Evolution of C4 phosphoenolpyruvate carboxylase in *Flaveria*- 
a conserved serine residue in the carboxyterminal part of the enzyme is a 
major determinant for C4-specific characteristics

Oliver E. Bläsin1, Peter Westhoff1 & Per Svensson2

1 Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität
Düsseldorf, D-40225 Düsseldorf, Germany

2 Department of Plant Biology, The Swedish University for Agricultural Sciences, S-750 07
Uppsala, Sweden

Corresponding author:
Dr. Per Svensson, Department of Plant Biology, The Swedish University for Agricultural
Sciences, P.O. Box 7080, S-750 07 Uppsala, Sweden.
Tel.: (46) 18 67 13 74 Fax.: (46) 18 67 32 79
E-mail: Per.Svensson@vbiol.slu.se

Running title: Molecular evolution of C4 phosphoenolpyruvate carboxylase
Summary

C4 phosphoenolpyruvate carboxylases have evolved from ancestral C3 isoforms during the evolution of angiosperms and gained distinct kinetic and regulatory properties compared to the C3 isozymes. To identify amino acid residues and/or domains responsible for these C4 specific properties the C4 phosphoenolpyruvate carboxylase of Flaveria trinervia (C4) was compared with its orthologue in the closely related C3 plant Flaveria pringlei. Reciprocal enzyme chimera were constructed and the kinetic constants $K_{0.5}$ and $k_{\text{cat}}$ as well as the Hill coefficient $h$ were determined for the substrate phosphoenolpyruvate both in the presence and absence of the activator glucose-6-phosphate. By this approach two regions were identified which determined most of the kinetic differences of the C4 and C3 ppcA phosphoenolpyruvate carboxylases with respect to the substrate PEP. In addition, the experiments suggest that the two regions do not act additively but interact with each other. The region between amino acids 296 and 437 is essential for activation by glucose-6-phosphate. The carboxyterminal segment between amino acids 645 and 966 contains a C4 conserved serine or a C3 invariant alanine at position 774 in the respective enzyme isoform. Site-directed mutagenesis shows that this position is a key determinant for the kinetic properties of the two isozymes.
Introduction

Phosphoenolpyruvate carboxylase (EC 4.1.1.31; PEPC) catalyzes the fixation of HCO$_3$\textsuperscript{-} to the receptor phosphoenolpyruvate resulting in the formation of oxaloacetate and inorganic phosphate. The enzyme uses Mg$^{2+}$ as a cofactor and is a homotetramer with four active sites and a molecular weight of about 100 kDa. PEPC has been found in prokaryotes, algae and higher plants but not in animals, fungi and yeast. The enzyme serves mainly to regenerate C4-dicarboxylic acids for the tricarboxylic acid cycle. In addition to this anaplerotic function other PEPC isozymes play an important role in C4 and CAM photosynthesis where they serve as the primary carboxylase (1). In C4 plants PEPC is compartmentalized in the mesophyll cells while the secondary carboxylase, ribulose-bisphosphate carboxylase/oxygenase, is confined to the bundle-sheath cells. The spatial separation of the C4 cycle reactions results in CO$_2$ pumping from mesophyll into bundle sheath cells which is responsible for the high efficiency of C4 photosynthesis (reviewed in (2,3)).

C4 plants have evolved several times independently from ancestral C3 plants due to selective environmental conditions (4). Today, only a few genera of dicots and monocots contain both C4 and C3 species, e.g. *Flaveria*, *Atriplex*, *Moricandia*, *Parthenium* and *Panicum* (5-7). Particularly interesting among these taxa is the dicotyledonous genus *Flaveria*, which comprises not only C3 and C4 species but also a large number of C3-C4 intermediates. This suggests that evolution towards C4 photosynthesis is still ongoing in this genus and makes *Flaveria* a good model system for the study of the underlying evolutionary processes (8,9).

To investigate the molecular evolution of C4 PEPC in the genus *Flaveria* we have chosen the C4 species *F. trinervia* and the C3 plant *F. pringlei* as the starting point. Cloning, sequencing and
genomic Southern blot experiments revealed that the nearest neighbor of the C4 enzyme of *F. trinervia* (gene designation: *ppcA*) is found in *F. pringlei*. Both *ppcA* PEPCs are 966 amino acids in size and share 96% identical residues. The comparison of these two orthologous *ppcA* PEPCs was therefore selected as the paradigm for studying the evolution of the ancestral C3 isoform towards the current C4 enzyme (10,11).

When expressed into functional protein in *Escherichia coli* the C4 and C3 *ppcA* PEPCs showed the expected kinetic and regulatory features of a C4 and a C3 isoform, respectively. The C4 *ppcA* isoform possesses a high $K_{0.5}$ for the substrate phosphoenolpyruvate (PEP) and a low sensitivity against feedback inhibition by malate, while the C3 *ppcA* isoform displays the opposite characteristics (12). These findings prompted us to investigate whether the determinants for the C4 specific properties could be located within segments of the primary structure. Since the high sequence similarity allowed the easy exchange of corresponding protein parts between the two isoforms a series of reciprocal exchanges was constructed. The kinetic properties of the chimerical enzymes were studied with respect to $K_{0.5}$-PEP, $k_{cat}$ and the Hill coefficient ($h$) in the presence or absence of the activator glucose-6-phosphate (Glc6P). In addition to these reciprocal *ppcA* PEPC chimera the functional role of a conserved amino acid residue in the carboxyterminus of the PEPCs was studied. Sequence comparison of plant PEPCs revealed that C4 isoforms of both mono- and dicot origin possesses a serine in the carboxyterminal region, which in *Flaveria* is equivalent to amino acid 774. In all C3 and CAM PEPC isoforms this serine is replaced by an alanine. This strict correlative behavior of the serine/alanine position suggested a functional importance of this amino acid residue (11) and therefore its role was investigated by site-directed mutagenesis.
Experimental Procedures

