Phospho*enol*pyruvate carboxylase purified from leaves of C_3, C_4, and C_3-C_4 intermediate species of *Alternanthera*: Properties at limiting and saturating bicarbonate

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Abstract

Phospho*enol*pyruvate carboxylase (PEPC) was purified from leaves of four species of *Alternanthera* differing in their photosynthetic carbon metabolism: *Alternanthera sessilis* (C_3), *A. pungens* (C_4), *A. ficoides* and *A. tenella* (C_3-C_4 intermediates or C_3-C_4). The activity and properties of PEPC were examined at limiting (0.05 mM) or saturating (10 mM) bicarbonate concentrations. The V_{max} as well as K_m values (for Mg^{2+} or PEP) of PEPC from *A. ficoides* and *A. tenella* (C_3-C_4 intermediates) were in between those of C_3 (*A. sessilis)* and C_4 species (*A. pungens*). Similarly, the sensitivity of PEPC to malate (an inhibitor) or G-6-P (an activator) of *A. ficoides* and *A. tenella* (C_3-C_4) was also of intermediate status between those of C_3 and C_4 species of *A. sessilis* and *A. pungens,* respectively. In all the four species, the maximal activity (V_{max}), affinity for PEP (K_m), and the sensitivity to malate (K_I) or G-6-P (K_A) of PEPC were higher at 10 mM bicarbonate than at 0.05 mM bicarbonate. Again, the sensitivity to bicarbonate of PEPC from C_3-C_4 intermediates was in between those of C_3- and C_4-species. Thus the characteristics of PEPC of C_3-C_4 intermediate species of *Alternanthera* are intermediate between C_3- and C_4-type, in both their kinetic and regulatory properties. Bicarbonate could be an important modulator of PEPC, particularly in C_4 plants.

Additional key words: glucose-6-phosphate; malate; proteins; species differences in enzyme activity.

Introduction

The C_3-C_4 intermediates constitute a group of plants with a syndrome of physiological, anatomical, and biochemical traits that fall between the features of C_3 and C_4 plants (Rawsthorne 1992, Raghavendra and Das 1993, Rawsthorne and Bauwe 1998). The extensive literature on PEPC established that the C_4 enzyme is distinct from that of C_3, as indicated by their biochemical properties and amino acid sequences. The genes for C_4, C_3, or CAM-PEPC are also different, both in structure and phylogeny (Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996).

The activities of PEPC in C_3-C_4 intermediates are significantly higher than in C_3 species (Ku *et al.* 1983, Edwards and Ku 1987), but the molecular properties of PEPC from C_3-C_4 intermediates were not studied in detail. The limited literature suggests that the PEPC from C_3-C_4 intermediates may be of C_3 type such as in *Panicum, Moricandia,* and *Flaveria* (Holaday *et al.* 1981, Adams *et al.* 1986, Bauwe and Chollet 1986), or intermediate type such as in *Flaveria* (Nakamoto *et al.* 1983). Thus, the nature of PEPC in C_3-C_4 intermediates is yet to be resolved clearly.

Alternanthera offers a unique and ideal opportunity of having species of C_3, C_4 as well as C_3-C_4 intermediates within the same genus (Rajendrudu *et al.* 1986, Raghavendra and Das 1993). We have been

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Abbreviations: G-6-P, glucose-6-phosphate; K_A, activator constant; K_I, inhibitor constant; MDH, malate dehydrogenase; PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase.
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examining the phenomena of photosynthesis and photorespiration in four species of *Alternanthera*: *A. sessilis* (C_3), *A. pungens* (C_4), *A. ficoides* and *A. tenella* (C_3-C_4 intermediates) (Devi and Raghavendra 1993a,b, 1994, Devi *et al.* 1995). As a part of these efforts, we studied the properties of PEPC purified from leaves of these four species.

