein analysis, protein extracts were loaded onto 4-12% bis-tris polyacrylamide gels (NuPAGE, ThermoFisher Scientific, Waltham, MA USA) and electrophoresed for a short time (10-15 min). The whole bands containing the entire protein extract (2-3 cm long) were excised and in-gel digested with trypsin followed by peptide desalt with C18 tips (Thermo Scientific, Rockford IL USA). For the phosphopeptide analysis of NADP-ME, protein extracts were loaded onto the same kind of gels (4-12% bis-tris polyacrylamide gels, NuPAGE, ThermoFisher Scientific, Waltham, MA USA), electrophoresed at a constant voltage of 25V for 10 min and then for 150V until the Bromophenol Blue reached the bottom of the gel. Then, the gel fractions containing the NADP-ME (ranging from just below the 63 kDa to just after the 75 kDa) were excised, in-gel digested with trypsin and further lyophilized to dryness. Vacuum dried peptides were first desalted using C18 tips (Thermo Scientific, Rockford IL USA) before being subjected to phosphopeptide enrichment using TiO₂ beads (TiO₂ Mag Sepharose, GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions.

LC-MS using an AB Sciex TripleTOF 6600

Samples were analyzed using a TripleTOF 6600 mass spectrometer (AB Sciex, Framingham, MA USA), coupled to a nanoLC Eksigent 425 system (AB Sciex, Framingham, MA USA). RP-HPLC was performed in a trap and elution configuration using a nano cHiPLC trap column (Eksigent ChromXP C18-CL, 3 μm particle size, 120 Å pore size, 0.5 mm x 200 μm I.D., AB Sciex, Framingham, MA USA) and an analytical column (Eksigent ChromXP C18-CL, 3 μm particle size, 120 Å pore size, 15 cm $\times$ 75 μm I.D., AB Sciex, Framingham, MA USA). Samples were loaded into the trap column at a flow rate of 2 $\mu\text{L min}^{-1}$ for 10 min using 100% solvent A (0.1% formic acid in water) and eluted at a flow rate of 300 nL/min using a stepwise gradient: 0-1min, 4.5% B (0.1% formic acid in acetonitrile); 1-91min, 29.7% B; 91-93, 79.2% B; 93-108min, 79.2% B; 108-110min, 4.5% B; 110-127min, 4.5% B. Samples were run in information-dependent acquisition (IDA) mode to perform peptide and protein identification in order to generate a spectral library for the SWATH quantification. This spectral library was created by combining all the IDA.wiff files in unison using ProteinPilot 5.0 (AB Sciex). Database search was performed using the Paragon algorithm, which is embedded in ProteinPilot software, v. 5.0 (AB Sciex, Framingham, MA USA). A Paragon search method was created with the following settings: sample type, identification; cysteine alkylation, acrylamide; digestion, trypsin; instrument, tripleTOF 6600; special factors, phosphorylation emphasis and gel-based ID; species, none; ID focus, biological modifications; search effort, thorough; detected protein threshold, >0.05. The searches were conducted using a UniProt database containing all reviewed protein sequences for the species *Zea mays*. A false discovery rate threshold was set to below 1%.

As for the SWATH-MS analysis, four replicates from each one of the seven time points collected were analyzed by LC-MS using the setup described for the IDA runs. SWATH-MS data were acquired with a standard SWATH acquisition method, using a set of 32 overlapping SWATH windows covering the precursor mass range of 400–1200 m/z. For each SWATH window, the Q1 transmission width was 26 m/z (containing 1 m/z for the window overlap). At the beginning of each cycle, a 50 ms survey scan (350-1250 m/z) was acquired for instrument calibration, and the subsequent SWATH windows were collected from 100 to 1800 m/z for 60 ms, resulting in a cycle time of 2.02 s. Data processing was performed using SWATH processing plug-in for PeakView 2.2 (AB Sciex, Framingham, MA USA). Briefly, peptides were confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide. Target fragment ions, up to 5, were automatically selected as previously described (Lambert et al., 2013) criteria: (i) fragment ions for a selected peptide were ranked

according to ion intensity; (ii) ions higher in m/z than the y4 fragment ion for each selected peptide were ranked highest; (iii) ions within the SWATH isolation window were excluded from selection; (iv) if insufficient target ions were found, ions lower than y4 but outside of the SWATH window were chosen; (v) if there were still insufficient ions, then fragment ions from within the SWATH window region were chosen. Peak group confidence threshold was determined based on an FDR analysis using the target-decoy approach, and 1% extraction FDR threshold was used for all the analyses. Peptides that met the 1% FDR threshold in all the four replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram window of 12 min. ZmC₄-NADP-ME protein levels were estimated by summing the areas of all the transitions from all the peptides for a given protein and normalized to the total area (Collins et al., 2013). As for the ZmC₄-NADP-ME phosphopeptide (acVWLVDpSK) levels, they were estimated by summing the areas of the transitions obtained for this phosphopeptide and normalized to the sum of the area of all the transitions from all the peptides within the same region (that include S419) for each of the time points.

Site-directed mutagenesis of ZmC₄-NADP-ME

As template for the PCR reaction (first step of the site-directed mutagenesis), pET32::WT NADP-ME expression construct (Detarsio et al., 2003) was used. Point mutations were introduced into the protein-coding region by performing site-directed mutagenesis with the following “back-to-back” primers: 5'-TCGTCCACCAGCCAAAC-3' and 5'-AAAGGGTTTGATTGTTGACTCTCG-3' (“for” and “rev” primers for the introduction of the S419E mutation; resulting construct has been termed pET32a::S419E NADP-ME) and 5'-GCGTCCACCAGCCAAACC-3' as the “for” primer and the same “rev” primer as used in case of the first mutagenesis for the introduction of the S419A mutation (resulting construct has been termed pET32a::S419A-NADP-ME). All primers used for the mutagenesis procedure were phosphorylated at the 5'-terminus.

