

Kinetic Properties of Phosphoenolpyruvate Carboxylase from C₃, C₄, and C₃-C₄ Intermediate Species of *Flaveria* (Asteraceae)¹

Received for publication April 22, 1986 and in revised form July 18, 1986

HERMANN BAUWE AND RAYMOND CHOLLET*

Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, DDR-4325 Gatersleben (GDR) (H.B.); and Department of Agricultural Biochemistry, East Campus, University of Nebraska-Lincoln, Lincoln, Nebraska 68583-0718 (R.C.)

ABSTRACT

Flaveria cronquistii (C₃), *F. chloraefolia* (C₃-C₄), *F. floridana* (C₃-C₄), *F. pubescens* (C₃-C₄), *F. anomala* (C₃-C₄), *F. linearis* (C₃-C₄), *F. brownii* (C₄), *F. palmeri* (C₄), *F. trinervia* (C₄) and *F. australasica* (C₄), comprising 10 out of the 21 known species of the genus *Flaveria* (Asteraceae), were included in a comparative study of the kinetic and regulatory properties of green leaf phosphoenolpyruvate (PEP) carboxylase. At least three kinetically distinct enzyme-forms were identified on the basis of their affinities for PEP and the degree of allostery with respect to this substrate. The kinetic properties of PEP carboxylase of most of the species seemingly were modified *in vivo* depending on the growth conditions of the plants. $K_m(\text{PEP}_{\text{free}})$ -values of the enzyme from the five C₃-C₄ intermediate species ranged from 6 micromolar (*F. chloraefolia*, low light-grown) to 38 micromolar (*F. pubescens*, high light-grown). In contrast, the K_m for PEP of PEP carboxylase from the C₃ species *F. cronquistii* (13 micromolar) apparently was not influenced by growth conditions. The response of the enzyme from the C₃ and C₃-C₄ species was hyperbolic in all cases. A second isoform with a lower affinity for PEP (88-100 micromolar), but also hyperbolic kinetics was found in the C₄ species *F. brownii*, whereas in the three other C₄ species examined a PEP carboxylase with a still lower affinity for PEP (187-221 micromolar) and sigmoidal kinetics was present. These isozyme-related kinetic data were supported by analyses of the elution behavior of the enzyme during anion-exchange chromatography on DEAE-Trisacryl M. The results are discussed with respect to the evolution of C₄ photosynthesis in the *Flaveria* genus.

Following the most recent comprehensive taxonomic and morphological description of the genus *Flaveria* (Asteraceae) by Powell (18), *Flaveria* species with different photosynthetic properties have been characterized by a variety of CO₂ exchange (2, 8, 12) and enzyme-activity (4, 12) measurements, as well as by ¹⁴CO₂ incorporation experiments (3, 20). From these studies and in contrast to the current situation with C₃-C₄ intermediate species of the genera *Panicum* and *Moricandia* (7, 15), there is now increasing evidence that in intermediate species of *Flaveria* a limited C₄ pathway of CO₂ assimilation may be operative. The existence of only 21 known *Flaveria* species with photosynthetic pathways ranging from C₃, C₃-C₄ intermediate, and C₄ makes this genus a most useful and promising tool for the elucidation of specific steps in the evolution of C₄ photosynthesis.

¹ Supported in part by National Science Foundation Grant No. DMB-8415292 and published as Paper No. 8049, Journal Series, Nebraska Agricultural Research Division.

In investigating the evolution of C₄ plants and the associated biochemical mechanisms of CO₂ assimilation, special attention should be devoted to PEP² carboxylase (EC 4.1.1.31). Given its role as a key enzyme of primary CO₂ fixation during C₄ photosynthesis and its complex regulatory properties (9, 10, 11, 13, 17), PEP carboxylase is likely to have been exposed to considerable evolutionary pressure during the evolution of C₄ plants. In addition, this enzyme is known to fulfill different functions in higher plant metabolism (11, 13, 17).

PEP carboxylase is regulated in an intricate manner and its enzymic properties have apparently undergone considerable change during the evolution of C₃ plants into species with more complex mechanisms of photosynthetic CO₂ assimilation, including CAM plants and C₄ species. Previous studies with PEP carboxylases from different sources indicated that there are kinetically distinct C₃- and C₄-specific isoforms of the enzyme (22). Similarly, Nakamoto *et al.* (16), in an intrageneric survey limited to four *Flaveria* species, concluded that in several respects the PEP carboxylases of *F. pubescens* (C₃-C₄) and *F. linearis* (C₃-C₄) have properties intermediate to those of related C₃ and C₄ species, with distinct kinetic differences also observed between the enzymes from the two intermediate species themselves.

