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A single serine to alanine substitution decreases bicarbonate affinity of phosphoenolpyruvate carboxylase in C4 Flaveria trinervia

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Abstract

Phosphoenolpyruvate (PEP) carboxylase (PEPc) catalyzes the first committed step of C4 photosynthesis generating oxaloacetate from bicarbonate (HCO3−) and PEP. It is hypothesized that PEPc affinity for HCO3− has undergone selective pressure for a lower $K_{\text{HCO}_3}$ ($K_{\text{m}}$ for HCO3−) to increase the carbon flux entering the C4 cycle, particularly during conditions that limit CO2 availability. However, the decrease in $K_{\text{HCO}_3}$ has been hypothesized to cause an unavoidable increase in $K_{\text{PEP}}$ ($K_{\text{m}}$ for PEP). Therefore, the amino acid residue S774 in the C4 enzyme, which has been shown to increase $K_{\text{PEP}}$, should lead to a decrease in $K_{\text{HCO}_3}$. Several studies reported the effect S774 has on $K_{\text{PEP}}$; however, the influence of this amino acid substitution on $K_{\text{HCO}_3}$ has not been tested. To test these hypotheses, membrane-inlet mass spectrometry (MIMS) was used to measure the $K_{\text{HCO}_3}$ of the photosynthetic PEPc from the C4 Flaveria trinervia and the non-photosynthetic PEPc from the C3 F. pringlei. The cDNAs for these enzymes were overexpressed and purified from the PEPc-less PCR1 Escherichia coli strain. Our work in comparison with previous reports suggests that $K_{\text{HCO}_3}$ and $K_{\text{PEP}}$ are linked by specific amino acids, such as S774; however, these kinetic parameters respond differently to the tested allosteric regulators, malate and glucose-6-phosphate.

Keywords: Bicarbonate kinetics, C4 photosynthesis, membrane-inlet mass spectrometry, phosphoenolpyruvate carboxylase.

Introduction

Phosphoenolpyruvate (PEP) carboxylase (PEPc) catalyzes the irreversible carboxylation of PEP using bicarbonate (HCO3−) to form the four-carbon sugar, oxaloacetate (OAA). In plants, this reaction generally influences stomatal conductance (Parvathi and Raghavendra, 1997; Cousins et al., 2007), seed development (Sangwan et al., 1992; O’Leary et al., 2011), pH regulation (Davies, 1986; Britto and Kronzucker, 2005), and the balance between carbon and nitrogen metabolism by providing intermediates for the tricarboxylic acid (TCA) cycle (Rademacher et al., 2002; Plaxton and Podestá, 2006). In mesophyll cells of C4 plants, PEPc catalyzes the first committed step of C4 photosynthesis by providing OAA that is subsequently modified to other four-carbon compounds before entering bundle sheath cells for decarboxylation, releasing CO2 at the site of Rubisco (Hatch et al., 1975; von Caemmerer and Furbank, 2003).

Higher plants contain multiple PEPc-encoding (ppc) genes comprising a multigene family where most of the....
genes encode a non-photosynthetic C3 PEPc (Christin and Besnard, 2009). C4 plants obtained a modified PEPc isofrom to power C4 photosynthesis through mutations to a native ppc coding region (Christin et al., 2007; Rosnow et al., 2014) and upstream promoter region (Schaffner and Sheen, 1992; Gowik et al., 2004). Changes to the C4 ppc promoter region led to strong, mesophyll-specific expression, resulting in high PEPc activity to drive the CO2-concentrating mechanism of C4 photosynthesis (Gowik et al., 2004). Work on PEPc peptide sequences from members of the Poaceae, Amaranthaceae, Asteraceae, and Cyperaceae families (Christin et al., 2007), as well as the Chenopodaceae family (Rosnow et al., 2014), identified amino acid residues predicted to be under positive selection in these C4 lineages. Comparing PEPc sequences of species within and between families shows that C4 PEPc isoforms from different species possess different combinations of amino acid residues under positive selection (Christin et al., 2007; Rosnow et al., 2014). These findings suggest that there are multiple ways the C4 PEPc kinetic properties can arise in different C4 origins or that there is diversity in the PEPc kinetics between species.

An increase in PEPc activity in the leaf mesophyll cytosol in the intermediate C3/C4 species would be likely to lead to selection for changes in kinetic properties (Westhoff and Gowik, 2004). This was previously tested in a variety of C3/C4 species that displayed a progression in altered K_{PP} (K_{m} for PEP) and decreased malate sensitivity (Westhoff and Gowik, 2004). C4 plants contain high levels of malate in the mesophyll cytosol, so there would be selection for amino acid substitutions that transition the malate-sensitive C3 PEPc to a less sensitive C4 PEPc (Blüising et al., 2002; Paulus et al., 2013). This is supported by the Gly884 substitution in the Flaveria trinervia C4 PEPc to the Flaveria prinilliseconds C3 PEPc arginine that caused the C4 PEPc to lose its resistance to malate (Paulus et al., 2013).

As PEPc transitioned from C3 to C4 function, it has been suggested that certain amino acid substitutions were under positive selection to alter K_{PP} and K_{HCO3} (K_{m} for HCO3^{-}). It is hypothesized that certain mutations in the C4 ppc coding region resulted from strong selective pressures to obtain a lower K_{HCO3} that of the C3 PEPc (Jacobs et al., 2008). The lower K_{HCO3} of the C4 PEPc may enhance the efficiency of C4 photosynthesis, especially when HCO3^{-} availability is low due to reduced stomatal conductance. Alternatively, the C4 PEPc has been shown to have a higher K_{PP}, with values typically reported between 100 µM and 590 µM (Svensson et al., 1997; Dong et al., 1998; Westhoff and Gowik, 2004; Lara et al., 2006; Rosnow et al., 2015), as compared with C3 non-photosynthetic PEPc K_{PP} values which range from 13 µM to 60 µM (Westhoff et al., 1997; Blüising et al., 2002; Gowik et al., 2006; Lara et al., 2006; Rosnow et al., 2015). It was hypothesized that this increase in C4 K_{PP} was an unavoidable consequence of the reduction of K_{HCO3} since the two kinetic traits may be linked by certain amino acids (Jacobs et al., 2008; Gowik and Westhoff, 2011). Alternatively, since the PEP pools in a C3 leaf are higher than in a C4 leaf, the high K_{PP} of the C4 PEPc may ensure stronger diurnal regulation of PEPc (Budde and Chollet, 1986; Hatch, 1987).