Site-directed mutagenesis has been performed as follows: usually, 5 similar 50 μ L PCR reactions were performed simultaneously. Obtained DNA was pulled together and (after digestion from the agarose gel, if needed) subjected to the self-ligation by using the T4 DNA ligase (ThermoFisher Scientific). Self-ligation was performed by incubation of the mix at 22 °C for 1 h. Subsequently, inactivation of the ligase by incubation of reaction mix at 65 °C for 10 min has been performed. In order to destroy the methylated WT (sample) DNA, the

reaction mix after self-ligation has been subjected to the digestion at 37°C for 2 h by the DpnI (Thermo Scientific). After digestion, the DpnI has been inactivated by incubation at 80°C for 5 min. Concluding, obtained constructs were used for the transformation of the chemically-competent *E. coli* DH5α cells. After plasmid preparation, the success of the site-directed mutagenesis procedure has been confirmed by sequencing with three primers covering the whole length of the protein-coding region.

Expression and purification of recombinant ZmC₄-NADP-ME

The WT and two obtained mutated pET32::ME constructs were used for the transformation of the *E. coli* strain Rosetta (DE3) (Novagen). For the heterologous protein production, transformed cells were grown in 400 mL LB medium at 37 °C and agitation of 110 rpm in the presence of 100 µg mL⁻¹ ampicillin and 34 µg mL⁻¹ chloramphenicol until an OD₆₀₀ of 0.6 - 0.8 was reached. To induce protein expression, final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and cells were grown for additional 20h under the same conditions. Harvest of the cells was performed by centrifugation at 4,000 *g* for 15 min. Obtained pellets were transferred into 50 mL Falcon tubes and were stored at -20°C until further usage. For the protein extraction, pellets were thawed on ice and resuspended in 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl, 5 mM imidazole, 2 mM phenylmethanesulfonyl fluoride (PMSF) and a spatula-tip amount of lysozyme, sonicated and centrifuged at 14,000 *g* for 20 min at 4°C to remove cell debris. Obtained supernatant has been deployed for protein purification using gravity-flow immobilized metal ion chromatography on nickel-nitrilotriacetic acid agarose (Ni-NTA Agarose, Qiagen). Prior to the supernatant loading, the column was equilibrated with 20 mM Tris-HCl buffer containing 500 mM NaCl and 5 mM imidazole. After the supernatant was loaded, columns were washed in four steps with 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl buffers containing increasing concentrations of imidazole (5, 30, 40 and 50 mM) in order to isolate the His-tagged NADP-MEs. Elution was performed using four times 500 µL of 20 mM Tris-HCl buffer containing 500 mM NaCl and 300 mM imidazole. Eluted protein from the first elution fraction has been used for further kinetic measurements.

For all constructs used in this work, the calculated molecular mass of the expressed protein corresponded to the expected molecular mass of the fusion protein as follows: mature ZmC₄-NADP-ME (63.4 kDa) plus 17.3 kDa encoded by the expression vector.

Protein quantification, gel electrophoresis and immunological detection

Protein concentration was determined using the PierceTM BCA Protein Assay Kit (Thermo Fischer Scientific). SDS-PAGE was performed using 12% (w/v) polyacrylamide gels according to Laemmli (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue or electroblotted onto a nitrocellulose membrane (Thermo Scientific) for immunological detection. The membranes were incubated for at least 1 h with the 1:7500 dilution of Anti-His-Tag antibody coupled to horseradish peroxidase (Anti-His-HRP; Miltenyi Biotec). After several washing step the membranes were incubated with a 1:2500 dilution of the Goat Anti-Rabbit IgG Antibody HRP-conjugate (Merck). The chemiluminescence signal was detected with ImmobilonTM Western Chemiluminescent HRP Substrate (Merck Millipore) with subsequent visualization on an LAS-4000 Mini Luminescent Image Analyzer (GE Healthcare Life Sciences formerly Fuji).

Determination of kinetic parameters

NADP-ME activity was determined using a Synergy HT Biotek[®] Plate Reader system by measuring the formation of NADPH at 340 nm (extinction coefficient = $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) at room temperature. The standard assay medium contained 0.5 mM NADP⁺, 10 mM MgCl₂, 4 mM L-malate, 50 mM Tris-HCl (pH 8.0) and 0.1 - 0.8 µg enzyme per well in a final volume of 200 µL. The dependence of the activity with the pH of the medium was determined with the standard assay medium using different buffer systems: 50 mM MES (pH 5.5-6.5), 50 mM Tricine-Mops (pH 7.0-7.5), and 50 mM Tris-HCl (pH 7.5-9.0). The Michaelis (K_m in case of WT and S419A) and apparent Michaelis (K_{mapp} in case of S419E) constant of the substrate were determined by varying the concentration of one substrate while keeping the other components constant at fixed concentrations as described for the standard assay medium. NADP concentrations were varied between 2 and 150 µM in case of WT, between 10 and 800 µM in case of S419A and between 10 and 5 mM in case of S419E. Malate concentrations were varied between 0.001 and 8 mM in case of WT and S419A and between 0.1 and 10 mM in case of the S419E. As S419 was not saturated with NADP at least until 5 mM NADP, the K_m NADP for S419E could not be estimated. Measurements of enzymatic activity using high NADP concentrations are technically impaired by the high absorption of this compound; thus, other kinetic parameters of S419E, apparent parameters (k_{catapp} and K_{mapp} malate) were estimated using 0.5 mM NADP. All kinetic parameters were calculated using at least three

biological replicates and adjusted to non-linear regression. Data were fitted with the Prism 6 (GraphPad Software).

Circular Dichroism

All recombinant versions of ZmC₄-NADP-ME were subjected to circular dichroism (CD) analysis in 20 mM NaP_i, pH 8.0 or 7.0; 5 mM MgCl₂. Protein concentration was determined at 280 nm using a Jasco V-650 spectrophotometer. The protein concentration (c , g L⁻¹) was calculated by the equation $c = A_{280} \cdot Mw / (\epsilon \cdot d)$, in which ϵ represents the molar extinction coefficient at 280 nm (76250 L mol⁻¹ cm⁻¹), d is the cell path (1 cm) and Mw is molecular weight (80.7 kDa for all three proteins).

CD spectra between 240 and 190 nm were obtained averaging 10 repetitive scans in a Jasco J-810 spectropolarimeter. Mean residue ellipticity ($[\Theta]$, deg cm² dmol⁻¹) was obtained by the equation $[\Theta] = MRW \cdot \Theta / (10 \cdot c \cdot d)$, where MRW (the mean amino acid residue weight) was calculated as relation of the protein's molecular weight (Mw) to the number of peptide bonds ($N - 1$; where N is the number of amino acids in the chain), d is the cell path (0.1 cm), c is the protein concentration and Θ is the observed ellipticity in millidegrees (machine units). Determination of the protein secondary structure contents was performed using the CONTIN-LL method (van Stokkum et al., 1990) and the reference dataset 4 (Sreerama and Woody, 2000) at the Dichroweb online server (<http://dichroweb.cryst.bbk.ac.uk>) (Whitmore and Wallace). Prism Software (<http://www.graphpad.com/scientific-software/prism/>) was used for visualization of the deconvolution by Dichroweb.

