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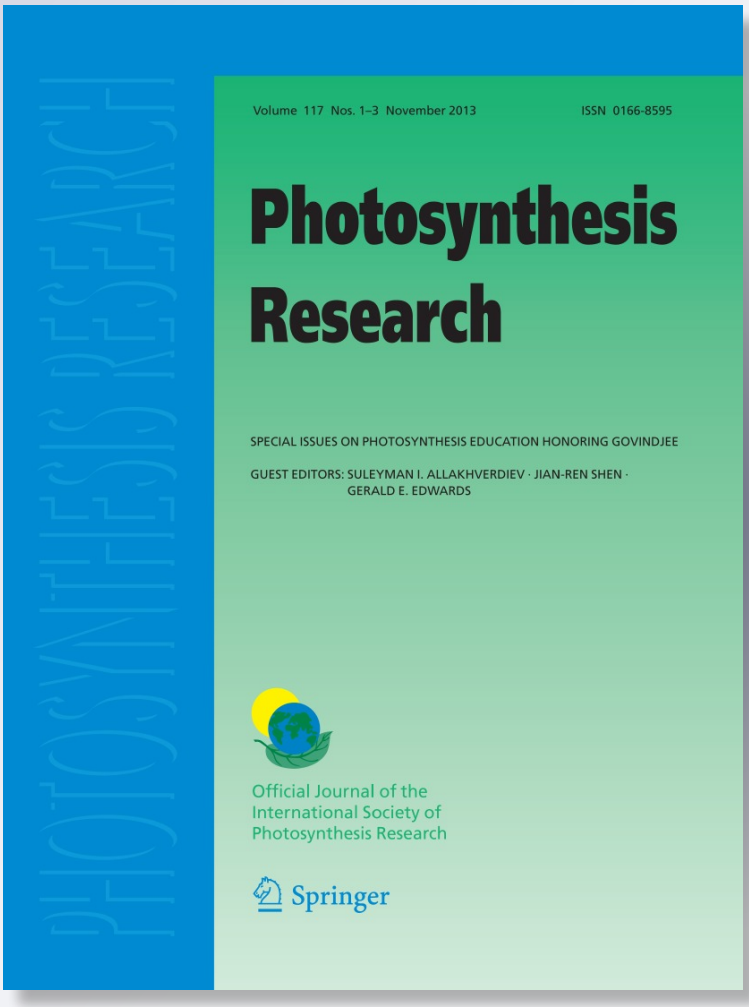

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REVIEW

Biochemical approaches to C_4 photosynthesis evolution studies: the case of malic enzymes decarboxylases

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Abstract C_4 photosynthesis enables the capture of atmospheric CO_2 and its concentration at the site of RuBisCO, thus counteracting the negative effects of low atmospheric levels of CO_2 and high atmospheric levels of O_2 (21 %) on photosynthesis. The evolution of this complex syndrome was a multistep process. It did not occur by simply recruiting pre-exiting components of the pathway from C_3 ancestors which were already optimized for C_4 function. Rather it involved modifications in the kinetics and regulatory properties of pre-existing isoforms of non-photosynthetic enzymes in C_3 plants. Thus, biochemical studies aimed at elucidating the functional adaptations of these enzymes are central to the development of an integrative view of the C_4 mechanism. In the present review, the most important biochemical approaches that we currently use to understand the evolution of the C_4 isoforms of malic enzyme are summarized. It is expected that this information will help in the rational design of the best decarboxylation processes to provide CO_2 for RuBisCO in engineering C_3 species to perform C_4 photosynthesis.

Keywords C_4 enzymes · Malic enzymes · Kinetic and structural properties · Molecular and biochemical technologies

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M. Saigo · M. A. Tronconi · M. C. Gerrard Wheeler · C. E. Alvarez · M. F. Drincovich · C. S. Andreo (✉)
Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Universidad Nacional de Rosario, Suipacha, 531, Rosario, Argentina
e-mail: carlosandreo@cefobi-conicet.gov.ar

Introduction

C_4 photosynthesis at a glance

C_4 photosynthesis represents an improvement in the function of the C_3 photosynthetic pathway to overcome adverse environmental conditions such as high light intensities, high temperatures and poor water availability (Walker and Edwards 1983). In these scenarios, the rate of carbon loss by photorespiration in C_3 plants is high due to the metabolization of 2-phosphoglycolate, a toxic product of ribulose-1,5-bisphosphate (RuBP) oxygenation catalysed by ribulose bisphosphate carboxylase oxygenase (RuBisCO) (Bowes et al. 1971). This process in which O_2 competes with CO_2 for reaction with RuBP is very expensive in terms of energy, and it is a major factor that lowers photosynthesis efficiency (Lorimer 1981). In most C_4 plants an auxiliary cycle pumps CO_2 from mesophyll cells (MC) to inner cells that surround the vascular bundle (bundle sheath cells, BSC) where RuBisCO initiates the incorporation of this CO_2 to carbohydrates (Fig. 1) (Hatch 1987, 1992). In this scheme, the ratio of carboxylation/oxygenation of RuBisCO is greatly increased thus diminishing the photorespiration. The metabolites involved in this CO_2 concentrating mechanism are three-carbon (C_3) and four-carbon (C_4) organic acids (Fig. 1). In MC, the CO_2 is converted to HCO_3^- by carbonic anhydrase (CA) and fixed by phosphoenolpyruvate (PEP) carboxylase (PEPC) using PEP as a substrate. The C_4 acid formed, oxalacetate (OAA), is then transformed to a more stable compound: malate and/or aspartate. These transitory storage carbon forms diffuse to BSC where they provide substrates for decarboxylation and thus CO_2 supply for RuBisCO. Three decarboxylating enzymes can operate in the C_4 cycle: NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic

enzyme (NAD-ME) and PEP carboxykinase (PEPCK) (for a recent review refer to Drincovich et al. 2010). Each one defines a C_4-subtype as the main decarboxylase. Then, C_3 compounds diffuse back to MC to be recycled back to PEP and initiate another turn of the C_4 cycle.

Biochemical adaptation: the final tuning in C_4 plants evolution

The evolution of this complex syndrome involved coordinated changes in leaf anatomy, such as increase in BSC size and organelle content, and metabolism including re-localization of key enzymes (for more detailed reviews see Gowik and Westhoff 2011; Sage et al. 2012; Ludwig 2013; Christin and Osborne 2013). Glycine decarboxylase complex (GDC) redistribution was a major event in C_4 evolution. In C_3 plants, this mitochondrial multienzyme system in MC releases CO_2 during the recycling of 2-phosphoglycolate, product of RuBisCO-catalysed ribulose-1,5-bisphosphate oxygenation. During the evolution of C_4 from C_3 plants, the restriction of GDC to BSC represents a resource of CO_2 for BSC chloroplasts thus increasing carbon fixation efficiency (Bauwe 2010). On the other hand, the genes for enzymes that make up the C_4 cycle were already present in the C_3 ancestors but they were fulfilling non-photosynthetic roles. Then, the novel C_4 enzymes arose from modified C_3 enzymes coding genes. In many cases the duplication of the C_3 genes was a previous event that facilitated the evolution of the C_4 genes, but there is evidence which indicates that this was not essential in C_4 evolution (Wang et al. 2009; Williams et al. 2012). The last step in C_4 evolution was the coordination of the whole photosynthetic process by optimizing the kinetic and regulatory properties of the key C_4 enzymes. These changes gave rise to enzymes that perform efficiently in the new metabolic environment especially under light conditions. For example, C_4 PEPCs bind PEP and bicarbonate with lower and higher affinity than C_3 PEPCs, respectively, and are less inhibited by malate and aspartate, both abundant metabolites in C_4 MC (Bauwe 1986; Engelmann et al. 2003; Svensson et al. 2003). PEPC activity raises upon illumination as a result of the phosphorylation catalysed by PEPC kinase, which in C_4 plants is transcriptionally regulated by light (for reviews, see Nimmo 2003; Gowik and Westhoff 2010). PEPCK is also subject to reversible phosphorylation in the leaves of some C_4 plants, such as Guinea grass (*Urochola maxima*). The enzyme is phosphorylated in darkened leaves and dephosphorylated in illuminated leaves (Leegood and Walker 2003). PEPCK dephosphorylation effectively acts to increase decarboxylase activity and substrate affinity as well as to enhance ATP activation (Walker et al. 2002). In the case of pyruvate orthophosphate dikinase (PPDK), which regenerates PEP from pyruvate inside the chloroplasts of MC, the transition into a C_4 enzyme involved minor changes in enzyme properties, as seen by the comparison of the catalytic and regulatory properties of maize C_4 PPDK and Arabidopsis C_3 PPDK (Chastain et al. 2011). From these observations, it was concluded that C_3 PPDK was suitable to fulfil the role in C_4 photosynthesis as PEP-regenerating enzyme, which probably facilitated its selection over other PEP-forming enzymes.

The failure in several earlier attempts to improve photosynthesis in C_3 plants by introducing C_4 enzymes was explained in part by the effect of the 'C_3 environments' on the exclusive regulatory properties of the C_4 enzymes (Haüsler et al. 2002). That said, the success of these genetic approaches depends on the molecular engineering of the C_4 counterpart to work efficiently in the C_3 metabolism, as was intended for maize C_4 PEPC (Endo et al. 2008). Hence, it becomes evident that it is essential to develop a comprehensive knowledge of the regulatory framework and the key residues that influence the biochemical properties of the target C_4 enzymes.

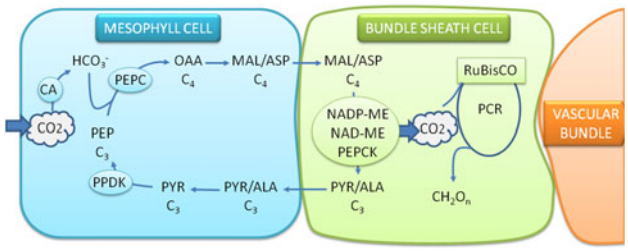

Fig. 1 C_4 photosynthesis basics. Carbon dioxide is transported from MC to BSC in the form of C_4 organic acids. The subsequent decarboxylation is catalysed by NADP-dependent malic enzyme (*NADP-ME*), NAD-dependent malic enzyme (*NAD-ME*) and/or phosphoenolpyruvate (*PEP*) carboxykinase (*PEPCK*), depending on the C_4-subtype. The CO_2 thus produced is fixed by RuBisCO and the C_3 acid diffuses back to MC to regenerate PEP, the substrate of PEP carboxylase (*PEPC*). *PCR* photosynthetic carbon reduction