Residue S774 in F. trinervia (S780 in maize) was shown to be under positive selection by Poetsch et al. (1991) and Hermans and Westhoff (1992), and substituting the conserved C4 serine for the conserved C3 alanine in F. trinervia (S774A) significantly decreased the K_{PP} of the C4 PEPc (Blüising et al., 2000; Engellmann et al., 2002). Since the S774A substitution affects the K_{PP} of PEPc, it is possible that it may also affect the K_{HCO3}, making S774 one of the residues potentially linking K_{PP} and K_{HCO3}. However, this serine residue was unimportant for the high K_{PP} in the C4 Chenopodaceae (Rosnow et al., 2014).

The only study to publish K_{HCO3} of both a C3 and C4 PEPc showed that the K_{HCO3} of five C4 species representing the Poaceae and Amaranthaceae families was ~26 µM compared with preliminary evidence suggesting that the K_{HCO3} of the C3 PEPc from Flaveria conquisitii (Asteraceae family) was 80 µM (Bauwe, 1986). Other studies reported C4 PEPc K_{HCO3} values ranging from 14 µM to 180 µM (Janc et al., 1992; Gao and Woo, 1995; Parvathi et al., 2000; Boyd et al., 2015), where the C3 K_{HCO3} of 80 µM falls within this reported range of C4 K_{HCO3} values. However, comparing K_{HCO3} for closely related C3 and C4 PEPc isoforms can provide a more accurate analysis of the change in C3 to C4 K_{HCO3} and whether there was a strong selective force on PEPc K_{HCO3}, but this date has not been performed. Additionally, the previously reported K_{HCO3} values were obtained by coupling PEPc activity to spectrophotometrically measured NADH oxidation rates. It is difficult to obtain accurate K_{HCO3} values using this method because it does not directly measure changes in HCO3^{-} concentration in the assay. This is problematic because measurements of K_{HCO3} require accurate determinations of PEPc activity and HCO3^{-} concentrations below the K_{HCO3}, which is in the micromolar range. To overcome this problem, membrane-inlet mass spectrometry (MIMS) can be used to measure HCO3^{-} consumption by PEPc accurately and directly in real-time over a wide range of inorganic carbon (C) concentrations, including concentrations well below the K_{HCO3}, without the complication of coupling PEPc activity to the NADH dehydrogenase reaction (Boyd et al., 2015).

In this study, we use MIMS to obtain K_{HCO3} values for the photosynthetic PEPc from the C4 plant F. trinervia and the non-photosynthetic PEPc from the C3 plant F. prinilliseconds that were overexpressed and purified from the PEPc-less PCR1 Escherichia coli strain (Sabe et al., 1984; Svensson et al., 1997). We found that the S774A substitution increases the C4 K_{HCO3}, whereas the A774S substitution did not affect the C3 K_{HCO3}, suggesting that additional amino acids besides S774 are involved in the C4 K_{HCO3} trait. Since previous studies reported PEPc K_{PP} changing in the presence of the allosteric activator glucose 6-phosphate (G6-P) and the inhibitor malate (Huber and Edwards, 1975; Wedding et al., 1990; Gupta et al., 1994; Blüising et al., 2002), we tested whether these allosteric regulators also affected K_{HCO3}. We report that G6-P and malate have a minimal effect on the K_{HCO3} of PEPc. We address how differences in calibration methods, assay conditions, and enzyme extractions can produce different in vitro kinetic values, and we report an improvement to the MIMS PEPc assay. Lastly, we demonstrate how the decrease in K_{HCO3} between the C3 and
C4 PEPc isoforms increases the modeled rates of C4 photosynthesis at low CO2 concentrations.

Materials and methods

Generating PCR1 PEPc-overexpressing lines

The PEPc-less E. coli strain, PCR1 (Sabe et al., 1984), and PEPc cDNA constructs used in Svensson et al. (1997) and Bläsiang et al. (2000) were generously provided by Professor Peter Westhoff’s lab. The plasmid, pTrc99A, carrying the cDNA coding for either the C4 F. trinervia PEPc, the C3 F. pringlei PEPc, or Flaveria PEPc with either an alanine or serine substitution at residue 774, C2774A or C2774S, respectively, was transformed into the PCR1 E. coli strain. PCR1 transformants producing plant PEPc were selected following the method of Svensson et al. (1997).

Growth of PCR1 PEPc-overexpressing lines for PEPc extraction

A 4 ml growth culture (Luria–Bertani broth; 0.1% w/v dextrose; 100 µg ml⁻¹ ampicillin) was inoculated with a glycerol stock of the PCR1 strain carrying a Flaveria PEPc construct and was incubated at 28 °C with shaking at 160 rpm overnight. The following morning, the 4 ml culture was centrifuged at 1530 g for 10 min at room temperature. The supernatant was discarded, and the bacterial pellets were resuspended and transferred to a large 500 ml growth culture which was incubated at 28 °C and shaken at 160 rpm. After 6 h of incubation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 µM to the 500 ml growth culture to induce PEPc production overnight.

