Greater efficiency of photosynthetic carbon fixation due to single amino-acid substitution

Judith Katharina Paulus¹,*, Daniel Schlieper¹,* & Georg Groth¹

The C4-photosynthetic carbon cycle is an elaborated addition to the classical C3-photosynthetic pathway, which improves solar conversion efficiency. The key enzyme in this pathway, phosphoenolpyruvate carboxylase, has evolved from an ancestral non-photosynthetic C3 phosphoenolpyruvate carboxylase. During evolution, C4 phosphoenolpyruvate carboxylase has increased its kinetic efficiency and reduced its sensitivity towards the feedback inhibitors malate and aspartate. An open question is the molecular basis of the shift in inhibitor tolerance. Here we show that a single-point mutation is sufficient to account for the drastic differences between the inhibitor tolerances of C3 and C4 phosphoenolpyruvate carboxylases. We solved high-resolution X-ray crystal structures of a C3 phosphoenolpyruvate carboxylase and a closely related C4 phosphoenolpyruvate carboxylase. The comparison of both structures revealed that Arg884 supports tight inhibitor binding in the C3-type enzyme. In the C4 phosphoenolpyruvate carboxylase isoform, this arginine is replaced by glycine. The substitution reduces inhibitor affinity and enables the enzyme to participate in the C4 photosynthesis pathway.
based on the type of CO₂ assimilation, plants can be divided into three photosynthetic types: the C3-type, the C4-type and the Crassulacean Acid Metabolism. In the classical C3-photosynthetic pathway, the primary CO₂ fixation is catalysed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) resulting in the formation of the three-carbon compound 3-phosphoglycerate. However, in high temperature conditions RuBisCO is prone to energy loss by a process called photorespiration. In C4 plants, this energy loss is minimized by an additional CO₂ concentrating mechanism. This new mechanism evolved to adapt to stress factors such as heat, high light and salinity in combination with low CO₂ availability in recent geological history.

The relevant key enzyme of this pathway, phosphoenolpyruvate (PEP) carboxylase (PEPC), catalyses the HCO₃⁻-dependent carboxylation of PEP to form the four-carbon molecule oxaloacetate. Subsequent to the carboxylation reaction, oxaloacetate is reduced to malate or transaminated to aspartate. Both C4 molecules form a reservoir pool for the malic enzyme or PEP carboxykinase. These enzymes generate a high CO₂ concentration at the active site of RuBisCO. Thereby RuBisCO’s oxygenase activity is reduced and the photosynthetic efficiency is increased in terms of use of water, nitrogen and other mineral nutrients for the production of valuable biomass. For the CO₂ concentration mechanism, it is necessary to spatially separate the primary CO₂ fixation by PEPC and the CO₂ release to RuBisCO. Most C4 plants realize this by a characteristic anatomical feature, the Kranz anatomy, which spatially separates RuBisCO in the bundle-sheath cells from the initial site of CO₂ assimilation in the mesophyll cells. Other mechanisms of compartmentation of the photosynthetic enzymes within cells have also been reported.

Another crucial step in the evolution of the C4 pathway is the recruitment of enzymes such as PEPC and the malic enzyme, which are required for initial CO₂ fixation and CO₂ release, respectively. The predecessors for these C4 enzymes are enzymes from C3 plants and are involved in non-photosynthetic metabolic processes. However, the C4-type enzymes have distinctly different kinetic and regulatory properties. For instance, C4 PEPC shows tenfold larger substrate saturation constants for PEP than the C3 PEPC and higher tolerance towards feedback inhibition by the C4-dicarboxylic acids malate and aspartate. Previous studies imply that the acquisition of this enhanced tolerance towards feedback inhibition is an essential achievement in the evolution of C4 PEPC from the C3 ancestor.

A prime example of the evolution of C4 photosynthesis is found in the genus Flaveria (yellowtops) in the Asteraceae family. It includes species that perform C3 photosynthesis (for example, F. pringlei), C4 photosynthesis (for example, F. trinervia) as well as C3–C4 intermediate metabolism (for example, F. pubescens). All species within the genus are closely related and therefore ideal model organisms to pinpoint the key mutations that led from C3 to C4 photosynthesis. In the case of PEPC, the C4 isoform from F. trinervia (encoded by the ppcA gene) and its corresponding non-photosynthetic C3 isoform, the orthologous ppcA gene of F. pringlei, share 94% amino-acid (aa) identity. But along with differences in location and function, they show distinct differences in their kinetic and regulatory properties.