Malic enzymes: a wide resource of C_3–C_4 diversity

Malic enzymes (ME) catalyse the oxidative decarboxylation of malate coupled to the reduction of NAD (NAD-ME) or NADP (NADP-ME). They are widely distributed among eukaryotes and prokaryotes where they perform diverse functions (Drincovich et al. 2010). This enzyme family is especially important in C_4 plants since in all C_4-subtypes an ME is implicated in the generation of CO_2 for RuBisCO: NADP-ME in NADP-ME subtype and NAD-ME in NAD- and PEPCK-subtype species. The discovery of special features of C_4 enzymes clearly depends on the comparison with C_3 isoenzymes, which are implicated in non-photosynthetic metabolisms not only in C_3 plants but also in C_4 plants. Accordingly, a

large number of studies have focused on the characterization of ME families, mainly in *Arabidopsis thaliana* (C_3 model) and maize (C_4 model) (Fig. 2), which will be described in the following sections to depict the biochemical toolset available to perform such studies.

Techniques used to study proteins

For the complete understanding of the mechanism underlying CO_2 fixation in C_4 plants a comprehensive kinetic, regulatory and structural characterization of the involved enzymes is clearly required. There are different methodologies to characterize an enzyme at the biochemical level. This information is often complemented with studies of gene expression, subcellular localization and mutant plants assays (Gerrard Wheeler et al. 2005; Tronconi et al. 2008). In turn, the currently available compilations of genomic sequences and ESTs databases allow addressing the objectives to study and to obtain data on the composition of isoform families, assign putative roles and predict signalling and regulatory sites (Saigo et al. 2013). In this review, some techniques that we have used in recent years are compiled, starting from the recombinant synthesis of the proteins, followed by classical biochemical tools and ending with more refined techniques. Moreover, by way of example, some results and conclusions obtained from their implementation are listed.

DNA technologies

The dark depths of C_4 biochemistry shine by the power of recombinant DNA technology

The determination of the biochemical properties of any enzyme implies that they must be first obtained in an isolated form. Before the genomic era, proteins were purified from organs or tissues by using bulky and time-consuming protocols. These conventional strategies included the release of proteins from plant material by mechanical disruption in an appropriate buffer system and their subsequent separation by sequential column chromatographic techniques. While some of the most representative enzymes were obtained in enough quantities for their biochemical characterization, these protocols were not suitable for less abundant proteins. In addition, protein copurification (i.e. more than one isoform) and enzymatic inactivation were problems that limited the applicability of conventional procedures in many cases. The emergence of modern molecular genetics era in the late 70s provided the recombinant DNA technology, through which proteins in quantities for assays are expressed and purified in a heterologous host. Figure 3 shows the basic steps for obtaining recombinant proteins in *Escherichia coli*. This strategy greatly increased the purification yields and reduced the initial biological material, as the desired product can comprise more than 50 % of the total host cell protein. In addition, in recombinant DNA technology the inactivation of the enzyme is prevented by reducing the time required for purification, as usually only one chromatographic step is used for separating the target enzyme from the bacterial lysate. Moreover, the chromatography column only binds the 'tag' protein so higher purification levels, commonly to homogeneity, are reached. Finally, since the protein is derived from a single cDNA clone, recombinant strategy makes it possible to discover whether the target protein is a biochemically active enzyme or if additional subunits are needed. This point was particularly important for the heterodimeric mitochondrial NAD-ME of *A. thaliana* because it allowed establishing that each individual subunits possessed enzymatic activity (Tronconi et al. 2008).

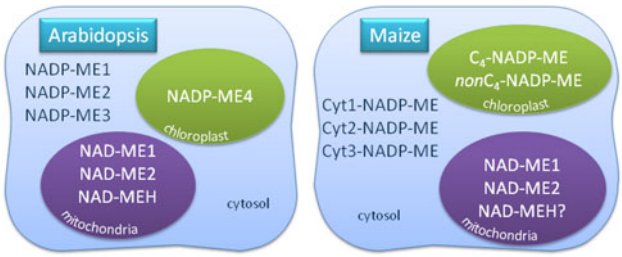

Fig. 2 NAD(P)-ME families in Arabidopsis and maize. Arabidopsis NADP-ME1-4 are described in Gerrard Wheeler et al. (2005) and NAD-ME in Tronconi et al. (2008). Maize C_4-NADP-ME is described in Detarsio et al. (2003), *non*-C_4-NADP-ME in Saigo et al. (2004), cyt1- and cyt2-NADP-ME in Alvarez et al. (2013), cyt3-NADP-ME in Detarsio et al. (2008) and NAD-ME are being characterized by Tronconi et al.