PEPc extraction and purification from E. coli

The 500 ml growth culture was centrifuged at 2600 × g for 10 min at room temperature and the bacterial pellets were resuspended in a total volume of 20 ml of ice-cold lysis buffer [50 mM Tris–HCl, pH 8.0; 0.5 M NaCl; 10 mM DTT; 1 mM EDTA, pH 8.0; 20 µl ml⁻¹ E. coli protease inhibitor (Sigma); 1 mg ml⁻¹ lysozyme (Biorwold); 10% (v/v) glycerol; 20% (v/v) sucrose]. The resuspended cells were placed in ice for 30 min and then lysed via sonication (BioLogics Ultrasonic Homogenizer 300 V/T). The sonicated cells were transferred to centrifuge tubes and were spun at 30 597 × g for 30 min at 4 °C. The supernatant was collected and MgCl₂ was added to the supernatant to a final concentration of 10 mM. Polyethylene glycol (50% PEG 8000) was added to the supernatant to a final concentration of 6% (v/v) before placing the supernatant on ice for 15 min with gentle mixing. The supernatant was again spun at 30 597 × g for 20 min at 4 °C. The protein pellets were discarded and 50% PEG 8000 was added to the final concentration of 12% (v/v). The supernatant was slowly stirred on ice for 15 min before centrifugation at 30 597 × g for 20 min at 4 °C.

The protein pellet was collected and resuspended in 6 ml of Buffer A [0.5 M (NH₄)₂SO₄, 20 mM Tris–HCl, pH 7.5; 0.1 mM DTT; 1 mM EDTA, pH 8.0; 5% (v/v) glycerol] supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Svensson et al., 1997). The protein sample was loaded onto a HiC phenyl–Sepharose column (1 cm × 5.5 cm) pre-incubated with Buffer A at a flow rate of 1.5 ml min⁻¹. The hydrophobic properties of PEPc were used for the partial purification of PEPc by following the protocol of Svensson et al. (1997).

Fractons from the phenyl–Sepharose column were analyzed for PEPc activity by coupling the PEPc and NADH dehydrogenase reactions following the protocol of Boyd et al. (2015). Fractions displaying the highest PEPc activity were pooled and desalted in Buffer B [100 mM HEPES-KOH, pH 7.6; 1 mM DTT; 1 mM EDTA, pH 8.0] and concentrated using Corning Spin-X UF columns (6 ml volume, 100 K molecular weight cutoff) according to the Corning procedure. Glycerol was added to a final concentration of 20% (v/v) before storage at −80 °C. Total protein content of the PEPc samples was measured by a modified Bradford assay (Bio–Rad Protein Assay Kit II) (Bradford, 1976) using the Bio–Rad procedure.

Extracting PEPc from F. trinervia and Setaria viridis

PEPc samples were extracted and desalted from leaves of F. trinervia and S. viridis following the procedure of Boyd et al. (2015). Once the desalted PEPc extracts were collected and moved through a Millex–GP 0.22 µm syringe filter (Millipore), the extracts were concentrated by spinning the samples at 2880 × g for 20 min at 4 °C in an Amicon Ultra-4 Ultracel-100K centrifugal filter (Millipore). Glycerol was added to the concentrated PEPc samples to a final concentration of 20% (v/v) and stored at −80 °C.

Obtaining Vₚmax, KₕCO₃, and Hill values for the different PEPc isoforms

The HCO₃⁻–dependent PEPc assays were run in a 600 µl cuvette attached to the inlet of a mass spectrometer as described by Cousins et al. (2010). A CO₂ calibration was conducted before each HCO₃⁻ response curve as reported by Boyd et al. (2015). The calibration consisted of three 2 µl injections of 10 mM NaHCO₃ into 0.1 N HCl and three 6 µl injections of 100 mM NaHCO₃ into the PEPc reaction mixture [100 mM HEPES-KOH, pH 7.6; 10 mM MgCl₂; 1 mM DTT; 50 µg ml⁻¹ carbonic anhydrase (CA); 5 mM G6-P, 5 mM PEP].

To measure PEPc HCO₃⁻ kinetics, seven NaHCO₃ concentrations (50, 100, 200, 350, 500, 750, and 1000 µM) were used for the assays. The CO₂ was removed from the assay buffer containing 100 mM HEPES-KOH, pH 7.6 and 10 mM MgCl₂ by continuously bubbling the buffer with humidified N₂ gas starting at least 1 h prior to initiating the assays. The assay buffer (600 µl) followed by 1 mM DTT, 50 µg ml⁻¹ CA, 5 mM G6-P, 5 mM PEP, and various NaHCO₃ concentrations were added to the reaction cuvette and held at a constant 25 °C with a temperature-controlled water bath.

A blank rate was obtained by measuring the change in the mass 44 (12C)O²⁻O³⁻) signal during a 30 s period before initiating the reaction with the addition of 10–15 µg of total protein of the PEPc extract. The PEPc reaction was run for 5 min but the first 20 s of the PEPc reaction was discarded to allow for enzyme mixing and rate stabilization. The MIMS reports a mass 44 signal every 0.8 s, so a robust 10 s running average of the change in mass 44 was used as a single data point for PEPc activity (Vₚ) at an averaged [HCO₃⁻]. Data points from 10 s running averages were taken immediately following the 20 s mixing phase. For the larger NaHCO₃ injections (350–1000 µM NaHCO₃), 10 s running averages were taken until a drop in PEPc activity was observed to avoid data points where there might be end-product inhibition of the reaction. For the lower NaHCO₃ concentrations (50–200 µM), 10 s running averages were taken until the reaction was depleted of CO₂. Once the CO₂ was depleted from the 50, 100, and 200 µM NaHCO₃ injections, as indicated by a zero slope for the mass 44 signal, a 30 s running average of the zero slope was taken to obtain a mass 44 zero. These mass 44 zeroes accounted for mechanical drift in the MIMS as the different zeroes were taken at various times throughout the HCO₃⁻ response curve.