Analytical Ultracentrifugation

Sedimentation velocity experiments were carried out using a Beckman Optima XL-A Analytical Ultracentrifuge. All recombinant versions of ZmC₄-NADP-ME were assayed in 10 mM Tris-HCl buffer (pH 7.0 and 8.0) with addition of 5 mM MgCl₂. Buffer exchange procedure was performed using the Amicon Ultra 0.5 mL centrifugal units with nominal molecular weight limit of 50 kDa (Merck) and final protein concentration was adjusted to 0.6 mg mL⁻¹. Samples (230 µg) and corresponding buffer solutions (400 µL) were loaded into aluminium double sector centerpieces separately and built up in a Beckman An-50 Ti rotor. Experiments were performed at 20 °C and a rotor speed of 35,000 rpm. Protein samples were monitored by UV absorbance at 280 nm in a continuous mode with a radial resolution of

0.003 cm. In time intervals of about 2 min scans of the radial concentration profile were collected until the protein was fully sedimented. Data were analysed using the c(s) model in the software package SEDFIT (Schuck and Rossmanith, 2000). For data analysis, a resolution of 0.1 S with a confidence level (F-ratio) of 0.95 was chosen for the appropriate s-value range within 0 to 30.0 S. Density and viscosity of the solvent had been calculated with the software Sednterp from tabulated values; $\rho = 0.99885 \text{ g cm}^{-3}$ and $\eta = 0.01006 \text{ g cm}^{-1}\text{s}^{-1}$. The protein partial specific volume $\bar{v}$ applied for WT and mutants was $0.7387 \text{ cm}^3 \text{ g}^{-1}$. Sedimentation coefficients are reported as $s_{20, w}$ values, i.e. normalized to 20°C and water as a solvent. Graphic output was generated by Gussi (Version 1.2.1) (Brautigam, 2015) and the final sedimentation coefficient distribution was normalized based on the maximum peak height.

NADP-ME activity measurements in extracts of maize leaves

Maize leaves were collected at ZT (Zeitgeber time) 0.5, 2, 4, 8, 15.5, 16.5, 20 and 23.5; where ZT0 = light on and ZT16 = light off. One-third (from leaf tip) of maize leaves (3rd and 4th leaf) from 12-day-old seedlings were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Soluble protein extracts were prepared by homogenizing 0.25 g of frozen maize leaves in 500 μL of ice-cold extraction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 0.1 mM EDTA, 10% (v/v) glycerol, 2 mM DTT, 1 mM PMSF. The homogenate was clarified by centrifugation at 20,000 g during 20 min at 4°C. Protein concentration of the protein extract was determined using the Amido Black assay (Schaffner and Weissmann, 1973). NADP-ME activity was measured in medium containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 0.5 mM NADP, 10 mM L-malate and 1.5 - 2.2 μg protein extract in a final volume of 0.2 mL. The reaction was started by addition of NADP^+ . Measurements were conducted using three independent replicates.

Gas Chromatography - Mass Spectrometry (GC-MS) analysis

Frozen maize leaves were ground in liquid nitrogen until a fine powder was obtained. Ground material (around 50 mg) was extracted in methanol:chloroform:water (5:2:2) with ribitol for internal standardization as described by Lee and Fiehn (Lee and Fiehn, 2008).

An aliquot of the supernatant (150 μL) was dried under vacuum, and the residue was derivatized for 120 min at 37°C (in 60 μL of 30 mg mL^{-1} methoxyamine hydrochloride in pyridine) followed by a 30 min treatment at 37°C with 120 μL of MSTFA. The GC-MS

system used was a gas chromatograph coupled to a time-of-flight mass spectrometer (Leco Pegasus HT TOF-MS). An auto sampler Gerstel Multi-Purpose system injected the samples. Helium was used as carrier gas at a constant flow rate of 2 mL s⁻¹ and gas chromatography was performed on a 30 m DB-35 column. The injection temperature was 230°C and the transfer line and ion source were set to 250°C. The initial temperature of the oven (85°C) increased at a rate of 15°C/min up to a final temperature of 360°C. After a solvent delay of 180 s mass spectra were recorded at 20 scans s⁻¹ with m/z 70-600 scanning range. Chromatograms and mass spectra were evaluated by using Chroma TOF 4.5 (Leco) and TagFinder 4.2 software (Ishizaki et al., 2005; Lisec et al., 2006).

Generation of the complex structures

For the complex preparation we used the monomeric *apo* structure of ZmC₄-NADP-ME (aa 132-628, PDB ID 5OU5). Based on the structural alignment with ME from pigeon liver (PDB ID 1GQ2) and SbC₄-NADP-ME (PDB ID 6C7N), a Mg²⁺ ion and NADP were placed into the active site of ZmC₄-ME. Subsequently, the complex was protonated using the Structure Preparation Tool and Protonate3D implemented in MOE (2019) at pH 8.0. The structures of the enzyme carrying a substitution or the phosphorylated residue were generated by replacing S419 either by glutamate or phosphoserine in LEaP (Case et al., 2005).

Molecular dynamics simulations of NADP/ZmME complexes

System setup

The following procedures were performed using the Amber18 software package (Case et al., 2018). We used tLEaP to solvate each of the complexes in a periodic box of TIP3P water (Jorgensen et al., 1983), in such a way that the distance between the edges of the box and a solute atom is at least 11 Å. The system was charge-neutralized by replacing random water molecules by sodium ions. The ff14SB force field (Hornak et al., 2006) was used for parameterizing the protein, and Mg²⁺ parameters were taken from the 12-6 LJ set of ion parameters by Li and Merz (Li et al., 2013). The phosphoserine parameters were taken from phosaa10 (Homeyer et al., 2006; Steinbrecher et al., 2012) and modified to be compatible with ff14SB. Partial charges according to the RESP procedure (Bayly et al., 1993) for NADP were derived from the R.E.DD.B. (Dupradeau et al., 2008) project F-91, while for bond parameters the GAFF force field (Wang et al., 2004) was used. In order to remove initial clashes due to

the introduction of the larger side chains replacing S419, for the modified systems an energy minimization of this residue was performed with pmemd (Le Grand et al., 2013;Case et al., 2018) prior to the thermalization.