Table 1 shows a comparison of purification characteristics and biochemical properties of purified photosynthetic NADP-ME from maize by conventional and recombinant strategies. The lower k_{cat} value obtained for the enzyme purified by conventional protocols indicates that enzyme inactivation is occurring during the large purification procedures (Drincovich et al. 1991; Detarsio et al. 2003). In addition, the similar values of K_m for substrates of the protein purified by both methods suggest that the recombinant isoform is correctly structured and highlights the use of the heterologous expression machinery for synthesis of eukaryotic proteins. However, it must be noted that a diversity of variations on the standard recombinant protocol described in Fig. 3 exists, in cases where the target protein is not properly expressed. These alternatives include the use of *E. coli* strains expressing tRNA for common codons in eukaryote cells when the target is very low or not synthesized; or strains expressing the T7 RNA polymerase in lower levels in those cases where the target

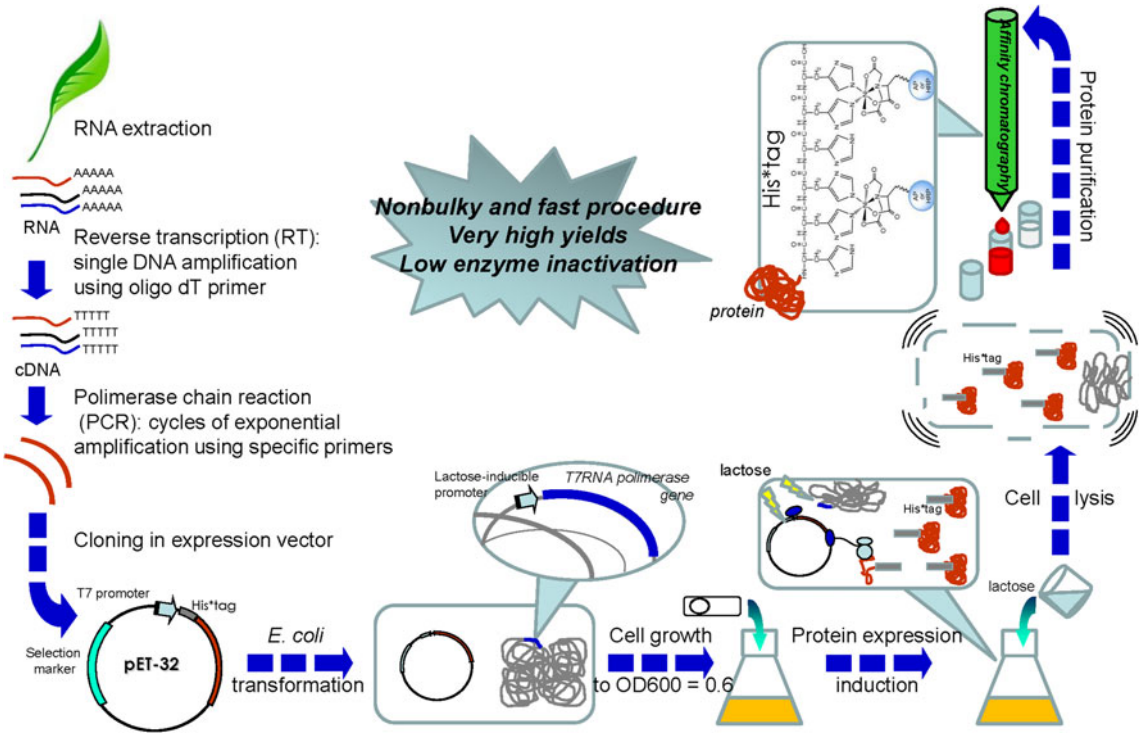

Fig. 3 Steps for synthesis of the recombinant proteins in *E. coli*. The cDNA encoding the protein is synthesized by RT-PCR using leaf-isolated mRNA as template and then cloned in an expression vector. The basic components of an expression vector include a selection marker (commonly an antibiotic resistance gene) that ensures the maintenance of the vector during bacteria duplication, and a strong and inducible promoter that will lead the cDNA transcription. The pET vectors (Novagen) are the most powerful system for protein expression in *E. coli*. Here, target genes are cloned downstream of bacteriophage T7lac promoter and in-frame for the expression of the enzymes as fusion proteins. The extra, vector-encoded, sequence introduces a 'tag' to purify (His*Tag) the recombinant enzyme from a bacterial lysate. The recombinant plasmid is transferred to an *E. coli* strain containing a copy of the gene for T7 RNA polymerase (*E. coli* (BL21 DE3)) under the control of an inducible promoter by lactose. Bacteria are grown in liquid rich medium in the presence of the appropriated antibiotic until OD_{600} reaches 0.6–0.8 and target protein expression is induced. After the cells are collected, they are lysated and the supernatant is loaded in an affinity nickel–nitriloacetic (Ni–NTA) chromatographic column. The target protein is purified by binding to the column through the coordination of nickel cation and His residues codified by the vector's extra sequence. After washes, protein is eluted from the column with a buffer containing imidazole

Table 1 Strategies for maize C_4-NADP-ME purification

	Biological material	Purification time	Yield	Purification (%)	k_{cat} (s^{-1})	K_m NADP (μM)	K_m malate (mM)
Conventional[a]	100–300 g leaves	2–3 days	3–4 mg protein/100 g	<95	70	8.6	0.19
Recombinant[b]	1 l cell suspension, $OD_{600} = 1$	2 h	1–3 mg protein/100 ml	>95	201	8.0	0.23

[a] Adapted from Drincovich et al. (1991)

[b] Adapted from Detarsio et al. (2003)

form precipitate aggregates (inclusion bodies). In addition, the use of an eukaryotic expression system (e.g. yeast) must be considered when the protein is post-translationally modified. For a detailed summary of recombinant methods and alternative strategies we suggest to refer Gräslund et al. (2008), and Demain and Vaishnav (2009).