The kinetic parameters Vₚmax, KₕCO₃, and Hill value (h) were obtained by using the Hill equation:

\[
V_p = \frac{V_{\text{max}} \times [\text{HCO}_3^\text{−}]^h}{(K_{\text{HCO}_3^\text{−}})^h + [\text{HCO}_3^\text{−}]^h}
\]

where the Hill equation was fit to the HCO₃⁻ response curve using Excel’s Solver function to produce the kinetic parameters listed above.
Measuring the impact of G6-P and malate on \(K_{HCO_3}\)

To measure the effect of G6-P on \(K_{HCO_3}\), G6-P was omitted from the assay described above to compare \(K_{HCO_3}\) values in the presence or absence of 5 mM G6-P. Alternatively, 2.5 mM malate (pH 7.6) was added to the assay described above to determine if malate affects PEPc \(K_{HCO_3}\) values in the presence or absence of G6-P.

Measuring PEP effects on malate inhibition

The MIMS assay described above was used to determine if PEP concentration affects malate inhibition of PEPc, in the absence of G6-P. Five malate concentrations were used (0, 1, 2, 3, and 4 mM) to determine the percentage change in enzyme activity of the C3 and C4 PEPc isoforms in the presence of saturating NaHCO3 (1000 µM) and when PEP was saturating (5 mM) or non-saturating (150 µM and 750 µM for the C3 and C4 isoforms, respectively). The non-saturating PEP concentrations were determined to be twice the reported \(K_{PEP}\) values (2\(\times K_{PEP}\)) in the absence of G6-P (Westhoff and Gowik, 2004).

Extraction source, pH, and calibration effects on PEPc \(K_{HCO_3}\)

Previously, MIMS measurements of desalted PEPc extracts from \(S. \text{viridis}\) reported a \(K_{HCO_3}\) of 62.8 ± 5.0 µM (Boyd et al., 2015). Therefore, we tested whether differences in PEPc source (plant versus \(E. \text{coli}\)), pH, or MIMS calibrations caused the \(K_{HCO_3}\) reported here to differ from those of Boyd et al. (2015). The MIMS assay described above containing 5 mM G6-P and no malate was used to test whether PEPc samples extracted from leaves of \(F. \text{tineoidea}\) and \(S. \text{viridis}\) produced different \(K_{HCO_3}\) values from the C3 PEPc extracted from \(E. \text{coli}\). For each assay, 5–10 µl of plant extract was added to initiate the PEPc reaction. PEPc extracts from \(S. \text{viridis}\) were used to compare \(K_{HCO_3}\) values obtained at pH 7.6, the pH used in this study, with \(K_{HCO_3}\) values obtained at pH 7.8 used by Boyd et al. (2015). These extracts were also used to compare \(K_{HCO_3}\) values obtained at pH 7.8 using either the current calibration method outlined above or the calibration method of Boyd et al. (2015).

Statistical analysis of experimental data

Statistical analyses of the kinetic data were performed using RStudio version 1.1.447 (RStudio Team, 2016). Homogeneity of variance was checked using Levene tests, and normality was checked using residual quantile plots and residual versus fitted value plots. Non-normal data were log transformed but they reported the same statistical outcomes as the transformed data for simplicity only non-transformed data analyses are presented. One-way ANOVA and Tukey HSD post-hoc tests were used to determine statistical significance (\(P<0.05\)) of \(K_{HCO_3}\) between PEPc isoforms. Two-way ANOVA (\(P<0.05\)) and Tukey HSD post-hoc tests were used to analyze statistically significant differences in \(K_{HCO_3}\) between isoforms and the impact of potential allosteric effectors. A two-way repeated measures ANOVA (\(P<0.05\)) was used to test if the change in PEPc activity in response to malate significantly differed between C3 and C4 PEPc isoforms at various PEP concentrations. One-way ANOVA and Tukey HSD post-hoc tests were used to determine significance differences between C3 PEPc extracted from \(E. \text{coli}\) and PEPc extracted from \(F. \text{tineoidea}\) and \(S. \text{viridis}\) leaves. Student’s \(t\)-tests (\(P<0.05\)) were separately used to determine significant differences in \(S. \text{viridis}\) PEPc \(K_{HCO_3}\) assayed at pH 7.6 and 7.8 and for \(S. \text{viridis}\) PEPc \(K_{HCO_3}\) assayed at pH 7.8 using the two calibration methods.

Modeling the effect of \(K_{HCO_3}\) on C4 photosynthesis

The modeled effect of \(K_{HCO_3}\) on the response of C4 enzyme-limited photosynthetic CO2 assimilation (\(A_4\)) to changing mesophyll CO2 concentrations (\(C_{\text{m}}\)) was determined by solving the quadratic formula using the set of equations as described by von Caemmerer (2000). The equations and input variables were taken from von Caemmerer et al. (1994), von Caemmerer (2000), Tholen and Zhu (2011), and Ubierna et al. (2013), and are presented in Supplementary Table S1 at JXB online.

Results

Kinetics of the C3, C4, and chimeric PEPc isoforms

The Hill equation was used to determine the maximum rate of PEPc carboxylation (\(V_{\text{max}}\)), the \(K_m\) for bicarbonate (\(K_{HCO_3}\)), and the co-operativity of the PEPc active sites (\(h\)) from 25 °C MIMS measurements of PEPc activity (\(V_p\)) in response to changes in HCO3− concentrations (Supplementary Fig. S1). Measurements were made on C4, C3, and chimeric Flaveria PEPc isoforms expressed and partially purified from \(E. \text{coli}\). The C4 PEPc had a significantly lower \(K_{HCO_3}\) than the C3 PEPc, 26.6 ± 1.7 µM and 64.0 ± 2.4 µM, respectively (Fig. 1). Additionally, the C4 PEPc had a lower \(V_{\text{max}}\) compared with the C4 PEPc, 5.1 ± 0.7 µmol mg protein−1 min−1 and 8.1 ± 0.7 µmol mg protein−1 min−1, respectively (Supplementary Table S2). Neither isoform displayed co-operativity towards HCO3− binding, with Hill values close to 1.0 under all assay conditions (Supplementary Table S2).