Materials

DNA modifying enzymes were purchased from Roche Diagnostics (Mannheim, Germany) or from MBI Fermentas (Vilnius, Lithuania). Glc6P and NADH were obtained from Sigma (München, Germany), NADH-malate dehydrogenase (porcine heart) from Serva (Heidelberg, Germany) and the trisodium salt of PEP from ICN (Eschwege, Germany). All chromatography materials were from Amersham Pharmacia Biotech (Freiburg, Germany).

Construction of chimerical ppcA sequences

The ppcA PEPC sequences from *F. trinervia* (13) and *F. pringlei* (12) had been inserted into the expression vector pTrc99A (Amersham Pharmacia Biotech, Freiburg, Germany) resulting in plasmids Ft966 and Fp966, respectively. In order to facilitate the construction of ppcA chimerical clones the 3’ ends of the inserted fragments were modified introducing a SacI restriction site. Plasmid Fp966 was restricted with XhoI and PstI and a SacI linker sequence was ligated to the blunted ends. Plasmid Ft966 was digested with EcoRI and PstI and the SacI linker was added correspondingly. The resulting plasmids were named Fp966* and Ft966*, respectively.

For constructing the chimerical ppcA sequences suitable fragments of plasmids Ft966, Ft966*, Fp966 and Fp966* were excised with the appropriate restriction endonucleases (Table I and Fig. 1) and recombined using standard methods. The successful construction of the intended chimerical ppcA sequences (see Table I) was confirmed by restriction and/or sequence analysis.

All ppcA chimerical expression plasmids were transformed (14) into the ppc− *E. coli* strain PCR1.
Site-directed mutagenesis of serine/alanine residue 774

The conversion of serine 774 of the *F. trinervia* PEPC into alanine and vice versa the exchange of alanine 774 of the *F. pringlei* protein into serine was carried out with the Chameleon" double-stranded site-directed mutagenesis kit (Stratagene, San Diego, USA) according to the manufacturer’s instructions. The mutagenic primer for the S774A conversion was 5’-CCATGGATCTTTGCATGGACTCAGACC-3’ and for the A774S conversion 5’-CCATGGATCTTTTCATGGACTCAGACC-3’. The selection primer was 5’-CCTCTAGAGTCGACGAGCTCGCATGCAAGCTTGG-3’. This primer converts the PstI restriction site of the vector into SacI. DNA sequencing of fragments containing the desired mutation confirmed the success of mutagenesis. The mutagenized fragments were then inserted into the chimerical ppcA sequences as described for the corresponding non-mutagenized fragments (Table I).

Expression and purification of recombinant ppcA PEPCs

The recombinant chimerical enzymes were produced in *E. coli* strain PCR1 essentially as described before. The PEPC proteins were purified by precipitation with polyethylene glycol 8000 and successive chromatography on Phenyl Sepharose CL-4B, Mono Q and Superdex 200 HR (12). Enzyme fractions obtained after the size exclusion chromatography were pooled and stored in 50 % (v/v) glycerol at −20°C.

In case of the chimerical enzyme FT296FP670 the purification through Phenyl Sepharose CL-
4B had to be replaced by Q Sepharose Fast Flow chromatography since no active enzyme could be eluted from the Phenyl Sepharose column. The polyethylene glycol-precipitated proteins were resuspended in buffer A [20 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 5 % (v/v) glycerol] and loaded at 1 ml/min onto a Q Sepharose column that was pre-equilibrated with the same buffer. PEPC activity was eluted by a linear 30 ml gradient of 0-0.5 M KCl in buffer A. Peak fractions of 1 ml were pooled, concentrated with a Centricon 30 micro concentrator (Millipore Amicon, Eschborn, Germany) and then subjected to size exclusion chromatography on the Superdex 200 HR column. Active fractions were stored as described above.

**PEPC activity assay**

PEPC activity was measured spectroscopically at 340 nm by coupling to exogenous NADH-malate dehydrogenase. The standard reaction mixture of 0.6 ml contained 50 mM Tricine/KOH, pH 8.0, 5 mM PEP, 10 mM MgCl₂, 10 mM KHCO₃, 0.15 mM NADH and 6 U NADH-malate dehydrogenase (porcine heart). The reaction was started by adding recombinant PEPC which was diluted in buffer B [10 mM Tricine/KOH, pH 8.0, 1 mM DTT, 50% (v/v) glycerol] to the desired activity. One Unit (U) enzyme was defined as the activity oxidizing 1 µmol NADH per minute at 25°C. In order to determine the kinetic parameters ($K_{0.5}$-PEP, $k_{cat}$ and the Hill coefficient $h$) the Hill-equation was fitted to the experimental data by non-linear regression analysis using the software package Kaleidagraph (Version 3.0.8, Synergy Software). For each chimerical enzyme two independent preparations were analyzed and each kinetic measurement was repeated at least once.
Miscellaneous

Standard molecular biological techniques were carried out essentially as described in (16).