The differences in kinetic efficiency have been addressed by reciprocal domain swapping and site-specific mutagenesis experiments. The increased PEP saturation kinetics of the C4 isoform depends on a single aa (Ser774). Less is known about the regulatory motif conferring the high malate/aspartate tolerance of C4 PEPC, which is imperative for the C4 cycle. Mutagenesis experiments indicate that the malate tolerance is largely mediated by the carboxy-terminal region of C4 PEPC (aa 645–966), and residues Arg641, Lys829, Arg888 and Asn964 (Flaveria numbering) together have been identified as the malate-binding motif in the crystal structure of a C4-type PEPC from maize. Mutagenesis of residues Lys829 and Arg888 was shown to completely disrupt the feedback inhibitor-binding site and results in enzymes with highly reduced malate sensitivity. However, as this malate-binding motif is also found in the C3-type ortholog, these residues cannot account for the different feedback inhibitor sensitivity of C3- and C4-type PEPCs. Despite intensive studies, no specific residue or motif was identified to account for the increased malate/aspartate tolerance of the photosynthetic C4 PEPC in comparison with the C3 PEPC isozyme.

As sequence analysis and mutagenesis studies failed to elucidate the molecular basis for malate/aspartate tolerance, we determined the crystal structures of PEPC isoforms from the C4 plant F. trinervia (2.5 Å) as well as from the C3 plant F. pringlei (2.7 Å) in their inhibited T-conformation. Our structures help to define the molecular adaptation that occurred when the housekeeping C3 isozyme mutated to the photosynthetic C4 PEPC.

Results

X-ray crystallography. Crystal structures of PEPC from Zea mays (maize), a representative C4 isozyme, and from Escherichia coli, a non-photosynthetic C3 PEPC catalysing typical housekeeping functions, are known. However, the low level of sequence similarity in these enzymes impedes the correlation of structural differences to C4-related features. Furthermore, the fact that the C4 structure was crystallized in the activated R-state and the C3 structure in the inhibited T-state may have resulted in the high level of backbone variation in multiple regions of the monomers. In contrast, any differences detected in the substrate site or the inhibitor-binding pocket of the PEPC isoforms from F. pringlei and F. trinervia can be attributed to a C3/C4-specific function. We crystallized PEPC from F. pringlei and F. trinervia with the inhibitor aspartate. We chose aspartate because malate and aspartate are equivalent feedback inhibitors and the addition of malate impeded crystal growth. The crystallographic data and the refinement statistics are shown in Table 1. The Ramachandran plot of the refined C3 PEPC structure showed that the backbone conformation of 97.2% of the residues lies in the favoured region, 2.5% in the allowed region and 0.3% in the outlier region. The C4 PEPC structure has 97.9% favoured residues, 1.8% allowed residues and 0.2% outliers.

The structural superposition of the PEPCs from F. pringlei and F. trinervia confirms the high level of structural similarity, which is also reflected in the low backbone root-mean-square deviation of 0.4 Å. A closer look at the inhibitor-binding site reveals that residue 884, which is located close to the feedback inhibitor-binding site, differs in the C3 and the C4 isoforms (Fig. 2 and corresponding stereo views in Fig. 3). The C3 PEPC isoform from F. pringlei has an arginine that is involved in inhibitor binding and functions as a molecular clamp tightening the inhibitory aspartate molecule in the binding pocket. In the C4 PEPC isoform from F. trinervia this arginine is replaced by glycine. The substitution reduces the number of protein–inhibitor interactions and provides more steric space for the inhibitor to dissociate from the binding pocket (Fig. 4). This important change is caused by a single-point mutation changing codon AGA to GGA. Thus, the structural comparison between the two Flaveria PEPC isoforms provides a unique way to analyse the function of these enzymes.