That said, a number of enzymes involved in the C_4 syndrome, as well as non-photosynthetic isoforms of C_4 and C_3 plants, have been expressed and purified through DNA recombinant technology (Table 2). Furthermore, several chloroplast envelope transporters of mesophyll or BSC have been characterized through this technique (Table 2). Given the highly hydrophobic nature, the target protein is usually obtained as inclusion bodies that are purified from bacterial pellet and solubilized using mild detergents (e.g. sarkosyl). Then, the soluble transporters are reconstituted into lipid vesicles generating proteoliposomes, on which they are functionally analyzed. An

alternative approach consists in the recombinant expression in yeast, followed by cellular disruption and incorporation of membrane fractions containing the expressed target in liposomes.

Recombinant DNA technology: a factory of tailor-made proteins

The recombinant DNA technology not only improved the proteins purification methods but also led to the design of new versions of proteins for comprehensive biochemical and structural characterization. In this regard, mutant and chimeric proteins were designed through cDNA site-directed mutagenesis (supplemental Fig. 1a, b). By using these strategies valuable data were obtained for several C_4 photosynthetic enzymes. For C_4 NADP-ME of maize the domains involved in subunits tetramerization and activity inhibition by the substrate were identified through chimeric assembly with the plastid non-photosynthetic isoform (Detarsio et al. 2007). Also for this enzyme, the relevance of Gly and Cys residues in the NADP binding site and in the activity redox regulation, respectively, was analysed by characterization of the mutated proteins (Detarsio et al. 2003; Alvarez et al. 2012).

Site-directed mutagenesis was also applied to maize C_4 PEPC, in which residues essential for allosteric inhibition by malate and aspartate (Endo et al. 2008) and for activation by glucose-6-phosphate (Takahashi-Terada et al. 2005) could be identified. Moreover, a study of enzyme chimeras between C_4 and C_3 PEPC isoforms from *Flaveria* species has revealed the major determinants for the specific properties of each enzyme form (Bläsing et al. 2000).

The power of recombinant DNA technology goes farther. Two proteins can be simultaneously expressed (co-expressed) in *E. coli* by a dual approach which uses two vectors with different selection markers (supplemental Fig. 1c). The dual approach was used for co-expression of both subunits of mitochondrial NAD-ME of *A. thaliana*. The NAD-ME1 subunit was expressed as protein fusion to His*tag for its binding to nickel–nitriloacetic (Ni–NTA) chromatographic column, while NAD-ME2 was expressed without this tag. Both proteins co-eluted from the affinity

Table 2 Recombinant approaches for C_4 and C_3 isoforms purification

	Isoform	Expression/purification system	References
Enzymes			
PEPC	• Maize C_4 • Maize non-photosynthetic isoform from roots	Cloned in pET32 prokaryotic vector and expressed as N-terminal fusion proteins to Txr*Tag and His*Tag in *E. coli*. Purified from bacterial lysate in NTA-Ni affinity chromatographic column	Dong et al. (1998)
NADP-ME	• Maize C_4		Detarsio et al. (2003)
	• Maize *non*-C_4		Saigo et al. (2004)
	• Arabidopsis NADP-ME1-4		Gerrard Wheeler et al. (2005)
NAD-ME	• Arabidopsis NAD-ME1-2		Tronconi et al. (2008)
PEPCK	• Maize C_4		Furumoto et al. (1999)
	• Cucumber isoform from cotyledons		Kim and Smith (1994)
PPDK	• Maize C_4	Cloned in prokaryotic vector, expressed in *E. coli*	Chastain et al. (1996)
Transporters			
Triose-P/Pi translocator (TPT)	• *Flaveria trinervia* C_4 • *Flaveria pringlei*	Cloned in eukaryotic vector, expressed in yeast and reconstituted in liposomes	Fischer et al. (1994)
Malate/OAA	• *Panicum miliaceum* C_4	Cloned in pET32 prokaryotic vector and expressed as C-terminal fusion protein His*Tag in *E. coli*. Solubilized form inclusion bodies and reconstituted in liposomes	Taniguchi and Sugiyama (1996)
Pyruvate/Na+	• *Flaveria bidentis* C_4 • *Flaveria trinervia* C_4	Cloned in pET32 prokaryotic vector, expressed as C-terminal fusion protein S*Tag and functionally characterized in *E. coli* cells	Furumoto et al. (2011)

column (supplemental Fig. 1c) indicate a true interaction between NAD-ME1 and NAD-ME2 for constituting a heteromeric isoform (NAD-MEH) with kinetic and regulatory properties which are different from those of homodimers (Tronconi et al. 2010a).