Double stranded plasmid DNAs were sequenced with the T7 sequencing kit from Amersham Pharmacia Biotech (Freiburg, Germany). Nucleic acid and protein sequences were analyzed with the software packages CLUSTAL V (17) and MacMolly® Tetra (Softgene GmbH, Berlin, Germany).
Results

Kinetic properties of the ppcA PEPCs of F. trinervia (FT966) and F. pringlei (FP966)

The basis for the study was the large difference in $K_{0.5}$-PEP observed between the C3 (FP966) and C4 (FT966) isoenzyme, respectively. Since the C4 isoenzyme has evolved from an ancestral C3 enzyme, we searched for positions that have changed to give rise to the C4 value of $K_{0.5}$-PEP. Under the assay conditions used the C3 enzyme displayed only small deviations from Michaelis-Menten kinetics both when investigated in its non-activated state and when activated with Glc6P. This was also true for the activated C4 enzyme. However, the non-activated C4 enzyme showed sigmoidal kinetics indicating an allosteric behavior. In order to be able to compare enzymes with different types of kinetics the $K_{0.5}$-PEP was used throughout this study and the Hill coefficient was calculated for all enzymes.

Experimental strategy

To localize C4/C3 determinants for the kinetic properties with respect to the substrate PEP within the ppcA PEPCs we took advantage of several conserved restriction sites in the ppcA PEPC sequences from F. trinervia and F. pringlei (Fig. 1). We interchanged progressively smaller parts of the C3 and C4 enzymes, produced active recombinant chimerical enzymes in E. coli and measured $K_{0.5}$-PEP and $k_{cat}$ both of the non-activated enzyme and when activated by 5 mM Glc6P (Tables II and III).

Turning a C3 enzyme into C4 type

Using the C3 enzyme (FP966) as the starting point we swapped parts of the enzyme with
corresponding parts of the C4 isozyme starting from the aminoterminus (Fig. 2), and asked the question when C4 properties appeared in the chimerical enzymes. The first chimerical enzyme, FT296FP670 (Fig. 2), was comprised of the 296 aminoterminal amino acids (region 1) of the C4 enzyme (FT966) while the remainder was of C3 type (FP966). This region contains the phosphorylation site for light activation (18), a domain suggested to be involved in oxaloacetate formation (19) and, additionally, two well-conserved domains of unknown function found in all PEPC (Fig. 1). When comparing the $K_{0.5}$-PEP of the non-activated and activated FT296FP670 chimera with that of the C3 enzyme (FP966) no significant change was found (Fig. 2). This result indicates that the first 296 amino acids do not contribute significantly to the differences in $K_{0.5}$-PEP. The second chimerical enzyme, FT437FP529, was investigated already in the previous study (11) and the results here are the same: the non-activated enzyme shows a small but significant alteration in $K_{0.5}$-PEP while the activated one is practically unchanged in comparison to the original C3 enzyme (Fig. 2). This implies that amino acids 296 to 437, called region 2 in Figure 2, bear a C4-relevant domain. In the next chimera, FT591FP375, the C4 PEPC sequences were extended by 154 amino acids (region 3) to comprise the first 591 aminoterminal amino acids while the 375 carboxyterminal amino acids were of C3 origin. The 154 additional amino acids contain several small domains (Fig. 1) with unknown function but which are conserved in all examined PEPCs. However, this extension of the C4 part had no significant influence either on the activated or on the non-activated enzyme. In the last construct of this series the C4 part was expanded up to amino acid position 645 (region 4) to create the chimerical enzyme FT645FP321 (Fig. 2). Region 4 contains two well-conserved domains where the second one is involved in PEP and PEP/HCO$_3$ binding (20), (Fig. 1). As can be seen in Figure 2 the $K_{0.5}$-PEP
for the non-activated and activated enzyme rises somewhat but this cannot be considered to be significant.

It follows from these experiments that region 5 of the enzyme must contain the major determinant(s) for the acquisition of a C4 specific $K_{0.5}$-PEP. As outlined already in the introduction, amino acid 774 in this carboxyterminal region is an interesting candidate in this respect, since all C4 enzymes examined possess a serine residue at this position while C3 as well as CAM enzymes instead have an alanine (11). To test if a serine at this position is essential for gaining C4 properties site-directed mutagenesis was performed and the alanine 774 of the construct FT645FP321 was replaced by serine creating the enzyme FT645FP321-A774S (Fig. 2). As can be seen from the kinetic data, this single amino acid change had a striking effect in comparison to both the non-activated and activated FT645FP321 enzyme and resulted in $K_{0.5}$-PEP values close to those of the C4 enzyme (Fig. 2).

To assess the importance of this position for C4 characteristics without interference from other regions of the C4 enzyme, alanine 774 in the C3 enzyme (FP966) was substituted by serine. The resulting enzyme, FP966-A774S, showed a significant increase in the $K_{0.5}$-PEP of the non-activated enzyme, however, the impact of this exchange on the activated enzyme was marginal (Fig. 2).