Thermalization

All simulations were carried out using the GPU version of pmemd. The Langevin thermostat (Pastor et al., 1988) was used for temperature control with a collision frequency of $\gamma = 2.0 \text{ ps}^{-1}$. For treatment of long-range electrostatic interactions the Particle Mesh Ewald method (Berendsen et al., 1984) was used with a cutoff of 8.0 Å. The SHAKE algorithm (Ryckaert et al., 1977) was used to constrain bond lengths involving hydrogen atoms, and hydrogen mass repartitioning (Hopkins et al., 2015) was used to allow for simulation steps of 4 fs.

Initially, an energy minimization was performed independently on solvent and solute atoms for 3,000 steps each with the steepest decent algorithm, followed by 2,000 steps with the conjugate gradient algorithm. Thereafter, all atoms were energy-minimized the same way. Then, the systems were heated to 300 K at a constant heating rate over 20 ps of NVT-MD and simulated for further 5 ps at 300 K. For density adaptation, 75 ps of NPT-MD were performed with a pressure relaxation time of $\tau_p = 1.0 \text{ ps}$, followed by 1,700 ps of NPT-MD with the same restraints and $\tau_p = 2.0 \text{ ps}$. For all these steps, positional restraints of $2.0 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$ were applied to the enzyme's C_α -atoms and NADP. Ten further iterations of minimization were performed on the system with iteratively decreasing restraints on the solute atoms (force constants ranging from 2 to 0 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$), with 500 steps of steepest decent minimization and 500 steps of conjugate gradient minimization each. Finally, the systems were heated again to 300 K over 100 ps of NVT-MD, followed by 3,100 ps NVT-MD. Again, positional restraints with a force constant of $2.0 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$ were applied to the enzyme's C_α -atoms and NADP.

Production

For each of the three systems ten individual NVT-MD simulations of 100 ns length were performed. Since especially in the mutated systems the binding pose of NADP was not stable, and large conformational rearrangements would compromise the effective binding energy calculations, we restrained NADP within the binding site in all three systems by a distance restraint between the C_α of S435 and the nicotinamide of NADP using a one-sided harmonic potential with a force constant of $2.0 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$. Coordinates for post-processing and further analysis were extracted every 100 ps. For geometric analyses, CPPTRAJ (Roe and Cheatham, 2013) was used.

Calculation of effective binding energies

The calculation was performed using MMPBSA.py from AmberTools18 (Miller et al., 2012;Case et al., 2018). For all systems the change of the effective energy due to binding of NADP was calculated using the single-trajectory MM-PBSA approach on the last 50 ns of trajectory (Gohlke and Case, 2004;Wang et al., 2016). All counter ions and water molecules were stripped from the trajectory, and the sum of electrostatic and van der Waals energies was calculated for each snapshot using the above-mentioned force fields (internal energies cancel in this approach). The polar part of the solvation free energy was calculated at the level of Poisson-Boltzmann theory, with the dielectric constants set to 80 for the solvent and 4 for the solute and an ionic strength of 300 mM. The nonpolar part of the solvation free energy was calculated by separating the attractive and repulsive terms, as shown by Tan *et al.* (Tan et al., 2007), using as a surface tension $0.0378 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. Previous studies have shown that inclusion of configurational entropy is crucial for calculating *absolute* binding free energies (Hou et al., 2011;Genheden and Ryde, 2015;Ben-Shalom et al., 2017). In the present study, however, we were interested in *relative* binding free energies and, thus, decided to neglect contributions due to changes in the configurational entropy of the ligand or the receptor upon complex formation in order to avoid introducing additional uncertainty in the computations (Gohlke and Case, 2004;Weis et al., 2006;Hou et al., 2011).

SUPPLEMENTAL DATA

Supplemental Figure 1. Alignment of plastidic C₄- and nonC₄-NADP-ME sequences in Poaceae showing conservation of S419 and the BARK substrate motif (DSKGL).

Supplemental Figure 2. SDS-PAGE stained with Coomassie-Blue (lines 1-4) and analysed by immunoblot (line 5) of protein fractions during the isolation of recombinant ZmC₄-NADP-ME variants.

Supplemental Figure 3. Representation of the bound cofactor NADP in SbC₄-NADP-ME chain B.

Supplemental Figure 4. Comparison of the CD spectra of ZmC₄-NADP-ME WT obtained at pH 7.0 and 8.0.

Supplemental Figure 5. Root mean square deviations of the enzyme's C_α atoms.

Supplemental Figure 6. Root mean square deviations of NADP in the binding site of ZmC₄-NADP-ME.

Supplemental Figure 7. Root mean square deviations of NADP in the binding site of ZmC₄-NADP-ME after restraining NADP to the respective binding sites.

Supplemental Table 1. Secondary structure contents of ZmC₄-NADP-ME WT, S419A and S419E estimated from the CD-spectra obtained at pH of 7.0 and 8.0.

Supplemental Table 2. Polar interactions/hydrogen bonds between monomer A of SbC₄-NADP-ME and the cofactor NADP with their distances.

Supplemental Table 3. P-values for figure 4.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.G.M.; Methodology, V.G.M. and I.A.A.; Investigation, A.B., B.M.A., A.H., C.E.A., I.M.L., C.D., S.A., D.B., H.G.; Writing – Original Draft, V.G.M., I.A.A., A.B., A.H., C.E.A., M.F.D., L.N-S., D.B., H.G.; Funding Acquisition, V.G.M. and I.A.A.; Resources, V.G.M., I.A.A., M.F.D, A.F; Supervision, V.G.M., I.A.A., M.F.D.

FIGURE LEGENDS

Figure 1. TripleTOF 6600 tandem MS data of the phosphopeptide acVWLVDpSK of ZmC₄-NADP-ME. The detected b (N-terminal, in red) and y (C-terminal, in blue) fragment ions are labeled in the spectrum. Ac denotes N-terminus acetylation and pS denotes phosphorylated serine. Precursor charge: +2; monoisotopic m/z: 484.7265 Da (-1.60 mmu/-3.30 ppm). Confidence (ProteinPilot): 96.4% (Confidence threshold for FDR≤1% was equal to 93.7%).

