

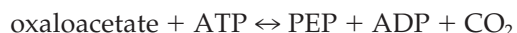
Phosphoenolpyruvate Carboxykinase Assayed at Physiological Concentrations of Metal Ions Has a High Affinity for CO₂¹

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The effect of Mn²⁺/Mg²⁺ concentration on the activity of intact, homogeneous phosphoenolpyruvate carboxykinase (PEPCK) from leaves of the C₄ grass, Guinea grass (*Panicum maximum*), have been investigated. Assay conditions were optimized so that PEPCK activity could be measured at concentrations of Mn²⁺/Mg²⁺ similar to those found in the cytosol (low micromolar Mn²⁺ and millimolar Mg²⁺). PEPCK activity was totally dependent on Mn²⁺ and was activated at low micromolar concentrations of Mn²⁺ by millimolar concentrations of Mg²⁺. Therefore, at physiological concentrations of Mn²⁺, PEPCK has a requirement for Mg²⁺. Assay at physiological concentrations of Mn²⁺/Mg²⁺ led to a marked decrease in its affinity for ATP and a 13-fold increase in its affinity for CO₂. The K_m (CO₂) was further decreased by assay at physiological ATP to ADP ratios, reaching values as low as 20 μM CO₂, comparable with the K_m (CO₂) of ribulose 1,5-bisphosphate carboxylase-oxygenase. This means that PEPCK will catalyze a reversible reaction and that it could operate as a carboxylase in vivo, a feature that could be particularly important in algal CO₂-concentrating systems.

Phosphoenolpyruvate carboxykinase (PEPCK-ATP; EC 4.1.1.49) is a Mn²⁺-dependent enzyme that catalyzes the reversible reaction:



This reaction is important in plant metabolism because it lies at an interface between organic acid, amino acid, and sugar metabolism. In keeping with the importance of this reaction the presence of PEPCK in a wide range of plant tissues is now emerging, including structures involved in plant defense, such as trichomes and oil and resin ducts, in flowers, fruits, and developing seeds, and in the phloem of some plants. In at least some of these tissues, it plays a role in nitrogen metabolism and its abundance may change greatly and rapidly in response to changes in the nitrogen status of the tissue (Leegood and Walker, 1999; Walker et al., 1999, 2001). In addition, it is well established that PEPCK is involved gluconeogenesis, converting stored fats to sugars after germination in oil-storing seeds (Leegood and ap Rees, 1978) and in the photosynthetic CO₂-concentrating mechanisms present in both PEPCK-type and some NADP-malic enzyme-type C₄ plants and in plants

with Crassulacean acid metabolism (Leegood et al., 1996; Walker and Leegood, 1996; Wingler et al., 1999). In higher plants it has always been thought that PEPCK acts as a decarboxylase in vivo because of its low affinity for CO₂ (Ray and Black, 1976; Urbina and Avilan, 1989). In both gluconeogenesis and in C₄ and Crassulacean acid metabolism photosynthesis, PEPCK certainly acts as a decarboxylase, but in some aquatic plants and algae it has also been proposed to act as a carboxylase (Reiskind and Bowes, 1991).

A difficulty with the suggestion that PEPCK acts as a carboxylase in some tissues has been the low affinity of the enzyme for CO₂ when measured in in vitro assay. It is possible that this low affinity is a result of assay of the enzyme at non-physiological concentrations of metal ions (Walker et al., 1997). For example, all previous studies have assayed PEPCK at completely unphysiological concentrations of Mn²⁺ (>0.5 mM) and in the absence of Mg²⁺. Mg²⁺ has also been shown to be inhibitory (Burnell, 1986). However, the concentration of Mn²⁺ in the cytosol of plant and animal cells is submicromolar as, for example, in maize roots (Quiquampoix et al., 1993), and the concentration of Mg²⁺ in the cytosol of plant cells is millimolar as, for example, in mung bean (*Vigna radiata*) roots (Yazaki et al., 1988). In addition, previous studies of PEPCK have used the proteolytically cleaved form of the enzyme, the properties of which may differ from the intact enzyme (Walker et al., 1997, 2002).