Since $K_{0.5}$ values only partially describe the kinetic properties of an enzyme and the turnover number $k_{cat}$ has to be taken into account, the specificity constants, $k_{cat}/K_{0.5}$, were calculated for each chimerical enzyme. Table II shows that the pattern of changes in $k_{cat}/K_{0.5}$ parallels that of the changes obtained by $K_{0.5}$ alone indicating a close relationship of these two kinetic parameters.
Taken together, this C3-to-C4 exchange series of chimerical enzymes defines two domains to be involved in the acquisition of C4 specific properties for both $K_{0.5}$-PEP and $k_{cat}/K_{0.5}$. Region 2 between positions 296 and 437 makes a small but significant contribution. Region 5 between amino acids 645 and 966, however, causes the major change of the C3 enzyme into C4 type. Serine 774 is the essential determinant for C4 characteristics in this region. It is necessary for C4 specific kinetics, but not sufficient.

Because the C3 enzyme follows Michaelis-Menten kinetics but the C4 enzyme displays sigmoidal behavior we analyzed when the C3-type Hill coefficient changed into C4 type while exchanging segment for segment. Table II shows that a major change is observed when region 2 is altered from C3 to C4. The full cooperative behavior, however, was only achieved when region 5 was of C4 type too, or, at least, the alanine774 was replaced by serine. This finding suggests that region 2 and 5 are not only involved in determining $K_{0.5}$-PEP but also the mode of enzyme kinetics.

**Turning a C4 enzyme into C3 type**

In order to test the conclusions drawn above we made the reciprocal experiments. We used the C4 enzyme (FT966) as the starting point, progressively interchanged parts of the C4 enzyme with C3 counterparts, and assayed for loss of C4 characteristics (Table III and Fig. 3). As was found for the C3-to-C4 exchange series, the exchange of region 1 had no significant effect on the $K_{0.5}$-PEP of either the non-activated or the activated enzyme. Further exchanges, however, differed in their effects on the kinetic properties of the non-activated and activated chimerical enzymes and are therefore described separately.
With respect to the non-activated enzyme the replacement of region 2 (FP437FT529) but also of region 3 (FP591FT375) led to significant decreases of $K_{0.5}$-PEP, while the last exchange (FP645FT321) had no effect on this kinetic parameter. However, only when the serine 774 of FP645FT321 was replaced by alanine (FP645FT321-S774A) a $K_{0.5}$-PEP close to that of the C3 enzyme (FP966) was obtained. It follows that for the non-activated enzymes regions 2, 3 and the serine/alanine 774 position of region 5 are the most important causes for the loss of C4 characteristics in $K_{0.5}$-PEP.

In contrast, when the $k_{cat}/K_{0.5}$ values of this series of non-activated chimerical enzymes were compared, region 3 and region 5 turned out to contain major determinants for the decrease in C4 characteristics, while the contribution of region 2 was neglectable. The replacement of serine 774 by alanine was not sufficient to bring about a C3 like $k_{cat}/K_{0.5}$ value in the FP645FT321 enzyme. This indicates that region 5 must bear additional amino acids influencing this kinetic constant.

The Hill coefficient was not significantly affected by this series of exchanges from C4-type enzyme to C3 except when the serine of FP645FT321 was mutagenized to an alanine (Table III). This result reinforces that serine774 is strongly involved in determining allosteric behavior.

When investigating the activated chimerical enzymes of this C4-to-C3 exchange series region 2 turned out to be the most important determinant for the change from a C4 to a C3 $K_{0.5}$-PEP value. The replacement of this region by its C3 counterpart in the FP296FT670 enzyme led to a C3 like $K_{0.5}$-PEP. All the further substitutions and the serine-alanine replacement had only minor effects.

Region 2 plays also a major role for explaining the increase in $k_{cat}/K_{0.5}$ when changing the C4
into a C3 enzyme. Its replacement in FP296FT670 by the corresponding segment of the C3 enzyme resulted in a doubling of $k_{\text{cat}}/K_{0.5}$ from $10 \times 10^5$ to $21 \times 10^5 \text{s}^{-1}\text{M}^{-1}$. This is about 50% of the difference between the C4 and the C3 enzyme. Approximately the same difference in $k_{\text{cat}}/K_{0.5}$ was observed when region 3 of the C4 enzyme was replaced by the corresponding part of the C3 enzyme suggesting that besides region 2 region 3 holds major determinants for $k_{\text{cat}}/K_{0.5}$. 
Discussion

We are interested in the events that led to the evolution of the C4 PEPC and are using the C4 PEPC of *F. trinervia* and its orthologue in the closely related C3 species *F. pringlei* as our experimental system (10,11). The two isozymes exhibit about the same turnover numbers ($k_{\text{cat}}$) but differ drastically in the $K_{0.5}$ for the substrate PEP both in the non-activated and the activated state. The two enzymes also differ in their degree of activation by the allosteric regulator Glc6P. While the C3 enzyme is activated only 1.5-fold, the C4 enzyme shows about a 5-fold activation. Finally, the two enzymes display different types of kinetics with respect to their substrate PEP. There is no difference in the activated enzymes which both show a typical Michaelis-Menten kinetic. However, the non-activated C4 enzyme, but not the C3 enzyme, shows a sigmoidal behavior. Taken together the evolution of the C4 isoform, therefore, must have involved changes in the steady-state interaction with its substrate PEP, the kinetic behavior and an increase in the activation by Glc6P.