In this paper we present additional observations about changes in kinetic and regulatory properties of PEP carboxylase during the evolution of C₄ photosynthesis in the genus *Flaveria*. We show that the PEP carboxylases of five C₃-C₄ intermediate *Flaveria* species differ kinetically only slightly, if at all, from the C₃-form of the enzyme. These findings are in total accord with the very recent related observations of Adams *et al.* (1) in which the enzymes from *F. cronquistii* (C₃) and *F. floridana* (C₃-C₄) were shown to be very similar, if not identical, to each other in terms of various electrophoretic analyses, including peptide mapping. A photosynthetic PEP carboxylase with truly intermediate kinetic properties has been detected in our study, however, in the unusual C₄ species, *F. brownii*.

MATERIALS AND METHODS

The following 10 *Flaveria* species were included in this study: *F. cronquistii* A. M. Powell (C₃), *F. chloraefolia* A. Gray (C₃-C₄), *F. floridana* J. R. Johnston (C₃-C₄), *F. pubescens* Rydb. (C₃-C₄), *F. anomala* B. L. Robinson (C₃-C₄), *F. linearis* Lag. (C₃-C₄), *F. brownii* A. M. Powell (C₄), *F. palmeri* J. R. Johnston (C₄), *F. trinervia* (Spreng.) C. Mohr (C₄), and *F. australasica* Hook. (C₄). The photosynthetic designations given in parentheses are based on data by Apel and Maass (2), Bauwe (4), and Holaday *et al.* (8).

² Abbreviations: PEP, phosphoenolpyruvate; G6P, glucose-6-phosphate; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase.

Plants were grown in Metromix potting medium either from seeds or vegetative cuttings in a growth chamber under the conditions described previously (8) (day/night: 16/8 h, 23/16°C, about 300 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, PAR). Where indicated, some plants were also grown during the summer months in a greenhouse at 25 to 35°C under natural light.

The entire extraction and concentration procedure was performed at about 2 to 5°C. Two g fresh weight of mature leaves were homogenized with a mortar in 10 ml of 50 mM Tricine-KOH (pH 8.0) containing 5 mM MgCl_2 , 5 mM DTT, 1 mM Na_2EDTA , 50 mM Na ascorbate, 10 mM Na diethyldithiocarbamate, and 0.4 g insoluble PVP. After filtration through Miracloth the homogenate was centrifuged for 10 min at 40,000g. To the supernatant fluid 0.39 g/ml of ultrapure $(\text{NH}_4)_2\text{SO}_4$ was dissolved and, after 10 min, the protein suspension was centrifuged for 15 min. The pellet was resuspended in a 2-fold volume of 50 mM Tricine-KOH (pH 8.0) containing 5 mM MgCl_2 and 1 mM DTT and clarified by centrifugation for 5 min at 40,000g. The supernatant fluid was desalted by passage through a Sephadex G-25 column (0.8 \times 10 cm) equilibrated with resuspension buffer. If too high, the PEP-saturated enzyme activity was reduced to about 1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$ by dilution with resuspension buffer.

The elution behavior of PEP carboxylase from several *Flaveria* species during anion-exchange chromatography was examined using the following experimental protocol. Ten (C_4 species) or 25 (C_3 and intermediate species) g fresh weight of leaf material and 20% (w/w) insoluble PVP were homogenized in a Waring Blendor with a 5-fold amount of 50 mM Hepes-KOH (pH 7.3) containing the same additives as in the extraction buffer described above. After filtration through Miracloth and centrifugation, the supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$ to yield the fraction precipitating between 30 and 60% saturation (4°C). This precipitate was dissolved in 50 mM Hepes-KOH (pH 7.0) containing 5 mM MgCl_2 , 5 mM DTT, and 1 mM Na_2EDTA , and desalted through a Sephadex G-25 column (2 \times 18 cm) equilibrated with the same buffer. After enzyme-activity determination, the eluate was applied to a DEAE-Trisacryl M (LKB) column (2 \times 15 cm), and protein was eluted at a flow rate of 50 $\text{ml}\cdot\text{h}^{-1}$ with a 200-ml linear gradient (0–300 mM) of KCl in the gel-filtration buffer.