The substitution of the conserved C4 serine at residue 774 (780 in maize) with the conserved C3 alanine (C4-S774A) significantly increased the \(K_{HCO_3}\) by 45% from 26.6 ± 1.7 µM to 38.6 ± 5.5 µM (Fig. 1). However, the C4-S774A substitution had no effect on \(V_{\text{max}}\) (Supplementary Table S2). The reverse substitution, C3-A774S made in the C3 PEPc, did not significantly change the \(K_{HCO_3}\) (from 64.0 ± 2.4 µM to 61.5 ± 9.1 µM; Fig. 1) nor did it affect \(V_{\text{max}}\) (Supplementary Table S2).

![PEPc Isoforms](https://academic.oup.com/jxb/article/70/3/995/5229914)

**Fig. 1.** The \(K_{HCO_3}\) of the C3, C4, and chimeric PEPc isoforms. The \(K_{HCO_3}\) values were obtained from the MIMS assayed in 100 mM HEPES-KOH buffer (pH 7.6) that contained 10 mM MgCl2, 5 mM PEP, 50 µg ml−1 CA, 1 mM DTT, and 5 mM G6-P. Error bars represent the mean ±SD of four independent extractions from \(E. \text{coli}\) for each PEPc isoform. Significance was determined by one-way ANOVA and Tukey HSD post-hoc tests. Bars with different letters are significantly different (\(P<0.05\)).
The impact of G6-P and malate on $K_{\text{HCO}_3}$

The $V_{\text{max}}$ did not change by omitting G6-P from the assay, regardless of the PEPc isoform (Supplementary Table S2). Additionally, the $K_{\text{HCO}_3}$ values of the PEPc isoforms were not significantly altered by the presence or absence of G6-P in the assay (Fig. 2).

Under the current measurement conditions of pH 7.6, 5 mM PEP, and the absence of G6-P, the addition of 2.5 mM malate decreased the $V_{\text{max}}$ in the C4, C$_4$-S774A chimeric, and the C$_3$ PEPc by 57.5, 24.4, and 6.8%, respectively (Supplementary Tables S2, S3). However, the $K_{\text{HCO}_3}$ values of the PEPc isoforms were not significantly altered by the presence of malate in the assay (Fig. 3A). $\text{HCO}_3^-$ response curves in the absence of G6-P were not obtained for the C$_3$-A774S PEPc due to severe inhibition of the chimeric PEPc by malate (Supplementary Fig. S2). When 5 mM G6-P and 2.5 mM malate were both present in the PEPc assay, $V_{\text{max}}$ decreased in the C$_4$, C$_4$-S774A chimeric, C$_3$, and C$_3$-A774S chimeric PEPc by 44.4, 28.6, 2, and 14%, respectively (Supplementary Tables S2, S3). However, the $K_{\text{HCO}_3}$ values of the PEPc isoforms were not significantly changed with both G6-P and malate in the assay (Fig. 3B).

Interestingly, decreasing the total PEP concentration in the assay from 5 mM to twice the reported $K_{\text{PEP}}$ of the C$_3$ and C$_4$ isoforms (Bläsing et al., 2002; Paulus et al., 2013), 150 µM and 750 µM PEP, respectively, caused the C$_3$ PEPc to lose activity dramatically in the presence of malate, whereas the change in PEP concentration had a smaller effect on malate inhibition of C$_4$ PEPc activity (Fig. 4).

**Effects of MIMS calibrations, assay conditions, and extraction sources on $K_{\text{HCO}_3}$**

At pH 7.6, the C$_4$ F. trinervia PEPc extracted from E. coli had a lower $K_{\text{HCO}_3}$ than desalted plant PEPc extracts from F. trinervia, 26.6 ± 1.7 µM and 35.2 ± 3.2 µM, respectively (Fig. 5A). Although not statistically significant, changing the pH of the assay buffer from 7.6 to 7.8 increased the $K_{\text{HCO}_3}$ of S. viridis PEPc by 21.3% from 30.0 ± 3.0 µM to 36.4 ± 5.3 µM (Fig. 5A, 5B). When using the Boyd et al. (2015) MIMS calibration method at pH 7.8, the S. viridis $K_{\text{HCO}_3}$ increased to 62.9 ± 8.7 µM (Fig. 5D).
Fig. 4. Malate resistance of the C3 PEPc is affected more by changing PEP concentrations than that of the C4 PEPc. At 5 mM PEP (filled circles and squares), the C3 PEPc (solid line) is more resistant to malate than the C4 PEPc (dashed line). When the PEP concentration was dropped to twice the reported K_{HCO3} for the C3 and C4 PEPc isoforms (open circles and squares), malate resistance of the C3 PEPc dropped drastically compared with the malate resistance for the C4 PEPc. Malate activity assays were performed in 100 mM HEPES-KOH (pH 7.6), 10 mM MgCl₂, 1 mM DTT, 50 µg ml⁻¹ CA, 2.5 mM malate (pH 7.6), 1 mM NaHCO₃, and various PEP concentrations. Shapes and error bars represent the mean ±SD of four independent extractions from E. coli for both the C3 and C4 PEPc isoforms. A two-way repeated measures ANOVA (P<0.05) determined that there was a significant difference between the increased sensitivity to malate of the C3 PEPc versus the C4 PEPc when PEP concentration was decreased to 2×K_{PEP} for each isoform.