To determine the structural elements that give the C4 PEPC its specific kinetic and regulatory properties a domain swapping strategy was pursued and two sets of chimerical enzymes were constructed. In the first series the C3 enzyme (FP966) was progressively interchanged with corresponding parts of the C4 enzyme (FT966) starting from the aminoterminus (Fig. 2). In the second series the reciprocal strategy was applied, i.e. regions of the C4 enzyme were swapped with corresponding segments of the C3 PEPC (Fig. 3). The enzymes, which were in their non-phosphorylated state (21), were investigated by performing saturation kinetics with the substrate PEP under non-activated and activated conditions, and the kinetic constants $K_{0.5}$, $k_{\text{cat}}$ and the
Hill coefficient were determined.

The main conclusion from these series of mosaic C3/C4 enzymes is that region 2 (positions 296 to 437) and region 5 (positions 645 to 966) contain the major determinants for C4 specific kinetic and regulatory properties. Region 5 is the key factor for $K_{0.5}$-PEP of the non-activated enzymes. The central determinant in this region is amino acid position 774, which holds a serine in all C4 enzymes but an alanine in all C3 and CAM PEPCs. Region 2 is essential for the allosteric regulation by Glc6P. However, in order to exert its effect region 2 of the C4 enzyme has to be combined with a region 5 that contains a serine residue at position 774. This indicates that region 2 and region 5 do not operate independently in the activated enzyme but interact with each other.

The interaction of the two regions becomes obvious when comparing the changes in $K_{0.5}$-PEP of the activated enzymes in the two exchange series. In the C4-to-C3 series (Fig. 3) the C4 $K_{0.5}$-PEP is lost when region 2 is exchanged from C4 to C3. If serine 774 of the intact C4 enzyme is altered to alanine the same effect is observed, i.e. the enzyme loses its C4 $K_{0.5}$-PEP and becomes C3 like. The conclusion is that both region 2 and position 774 of region 5 must be of C4 type in order to get a C4 $K_{0.5}$-PEP of the activated enzyme. As this mutual dependency is true only for the activated enzyme we suggest that at least one of these regions is involved in the allosteric behavior of the C4 PEPC. The C3-to-C4 exchange series is consistent with this interpretation.

The addition of a C4 type region 2 to a C3 enzyme affects the $K_{0.5}$-PEP of the activated enzyme only if alanine 774 is converted to serine.

There is evidence that other regions are also involved in C4 specific properties. The analysis of the kinetic parameter $k_{\text{cat}}/K_{0.5}$ suggests that besides region 2 and 5 region 3 may contain determinants for C4 characteristics. However, the contribution of region 3 to the C4 specific
properties becomes only apparent in the C4-to-C3 exchange series.

How do these suggestions of the effect of region 2 and 5, and especially position 774 of region 5, suit with the three-dimensional structure of the \textit{E. coli} PEPC that recently became available (22)? The \textit{E. coli} PEPC is very similar to the plant enzyme in the primary structure suggesting that the three-dimensional structure of the bacterial enzyme can be directly applied to plant PEPC (22). The position 774 (corresponding to alanine-720 in the \textit{E. coli} PEPC) is located above and very close to the active site suggesting influence on the catalysis (Fig. 5). The substitution of this alanine to serine might give rise to a hydrogen bond that interacts with the substrate PEP or with other parts of the enzyme. Such a change may very well result in weaker steady-state interactions revealed as a higher $K_{0.5}$-PEP, which is typical for C4 PEPCs (23). Thus, the three-dimensional structure of the enzyme is in accordance with our view of position 774 as a determinant for C3/C4 characteristics.

As the exact location of the C4 determinant(s) in region 2 is not known it is more difficult to grasp how this region interacts with the catalytic center and with position 774. Comparison of the two \textit{ppcA} PEPCs reveals 13 amino acids that differ in this region (Fig. 1). Some of these positions can be located by a comparison with the crystallized \textit{E. coli} enzyme but they are far away from the catalytic center. However, this region contains a 13 amino acid long sequence in plant PEPCs that is missing in the bacterial enzyme (Fig. 5). Two of the positions in this stretch differ between the investigated PEPCs. Amino acid 347 in the \textit{Flaveria} PEPC is an interesting candidate. This position harbors a lysine in FT966 (C4) but an arginine in FP966 (C3) and was suggested as a candidate of C3/C4 difference (11). However, with more C4 sequences available (i.e. \textit{Amaranthus hypochondriacus}; Acc. L49175) it became apparent that this lysine residue is
not 100% C4 unique. This might indicate that the conversion of a C3 enzyme into C4 type depends on one strictly conserved site in the carboxyterminus, serine/alanine at position 774, and a second determinant in the aminoterminal half whose position is more flexible. The second determinant may be composed of several positions but we suggest that one of them is position 347 arginine/lysine at least what concerns the *Flaveria* PEPCs. Preliminary sequence data from other C3 and C4 PEPCs in the *Flaveria* genus support this suggestion (unpublished results). The fact that none of the 13 amino acid candidates in region 2 are in close contact to the active site (Fig. 5) may indicate that the C3/C4 determinant in region 2 is involved in the allosteric regulation of the enzyme. The strong influence that this region has on the Glc6P activation is in favor of this suggestion.