Kinetic assays were performed as initial-rate determinations at 25°C in a total volume of 1 ml containing 50 mM Tricine-KOH (pH 8.0), 5 mM MgCl_2 , 5 mM NaHCO_3 , 1 mM DTT, 0.15 mM NADH, and about 2 units NADH-malic dehydrogenase (bovine heart, Sigma). Immediately after addition of 50 μl of eluate the reaction was initiated with varying amounts of Na_3PEP . Total PEP concentrations were varied between 5 and 300 μM for all C_3 and C_3 - C_4 intermediate species, between 10 and 600 μM for *F. brownii* (C_4), and between 50 and 3000 μM for all other C_4 species. All initial rates refer to the first 20 to 30 s after the addition of PEP. The actual PEP concentrations in the stock solutions were determined experimentally using the same assay with limiting PEP and partially purified PEP carboxylase from maize.

Concentrations of free PEP were calculated using dissociation constants of $K_p = 4.35$ mM (14) and $K_t = 63$ mM (6) for the PEP-Mg and Tricine-Mg complexes, respectively, according to:

$$a \cdot \text{PEP}^3 + b \cdot \text{PEP}^2 + c \cdot \text{PEP} + d = 0$$

where,

$$\begin{aligned} a &= K_p - K_t \\ b &= K_p \cdot (K_p - K_t + M_t - T_t - 2 P_t) + K_t \cdot (P_t - M_t) \\ c &= K_p \cdot P_t \cdot (P_t + T_t - M_t - 2 K_p + 1) \\ d &= (K_p \cdot P_t)^2 \end{aligned}$$

and $P_t = \text{PEP (total)}$, $M_t = \text{Mg (total)}$, and $T_t = \text{Tricine (total)}$. All K_m values have been calculated on this basis. For an approx-

imate comparison with literature values referring to the total concentration of PEP, a factor of 1.7 may be used to multiply our values for PEP(free).

Kinetic parameters were calculated by nonlinear regression analysis according to the equation:

$$v = \frac{V \cdot \text{PEP}^n}{K^n + \text{PEP}^n}$$

with n -values fixed to 1.0 in the case of hyperbolic kinetics.

RESULTS AND DISCUSSION

Nakamoto *et al.* (16) reported that the PEP carboxylases from *F. pubescens* and *F. linearis* are in several respects kinetically intermediate to those of C_3 and C_4 *Flaveria* species. Both of these C_3 - C_4 intermediate species, as well as *F. floridana* and *F. chloraefolia*, belong to the five to six phyllary line of the phylogenetic scheme for *Flaveria* proposed by Powell (18). The only known C_4 species in this subgroup of the genus is *F. brownii*, and we have previously reported that this species has certain features indicative of a not yet fully evolved C_4 photosynthetic apparatus (4, 19) and that PEP carboxylase of *F. brownii* might be similar to those of C_3 and C_3 - C_4 intermediate *Flaveria* species (4).

To achieve a more comprehensive kinetic analysis of PEP carboxylase in *Flaveria*, we not only included these five species from the five to six phyllary line, but also several members of the three to four phyllary group. *F. cronquistii* is the only physiologically well-characterized C_3 species of the genus. *F. anomala* is an additional C_3 - C_4 intermediate species, whereas *F. palmeri*, *F. trinervia*, and *F. australasica* are all C_4 plants. The latter two species are regarded by Powell (18) to be, perhaps, the most derived species of the entire genus.

The evaluation of kinetic properties of enzymes using relatively crude preparations always gives rise to uncertainties due to the possible simultaneous occurrence of isoforms with different catalytic and/or regulatory properties. However, it is a relatively rapid method and amenable to comparisons between species. Table I summarizes the $K_m(\text{PEP})$ -values and Hill coefficients (n)

Table I. $K_m(\text{PEP})$ -Values and Hill Coefficients of PEP Carboxylase from C_3 , C_4 , and C_3 - C_4 Intermediate *Flaveria* Species