The present availability of DNA genomic sequences for more than 25 plant species makes it possible to express through recombinant techniques each member of an enzymatic family for their biochemical analysis. Then, kinetic and regulatory data can be globally analyzed and integrated to the comparative sequence analysis. For enzymes involved in the C_4 syndrome, this approach allows discovering special function–structure relationships of C_4 isoforms that could provide clues about the evolutionary path that led to C_4 metabolism.

Kinetic parameters

When the factors affecting the rate of a reaction are appropriately analyzed, it is possible to make inferences about the nature of the enzyme, such as mechanism, physiological reaction direction, in vivo regulation and active site residue identities (Dixon and Webb 1979). The enzymatic activity can be determined spectrophotometrically by following the appearance of a product or consumption of a substrate of the reaction. In the particular case of dehydrogenases, the property of NAD(P)H to absorb at 340 nm ($\varepsilon_{340nm} = 6.22\ mM^{-1}\ cm^{-1}$) is used. Thus, 1 unit (U) is defined as the amount of enzyme that catalyses the formation or disappearance of 1 μmol of NAD(P)H min^{-1} under specific conditions.

Optimum pH

The optimum pH value of a purified protein is determined by measuring the initial reaction rate at different pH values in the presence of the necessary cofactors and substrates. The active and regulatory sites of the enzymes often consist of ionizable groups, which must be in the proper ionic form in order to maintain the native conformation, bind the ligands and catalyse the reaction or transmit a signal to the active site. Thus, studying the pH effect on the activity or regulation of an enzyme allows us to understand how these processes occur and which amino acid side chains are possibly involved (Arias et al. 2012). It is worth mentioning that the pH effect on enzyme stability also should be taken into account. In a physiological context, the optimum pH is often related to the compartment where the enzyme is functional. Usually, chloroplastic stromal enzymes are most active at pH close to 8 (Detarsio et al. 2003).

Kinetic constants

The kinetic constants are estimated by measuring the initial reaction rate and varying the concentration of each substrate while keeping the other substrate at saturating levels. The determinations are made at least in triplicate assays using different enzyme preparations. Then, the results are adjusted by non-linear regression to the proper equation. For enzymes with hyperbolic response, the initial velocity (v) depends on the variable substrate concentration (s) according to the Michaelis–Menten equation:

$$v = V_{max} s/(K_{0.5} + s),$$

where V_{max} is the maximum reaction rate and $K_{0.5}$ is the substrate concentration for which $V_{max}/2$ is obtained. k_{cat} is the catalytic rate constant equal to the micromoles of substrate converted to product per minute under optimal conditions (V_{max}) per micromole of active sites. In the case of enzymes with sigmoid kinetics, the following equation is often applied:

$$v = V_{max} s^{nH}/\left(K_{0.5}^{nH} + s^{nH}\right),$$

where nH is the Hill coefficient. This is the case of enzymes with allosteric sites or oligomers with multiple non-independent active sites, where the substrate itself acts as an effector. The sigmoid response provides a more sensitive control of the reaction rate by varying the substrate concentration.

The $K_{0.5}$ numerical value is of great interest because it is a measure of the affinity of the enzyme for its substrate. In turn, k_{cat} gives the inverse of the time required for a single catalytic cycle. Finally, the catalytic efficiency ($k_{cat}/K_{0.5}$) provides a means to compare the performance of different isoforms between tissues, developmental stages and organisms (Segel 1994).

$K_{0.5}$ values reported for NADP-ME plant isoforms are in the range 7–72 μM for NADP and 0.1–3 mM for malate (Gerrard Wheeler et al. 2005; Detarsio et al. 2008; Müller et al. 2008; Saigo et al. 2013). It has been determined that $K_{0.5}$ constants are higher in non-photosynthetic isoforms, which have a lower k_{cat} than their photosynthetic counterparts (Detarsio et al. 2003, 2008; Saigo et al. 2004, 2013). As indicated by its particular structural, kinetic and regulatory properties, photosynthetic NADP-ME proteins have acquired unique and specialized features after their creation from the enzymatic forms present in C_3 species (Drincovich et al. 2010).

Reaction reversibility

For thermodynamically reversible enzymes, comparative tests are performed by determining the two reaction activities. According to its role in providing CO_2, maize

NADP-ME involved in photosynthesis is a basically decarboxylating enzyme (Spampinato and Andreo 1995). However, NADP-ME isoforms from the C_3 species Arabidopsis were able to catalyse not only the malate oxidative decarboxylation but also the pyruvate reductive carboxylation with high performance and $K_{0.5}$ values similar to substrate levels found in the plant (Gerrard Wheeler et al. 2008). This result is indicating that both the reactions may be relevant in vivo in *A. thaliana* (Maurino et al. 2009) and could be related to the organic acid hyperaccumulator capacity of this plant (Fernie and Martinoia 2009; Araújo et al. 2011; Arias et al. 2012).

Cofactor specificity

In the case of ME, the activity may be measured in the presence of NAD or NADP, but they show a strong preference for one cofactor over the other (Detarsio et al. 2003; Maurino et al. 2009). The structural basis of cofactor specificity is poorly understood, although both activities would be catalysed by the same active site (Detarsio et al. 2004).