In conclusion, our results show that the genus *Flaveria* offers the opportunity to get insight in the evolutionary process leading from C3 to C4 metabolism. The fact that this genus contains C3, C4 as well as C3/C4 intermediate species suggests that it should be possible to ascertain both the stepwise evolutionary changes as well as the order of these steps. In this paper we have put a small piece of work towards this goal what concerns PEPC, the key enzyme of C4 metabolism.

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Figure Legends

Fig. 1. Amino acid sequence comparison of the ppcA PEPCs of F. trinervia (FT966) and F. pringlei (FP966). Conserved restriction sites, which were used to construct the reciprocal chimerical enzymes, are indicated at their position within the polypeptide chain. Highly conserved amino acid sequences among all known PEPCs of bacteria and plants are labeled by boxes in gray. Regions with known functions are designated accordingly. The stars show those amino acid residues that are identical in the two PEPCs.

Fig. 2. Localization of C4 characteristics by C3-to-C4 chimerical enzymes.

The appearance of C4 properties was studied by a progressive replacement of segments of the C3 enzyme (gray) by its C4 counterparts (white), starting from the aminoterminus. Note the drastic change in $K_{0.5}$-PEP when region 5 is changed from C3 type (FT645FP321) to C4 (FT966). Site–directed mutagenesis of alanine 774 into a serine resulted in a substantial change of $K_{0.5}$-PEP, indicating that this position is a major determinant for C4 characteristics in this region (see lower part of the figure). The enzymes were divided into 5 regions according to common restriction sites. The black boxes denote the highly conserved amino acid sequences in all PEPCs (see Fig. 1). Amino acid 774 is indicated by a circle. The figures presented are mean values of four measurements from two independent enzyme preparations. For standard errors see Table II.

Fig. 3. Localization of C4 characteristics by C4-to-C3 chimerical enzymes.

The loss of C4 properties was investigated by a progressive replacement of segments of the C4
enzyme (white) by its C3 counterparts (gray), starting from the aminoterminus. Note the profound drop in $K_{0.5}$-PEP of the activated enzyme (+Glc6P) when region 2 of FP296FT670 is changed to its C3 counterpart creating the chimerical enzyme FP437FT670. This suggests that region 2 is of utmost importance for the Glc6P activation. The $K_{0.5}$-PEP of the non-activated enzyme drops significantly when region 2 or region 5 is changed from C4 to C3, indicating that these regions harbor determinants for C4 characteristics in the non-activated enzyme. In addition, site-directed mutageneses (lower part of the figure) show that the replacement of serine 774 in region 5 by an alanine drastically lowers the $K_{0.5}$-PEP indicating that this position is a major determinant for C4 properties of the non-activated enzyme. The black boxes denote the highly conserved amino acid sequences in all PEPCs (see Fig. 1). Amino acid 774 is indicated by a circle. The figures presented are mean values of four measurements from two independent enzyme preparations. For standard errors see Table III.

Fig. 4. Three-dimensional view of the *E. coli* PEPC corresponding to the C4 specific serine and region 2 of the *Flaveria* enzymes.

The picture was created from the three-dimensional structure of the *E. coli* enzyme published recently (20) by using SwissPdbViewer version 3.5 (24). The Ala-720 marked with an arrow in the upper part of the figure corresponds to position 774 of the *F. trinervia* enzyme that holds the C4 specific serine residue. The amino acids located above the beta barrel (in green) are involved in enzyme catalysis (1,49,25,26). Region 2 of the *Flaveria* enzymes corresponds to helices a11 to a15 of the *E. coli* enzyme. The lysine 347 of the C4 PEPC which is suggested to be involved in
Glc6P activation is located between a12 and a13 in a small domain that is missing in the bacterial enzyme.
### Table I

**Construction of PEPC expression plasmids**

The expression plasmids were constructed by double- or triple ligations of the restriction fragments as listed below. The plasmids used to prepare the restriction fragments are indicated in parentheses. See “Experimental Procedures” for details.