Fig. 5. The K_{HCO3} measured under different assay conditions for PEPc isoforms extracted from F. trinervia, S. viridis, and E. coli. (A) The K_{HCO3} values of the C3 PEPc extracts from E. coli (black bar; value from Fig. 1) and desalted PEPc extracts from F. trinervia and S. viridis (gray bars) were obtained from the MIMS assay in 100 mM HEPES-KOH buffer (pH 7.6) with 10 mM MgCl₂, 5 mM PEP, 50 µg ml⁻¹ CA, 1 mM DTT, and 5 mM G6-P. Bars represent the mean ±SD of four independent PEPc extractions. Significance was determined by one-way ANOVA and Tukey HSD tests. Bars with different letters are significantly different (P<0.05). (B) S. viridis PEPc K_{HCO3} values measured from assays at pH 7.8 using either the current MIMS calibration or the Boyd et al. (2015) calibration. Significance between the K_{HCO3} of S. viridis PEPc assayed at (A) pH 7.6 and (B) 7.8 was determined by a Student’s t-test (P<0.05). Significance between the K_{HCO3} of S. viridis PEPc assayed at pH 7.8 obtained by either the current calibration method or the Boyd et al. (2015) calibration method was determined by a Student’s t-test (P<0.05).

Modeling the effect of different K_{HCO3} values on C₄ photosynthesis

The C3 and C4 PEPc K_{HCO3} values from Fig. 1 were input into the C4 photosynthesis model from von Caemmerer (2000) to determine how differences in K_{HCO3} would impact modeled rates of C4 photosynthesis. Varying K_{HCO3} with a constant V_{Pmax} significantly changed the modeled rates of C4 photosynthesis under CO₂ conditions below 20 Pa. The lower K_{HCO3} of the C4 PEPc resulted in higher modeled rates of C4 photosynthesis at these low mesophyll CO₂ concentrations (C₅, Fig. 6). However, the difference between the C3 and C4 K_{HCO3} modeled no differences in net CO₂ assimilation above ~20 Pa C₅ (Fig. 6).

Discussion

Kinetic changes during the evolution of the C₄ PEPc

We and others (Jacobs et al., 2008; Gowik and Westhoff, 2011) have hypothesized that there was a strong selective pressure to reduce the K_{HCO3} of the C₄ PEPc isoform. Additionally, previous studies have reported that changing the PEPc amino acid residue 774 in Flaveria spp. (780 in maize) influences K_{HCO3} and its allosteric regulation (Engelmann et al., 2002; Endo et al., 2008). Therefore, the aim of this research was to test the hypotheses that the K_{HCO3} of the C₄ PEPc isofrom from F. trinervia would be lower than the K_{HCO3} of the C₃ PEPc isofrom from F. pringlei and that changes to residue 774 will impact K_{HCO3} and its allosteric regulation. Residue 774 was chosen because others have shown the C₄ F. trinervia S774A substitution reduces K_{PEP} (Bläsing et al., 2000; Endo et al., 2008). Furthermore, residue 774 is near both the PEP- and HCO₃⁻-binding sites, and may also influence K_{HCO3}. We have analyzed the influence of this residue on K_{HCO3} and showed that the C₄-S774A chimeric PEPc had a significantly higher K_{HCO3} compared with the C₄ PEPc (Fig. 1). This fits with previous data that suggest that K_{PEP} and K_{HCO3} are inversely linked through specific amino acid residues near the two binding sites. For example, the K829G substitution in the F. trinervia C₄ PEPc resulted in a small decrease in K_{HCO3} and a simultaneous increase to K_{PEP} (Gao and Woo, 1996). Alternatively, swapping Lys600 with either an arginine or threonine in F. trinervia led
to increases in both $K_{\text{PEP}}$ and $K_{\text{HCO}_3}$, but this residue is one of the four conserved amino acids comprising the HCO$_3^−$-binding site (Gao and Woo, 1995; Kai et al., 2003).

The chimeric C$_3$ PEPc of F. pringlei, C$_3$-A774S, had a minimal effect on $K_{\text{HCO}_3}$ (Fig. 1). This same amino acid substitution was also shown not to influence the $K_{\text{PEP}}$ of the C$_3$ PEPc with G$_6$-P present in the assay. However, in the absence of G$_6$-P, the same A774S substitution did increase $K_{\text{PEP}}$ (Bläsing et al., 2000). Taken together, these results support the analysis that multiple amino acid residues, in addition to S774 (S780, 2000). Taken together, these results support the analysis suggested by Schlieper et al. (2014), the HCO$_3^−$-binding site is further from the G$_6$-P and aspartic acid/malate allosteric binding sites than the PEP-binding site is from the allosteric sites, so any structural changes to PEP caused by allosteric binding may affect the PEP-binding site more than the HCO$_3^−$-binding site. Additionally, the S774A and A774S substitutions did not influence the allosteric regulation of PEPc to the extent that the R884G and G884R substitutions affected malate sensitivity of the F. pringlei and F. trinervia PEPc isofoms, respectively (Paulus et al., 2013). This discrepancy may be due to residue 884 being closer to the residues of the aspartate/malate-binding sites compared with residue 774, whereas residue 774 is closer to the PEP- and HCO$_3^−$-binding sites (Kai et al., 2003; Paulus et al., 2013).