Mutagenesis and enzyme kinetics. To substantiate these findings, we assessed the interactions in the malate/aspartate-binding site.
We generated a series of mutations and examined their effect on the catalytic function of PEPC. When the key residue arginine 884 in C3 PEPC from F. pringlei was replaced by glycine as found in C4 PEPC, the malate tolerance of the engineered enzyme was increased by a factor of ~3. This increase corresponds to the IC₅₀ value obtained with C4 wild-type PEPC from F. trinervia. The reverse test is the mutation from glycine to arginine in F. trinervia (C4-type, yellow). The Flaveria PEPCs have a high overall topological similarity with a r.m.s.d. of 0.4 Å. Structures are shown as ribbons with the aspartate/malate-binding site on top. The bound inhibitors are shown as spheres representing van der Waals radii.

Previous attempts to pinpoint the molecular origin of the different kinetic and regulatory characteristics of C4 PEPC suggest that multiple residues are involved in the transfer of a C3- to a C4-type enzyme. Based on phylogenetic analysis of PEPC in grasses (Poaceae), Christin et al. identified 21 codons that have undergone positive selection in C4 species. However, all but two of the aas concerned are identical in the C3 plant F. pringlei and the C4 plant F. trinervia. One of the C4-adaptive aa is Ser774 (Flaveria numbering), which defines the PEPC therefore functions in C4-like photosynthesis but shows only moderate tolerance towards feedback inhibition. The full potential of malate tolerance in such enzymes would be obtained by the glycine–arginine substitution at position 884 as realized in the C4 PEPC of F. trinervia, though kinetic data to test this hypothesis are not available.

In summary, it is obvious that during evolution the main kinetic and regulatory changes of the C4 enzyme are the result of two point mutations rather than extensive modular changes such as recombination or exon shuffling. This notion is consistent with
the idea that the evolution of new enzymatic activity is often achieved by the duplication of an existing gene that is capable of catalysing the chemical reaction required.22

Within the next 50 years, the world’s population will increase by a third25 and a corresponding increase of crop yields is needed. A concerted effort of research and development is necessary to drastically increase crop yields—similar to the 'green revolution'26. One approach is to turn C3 plants into C4 plants and, as a result, to increase the photosynthesis efficiency27. The best candidates for this change are the C3 plants rice and wheat, two of the most important crops of the world. Attempts were made to introduce C4 enzymes into C3 plants28,29. However, the key trigger that changes plants in anatomy as well as enzyme function and regulation has not yet been found.

With our data, we complement the knowledge about PEPC, the key enzyme of C4 photosynthesis. A single mutation in the active site (774)14 and a single mutation at the inhibitory site (884) are sufficient to switch the function from C3 to C4 activity. Now, physiological experiments could test if instead of introducing the maize PEPC into rice 28,29, the rice PEPC itself could be engineered to function as a C4 enzyme.

### Methods

**Recombinant PEPC.** The coding sequences of PEPC from *F. trinervia* (ppcA, EMBL-Bank X61304) and *F. pringlei* (ppcA, EMBL-Bank Z48966) were cloned in pETEV16b (Novagen), together with the coding sequence of an amino-terminal His10-tag and a TEV cleavage site. Plasmids were transformed in *E. coli* BL21(DE3) (*F. trinervia*) or BL21-Gold(DE3) (*F. pringlei*) (Agilent Technologies).

| PEPC crystal | C3 (*F. pringlei*) | C3 truncated | C4 (*F. trinervia*) |
|--------------|-------------------|--------------|-------------------|
| **Data collection** | 
| **Space group** | P2_12 12 | P2_12 12 | P2_12 12 |
| **Cell dimensions** | 
| *| * | * |
| *a*, *b*, *c* (Å) | 164.8, 121.6, 132.9 | 165.6, 121.7, 132.0 | 162.5, 122.1, 131.9 |
| *α*, *β*, *γ* (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| **Resolution (Å)** | 48.93 – 3.20 (3.37 – 3.20) | 165.55 – 2.71 (2.86 – 2.71) | 37.83 – 2.49 (2.62 – 2.49) |
| **R*-factor** | 0.130 (0.438) | 0.102 (0.344) | 0.099 (0.397) |
| **I/σI** | 6.0 (1.9) | 6.3 (2.0) | 5.9 (2.0) |
| **Completeness (%)** | 99.9 (100) | 100 (100) | 100 (100) |
| **Redundancy** | 3.7 (3.8) | 15.8 (16.4) | 26.2 (24.0) |
| **Refinement** | 
| **Resolution (Å)** | 70.1 – 2.71 (2.78 – 2.71) | 37.0 – 2.49 (2.55 – 2.49) |
| **No. of reflections** | 71548 (1486) | 90203 (1845) |
| **R*-factor/R*-free** | 0.188 (0.226)/0.230 (0.275) | 0.204 (0.241)/0.237 (0.292) |
| **No. of atoms** | 
| **Protein** | 14750 | 14710 |
| **Ligand/ion** | 46 | 36 |
| **Water** | 56 | 198 |
| **B-factors** | 
| **Protein** | 75.0 | 60.4 |
| **Ligand/ion** | 51.8 | 39.1 |
| **Water** | 34.3 | 37.3 |
| **r.m.s.d.** | 
| **Bond lengths (Å)** | 0.016 | 0.017 |
| **Bond angles (°)** | 1.758 | 1.794 |