| Plasmid          | Constructed by ligation of the following fragments: |  |
|------------------|---------------------------------------------------|---|
|                  | Fragment I                                | Size (bp) | Fragment II | Size (bp) | Fragment III | Size (bp) |
| Fp296Ft670       | AocI-PstI(Ft966)                          | 2323 | PstI-AocI(Fp966) | 5061 |
| Ft296Fp670       | AocI-PstI(Fp966)                          | 2299 | PstI-AocI(Ft966) | 5016 |
| Fp437Ft529       | SalI-SacI(Ft966*)                         | 1830 | SacI-SalI(Fp966*) | 5443 |
| Ft437Fp529       | SalI-SacI(Fp966*)                         | 1839 | SacI-SalI(Ft966*) | 5443 |
| Fp591Ft375       | SacI-BclII(Ft966*)                        | 3139 | BclII-BspHI(Fp966) | 2795 | BspHI-SacI(Ft966*) | 1369 |
| Ft591Fp375       | SacI-BclII(Fp966*)                        | 3139 | BclII-BspHI(Ft966) | 2795 | BspHI-SacI(Fp966*) | 1378 |
| Fp645Ft321       | ApaI-SacI(Ft966*)                         | 1207 | SacI-NarI(Ft966*) | 3735 | NarI-ApaI(Fp966) | 2331 |
|          | Restriction                      |    | Restriction                      |    | Restriction                      |    |
|----------|----------------------------------|----|----------------------------------|----|----------------------------------|----|
| Ft645Fp321 | ApaI-SacI(Fp966*)                | 1216 | SacI-NarI(Fp966*)                | 3735 | NarI-ApaI(Ft966)                | 2331 |
| Ft966-S774A | BcII-BspHI(Ft966)               | 2795 | BspHI-SacI(Ft966-S774A)          | 1438 | SacI-BcII(Ft966*)               | 3139 |
| Fp966-A774S | BcII-BspHI(Fp966)               | 2795 | BspHI-SacI(Fp966-A774S)          | 1414 | SacI-BcII(Ft966*)               | 3139 |
| Fp645Ft321-S774A | AocI-ApaI(Fp966)         | 1047 | ApaI-AgeI(Ft966-S774A)          | 959  | AgeI-AocI(Fp966)                | 5309 |
| Ft645Fp321-A774S | AocI-ApaI(Ft966)         | 1047 | ApaI-AgeI(Fp966-A774S)          | 959  | AgeI-AocI(Ft966)                | 5333 |
**Table II**

**Kinetic constants for the C3-to-C4 exchange series of chimerical phosphoenolpyruvate carboxylases**

Kinetic parameters were obtained as described under “Experimental Procedures”. Purified enzyme fractions exhibiting 40-50 milliunit (U) activity were assayed with at least eight different concentrations of the substrate PEP. The kinetic parameters $K_{0.5}$, $V_{\text{max}}$ and the Hill coefficient were determined using nonlinear regression analysis. One U of enzyme catalyzes the oxidation of 1 µmol NADH per minute in a MDH coupled assay at 25° C.

| Enzyme      | $K_{0.5}$ (PEP) | Hill coefficient $h$ | $V_{\text{max}}$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{0.5}$ |
|-------------|-----------------|----------------------|------------------|------------------|-------------------------|
|             | µM              |                      | U/mg             | s$^{-1}$         | s$^{-1}$ M$^{-1}$        |
| FP966 (C3)  | 29 ± 2.2        | 19 ± 1.5             | 0.9 ± 0.03       | 28 ± 2.6         | 51 ± 4.8                | 60 ± 5.8                | 18 x 10$^5$ | 31 x 10$^5$ |
| FT296FP670  | 25 ± 1.2        | 17 ± 0.9             | 0.9 ± 0.05       | 27 ± 1.5         | 50 ± 2.7                | 58 ± 3.3                | 20 x 10$^5$ | 34 x 10$^5$ |
| FT437FP529   | 55 ± 1.7 | 22 ± 0.7 | 1.3 ± 0.03 | 1.2 ± 0.05 | 35 ± 1.4 | 39 ± 1.5 | 65 ± 2.6 | 72 ± 2.7 | 12 x 10⁶ | 33 x 10⁵ |
| FT591FP375   | 51 ± 0.7 | 19 ± 0.4 | 1.2 ± 0.04 | 1.1 ± 0.02 | 31 ± 1.8 | 34 ± 2.0 | 57 ± 3.2 | 63 ± 3.7 | 11 x 10⁵ | 34 x 10⁵ |
| FT645FP321   | 78 ± 3.1 | 25 ± 1.2 | 1.2 ± 0.04 | 1.1 ± 0.02 | 33 ± 1.0 | 35 ± 1.5 | 61 ± 1.8 | 65 ± 2.7 | 8.0 x 10⁵ | 27 x 10⁵ |
| FT966 (C4)   | 278 ± 4.0 | 58 ± 1.8 | 1.5 ± 0.02 | 1.1 ± 0.05 | 27 ± 1.3 | 30 ± 1.5 | 49 ± 2.3 | 55 ± 2.8 | 1.8 x 10⁵ | 9.5 x 10⁵ |
| FT645FP321-A774S | 252 ± 1.9 | 47 ± 0.4 | 1.4 ± 0.02 | 1.0 ± 0.04 | 27 ± 1.3 | 27 ± 1.4 | 59 ± 2.4 | 50 ± 2.5 | 1.9 x 10⁵ | 11 x 10⁵ |
| FP966-A774S   | 97 ± 9.3 | 22 ± 0.3 | 0.9 ± 0.02 | 1.1 ± 0.08 | 24 ± 1.1 | 29 ± 1.9 | 45 ± 2.1 | 54 ± 3.5 | 4.7 x 10⁵ | 25 x 10⁵ |
Table III

Kinetic parameters for the C4-to-C3 exchange series of chimerical phosphoenolpyruvate carboxylases

Kinetic parameters were obtained as described under “Experimental Procedures”. Purified enzyme fractions exhibiting 40-50 milli unit (U) activity were assayed with at least eight different concentrations of the substrate PEP. The kinetic parameters $K_{0.5}$, $V_{\text{max}}$ and the Hill coefficient were determined using nonlinear regression analysis. One U of enzyme catalyzes the oxidation of 1 µmol NADH per minute in a MDH coupled assay at 25° C.