Studies in our laboratory have revealed that the kinetic and regulatory properties of PEPC from *Amaranthus hypochondriacus* are markedly modulated by bicarbonate. High concentration of bicarbonate (10 mM) decreased the sensitivity of PEPC to malate or G-6-P while increasing the affinity for Mg^{2+} (Parvathi *et al.* 1998). We therefore evaluated the activity and properties of PEPC from four species of *Alternanthera* at limiting (0.05 mM) and saturating bicarbonate (10 mM) concentrations.

Materials and methods

Plants of four *Alternanthera* species, *A. sessilis* (L.) R.Br. (C_3), *A. pungens* H.B.K (C_4), and *A. ficoides* (L.) R.Br. (C_3-C_4 intermediate), *A. tenella* Colla (C_3-C_4 intermediate) were propagated through cuttings. The plants were grown in earthen pots filled with red soil supplemented with farmyard manure (in a ratio of 3 : 1). The plants were grown outdoors under approximately 12-h photoperiod (temperature 35-40/25-30 °C, day/night). The second to fourth leaves, beginning from youngest fully matured leaf, were harvested from 4- to 6-week-old-plants. Leaves were usually collected at 09:00 to 10:00 h, about 2-3 h after sunrise, and used for the purification of PEPC.

Purification of PEPC was partially done from leaves of different species using a rapid 3-step procedure described by Gayathri *et al.* (2000). After purification, PEPC was concentrated using *PEG-20 000* and stored in 50 % glycerol until use. At this stage, the purified PEPC was stable for more than 3 months in liquid nitrogen (with no significant loss in specific activity). Typical steps of purification from leaves of *A. pungens* (C_4) are described in Table 1.

The activity of PEPC was assayed by coupling the PEPC activity enzymatically to NAD-malic dehydrogenase and measured as NADH oxidation (340 nm) at 30 °C in a dual-beam UV-Vis spectrophotometer (*Shimadzu UV-160A*). The standard assay medium (1 cm^3) contained 50 mM Hepes-KOH, pH 7.3, 5 mM $MgCl_2$, 2.5 mM PEP, 0.2 mM NADH, 2 units of NAD-MDH, and 0.05 or 10 mM $NaHCO_3$.

The maximum velocity of the enzymic rection (V_{max}) and K_m for PEP or Mg^{2+} were examined by varying the concentration of PEP or $MgCl_2$ (0.01 to 5 mM) in the assay medium. The sensitivity of PEPC to malate or G-6-P was determined using the assay mixture (with 1 mM PEP) containing either malate or G-6-P (0.01 to 5 mM). K_I and K_A values were calculated by using an *Enzkinet* computer programme.

Protein was estimated by using Folin-phenol reagent (Lowry *et al.* 1951). The experiments were repeated three or four times at different days.

Table 1. A typical pattern of the steps during the purification of PEPC from leaves of *Alternanthera pungens*, a C_4 species. The activity was assayed at 10 mM bicarbonate.

Step	Total protein [mg]	Total activity [μmol s^{-1}]	Specific activity [mmol s^{-1} kg^{-1}(protein)]	Purification [fold]	Yield [%]
Crude extract	791	29.02	36.7	-	100
40-60 % $(NH_4)_2SO_4$	228	13.32	58.3	1.6	46
DEAE-*Sepharose*	66	6.25	95.0	2.6	22
Hydroxylapatite	4	2.57	675.0	18.4	9

Results

Kinetic properties: The maximal velocity (V_{max}) of PEPC from C_4 species (*A. pungens*) was much greater (almost 3-4 times) than that of C_3 species (*A. sessilis*) at both limiting (0.05 mM) as well as saturating (10 mM) concentrations of bicarbonate (Table 2). The V_{max} of PEPC from the two C_3-C_4 intermediates (*A. ficoides* and *A. tenella*) was also much greater than that of C_3 species, particularly at 10 mM bicarbonate. The K_m (PEP) values of PEPC in *A. tenella* (C_3-C_4) were 2-3 times that of *A. sessilis* (C_3). The K_m (PEP) of PEPC from *A. pungens* (C_4) was several times greater than that of *A. sessilis* (C_3). The K_m (Mg^{2+}) of PEPC from leaf extracts of

Table 2. Catalytic and regulatory properties of PEPC purified from leaves of four species of *Alternanthera*. The enzyme was assayed at 0.05 or 10 mM bicarbonate. V_{max} [mmol kg^{-1}(protein) s^{-1}], K values [mM]. Figures in parentheses in the lower part of the Table give % increase at 10 mM bicarbonate over that in 0.05 mM bicarbonate.