In this paper we show how PEPCK activity can be measured in in vitro assay at physiological concentrations of Mn²⁺/Mg²⁺ and that under such condi-

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tions the affinity of PEPCK for CO_2 is greatly increased.

RESULTS

The effects of Mn^{2+} and Mg^{2+} concentration on the carboxylation and decarboxylation activities of pure, intact PEPCK from illuminated leaves of the C_4 plant, Guinea grass (*Panicum maximum*) were characterized (Fig. 1). For the carboxylation reaction, in the absence of 2-mercaptoethanol (ME), there was no activity at $10 \mu\text{M Mn}^{2+}$, little activity at $100 \mu\text{M Mn}^{2+}$ and maximum activity at millimolar concentrations of Mn^{2+} . Under these conditions, Mg^{2+} was inhibitory, with more than 50% inhibition at 5 mM Mg^{2+} . In the presence of high concentrations of ME (500 mM), the characteristics of the carboxylation reaction were different. First, there was some activity at $10 \mu\text{M Mn}^{2+}$, and this was greatly increased by inclusion of Mg^{2+} . Second, there was substantial activity at $100 \mu\text{M Mn}^{2+}$, but Mg^{2+} was inhibitory, as at 5 mM Mn^{2+} . For the decarboxylation reaction, in the absence of ME, there was essentially no activity at 10 or $100 \mu\text{M Mn}^{2+}$, but activity was greatly stimulated by the presence of Mg^{2+} . Maximum activity was still observed at 5 mM Mn^{2+} and Mg^{2+} was inhibitory. In the presence of ME, the pattern of response to Mn^{2+} and Mg^{2+} was much the same, except that there was an inhibition of PEPCK activity compared with the absence of ME. ME was therefore inhibitory to the decarboxylation reaction, but stimulated the carboxylation reaction dramatically at low micromolar concentrations of Mn^{2+} in the presence of millimolar concentrations of Mg^{2+} (Fig. 2).

Kinetic constants were determined for PEPCK from illuminated leaves (light) at both 5 mM Mn^{2+} and 10

$\mu\text{M Mn}^{2+}/4 \text{ mM Mg}^{2+}$. There were decreases in the affinities of PEPCK for phosphoenolpyruvate (PEP) and ADP in the carboxylation reaction and for oxaloacetate (OAA) and ATP in the decarboxylation reaction when assayed at micromolar concentrations of Mn^{2+} in the presence of 4 mM Mg^{2+} . The increase in K_m was substantial in the case of ATP. However, there was a large increase in the affinity of PEPCK for CO_2 when Mn^{2+} was lowered from 5 mM to $10 \mu\text{M}$. Figure 3 shows that at least part of the decrease in K_m (CO_2) was the result of the inclusion of Mg^{2+} (compare plots with 5 mM Mn^{2+} and $5 \mu\text{M Mn}^{2+}/4 \text{ mM Mg}^{2+}$). There was no further decrease in the K_m (CO_2) when PEPCK was assayed in the presence of $1 \mu\text{M Mn}^{2+}$ (Fig. 3). However, inclusion of ATP as well as ADP in the carboxylation reaction resulted in a further 3-fold reduction in the K_m (CO_2) as the ATP to ADP ratio was increased (Fig. 4).

When the properties of PEPCK purified from darkened leaves (dark) were compared with that purified from illuminated leaves (light) at $10 \mu\text{M Mn}^{2+}/4 \text{ mM Mg}^{2+}$ (Table I), there was no difference in the affinity of the light and dark enzymes for CO_2 and there were no significant changes in affinities for PEP and ADP in the carboxylation reaction. There were substantial decreases in the affinities for OAA and ATP in the decarboxylation reaction with an approximate doubling of the K_m . These are the result of light-dependent changes in the phosphorylation state of PEPCK, as discussed by Walker et al. (2002).