Figure 2. Biochemical characterization of recombinant ZmC₄-NADP-ME versions. A, Dependence of the activity, measured using 0.5 mM NADP and 4 mM malate, on the pH of the assay. The values represent the mean ± standard error of at least four independent enzyme preparations, each measured in triplicate. **B,** Kinetic parameters at pH 8.0. Kinetic data were best fitted by nonlinear regression analysis. The values represent the mean ± standard error of at least three independent enzyme preparations, each measured in triplicate.

Figure 3. Exploration of the secondary structure organization and quaternary composition of recombinant ZmC₄-NADP-ME versions. A and B, CD spectra of ZmC₄-NADP-ME WT and the two mutated versions obtained at pH 7.0 (A) and 8.0 (B). Ten accumulations each were collected from 190 to 260 nm for 0.16 mg mL⁻¹ enzyme, at 20°C. Each graph is showing the reconstructed curves obtained by applying CONTIN/LL algorithm for data evaluation as provided by the Dichroweb server. **C and D,** Continuous sedimentation coefficient distribution of ZmC₄-NADP-ME WT and the mutated versions at pH 7.0 (C) and 8.0 (D). Data was fitted with the ls-g*(s) model in the software package SEDFIT.

Figure 4. Measurements of different parameters in extracts of maize leaves during a diurnal cycle. A, Quantification of total ZmC₄-NADP-ME protein by SWATH-MS. **B,** Quantification of the phosphopeptide VWLVDpSK, corresponding to the phosphorylation of

ZmC₄-NADP-ME at S419. **C**, Total NADP-ME activity measured with 1.5 - 2.0 µg protein extracts. **D**, Malate concentration assayed by GC-MS analysis. Values represent mean ± standard error of four (A and B) or three (C and D) biological replicates. The dark period is highlighted in grey. Statistical analyses were performed against the first time point in the night (ZT16.5). Letters indicate that the value is significantly different from the reference value evaluated by two-tailed t-test at a, 0.05; b, 0.01; c, 0.001 levels (the precise p-values are shown in Supplementary Table 3). ZT, Zeitgeber Time.

Figure 5. SbC₄-NADP-ME crystal structure and amino acids involved in cofactor binding. **A**, Cartoon and ribbon representation of SbC₄-NADP-ME. Monomer A (red) and B (blue) with bound cofactor NADP (colored sticks). Large parts of chain C and D are not well resolved by electron density; therefore, we depicted these chains as gray ribbons only to clarify the tetrameric assembly. **B**, Monomer A from SbC₄-NADP-ME as surface representation with the bound cofactor NADP (represented by sticks). **C**, Residues involved in cofactor binding are depicted as sticks with labels (distances are omitted for clarity and are listed in Table S2).

Figure 6: Effective free energies of binding of NADP. **A**, Effective binding energies computed according to the MM-PBSA approach for WT ZmC₄-NADP-ME, the phosphomimetic variant (S419E), and the phosphorylated enzyme (pS419). The error bars indicate the standard error of the mean over 10 individual trajectories. Statistical significance was calculated according to Student's *t*-test. **B**, The contribution of the different energy terms, computed as variant's energy term minus the respective energy term of the WT enzyme ($\Delta\Delta G_{\text{eff.}} = \Delta G_{\text{eff}}(\text{Var}) - \Delta G_{\text{eff}}(\text{WT})$). Positive terms indicate more favorable binding to the WT enzyme, negative ones to the variant. The error bars show the standard error of the mean of the differences in the individual terms over the trajectories. **C**, Molecular representation of NADP bound to the WT ZmC₄-NADP-ME, the phosphomimetic variant (S419E), and the phosphorylated enzyme (pS419). Interactions with residue 419 are highlighted, showing hydrogen bonds for the WT enzyme with green dashed lines, and charge-charge repulsion for the phosphomimetic and phosphorylated variants with red dashed lines.

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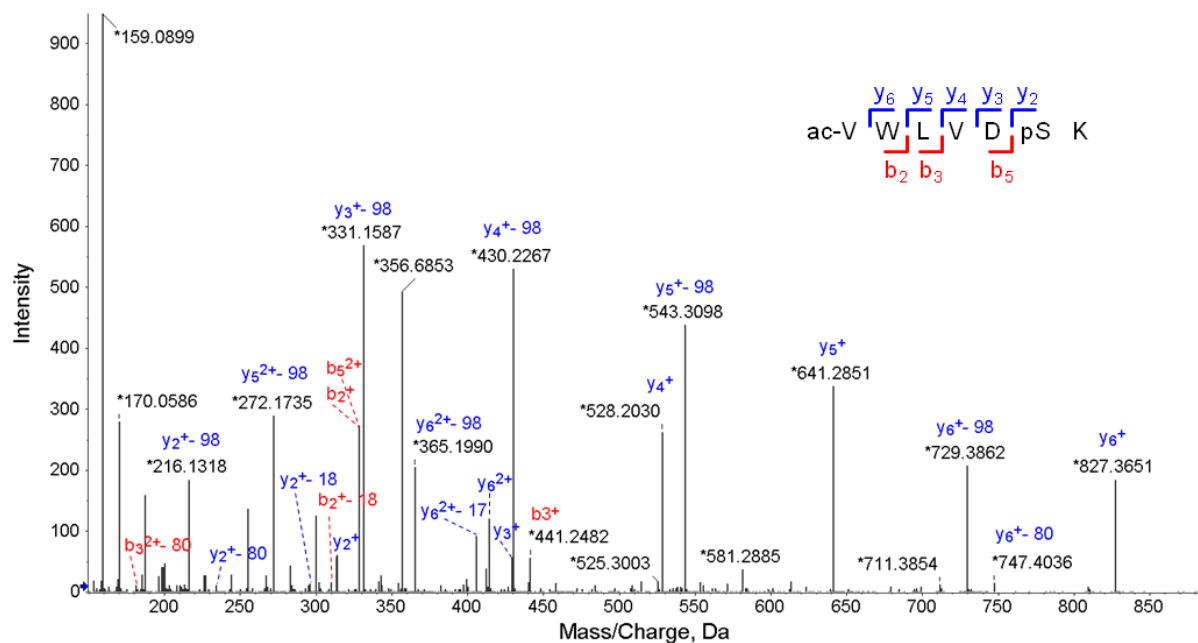


Figure 1. TripleTOF 6600 tandem MS data of the phosphopeptide acVWLVDpSK of ZmC₄-NADP-ME. The detected b (N-terminal, in red) and y (C-terminal, in blue) fragment ions are labeled in the spectrum. Ac denotes N-terminus acetylation and pS denotes phosphorylated serine. Precursor charge: +2; monoisotopic m/z: 484.7265 Da (-1.60 mmu/-3.30 ppm). Confidence (ProteinPilot): 96.4% (Confidence threshold for FDR≤1% was equal to 93.7%).