Another possibility is that PEP binds before HCO$_3^−$ (Janc et al., 1992), potentially conferring the primary allosteric regulation of PEPc to the binding of PEP. Alternatively, under our assay conditions, the high PEP concentration may have reduced the impact G$_6$-P and malate had on $K_{\text{HCO}_3}$. For example, G$_6$-P has a greater activating effect on PEPc under limiting PEP concentrations at 0.5 mM (Gupta et al., 1994). In addition, multiple studies suggest that there is a regulatory PEP-binding site different from the PEPc active site (Rustin et al., 1988; Rodríguez-Sotres and Muñoz-Clares, 1990; Mújica-Jiménez et al., 1998; Yuan et al., 2006), and it is possible this regulatory PEP site may not be saturated under low PEP concentrations. Saturating this regulatory PEP-binding site might supersede G$_6$-P activation and overcome malate inhibition of PEPc (Huber and Edwards, 1975). The assay conditions used in this study contained saturating (5 mM) levels of PEP, which were well above the $K_{\text{PEP}}$ of both PEPc isoforms, since limiting PEP would complicate the response to changes in HCO$_3^−$ concentrations. The C$_3$ PEPc was more resistant to malate inhibition than the C$_4$ PEPc under these saturating PEP conditions, which is in contrast to previous reports (Bläsing et al., 2002; Paulus et al., 2013). However, we found that the C$_3$ PEPc was more sensitive to malate than the C$_4$ PEPc when the PEP concentration in the assay was reduced to 2×$K_{\text{PEP}}$ (Fig. 4). This suggests that the PEP regulatory site for the C$_3$ PEPc may be less sensitive than the C$_4$ PEPc to changes in free PEP availability. Alternatively, the C$_4$ PEP regulatory site may not have as much influence on malate tolerance as the C$_3$ PEP regulatory site under the current assay conditions.

We were unable to obtain kinetic data for the C$_3$-A774S chimeric PEPc due to drastic inhibition of the enzyme by malate when G$_6$-P was absent from the assay (Supplementary Fig. S2). This result was unexpected since the addition of 2.5 mM malate had a small effect on the activity of the C$_3$ PEPc (Supplementary Tables S2, S3). However, since PEP and

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**Fig. 6.** Modeled rates of C$_4$ photosynthesis with C$_3$ and C$_4$ $K_{\text{HCO}_3}$. The $K_{\text{HCO}_3}$ values for the C$_3$ PEPc (filled circles) and C$_4$ PEPc (open circles) were input into the C$_4$ photosynthesis model from von Caemmerer (2000) to determine the modeled rate of CO$_2$ assimilation ($A_{\text{max}}$) at various mesophyll CO$_2$ concentrations ($C_m$). Symbols represent means ±SD of four independent $K_{\text{HCO}_3}$ values presented in Fig. 1 input into the model, where all other variables in the C$_4$ model were held constant. The maximal rates of PEP regeneration ($V_{\text{q}}$), Rubisco carboxylation ($V_{\text{r}}$), and maximum PEPc carboxylation per unit leaf area [V$_{\text{max\,carboxy}}$] were set to 80, 60, and 120 µmol m$^{-2}$ s$^{-1}$, respectively (von Caemmerer, 2000), and all other values are presented in Supplementary Table S1. A $pK_a$ of 6.12 and assumed a mesophyll cytosol pH of 7.2 were used to convert µM HCO$_3^−$ to µM CO$_2$. Pa CO$_2$ was obtained by using Henry’s constant for CO$_2$ (0.034 mol l$^{-1}$ atm$^{-1}$) and assumed standard pressure (101325 Pa atm$^{-1}$).
malate interact differently with the C3 and C4 PEPc isoforms, it is possible that the A774S substitution in the C3 PEPc modified these interactions to allow potent inhibition of the C3–A774S chimeric PEPc. Further analysis is needed to test the extent of malate inhibition on the C3-A774S PEPc and other chimeric PEPc isoforms under various assay conditions. It is worth noting that malate has a stronger inhibitory effect on PEPc at pH 7.0 than at pH 8.0 (Huber and Edwards, 1975; Gupta et al., 1994). As discussed below, pH and other assay conditions used to measure PEPc activity can influence the absolute values of the kinetic parameters.

Assay conditions, extraction method, and source can affect PEPc kinetics

MIMS can directly measure dissolved CO2 even at very low Ci concentrations below the KHCO3 of PEPc (Beckmann et al., 2009; Cousins et al., 2010). Previously, Boyd et al. (2015) reported a MIMS-measured KHCO3 value of 62.8 µM for the S. viridis C4 PEPc which is higher than our MIMS-measured KHCO3 value of 26.6 µM for the F. trinervia C4 PEPc extracted from E. coli. This difference in KHCO3 between the S. viridis and F. trinervia C4 PEPc may be due to any combination of species differences in enzyme kinetics, enzyme purity, pH of the assay, and MIMS calibrations. Plant PEPc extracted from F. trinervia, a dicot in the Asteraeaceae family, and S. viridis, a monocot in the Poaceae family, had similar KHCO3 values at pH 7.6 (Fig. 5A). Bauwe (1986) also reported similar KHCO3 values for different C4 PEPc isoforms extracted from multiple grasses and Glomphrena globosa, a dicot from the Amaryllidaceae family.

The F. trinervia C4 PEPc partially purified from E. coli was reported to be unphosphorylated at the N-terminal serine residue (Svensson et al., 1997) and had a significantly lower KHCO3 than the desalted plant PEPc extracts taken from F. trinervia leaves during the day (Fig. 5). This suggests that potential differences in post-translation modifications might influence the kinetic properties of PEPc. Parvathi et al. (2000) observed a decrease in KHCO3 as PEPc changed from the unphosphorylated to the phosphorylated state, and that PEPc extracts from illuminated leaves had lower KHCO3 values than PEPc extracted in the dark. In the current study, the phosphorylation status of the PEPc extracts was not tested, so it cannot be confirmed that the difference in KHCO3 between the plant and E. coli extracts is due to changes in PTMs. Alternatively, Bauwe (1986) observed that unphosphorylated C4 PEPs had higher KHCO3 values than purified C4 PEPc extracts. It is possible that the impurity of our desalted plant PEPc extracts from F. trinervia contributed to the increased KHCO3 relative to the C4 PEPc purified from E. coli. The potential differences in PTMs and enzyme purity do not completely explain why the KHCO3 for the S. viridis PEPc reported here and by Boyd et al. (2015) differ; however, this discrepancy in KHCO3 can be explained by differences in assay conditions and MIMS calibrations.