DISCUSSION

Previous investigators of the assay conditions and kinetics of PEPCK from plants have assayed it at unphysiological concentrations of Mn^{2+} ($> 0.5 \text{ mM}$)

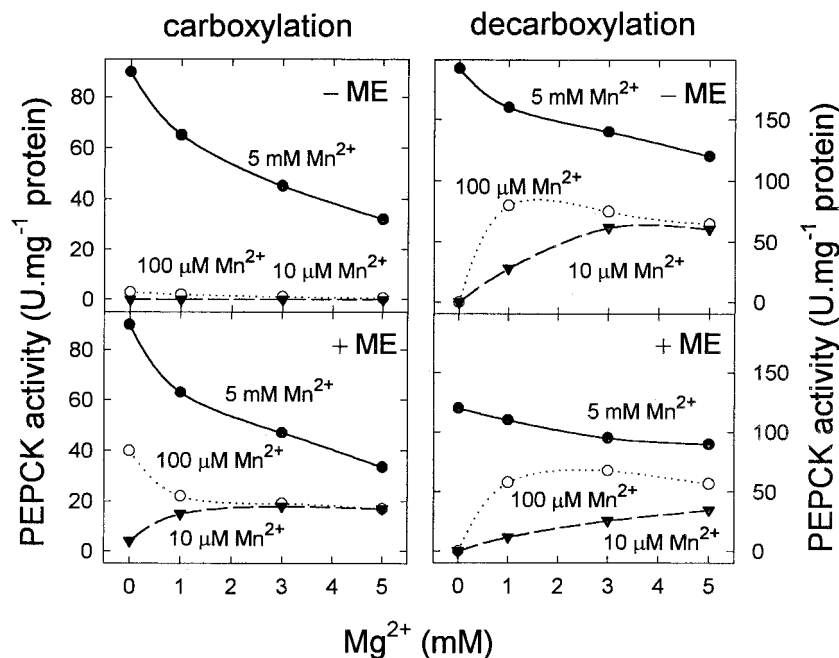


Figure 1. Effect of Mn^{2+} and Mg^{2+} concentration on the carboxylation and decarboxylation activities of Guinea grass PEPCK, measured in either the presence or absence of ME. Substrate concentrations for the carboxylation assay were $0.5 \text{ mM ADP}/5 \text{ mM PEP}$ and for the decarboxylation assay $0.5 \text{ mM ATP}/200 \mu\text{M OAA}$.

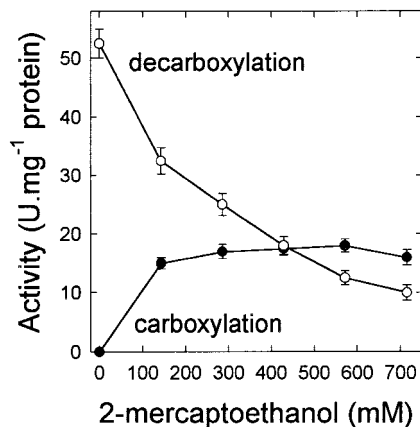


Figure 2. Effect of ME concentration on both the carboxylation and decarboxylation activities of Guinea grass PEPCK, measured at $10 \mu\text{M Mn}^{2+}/5 \text{ mM Mg}^{2+}$. Substrate concentrations for the carboxylation assay were $0.5 \text{ mM ADP}/5 \text{ mM PEP}$ and for the decarboxylation assay $0.5 \text{ mM ATP}/200 \mu\text{M OAA}$.

and have concluded that plant PEPCK is strongly inhibited by millimolar concentrations of Mg^{2+} (Burnell, 1986; Walker et al., 1997). This behavior is different to that reported for the enzyme from non-plant tissues, in which a synergistic activation by a combination of Mn^{2+} and Mg^{2+} occurs, e.g. the enzyme from yeast (Cannata and Stoppani, 1963) and *Trypanosoma cruzii* (Jurado et al., 1996), which show substantial sequence similarity to the plant enzyme, and the enzyme from rat liver, which shows no significant sequence similarity (Foster et al., 1967). It has been suggested that, in the rat liver enzyme, Mg^{2+} forms a MgATP^{2-} complex that acts as a substrate for the reaction, whereas Mn^{2+} acts as an activator at a separate site (Foster et al., 1967). The present results resolve this discrepancy between PEPCK from plant and non-plant tissues and show that PEPCK from