|                | $K_{0.5}$ (PEP) | Hill coefficient $h$ | $V_{\text{max}}$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{0.5}$ |
|----------------|-----------------|----------------------|-------------------|-----------------|--------------------------|
|                | µM              |                      | U/mg              | s⁻¹             | s⁻¹ M⁻¹                  |
| Enzyme         | - Glc6P         | +Glc6P               | - Glc6P           | +Glc6P          | - Glc6P                  | +Glc6P                  | - Glc6P                  | +Glc6P                  |
| FT966 (C4)     |                 |                      |                   |                 |                          |                         |                          |                         |
|                | 278 ± 4.0       | 58 ± 1.8             | 1.5 ± 0.02        | 1.1 ± 0.05      | 27 ± 1.3                | 30 ± 1.5                | 49 ± 2.3                | 55 ± 2.8                |
|                |                 |                      |                   |                 |                          |                         |                          |                         |
| FP296FT670     |                 |                      |                   |                 |                          |                         |                          |                         |
|                | 281 ± 9.9       | 63 ± 1.0             | 1.4 ± 0.04        | 1.1 ± 0.07      | 30 ± 1.1                | 34 ± 1.4                | 56 ± 2.1                | 63 ± 2.6                |
|                |                 |                      |                   |                 |                          |                         |                          |                         |
|     | Value 1 | Value 2 | Value 3 | Value 4 | Value 5 | Value 6 | Value 7 | Value 8 | Value 9 | Value 10 |
|-----|---------|---------|---------|---------|---------|---------|---------|---------|---------|----------|
| FP437FT529 | 189 ± 7.3 | 24 ± 1.0 | 1.5 ± 0.02 | 1.1 ± 0.01 | 23 ± 1.1 | 27 ± 1.1 | 43 ± 2.0 | 50 ± 1.9 | 2.3 x 10^5 | 21 x 10^5 |
| FP591FT375  | 107 ± 2.3 | 24 ± 0.4 | 1.3 ± 0.02 | 1.2 ± 0.04 | 30 ± 2.0 | 39 ± 2.5 | 55 ± 3.6 | 71 ± 4.5 | 5.1 x 10^5 | 30 x 10^5 |
| FP645FT321  | 105 ± 2.2 | 21 ± 0.3 | 1.3 ± 0.02 | 1.2 ± 0.04 | 25 ± 0.5 | 33 ± 0.7 | 46 ± 0.9 | 60 ± 1.2 | 4.5 x 10^5 | 29 x 10^5 |
| FP966 (C3)  | 29 ± 2.2  | 19 ± 1.5 | 0.9 ± 0.03 | 1.2 ± 0.13 | 28 ± 2.6 | 32 ± 3.1 | 51 ± 4.8 | 60 ± 5.8 | 18 x 10^5 | 31 x 10^5 |
| FP645FT321-S774A | 44 ± 2.2 | 18 ± 0.4 | 0.8 ± 0.07 | 1.2 ± 0.04 | 20 ± 0.5 | 24 ± 0.7 | 37 ± 0.9 | 43 ± 1.2 | 8.5 x 10^5 | 24 x 10^5 |
| FT966-S774A | 104 ± 3.0 | 23 ± 1.0 | 1.3 ± 0.04 | 1.1 ± 0.04 | 26 ± 0.5 | 27 ± 0.3 | 47 ± 0.8 | 51 ± 0.6 | 4.5 x 10^5 | 22 x 10^5 |
**Figure 1 (Bläsing et al.)**
| Construct     | AocI | Sall | Rcal | Apal | PEP [µM] |
|---------------|------|------|------|------|----------|
|               | 296  | 437  | 591  | 645  | - Glc6P  |
| FP966 (C3)    |      |      |      |      | 29       |
| FT296FP670    |      |      |      |      | 25       |
| FT437FP529    |      |      |      |      | 55       |
| FT591FP375    |      |      |      |      | 51       |
| FT645FP321    |      |      |      |      | 78       |
| FT966 (C4)    |      |      |      |      | 278      |
| FT645FP321-A774S |      |      |      |      | 252      |
| FP966-A774S   |      |      |      |      | 97       |
| Protein          | Domain 1 | Domain 2 | Domain 3 | Domain 4 | Domain 5 | PEP [-Glc6P] | PEP [+Glc6P] |
|------------------|----------|----------|----------|----------|----------|-------------|-------------|
| FT966 (C4)       | 1        |          |          |          |          | 278         | 58          |
| FP296FT670       | 1        |          |          |          |          | 281         | 63          |
| FP437FT529       | 1        |          |          |          |          | 189         | 24          |
| FP591FT375       | 1        |          |          |          |          | 107         | 24          |
| FP645FT321       | 1        |          |          |          |          | 105         | 21          |
| FP966 (C3)       | 1        |          |          |          |          | 29          | 19          |
| FP645FT321-S774A | 1        |          |          |          |          | 44          | 18          |
| FT966-S774A      | 1        |          |          |          |          | 104         | 23          |

Fig. 3 (Bläsing et al.)
Fig. 4 (Bläsing et al.)
Evolution of C4 phosphoenolpyruvate carboxylase in Flaveria-a conserved serine residue in the carboxyterminal part of the enzyme is a major determinant for C4-specific characteristics

Oliver E Bläsing, Peter Westhoff and Per Svensson

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