Species	$K_m(\text{PEP})$ Free		
	A ^a	B	C
	$\mu\text{M} \pm \text{SE}$		
C_3			
<i>F. cronquistii</i>	13 \pm 1	13 \pm 2	8 \pm 1
C_3 - C_4			
<i>F. chloraefolia</i>	13 \pm 2	6 \pm 2	8 \pm 2
<i>F. floridana</i>	18 \pm 2	13 \pm 2	9 \pm 2
<i>F. linearis</i>	15 \pm 2	12 \pm 2	ND ^c
<i>F. anomala</i>	22 \pm 2	15 \pm 2	ND
<i>F. pubescens</i>	38 \pm 6	14 \pm 2	8 \pm 1
C_4			
<i>F. brownii</i>	100 \pm 10 (1.0 \pm 0.1) ^b	88 \pm 19 (1.0 \pm 0.1)	29 \pm 8 (1.0 \pm 0.1)
<i>F. palmeri</i>	193 \pm 6 (1.4 \pm 0.1)	ND	ND
<i>F. trinervia</i>	221 \pm 10 (1.3 \pm 0.1)	215 \pm 19 (1.1 \pm 0.1)	47 \pm 6 (1.0 \pm 0.2)
<i>F. australasica</i>	211 \pm 9 (1.4 \pm 0.1)	187 \pm 14 (1.3 \pm 0.1)	55 \pm 3 (1.1 \pm 0.1)

^a Column A, greenhouse-grown plants; column B, growth chamber-grown plants; column C, extracts from 'B' assayed in the presence of 5 mM G6P. ^b Values in parentheses are Hill coefficients (n) \pm SE. ^c Not determined.

determined for the 10 *Flaveria* species examined and presents a qualitative description of the influence of the activator G6P on these parameters. In accord with the relatively limited data of Nakamoto *et al.* (16), our results show that there are several kinetically distinct isozymes of PEP carboxylase in *Flaveria*. With perhaps only one exception (*F. chloraefolia*), there are no significant differences in the affinities for PEP between the enzyme from different C₃-C₄ intermediate species when measured with plant material grown in a growth chamber (Table I, column B), *i.e.* under a relatively low light intensity. Under these conditions, the PEP carboxylase of most C₃-C₄ intermediate *Flaveria* species is similar, if not identical, to that of the C₃ species, *F. cronquistii*. *F. chloraefolia* has an isoform of PEP carboxylase with a $K_m(\text{PEP})$ of about 6 μM , which is only about half that found for the enzyme from *F. cronquistii*. With these more extensive data we are not able to totally confirm the previous claim (16) that PEP carboxylases of the C₃-C₄ *Flaveria* species have intermediate or C₄-like kinetic properties. Our findings are in complete accord with the very recent results of Adams *et al.* (1) who, in a study of the electrophoretic/molecular properties of PEP carboxylase from three *Flaveria* species, found that the enzyme from greenhouse-grown *F. cronquistii* (C₃) is very similar, if not identical, to that of *F. floridana* (C₃-C₄) and markedly distinct from the *F. trinervia* (C₄) PEP carboxylase. In making a critical comparison between our kinetic data (Table I, column B) and those reported by Nakamoto *et al.* (16), it should be noted, however, that our growth chamber-grown plants were propagated under both lower light intensities (about 300 *versus* 800 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and temperatures (23/16°C *versus* 27/22°C) than their chamber-grown material. Our comparative results (Table I, columns A *versus* B) clearly show that growth conditions, for yet unknown reasons, modify the apparent kinetic properties of the enzyme of most of the *Flaveria* species included in this study. Exclusive of *F. cronquistii* with which there was no change, for all other species of the genus examined we found an increase in the $K_m(\text{PEP})$ -values at high light, which in the case of sigmoidal kinetics was also correlated with an increase in the Hill coefficient. Although this effect was relatively small with most of the species examined, a considerable difference in the $K_m(\text{PEP})$ -values was observed with *F. pubescens*. The results of native isoelectric focusing analyses of PEP carboxylase from C₃, C₃-C₄, and C₄ *Flaveria* species (1) suggest that these kinetic changes are, perhaps, due to changing ratios of different isoforms of the enzyme. This might also account for the different results reported in (16).