Raising the pH of the PEPc assay from 7.6 to 7.8, the pH used by Boyd et al. (2015), increased the S. viridis PEPc KHCO3 by ~21% (Fig. 5A, B). In addition to the pH of the assay buffer, the MIMS calibration method can alter the measured KHCO3. This is because two MIMS calibrations are required to convert a voltage signal of mass 44 to a micromolar concentration of CO2 and to determine the HCO3− concentration in the reaction cuvette. Since the development of a novel MIMS technique to measure KHCO3 of PEPc (Boyd et al., 2015), we have improved the MIMS calibration method to obtain more accurate KHCO3 values to analyze kinetic differences between PEPc isoforms. The calibration method presented here differed from that of Boyd et al. (2015) because all reaction components except the enzyme extract were included in the calibration. This would account for slight pH changes to the assay when adding DTT, G6-P, or PEP, which is important for determining the CO2/HCO3− ratio. If there is a slight reduction in pH from adding assay components that is not accounted for during the calibrations, then the CO2/HCO3− ratio can be slightly overestimated, leading to higher estimations of KHCO3. Using the calibration method and pH of 7.8 from Boyd et al. (2015), the KHCO3 values reported here (62.9 ± 8.7 µM) and by Boyd et al. (2015) (62.8 ± 5.0 µM) were nearly identical (Fig. 5B), suggesting that assay conditions such as pH and differences in MIMS calibration methods can affect the estimated KHCO3. These findings also highlight the important consideration of how well in vivo assay conditions reflect the in vivo conditions where PEPc operates. So far, in vivo PEPc kinetics can only be obtained by models using gas exchange (von Caemmerer, 2000). Further research is needed to compare in vivo and in vitro PEPc kinetics, since accurate PEPc kinetics are needed to model C4 photosynthesis.

KHCO3 affects modeled rates of C4 photosynthesis

The C4 photosynthesis model developed by von Caemmerer (2000) was used to test if differences in KHCO3 between the C3 and C4 PEPc isoforms were enough to influence rates of net CO2 assimilation during C4 photosynthesis. The C4 model predicts that a lower KHCO3 may not affect photosynthetic rates under high CO2 partial pressures (Fig. 6). This is expected since C4 photosynthesis rates are typically not limited by PEPc under these conditions (von Caemmerer, 2000). However, a large KHCO3 may limit rates of C4 photosynthesis under low CO2 partial pressures (Fig. 6), for example when reduced stomatal conductance limits CO2 movement into the leaf. Flux control analysis found that PEPc has substantial control of C4 photosynthesis under low CO2 partial pressures (Dever et al., 1997; Bailey et al., 2000). Therefore, there is likely to be strong selective pressure to increase PEPc affinity for HCO3− in C4 plants to increase the amount of Ci entering C4 photosynthesis. Our results, combined with those of others (Bläsig et al., 2000; Engelmann et al., 2002; Endo et al., 2008), show that as Ci KHCO3 dropped, there was a concurrent increase in Ci KPEP. Due to the higher PEP levels observed in leaves of C4 plants compared with C3 plants (Leegood and von Caemmerer, 1994), it can be argued that there was stronger selective pressure to increase PEPc affinity for HCO3− than to maintain high affinities for PEP since an increase in KPEP may not negatively impact C4 photosynthesis rates to the extent that changes in KHCO3 can under low CO2 partial pressures (Fig. 6).

Conclusion

The direct comparison of closely related C3 and C4 PEPc isoforms from Flaveria demonstrates that the photosynthetic C3 PEPc isoform has a significantly higher affinity for HCO3− than its closely
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related C3 PEPc isoform. This reduced $K_{\text{HCO}_3}$ impacts net CO2 assimilation rates, particularly at low CO2 availability, suggesting selective pressure to reduce the C4 $K_{\text{HCO}_3}$ to optimize inorganic carbon flux through C4 photosynthesis. Alternatively, the increase in $K_{\text{PEP}}$ can be seen as strengthening the diurnal regulation of C4 PEPc but residue S774 appears to link $K_{\text{HCO}_3}$ and $K_{\text{PEP}}$, indicating that the increase in $K_{\text{PEP}}$ could be a negative consequence of reducing $K_{\text{HCO}_3}$. Testing different plant species will provide new insights into which amino acids control $K_{\text{HCO}_3}$ and provide a better understanding of the structure and function relationship of the enzyme. Obtaining a better understanding of what controls $K_{\text{HCO}_3}$ will also lead to enhancing C4 photosynthesis, particularly at low CO2 partial pressures when stomata are partially closed. This raises interesting questions of whether there is a range in $K_{\text{HCO}_3}$ across the diverse lineages of C4 plants and finding relationships between certain amino acid residues and ranges of $K_{\text{HCO}_3}$ values can be beneficial for promoting strategies to optimize C4 photosynthesis in crop species for drought conditions.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Variable descriptions and values used to model C4 photosynthesis.

Table S2. Kinetic properties of PEPc isoforms from Flaveria trinervia and F. pringlei.

Table S3. Kinetic properties of PEPc isoforms in the presence of 2.5 mM malate.

Fig. S1. Representative MIMS responses of the C3 and C4 PEPc activities with changing HCO3− concentrations.

Fig. S2. HCO3− response curves for the C3, C3-A774S, C4, and C4-S774A PEPc isoforms in the presence of 2.5 mM malate.

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