r.m.s.d., root-mean-square deviation.
A single crystal was used for each data set.

*Highest resolution shell is shown in parenthesis.
†Signal-to-noise ratio of merged intensities.
§Protein B factors are calculated as the sum of the contribution from the TLS parameters and the residual B factors.

### Figure 2

**Comparison of the inhibitor-binding sites.** (a) C3 PEPC from *F. pringlei*. (b) C4 PEPC from *F. trinervia*. Arg884 in C3 PEPC functions as a clamp by providing an additional hydrogen bond for inhibitor binding, while Gly884 in C4 PEPC is more than 6 Å away from the inhibitor. Structures and 2Fo–Fc electron densities at 1.2σ of the malate/aspartate-binding pockets.
Inhibitor studies. The IC$_{50}$ of malate inhibition was determined by a coupled spectrometric assay as described.$^8$ In this assay, conversion of PEP to oxaloacetate was measured at 0.8726 Å wavelength.$^8$ Data of C4 PEPC with the inhibitor aspartate were integrated with Mosflm$^{30}$ and further processed with tools of the CCP4 suite$^{31}$, Pointless$^{32}$ and Scala$^{33}$ in particular. Initial phases of C4 PEPC (F. trinervia) were obtained by molecular replacement with Z. mays C4 PEPC (PDB Code: 1IJO, 73% sequence identity) using Phaser$^{33}$. Model building was initially done automatically with ARP/wARP$^{34}$. This step efficiently reduced model bias. Several rounds of manual building followed using Coot$^{35}$ and refinement by Refmac5$^{36}$ using non-crystallographic restraints and partially describing the atomic displacement parameters in groups of translation, libration and screw-motion (TLS). The structure comprises two monomers within the asymmetric unit. Crystallographic symmetry completes the tetramer. Additional electron density was found at the inhibitor-binding site. However, to avoid model bias the aspartate molecule was not fitted in the structure until model building was almost complete. The structures were validated using the tools provided by Coot, CCP4 and Rampage$^{37}$. The C3 PEPC structure from F. pringlei was obtained by essentially the same workflow. Model building started using a 3.2 Å data set collected at ESRF ID23-2. Molecular replacement was done using the C4 PEPC structure from F. trinervia. As the region comprising the His$_{10}$-tag, the TEV protease recognition site and the first five aas (construct C3truncated) were disordered in this structure, we deleted the TEV site and the first five aas (construct C3truncated$_{2013}$) were obtained under identical conditions and diffracted to 2.7 Å (EMBL/DESY PETRA P14). These data were integrated with XDS$^{38}$ and the model was built as described including ARP/wARP autobuilding to reduce model bias. Figures of the atomic structures were generated with Pymol (Schrödinger). Schematic diagrams of protein–ligand interactions were made using LigPlot$^{39}$.