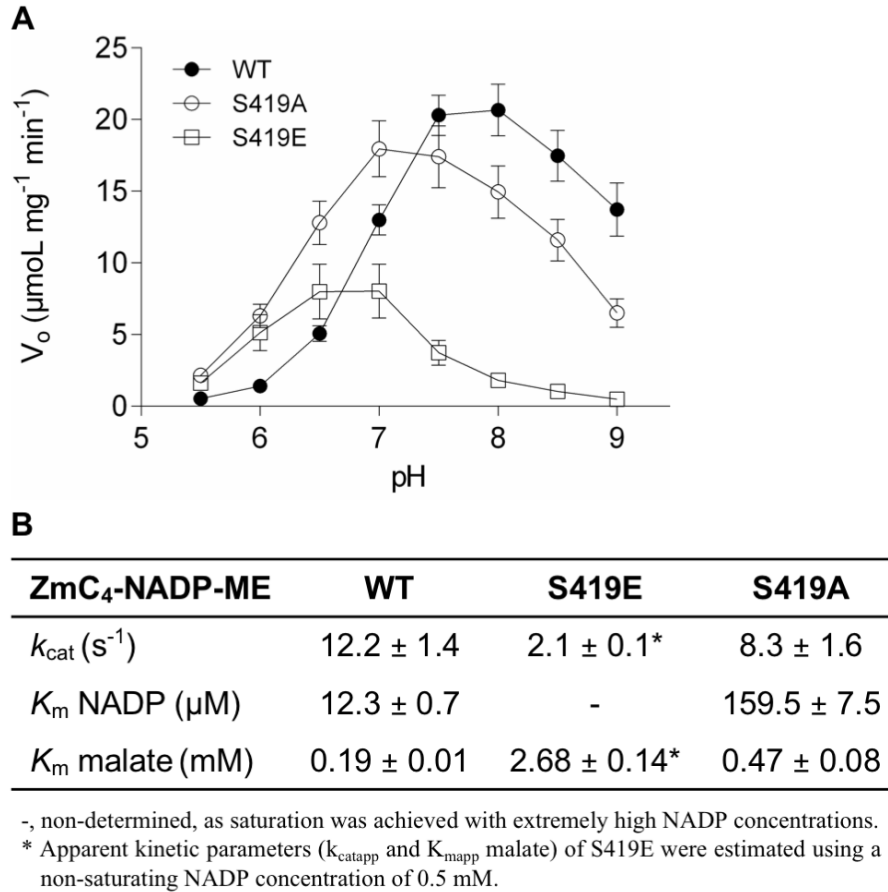


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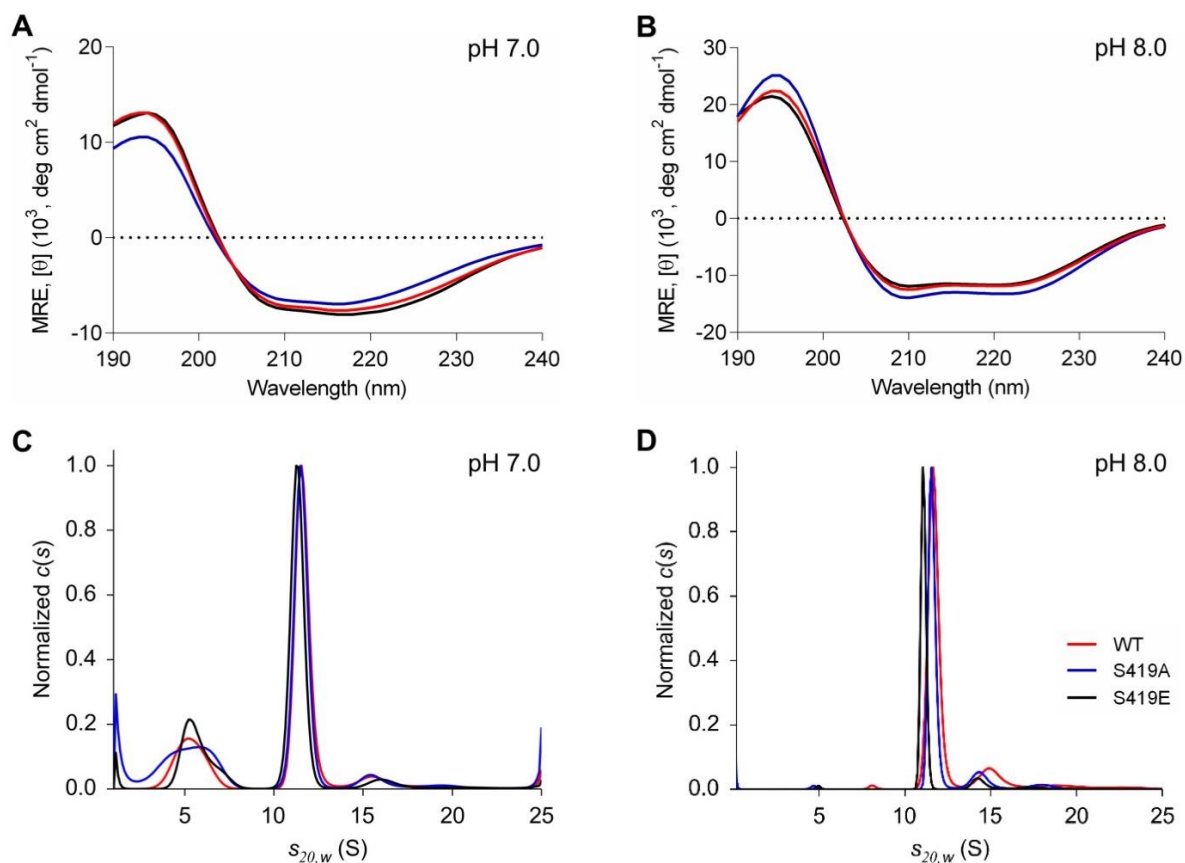


Figure 3. Exploration of the secondary structure organization and quaternary composition of recombinant ZmC₄-NADP-ME versions. **A and B:** CD spectra of ZmC₄-NADP-ME WT and the two mutated versions obtained at pH 7.0 (A) and 8.0 (B). Ten accumulations each were collected from 190 to 260 nm for 0.16 mg mL⁻¹ enzyme, at 20°C. Each graph is showing the reconstructed curves obtained by applying CONTIN/LL algorithm for data evaluation as provided by the Dichroweb server. **C and D:** Continuous sedimentation coefficient distribution of ZmC₄-NADP-ME WT and the mutated versions at pH 7.0 (C) and 8.0 (D). Data was fitted with the ls-g*(s) model in the software package SEDFIT.