Alternative reactions

It is also possible to evaluate the ME ability to reduce pyruvate and to decarboxylate oxaloacetate. These activities are side reactions derived from the ME mechanism which determine the classification of ME group by the International Union of Biochemistry and Molecular Biology (Spampinato and Andreo 1995; Gerrard Wheeler et al. 2008; Tronconi et al. 2010b). So far, it is unknown whether these alternative reactions would have in vivo physiological significance (Maurino et al. 2009).

Reaction mechanism

From kinetic studies, it is possible to identify the mechanism of a reaction involving two or more substrates and two or more products (Schimerlik and Cleland 1977). According to our results, NAD-ME isoforms of *A. thaliana* display distinct kinetic mechanisms that support a differential contribution of these enzymes to organic acid mitochondrial metabolism (Tronconi et al. 2010b). Product inhibition and substrate analogue analyses indicated that NAD-ME2 follows a sequential ordered mechanism, NAD being the leading substrate followed by malate, similar to the photosynthetic NADP-ME from maize (Spampinato and Andreo 1995). On the other hand, NAD-ME1 can bind both substrates randomly in order to form the ternary complex with the enzyme (Tronconi et al. 2010b).

Regulatory properties

Modulation of enzyme content through synthesis and/or degradation is a common long-term acclimation strategy of all living organisms (Kilian et al. 2007). However, fast enzyme activity changes are critical and can be achieved by allosteric regulation (Laskowski et al. 2009) or covalent post-translational modifications (Alvarez et al. 2012).

Partial inhibition by substrate

Malic enzymes involved in C_4 photosynthesis display a characteristic inhibition by high malate concentrations at pH 7 (Detarsio et al. 2003). This regulatory mechanism, not exhibited at pH 8, could be caused by the presence of two malate binding sites: one catalytic and other allosteric. A combination of kinetic assays and protein characterization of mutant versions support this hypothesis (Detarsio et al. 2007). It has been proposed that the C_4 cycle is regulated through malate by the pH change that occurs in illuminated chloroplasts, so that NADP-ME is fully active when photosynthesis is in progress. However, more recent studies indicate that this behaviour is also present in some C_3 isoforms (Müller et al. 2008; Gerrard Wheeler et al. 2009). Subsequent studies will reveal whether this type of regulation is occurring in vivo also in C_3 plants and under which conditions.

Study of putative metabolic effectors

Determining the activity of an enzyme in the presence of various key compounds can be very useful to study its relationship with different metabolic pathways. In general, the assays are performed in the presence of substrate concentrations equal or near the $K_{0.5}$ values for better simulating cellular conditions and for testing the possible regulators on a non-saturated enzyme.

Despite extremely high values of sequence identity, our studies showed different regulatory patterns for many ME isoforms. This diversity could reflect their non-overlapping functions depending on the organ, intracellular location and temporal context. In turn, it may reveal a functional specificity even when several isoforms are co-expressed. This seems to be the case of the NAD(P)-ME family from *A. thaliana*, where the same metabolite produces opposite modulation of activity in some members (Maurino et al. 2009; Tronconi et al. 2010a, 2012).

The modulators can also influence the direction of the reaction. In this regard, a recent study showed that the forward and reverse activities of the major Arabidopsis NADP-ME isoform are differentially modulated by fumarate, suggesting a specific metabolic flux control leading to

the synthesis or consumption of C_4 acids under particular conditions (Arias et al. 2012).

The interactions of the recombinant purified proteins with the effectors can also be investigated by kinetic approaches and fluorescence quenching or urea denaturation assays. Kinetic determinations allowed us to find potential physiological activators or inhibitors of the enzyme activity and to infer the mode of action of a particular effector (Gerrard Wheeler et al. 2008; Arias et al. 2012). It is also possible to estimate the regulatory constants which provide an idea of whether the regulation could occur in vivo (Tronconi et al. 2010a). On the other hand, experiments of intrinsic fluorescence quenching of the Trp residues are very useful for determining the binding sites of the enzyme and its affinity constants (Tronconi et al. 2012). Fluorescence intensities are measured after sequential addition of small volumes of ligand to a cuvette containing the protein. The quenching data are corrected for buffer spectra and dilution and are fitted to the Stern–Volmer equations (Lehrer and Leavis 1978). Finally, binding properties can be evaluated by urea-induced denaturation experiments. The proteins are incubated with increasing urea concentrations in the absence or presence of the putative modulator. Then, overall protein integrity is analyzed by measuring residual enzymatic activity (Tronconi et al. 2012; Arias et al. 2012).

Redox-dependent activity modulation

The sensitivity of a recombinant enzyme to changes in the redox conditions provides clues to its modulation in a cellular environment. For this purpose, the proteins are incubated with chemical oxidants with variable redox potentials. As indicated by our results, the photosynthetic sorghum and maize NADP-ME isoforms display a time-dependent decrease in their activities following the oxidation, which can be reversed with the addition of reductant agents (Alvarez et al. 2012; Saigo et al. 2013). This behaviour, not observed in the non-photosynthetic counterparts, was related to the presence of Cys residues capable of forming disulphide bridges (Alvarez et al. 2012). From these in vitro results, we hypothesize that activation of photosynthetic NADP-ME by reduction of thiol groups may occur in the light in BSC to support flux through the C_4 pathway.