Figure 3 | Electron density maps and final model structures. (a) C3 PEPC from F. pringlei, (b) C4 PEPC from F. trinervia. Shown are the refined 2F$_{o}$ − F$_{c}$ electron density maps around the inhibitor-binding sites. The maps are contoured at 1.2σ, where σ represents the root mean square of the density fluctuation of the unit cell. The model structures are shown as sticks. Carbon atoms are coloured green, yellow and purple for F. pringlei PEPC, F. trinervia PEPC and the inhibitor Asp, respectively. Nitrogen atoms are coloured blue, oxygens red and sulphurs yellow.
is monitored via the reduction of oxaloacetate to malate by malate dehydrogenase. The reaction velocity is measured as a decrease in OD340 resulting from the oxidation of NADH in this reaction. The inhibition of the PEPC by malate was measured in the presence of PEP (2 \times \text{K}_m), at malate concentrations from 0–20 mM. The half maximal inhibitory concentrations (IC50) of wild-type and mutant enzymes were calculated from the experimental data using the Grafit software package (Erithacus Software).

Figure 4 | Side by side comparison of the feedback inhibitor-binding site. (a) Inhibitor-binding site of C3 PEPC (F. pringlei). (b) Inhibitor-binding site of C4 PEPC (F. trinervia). The distances between the inhibitor aspartate and amino acids of the binding pocket are given in Å. The estimated coordinate errors are 0.24 Å (F. pringlei C3 PEPC) and 0.29 Å (F. trinervia C4 PEPC). Residues Arg641, Lys829, Arg888 and Asn964 have been identified as the malate-binding motif15. In C3 PEPC, Arg884 provides an additional hydrogen bond for inhibitor binding. In C4 PEPC, this residue is replaced by glycine, which is 6.9 Å away from the inhibitor molecule.

Figure 5 | Determination of IC50 values for wild-type and mutant PEPC from F. pringlei (C3) and F. trinervia (C4). (a) The mean activity values of F. pringlei wild-type C3 PEPC (open circles) and F. pringlei R884G mutant (solid circles) are plotted against the concentration of the feedback inhibitor malate. The calculated IC50 values are 2.9 mM for the wild-type (solid curve) and 8.7 mM for the R884G mutant C3 PEPC (dashed curve). (b) Plot of mean activity values of F. trinervia wild-type C4 PEPC (open circles) and F. trinervia G884R mutant (closed circles). IC50 values calculated from the experimental data are 8.4 mM for wild-type and 2.8 mM for the mutant C4 PEPC. S.d. was calculated from three independent experiments. Activities were measured in triplicates for each experiment.

Figure 6 | Sequence alignment of C3 and C4 determining regions in various PEPCs. Amino acids that determine C3/C4-specific function are highlighted. In the substrate-binding centre, Ala774 (Flaveria numbering) mediates C3 specificity, while Ser774 determines the increased kinetic efficiency of C4 PEPC (black boxes). Malate binding in the inhibitory site is controlled by Arg884 in the C3 enzyme, while the increased tolerance of the C4 PEPC towards the feedback inhibitor is mediated by Gly884 (grey boxes). The sequences are: Ft (F. trinervia UniProt No. P30694), So (Saccharum officinarum Q9FS96), Sb (Sorghum bicolor P15804), Zm (Zea mays P04711), Fp (F. pringlei GenBank CAA88829.1), Os (Oryza sativa subsp. indica Q84XHO), Nt (Nicotiana tabacum P27154) and At (Arabidopsis thaliana Q84VW9).
Table 2 | Constructs in pETEV16b.

| Construct | Source | Modification                  |
|-----------|--------|-------------------------------|
| C3        | F. pringlei | —                            |
| C3(R884G) | F. pringlei | Point mutation GGA to AGA on aa position 884 |
| C3truncated | F. pringlei | Deletion of TEV site and linker region, deletion of the first five aa in the coding region |
| C4        | F. trinervia | —                            |
| C4(G884R) | F. trinervia | Point mutation AGA to GGA on aa position 884 |

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Author contributions

J.K.P. expressed, purified and crystallized PEPC from F. trinervia and F. pringlei, collected X-ray diffraction data, built the structure, performed mutagenesis and enzyme kinetics studies, and wrote the manuscript. D.S. collected X-ray diffraction data, solved and refined the crystal structures, and wrote the manuscript. G.G. designed and led the project, analysed data, designed mutants and wrote the manuscript.

Additional information

Accession codes: Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 3zgb (F. pringlei) and 3ggg (F. trinervia).

Competing financial interests: The authors declare competing financial interests: J.K.P., D.S. and G.G. have a patent pending based on the work reported in this paper (EP 11 195 367.5).

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