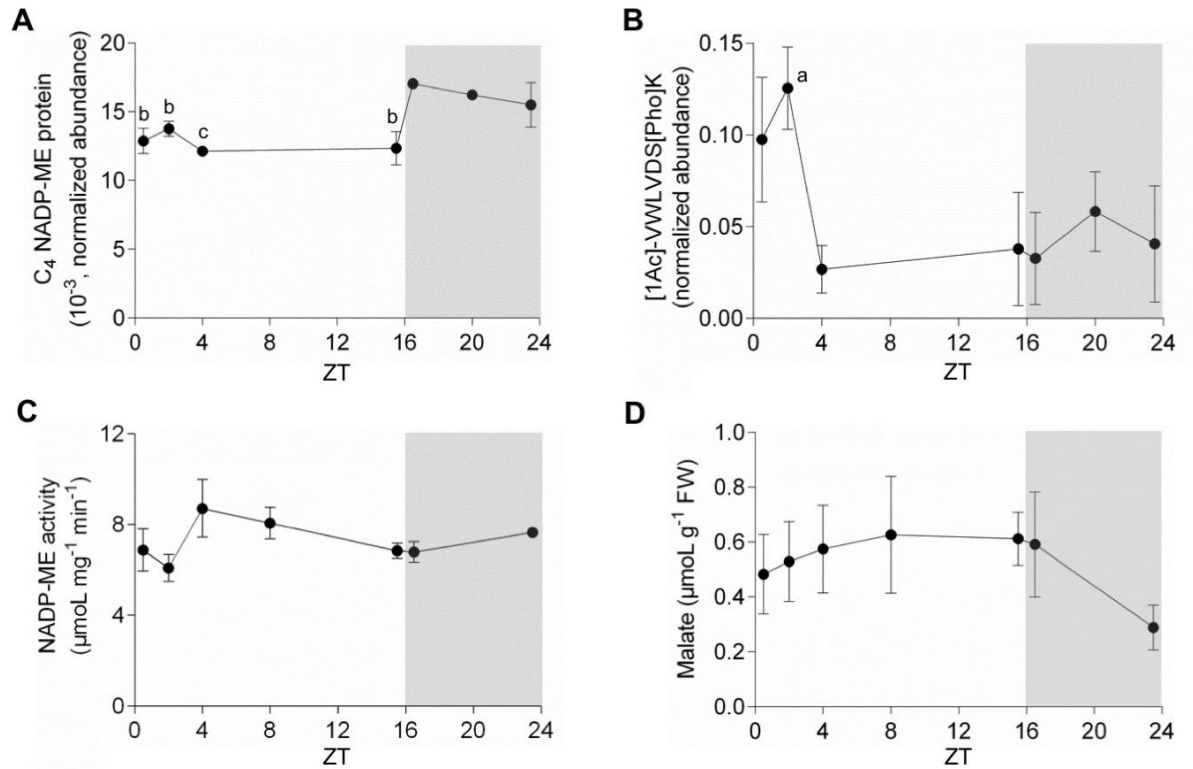


Figure 4. Measurements of different parameters in extracts of maize leaves during a diurnal cycle. **A**, Quantification of total ZmC₄-NADP-ME protein by SWATH-MS. **B**, Quantification of the phosphopeptide VWLVDpSK, corresponding to the phosphorylation of ZmC₄-NADP-ME at S419. **C**, Total NADP-ME activity measured with 1.5 - 2.0 µg protein extracts. **D**, Malate concentration assayed by GC-MS analysis. Values represent mean ± standard error of four (A and B) or three (C and D) biological replicates. The dark period is highlighted in grey. Statistical analyses were performed in all cases (A to D) against the first time point in the night (ZT16.5 = 16.5 h). Letters indicate that the value is statistically significant at a, 0.05; b, 0.01; c, 0.001 levels (the precise p-values are shown in Supplementary Table 3). ZT, Zeitgeber Time.