	V_{max}	K_m(PEP)	K_m(Mg)	K_I(malate)	K_A(G-6-P)
0.05 mM bicarbonate					
A. sessilis (C_3)	100± 3	0.05±0.01	0.20±0.04	0.004±0.001	0.04±0.01
A. ficoides (C_3-C_4)	167±13	0.11±0.01	0.44±0.09	0.013±0.001	0.07±0.01
A. tenella (C_3-C_4)	233±20	0.15±0.02	0.50±0.03	0.014±0.001	0.09±0.01
A. pungens (C_4)	267±32	0.28±0.04	1.20±0.16	0.015±0.002	0.15±0.02
10 mM bicarbonate					
A. sessilis (C_3)	183± 7 (83)	0.07±0.01 (40)	0.04±0.01	0.013±0.001 (225)	0.05±0.01 (25)
A. ficoides (C_3-C_4)	383± 7 (130)	0.20±0.01 (82)	0.14±0.02	0.046±0.006 (283)	0.17±0.03 (142)
A. tenella (C_3-C_4)	533± 3 (130)	0.29±0.03 (93)	0.15±0.02	0.052±0.008 (271)	0.20±0.03 (122)
A. pungens (C_4)	700±12 (163)	0.62±0.04 (121)	0.42±0.06	0.070±0.009 (367)	0.40±0.02 (167)

A. ficoides or *A. tenella* (C_3-C_4) was also nearly twice of that in *A. sessilis*, and was much less than that of *A. pungens*.

Regulatory properties: The activities of PEPC from *A. pungens*, *A. ficoides* and *A. tenella*, and *A. sessilis* were inhibited by L-malate. The apparent K_I (malate) values of PEPC from *A. ficoides* and *A. tenella* were similar to that of *A. pungens* and were much higher than that of *A. sessilis* (Table 2).

The activity of PEPC in leaf extracts was enhanced in presence of G-6-P. However, the degree of G-6-P activation differed between the species of *Alternanthera*. The apparent K_A (G-6-P) values of PEPC from leaf extracts of *A. ficoides* and *A. tenella* were much higher than that of *A. sessilis*, but were less than that of *A. pungens*.

Modulation by bicarbonate: The catalytic activity of PEPC was higher and the extent of inhibition by malate (or stimulation by G-6-P) was lower at saturating (10 mM) levels of bicarbonate than those at limiting (0.05 mM) bicarbonate in all the four species of *Alternanthera* (Table 2). However, there were quantitative differences in the extent of response. The increases in V_{max}, K_m(PEP), K_I(malate), or K_A(G-6-P) at 10 mM bicarbonate (compared to that 0.05 mM) were more with PEPC of *A. pungens* than with that of *A. sessilis*. The extent of response of PEPC from the two C_3-C_4 intermediates (*A. ficoides* and *A. tenella*) was again in between those of C_3 and C_4 species.

Discussion

Kinetic characteristics of C_4-PEPC are usually distinct from those of C_3 species (Lepiniec *et al.* 1994, Rajagopalan *et al.* 1994, Chollet *et al.* 1996). The present article is the first detailed report on comparative properties of PEPC purified from leaves of three photosynthetic types within the same genus of *Alternanthera*. Ting and Osmond (1973) made a similar attempt with *Atriplex* but their study was limited. Another novelty is the use of limiting (0.05 mM) concentration of bicarbonate to assess the properties of PEPC. As indicated in Table 2, the responses of PEPC to inhibition (*e.g.*, malate) and activation (*e.g.*, G-6-P) were highly dampened at saturating concentration (10 mM) of bicarbonate.