As has been reported for *F. trinervia* (16) and C₄ plants in general (22), the affinities for PEP are distinctly lower with the four C₄ *Flaveria* species included in this intrageneric study when compared with the C₃ PEP carboxylase. However, in contrast to the three other C₄ species of the genus which all show a significant degree of cooperativity in the kinetics of PEP carboxylase, the enzyme from *F. brownii* is unique among the C₄ PEP carboxylases of *Flaveria* in having a significantly higher affinity for PEP. Moreover, as with the enzyme from the C₃ and C₃-C₄ intermediate species, there is no indication of positive cooperativity when PEP carboxylase from *F. brownii* is assayed at pH 7 (data not shown) or pH 8. Although the enzyme's affinity for PEP is increased by about 10% at pH 7, the Hill coefficient still does not significantly deviate from unity. This is clearly different from the enzyme from the other C₄ *Flaveria* species, where the sigmoidicity is increased at neutral pH (Ref. 16 and our own observations). These findings support the view that the photosynthetic PEP carboxylase operative in *F. brownii* is different from those in the more advanced C₄ species of the genus.

It is known that PEP carboxylase from C₄ plants is more sensitive to activation by G6P than the C₃ enzyme. This effect is mainly due to a lowering of the K_m for PEP (23). We found that

the same is true for PEP carboxylase from *Flaveria* species (Table I, columns B *versus* C). Except for the enzyme from *F. chloraefolia* where there is no significant effect of G6P, inclusion of this activator at a concentration of 5 mM in the assay medium resulted in a reduction of the K_m for PEP by a factor ranging from about 0.6 for the C₃ and intermediate species to about 0.3 for *F. brownii* and 0.25 for the two other C₄ species examined. With the C₄ enzyme from *F. trinervia* and *F. australasica*, G6P induced a change in cooperativity resulting in lower Hill coefficients. The effect of G6P is difficult to evaluate quantitatively, however, because of a distinct concentration maximum for activation, at least with the C₄ leaf enzyme. For example, in Figure 1 PEP carboxylase activities have been measured at near- K_m PEP concentrations and varying levels of G6P. It can be seen that the activating effect is half-saturated at concentrations much lower than 1 mM for PEP carboxylase from *F. pubescens* (C₃-C₄). In contrast, the enzyme from *F. australasica* (C₄) requires much higher levels of G6P, with a maximum effect at about 3 mM. The results of similar measurements with PEP carboxylases from other *Flaveria* species (data not shown) allow a general classification with respect to the activating effect of G6P—being half-saturated above 1 mM for PEP carboxylase from *F. palmeri*, *F. trinervia*, and *F. australasica*, and far below 1 mM for the enzyme from the seven other species listed in Table I. Once again, PEP carboxylase from *F. brownii*, by approximating the C₃-form of the enzyme, behaves differently from the other C₄ PEP carboxylases.

With respect to the interpretation of the kinetic data presented in Table I, the presence of a noncooperative isoform of PEP carboxylase in *F. brownii* with an intermediate affinity for PEP raises the possibility of the simultaneous occurrence of kinetically distinct isoforms in the same species. Although there appears to be at least three kinetically different isoforms of PEP carboxylase in the *Flaveria* genus, it cannot yet be completely excluded that at least some of the differences in kinetic properties observed in the present and previous (16) studies are due to the simultaneous presence of varying ratios of kinetically distinct PEP carboxylases in certain species of *Flaveria*. As discussed above, this explanation seems even more plausible in view of the changes in the

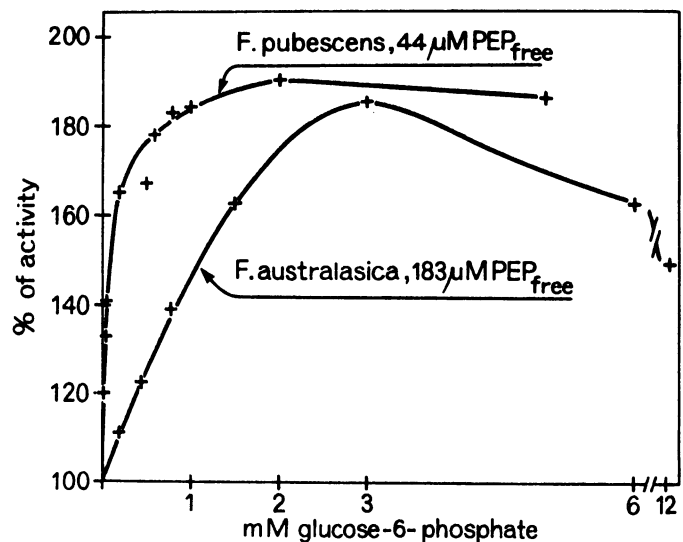


FIG. 1. Influence of glucose-6-P concentration on PEP carboxylase activity from growth chamber-grown *F. pubescens* (C₃-C₄) and *F. australasica* (C₄) at pH 8.0 and either 44 (C₃-C₄) or 183 (C₄) μM free PEP. Measurements were performed using enzyme partially purified as described in "Materials and Methods," but using DEAE-Sepharose instead of DEAE-Trisacryl M.

kinetic behavior of PEP carboxylases from most of the *Flaveria* species which appeared when the plants were grown in a greenhouse at much higher light intensities than prevailing in the growth chamber.