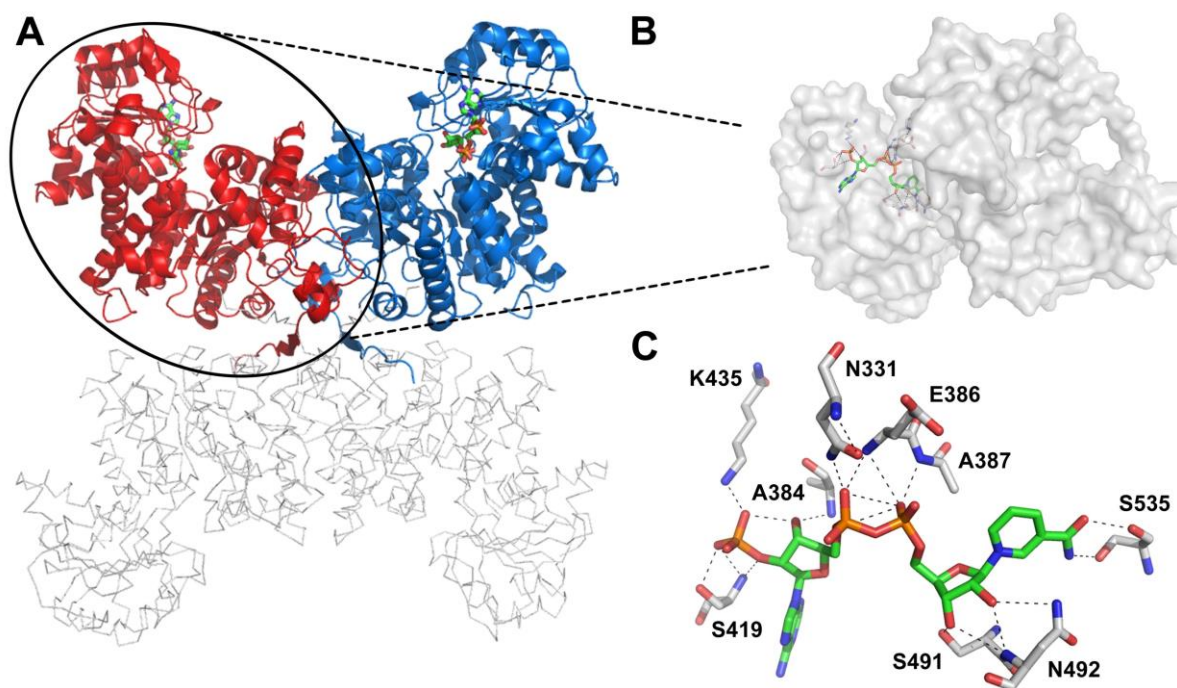


Figure 5. SbC₄-NADP-ME crystal structure and amino acids involved in cofactor binding. **A**, Cartoon and ribbon representation of SbC₄-NADP-ME. Monomer A (red) and B (blue) with bound cofactor NADP (colored sticks). Large parts of chain C and D are not well resolved by electron density; therefore, we depicted these chains as gray ribbons only to clarify the tetrameric assembly. **B**, Monomer A from SbC₄-NADP-ME as surface representation with the bound cofactor NADP (represented by sticks). **C**, Residues involved in cofactor binding are depicted as sticks with labels (distances are omitted for clarity and are listed in Table S2).

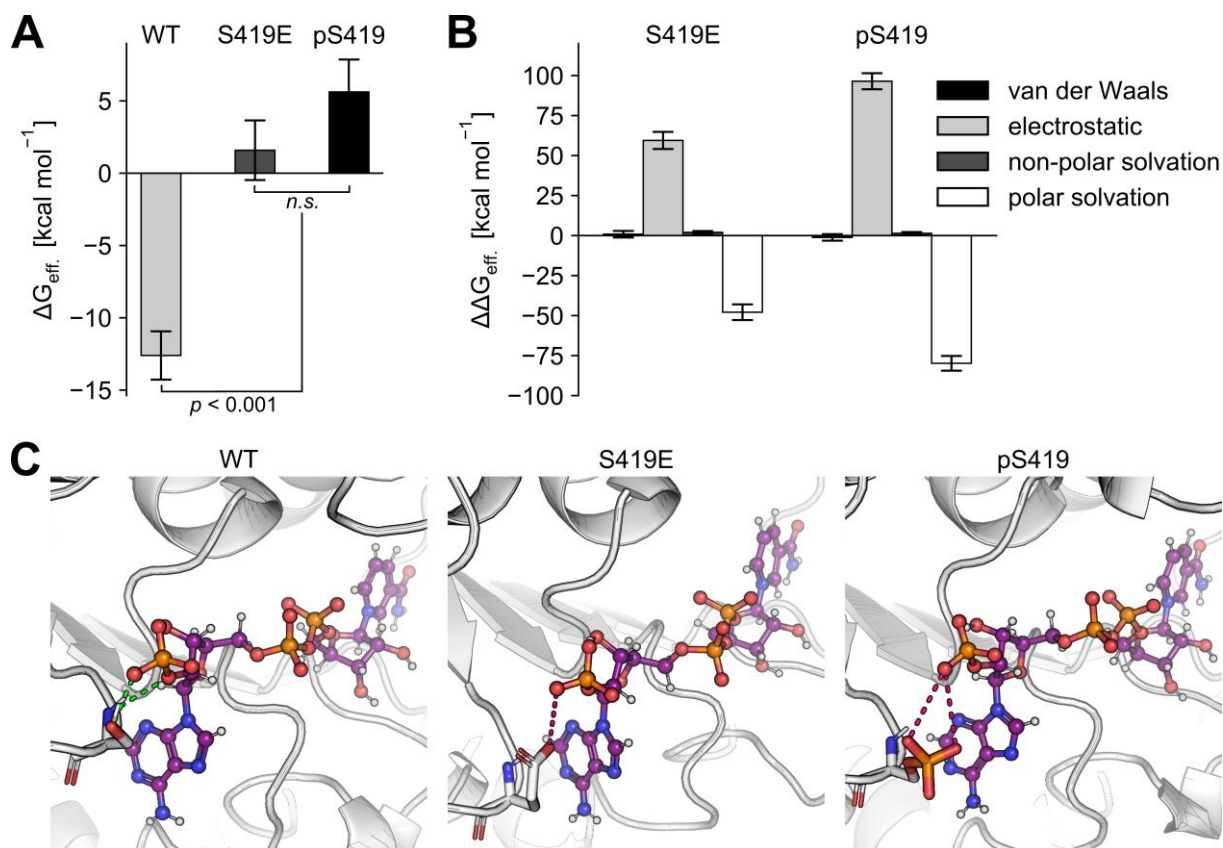


Figure 6: Effective free energies of binding of NADP. **A**, Effective binding energies computed according to the MM-PBSA approach for WT ZmC₄-NADP-ME, the phosphomimetic variant (S419E), and the phosphorylated enzyme (pS419). The error bars indicate the standard error of the mean over 10 individual trajectories. Statistical significance was calculated according to Student's *t*-test. **B**, The contribution of the different energy terms, computed as variant's energy term minus the respective energy term of the WT enzyme ($\Delta\Delta G_{\text{eff.}} = \Delta G_{\text{eff.}}(\text{Var}) - \Delta G_{\text{eff.}}(\text{WT})$). Positive terms indicate more favorable binding to the WT enzyme, negative ones to the variant. The error bars show the standard error of the mean of the differences in the individual terms over the trajectories. **C**, Molecular representation of NADP bound to the WT ZmC₄-NADP-ME, the phosphomimetic variant (S419E), and the phosphorylated enzyme (pS419). Interactions with residue 419 are highlighted, showing hydrogen bonds for the WT enzyme with green dashed lines, and charge-charge repulsion for the phosphomimetic and phosphorylated variants with red dashed lines.

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