The studies on the properties of PEPC from the species of *Panicum*, *Moricandia*, or *Flaveria* suggest that the PEPC enzyme of C_3-C_4 intermediates is of C_3 type (Holaday and Black 1981, Holaday *et al.* 1981, Adams *et al.* 1986, Bauwe and Chollet 1986). However, in an intrageneric survey covering four *Flaveria* species, Nakamoto *et al.* (1983) concluded that the PEPC of *Flaveria pubescens* (C_3-C_4) has properties intermediate in several respects to those of related C_3 and C_4 species. Our results endorse the opinion of Nakamoto *et al.* (1983) and add another instance of C_3-C_4 intermediate nature of PEPC in two species of *Alternanthera* (Table 2).

There are very few reports on the comparative study of K_m (Mg^{2+}) of PEPC from C_3 and C_4 plants (Ting and Osmond 1973, Gavalas and Manetas 1980), and none on PEPC of C_3-C_4 intermediates. The present work (Table 2) reveals that the K_m for Mg^{2+} of PEPC from *A. ficoides and A. tenella* (C_3-C_4) is 2-3 times that of the enzyme from *A. sessilis* (C_3). However, the K_m (Mg^{2+}) values of the two C_3-C_4 intermediate species were closer to that of C_3 species rather than that of C_4 species. The K_m(PEP) or $K_m(Mg^{2+})$ values for C_3 and C_4 *Alternanthera* species obtained in the present study at 10 mM bicarbonate are similar to the values reported for C_3 and C_4 species (Ting and Osmond 1973).

L-malate is a typical inhibitor while G-6-P is a typical activator of PEPC in C_4 leaves (Andreo *et al.* 1987). The degree of inhibition by malate or activation by G-6-P of PEPC is usually more in extracts of C_4 species than in C_3 extracts (Gupta *et al.* 1994). In the present study, the K_I (malate) values of PEPC from *A. tenella* or *A. ficoides* (C_3-C_4) were higher than that of *A. sessilis* (C_3) and close to those of *A. pungens* (C_4). Svensson *et al.* (1997) observed that the response to G-6-P of PEPC from C_4 species (*Flaveria trinervia*) was more pronounced than that of C_3 species (*F. pringlei*). Previous studies on G-6-P activation in C_3-C_4 intermediates of *Moricandia* and *Flaveria* are contradictory and confusing (Holaday *et al.* 1981, Nakamoto *et al.* 1983, Bauwe and Chollet 1986). The K_A (G-6-P) values of PEPC from C_3-C_4 intermediates were also closer to those of C_4 than C_3 species. Thus, the regulatory properties of PEPC from the intermediates exhibited distinctly an intermediate status between the C_3 and C_4 species of *Alternanthera* (Table 2).

Another interesting feature hitherto not emphasised is the distinct difference between C_3 and C_4 plants in the extent of modulation by bicarbonate of the properties of PEPC. The increases in V_{max}, K_m(PEP), K_I(malate), or K_A(G-6-P) of PEPC at 10 mM bicarbonate were more pronounced with the C_4 enzyme than with that of the C_3 one (Table 2). The response to bicarbonate can therefore be taken as another criterion to distinguish C_3 and C_4 enzymes. The K_m for bicarbonate of PEPC from C_4 plants is lower than that of C_3 species (Bauwe 1986). Again, the response to bicarbonate of PEPC from C_3-C_4 intermediates was clearly in between those of C_3 and C_4 species.

The present report on the properties of PEPC from C_3, C_4, or C_3-C_4 intermediate species of *Alternanthera* genus is of particular interest, since all the three species belong to the same genus. We conclude that PEPC of C_3-C_4 intermediate species of *Alternanthera* is indeed of an intermediate nature between C_3 and C_4 types. We also suggest that bicarbonate could be an important modulator of PEPC in C_4 plants. There is a marked modulation of PEPC properties by bicarbonate (Parvathi *et al.* 1998). Further, the affinity of PEPC in *A. hypochondriacus*, a C_4 plant, decreases on irradiation of leaves (Parvathi *et al.* 2000). Thus, there is a marked interaction between PEPC, bicarbonate presence, and irradiation in leaves of C_4 plants.

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