Structural characterization

Classical methods

It is possible to estimate the monomeric molecular mass of a protein by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE; Laemmli 1970). These data are analyzed together with the native molecular mass, in order to infer their quaternary structure, namely the number and type of oligomers forming the protein. The most common technique to determine the native mass is the gel filtration chromatography, using columns calibrated with standards. These results are well-correlated in our lab with the mobilities obtained by electrophoresis under non-denaturing conditions. Proteins are visualized with dyes, biological activity staining or Western blot assays using specific antibodies. This methodology allowed us to further investigate the effect of several conditions such as pH, the presence of metals, substrates or effectors on oligomerization.

NADP-ME is an oligomeric protein composed of identical subunits. The most common form is a tetramer formed by a dimer of dimers (Chang and Tong 2003; Detarsio et al. 2003; Müller et al. 2008; Maurino et al. 2009). However, the recombinant plastidic *non*-C_4-NADP-ME from maize assembles as a dimer (Saigo et al. 2004). In addition, our results suggest a native state higher than a tetramer for maize cytosolic isoform found in embryo and emerging roots (Detarsio et al. 2008). The values of molecular mass of the monomer are in the range of 62–66 kDa.

On the other hand, it has been observed that the products of the two *nad-me* genes may form heterodimers and homodimers in *A. thaliana*, which could be distinguished by non-denaturing gel electrophoresis (Tronconi et al. 2008). The presence of three different NAD-ME entities, which originate by alternative associations of two subunits, is suggested to be a novel phenomenon unique to plant mitochondria (Tronconi et al. 2010a).

Other parameters of interest are the denaturing and native isoelectric points obtained by isoelectrofocusing under different conditions. They are estimated from a calibration curve of pH versus migration distance and represent useful parameters to identify isoforms in tissues or organs that express more than one gene per family (Drincovich et al. 1998).

Structural techniques for a more detailed view

Sometimes it is necessary to use more accurate and specific structural techniques in order to understand in detail the intimate connection between the structure and function of proteins.

UV circular dichroism (UV-CD)

This spectroscopy technique measures differences in the absorbed polarized UV light which is due to the optical properties of the secondary structures of proteins (α-helix, β-sheet and random coil). This method is very reliable for

monitoring changes in the conformation of proteins under different conditions or to detect misfoldings. For ME, UV-CD has been routinely used to verify the secondary structure conservation when point mutant and chimeric proteins were constructed (Detarsio et al. 2003; Alvarez et al. 2012; among many others).

Dynamic light scattering (DLS)

This technique detects the scattered light from the particles in the sample solution and provides a rapid estimate of the protein molecular size. Typically, DLS monitoring is used prior to other methodologies requiring only one type of conformation structure in the protein (Wilson 2003).

X-ray crystallography

This is a technique with an unsurpassed power to solve the 3D configuration of amino acids within the proteins, reaching an atomic level of resolution. Crystallography basically consists of irradiating an ordered arrangement of multiple copies of proteins which form a crystal, with X-ray. Two important points are the requirement of large amounts of high-quality protein and the need to set the crystallization process conditions (McPherson 2004; Berry et al. 2006). Additionally, different techniques can be used to get the diffraction pattern. In this regard, our study of the structure of a plant ME (the authors, unpublished results) takes advantage of the solved structures of human NAD(P)-ME (Xu et al. 1999), pigeon NADP-ME (Yang et al. 2002) and *Ascaris suum* NAD-ME (Coleman et al. 2002) having high similarity with the target protein.

Isothermal titration calorimetry (ITC)

This approach measures directly the thermodynamic parameters of the binding between a ligand and a macromolecule (Minor 2007). Basically, it consists in measuring the current necessary to maintain the reaction chamber at a constant temperature when one component of the binding experiment is added to the other. From these data, the dissociation constants and binding stoichiometry are determined. The use of this technique may help to elucidate the activation mechanism of fumarate to both forward and reverse reactions of *A. thaliana* NADP-ME2 (Arias et al. 2012; unpublished results).

Alanine scanning mutagenesis

This method allows determining if a lateral side chain contributes to protein–protein interaction. It consists in selecting each residue that putatively contributes to the interface contact between two proteins and changing it to alanine (Wells 1991). The use of this technique may be promising to elucidate the structural determinant for the different oligomeric behaviour present in homo- or hetero-dimeric structures of NAD-ME (Tronconi et al. 2010a).

Final remarks

The higher photosynthetic efficiency of C_4 plants compared to C_3 plants is appealing, which results in concerted efforts to determine means of introducing this feature into C_3 crops (for recent reviews refer to Sage and Zhu 2011 and articles in the same issue). After many years of research in this field it has become clear that biochemical characteristics which govern the response of an enzyme in a novel cellular environment have to be well known to succeed (Peterhansel 2011). For example, the lack of progress from introducing several C_4 enzymes into rice has been largely attributed to the misregulation and poor kinetic fitness under conditions in rice MC (Miyao et al. 2011). Thus, features of enzymes must be considered, from the basic kinetic properties, such as catalytic efficiency and substrate affinity, to the existence of means of regulation, either allosteric or covalent. All of these information represent fundamental issues that have to be thoughtfully addressed in a dynamic working programme (supplemental Fig. 2) when exploring the feasibility of introducing C_4 traits into C_3 plants.

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