Although the electrophoretic analyses of Adams *et al.* (1) clearly demonstrate the existence of several PEP carboxylase charge-variants in C_3 , C_4 , and intermediate species of *Flaveria*, a preparative separation of these isoforms is necessary for a detailed kinetic analysis. In attempts to devise a suitable isolation technique, we found that neither chromatofocusing (pH 4–7 [Pharmacia]) nor ion-exchange chromatography on DE-52 cellulose (Whatman) or DEAE-Sephadex (Pharmacia) effected the separation of kinetically different isozymes. With the latter method, PEP carboxylase from *F. cronquistii*, *F. pubescens*, *F. brownii*, and *F. australasica* all eluted at the same KCl concentration of about 200 mM. However, differences in charge-related properties of the enzyme were suggested by chromatofocusing experiments in which PEP carboxylase from *F. palmeri* and *F. australasica* eluted between pH 5.2 to 5.8 in contrast to the enzyme from *F. chloraefolia*, which eluted at a slightly more acidic pH value (4.5–5.1). With DEAE-Trisacryl M (LKB) we were able to effect a separation of kinetically different PEP carboxylases using mixtures of extracts from different *Flaveria* species. As an illustration, the elution profiles of experiments with *F. anomala* and *F. australasica* are shown in Figure 2. When examined in this way, we could not achieve a preparative separation of different PEP carboxylase isoforms in a given species. In each case, except for *F. australasica* where at least two kinetically similar activity-peaks eluting between 90 and 130 mM KCl were observed (Fig. 2), PEP carboxylase activity eluted as a single peak at 130 to 170 mM KCl (*F. brownii*) or 150 to 180 mM KCl (*F. anomala* [Fig. 2], *F. pubescens*, *F. chloraefolia*, and *F. cronquistii*). Essentially the same results were obtained with plant material grown in the growth chamber or under greenhouse conditions. Therefore, it seems reasonable to propose that the kinetic differences summarized in Table I are caused by the presence of at least three kinetically distinct PEP carboxylases in the *Flaveria* genus, one representing the C_3 -form which is also found in four of the intermediate species, one isoform specific for *F. brownii*, and a third operating in the more advanced C_4 species of the genus. The changes in PEP affinity induced by different growth conditions suggest that PEP carboxylases of *Flaveria*, like those of species belonging to other genera, may be subject to a complex regulatory mechanism triggered by environmental conditions. This regulation could be directed at either the expression of genes encoding kinetically different isozymes or at posttranslational modifications of the protein. Along these lines, Karabourniotis *et al.* (10) and Huber and Sugiyama (9) have recently reported that PEP carboxylase from certain C_4 plants is subject to photoactivation *in situ*, resulting in a modulation of activity, PEP affinity, and effector sensitivity.

Based on our collective findings, we propose that PEP carboxylase from *F. brownii* is actually an intermediate isoform representing one step during the evolution of the C_3 - to the C_4 -form(s) of this key C_4 -photosynthesis enzyme. This supports an earlier view (4) that *F. brownii* likely represents a C_4 species with a less advanced capacity for C_4 photosynthesis when compared to other C_4 members of the genus. In comparing *F. brownii* with the other C_4 *Flaveria* species it should be remembered that *F. trinervia* and *F. australasica* are closely related and presumably represent the most derived species of the genus (18). As with *F. palmeri*, they belong to the three to four phyllary subgeneric group and are annual species. In contrast, *F. brownii* belongs to the five to six phyllary group and is the only perennial C_4 species of the entire genus, which also can be taken as an indication of a relatively ancient origin. This species is also unique among the other C_4 *Flaveria* species in having a higher CO_2 compensation