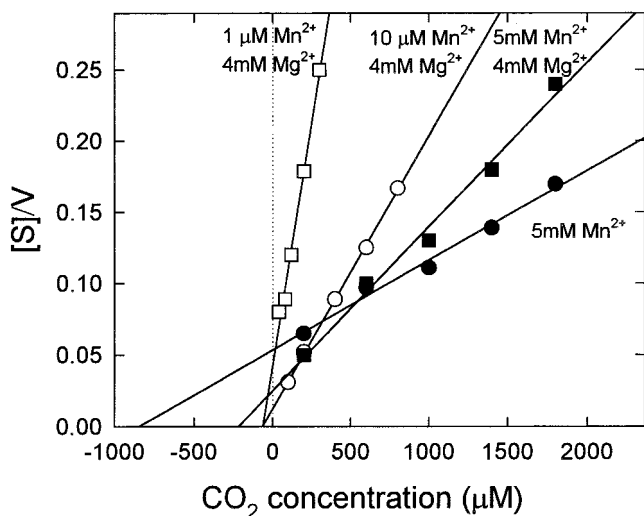


Figure 3. Hanes-Woolf plots, which show the effect of Mn^{2+} and Mg^{2+} concentration on the affinity of Guinea grass PEPCK for CO_2 . Substrate concentrations were $5 \text{ mM PEP}/0.5 \text{ mM ADP}$.

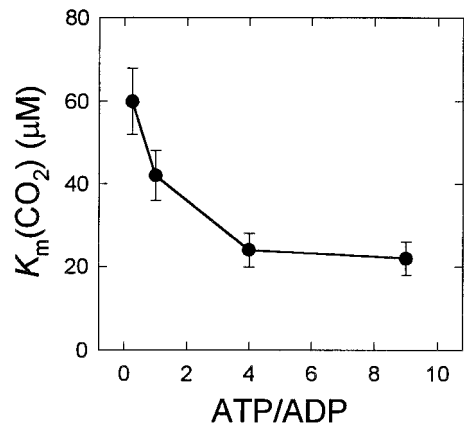


Figure 4. Effect of ATP to ADP ratio (total adenylate concentration 1 mM) on the affinity of Guinea grass PEPCK for CO_2 . These assays used $10 \mu\text{M Mn}^{2+}/4 \text{ mM Mg}^{2+}/5 \text{ mM PEP}$. Michaelis constants were determined from Hanes-Woolf plots and are the means $\pm \text{SE}$ of three separate determinations.

Guinea grass is (a) totally dependent on Mn^{2+} , (b) that it can operate at physiological (μM) concentrations of Mn^{2+} , and (c) that physiological (mM) concentrations of Mg^{2+} activate the enzyme at physiological concentrations of Mn^{2+} (presumably forming an MgATP^{2-} substrate complex). PEPCK thus has a dual requirement for both Mn^{2+} and Mg^{2+} . However, it is also clear that measurements of the maximum activity of PEPCK in plant extracts should still be made under conditions of saturating Mn^{2+} in the absence of Mg^{2+} .

The improvements made to the assay of PEPCK involve the inclusion of high concentrations of ME. A notable property of both ATP-dependent (plant) and GTP-dependent PEPCKs is their inactivation by thiol-modifying reagents (Chang and Lane, 1966), affecting nucleotide binding at the active site (Lewis et al., 1993). Although the sequence of the ATP-dependent PEPCK from higher plants, yeast, and many bacteria shows little similarity to the GTP- or ITP-dependent enzyme found in animals and other bacteria, the active site has considerable homology in both ATP- and GTP- or ITP-dependent PEPCKs. Reactive thiol groups in ATP-dependent PEPCKs occur in yeast (Cardemil et al., 1990) and *T. cruzii* (Jurado et al., 1996). Cardemil et al. (1990) reported that the inactivation of yeast PEPCK by thiol reagents is caused by modification of both thiol and vicinal dithiol groups within the active site of each subunit and that loss of activity was effectively prevented by the combined presence of ATP plus Mn^{2+} (see also Walker et al., 1997). Whether or not this loss of activity, that can be more severe for the carboxylation than for the decarboxylation reaction (e.g. Ray and Black, 1976), is related to the changes in metal ion dependence with ME for the carboxylation reaction, as shown in Figure 1, has yet to be resolved. Another possibility is that ME is acting to chelate inhibitory trace metals or to stabilize the concentration of Mn^{2+} .

Table 1. Kinetic constants for PEPCK from illuminated leaves of *Panicum maximum*

Carboxylase activity was measured using 10 mM CO₂/0.5 mM ADP/5 mM PEP) and decarboxylase activity using 0.5 mM ATP/200 μM OAA. Michaelis constants were determined from Hanes-Woolf plots and are the means ± SE of three separate determinations. Measurements are for the enzyme purified from illuminated leaves except for the row labelled dark, which are for the enzyme purified from darkened leaves.

Metal Ions in Assay	K_m				
	CO ₂	PEP	ADP	OAA	ATP
	μM	mM	mM	μM	μM
5 mM Mn ²⁺	732 ± 90	1.38 ± 0.21	18 ± 2.0	85 ± 15	1.5 ± 0.2
5 mM Mn ²⁺ + 4 mM Mg ²⁺	238 ± 55	1.67 ± 0.30	17 ± 2.5	100 ± 16	1.1 ± 0.3
10 μM Mn ²⁺ + 4 mM Mg ²⁺					
Light	55 ± 18	2.10 ± 0.25	36 ± 3.3	156 ± 20	25.7 ± 3.5
Dark	55 ± 19	2.65 ± 0.31	44 ± 3.1	304 ± 25	43.5 ± 3.3

However, the fact that ME stimulated the carboxylation reaction but was inhibitory to the decarboxylation reaction suggests that this explanation is less likely.

An important consequence of assay of PEPCK at physiological concentrations of metal ions is the effect on its substrate affinities, in particular a marked decrease in its affinity for ATP and a 13-fold increase in its affinity for CO₂. PEPCK from higher plant and algal sources has always been notable for its low affinity for CO₂. For example, the K_m (CO₂) in Guinea grass has been estimated at 1.36 and 1.61 mM (calculated from Urbina and Avilan [1989] and Ray and Black [1976], respectively), 0.497 mM in pineapple (calculated from Daley et al. [1977]) and 0.175 to 1.21 mM in a range of brown and green algae (Table II in Johnston and Raven, 1983). This low affinity for CO₂ has been particularly controversial in the algae in which it has been proposed that PEPCK may sometimes act as a carboxylase (Reiskind and Bowes, 1991; Johnston and Raven, 1983). The present results suggest that the K_m (CO₂) of PEPCK can be many-fold lower than previous estimates, reaching as low as 20 μM CO₂ at physiological ATP to ADP ratios (an ATP to ADP ratio above 4:1 is typical in the cytosol of wheat leaf mesophyll protoplasts in the light; Stitt et al., 1982). It is not clear how the ATP to ADP ratio affects the K_m (CO₂). Although chelation of metal ions could be involved, ATP is known to be an effector of PEPCK (Walker et al., 2002). The K_m (CO₂) of PEPCK at high ATP to ADP ratios is comparable with the K_m (CO₂) of Rubisco from C₃ and Crassulacean acid metabolism plants (12–26 μM) or from C₄ plants (28–63 μM; Yeoh et al., 1980, 1981). This means that PEPCK will catalyze a reversible reaction and that it could operate as a carboxylase in vivo. Clearly the balance between carboxylation and decarboxylation will be strongly influenced by the ATP to ADP ratio, that will affect both the equilibrium of the reaction and the K_m (CO₂), and by the availability of reductant or amino donors that will determine the concentration of OAA. Further studies of algal PEPCK are now required.

MATERIALS AND METHODS

Plant Material

Seeds of Guinea grass (*Panicum maximum*) were obtained from the Kew Seed Bank (Royal Botanical Gardens, Kew, UK). Plants were grown in soil in a greenhouse during the summer with no supplementary light.

Purification of PEPCK

PEPCK was purified from both darkened and illuminated leaves of Guinea grass as described by Walker et al. (2002).

Assay of PEPCK

Carboxylase activity was measured as described by Walker et al. (1995), and decarboxylase activity was measured as described by Lee et al. (1981). Modifications to these procedures in individual experiments are described in the text. For determination of CO₂ affinity, solutions were made up in boiled acidified water that had been purged with N₂. The CO₂ concentration was calculated using a pK of 6.365 at 25°C (Umbreit et al., 1972). One unit of PEPCK activity corresponds to the production of 1 μmol product/min at 25°C.

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