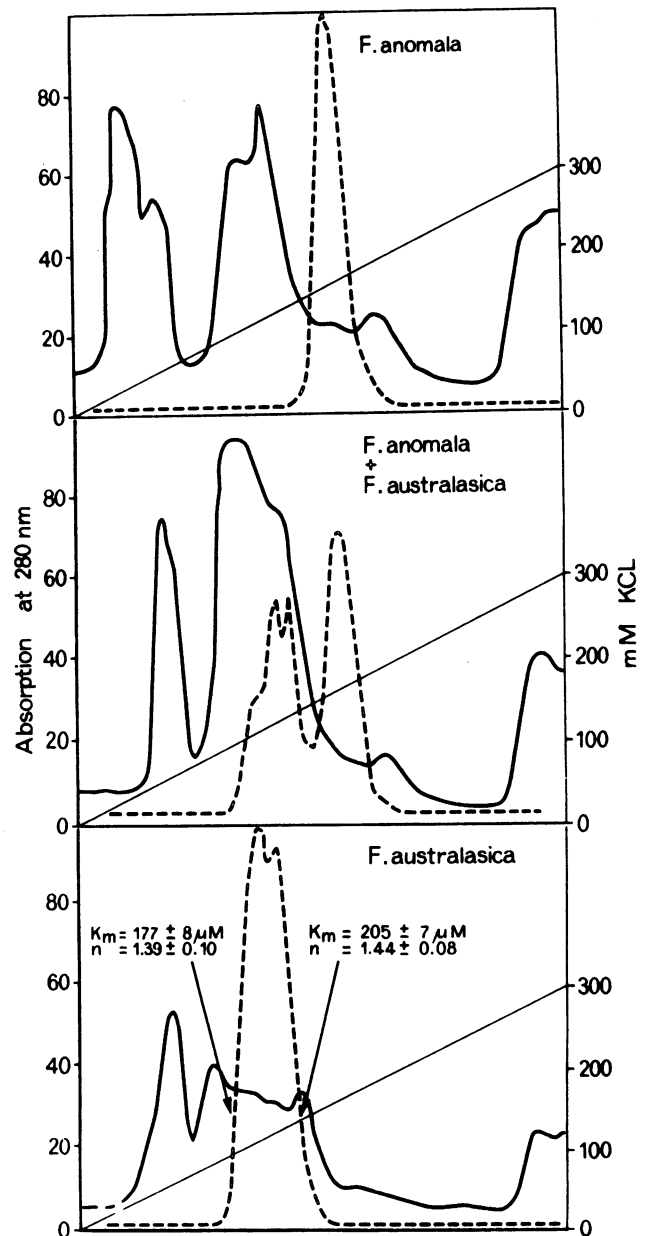


FIG. 2. Anion-exchange chromatography on DEAE-Trisacryl M of $(NH_4)_2SO_4$ -fractionated (30–60% saturation) extracts from growth chamber-grown *F. anomala* (C_3 - C_4), *F. australasica* (C_4), and co-chromatography of a mixture of both extracts. Dashed lines represent PEP carboxylase activity in arbitrary units. For PEP carboxylase from *F. australasica*, half-saturating PEP (free) concentrations and Hill coefficients \pm SD are given for the leading and trailing fractions eluted at the positions marked by arrows.

point and a higher rate of photorespiration (2), an incomplete intercellular compartmentation of Rubisco and PEP carboxylase (4, 19), and the most negative carbon isotope discrimination ratio (2, 21). With respect to PEP carboxylase, the adaptation of its kinetic and regulatory properties to changing metabolic needs during the evolution of C_4 photosynthesis in *Flaveria* apparently proceeded first via a reduction in the PEP affinity connected with higher enzyme activities in the leaf, and subsequently by a further decrease in PEP affinity accompanied by the appearance of sigmoidal kinetics. Obviously, the evolution of C_4 photosynthesis in its early stages is not necessarily paralleled by a dramatic change in the kinetic or structural (1) properties of PEP carbox-

ylase, although such changes apparently are of metabolic advantage for the full development of a C₄-photosynthesis system. The detailed metabolic reasons and implications of this change in the enzyme's properties remain speculative, however. It is our opinion that the kinetic and regulatory properties of PEP carboxylase must be viewed as an integral part of the whole C₄ regulatory network. Therefore, major attention presumably should be given to the regulatory interrelations with such enzymes as pyruvate, Pi dikinase and NADP-malate dehydrogenase which are involved in the light/dark regulation of C₄ photosynthesis *in vivo* (5).

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