Serine 774 and amino acids 296 to 437 comprise the major C4 determinants of the C4 phospho*enol*pyruvate carboxylase of *Flaveria trinervia*

Sascha Engelmann[a], Oliver E. Bläsing[a,1], Peter Westhoff[a], Per Svensson[b,*]

[a] *Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany*
[b] *Department of Plant Biology, The Swedish University of Agricultural Sciences, P.O. Box 7080, SE-750 07 Uppsala, Sweden*

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Abstract C4 phospho*enol*pyruvate carboxylases have evolved several times independently from non-photosynthetic C3 ancestral enzymes. To identify C4-specific determinants at the amino acid level the two orthologous *ppcA* PEPCases of *Flaveria trinervia* (C4) and *Flaveria pringlei* (C3) were used as a model system. In a previous publication [Bläsing et al., J. Biol. Chem. 275 (2000) 27917–27923] it was reported that the serine at position 774 is an invariant residue in all C4 phospho*enol*pyruvate carboxylases. Here we show by swapping experiments and site-directed mutagenesis that the serine 774 and amino acids 296–437 explain two thirds of the C4 characteristic phospho*enol*pyruvate saturation kinetics when investigated in the C3 background. In addition, the results indicate that the determinants functionally interact with each other.

Key words: Phospho*enol*pyruvate carboxylase; Evolution; C4 photosynthesis; *Flaveria*

1. Introduction

Phospho*enol*pyruvate carboxylase (PEPCase, EC 4.1.1.3) is a key enzyme of the C4 photosynthetic pathway [2]. This extra metabolic cycle efficiently pumps CO_2 to the site of Rubisco thereby practically eliminating photorespiration in these plants. In addition, PEPCase is essential for anaplerosis and regulation of opening of the stomata in both C3 and C4 species as well as for several other important functions in basic plant metabolism [3–5]. The PEPCase involved in the C4 cycle has distinct kinetic and regulatory properties compared to the other isoforms suggesting that they may be encoded by specific genes. And, in fact, in all plants studied so far a small gene family encodes PEPCases [5]. It is therefore generally accepted that C4 PEPCase has evolved from ancestral non-photosynthetic enzymes (C3 enzymes) in parallel to the overall evolution of C4 photosynthesis [6–8].

We are interested in the molecular basis of the specific properties of the C4 enzyme and the evolutionary steps that the enzyme went through on its way from the ancestral C3 isoform into a full-fledged C4 PEPCase. For this purpose we use the genus *Flaveria* (Asteraceae) as our model system [9]. This dicot genus contains C3 and C4 plants as well as several species of C3/C4 intermediate characteristics indicating that the evolution towards C4 photosynthesis is still proceeding in *Flaveria* [10–12].

Earlier investigations have shown that the C4 PEPCase of *Flaveria trinervia* (gene designation *ppcA*) displays an about 10 times higher $K_{0.5}$-phospho*enol*pyruvate (PEP) value than its orthologue in the C3 plant *Flaveria pringlei*. In addition, the activation by glucose 6-phosphate (Glc6P) is significantly higher for the C4 PEPCase compared to its C3 counterpart [1,13]. Despite their clear-cut differences in these enzymatic properties the two *ppcA* proteins are 96% identical. These results prompted us to map determinants for C4 characteristics within the primary structure of the *ppcA* PEPCases by constructing and analyzing reciprocal enzyme chimeras. The results obtained suggested that the major determinants were located in two regions, i.e. the so-called regions 2 (amino acids 296–437) and 5 (positions 645–966) [1]. By site-directed mutagenesis the major C4 determinant of region 5 could be confined to the serine at position 774. A serine residue at this position is characteristic for all C4 PEPCases regardless of their taxonomic origin [1]. In contrast, all C3 and CAM PEPCases contain an alanine at that position demonstrating that this position is of prime importance for the C3–C4 evolution.

In the present paper we extend the investigation of C4 determinants by examining the influence of region 2 and the corresponding amino acid residue 774 in the background of the other *ppcA* enzyme. We found that position 774 and region 2 explain two thirds of the differences in the PEP saturation kinetics ($K_{0.5}$-PEP) of the activated and non-activated enzyme. It follows that the two regions harbor the most important determinants for the evolution of a C4-type $K_{0.5}$-PEP.

2. Materials and methods

The chimeric enzymes were constructed using standard biological cloning procedures [14] as described elsewhere [1]. The enzymes were purified and analyzed as reported previously [1].

*Corresponding author. Fax: (46)-18-673279.
E-mail address: per.svensson@vbiol.slu.se (P. Svensson).

[1] Present address: Max Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Golm, Germany.

Abbreviations: PEPCase, phospho*enol*pyruvate carboxylase; Glc6P, glucose 6-phosphate; *ppc*, phospho*enol*pyruvate carboxylase gene

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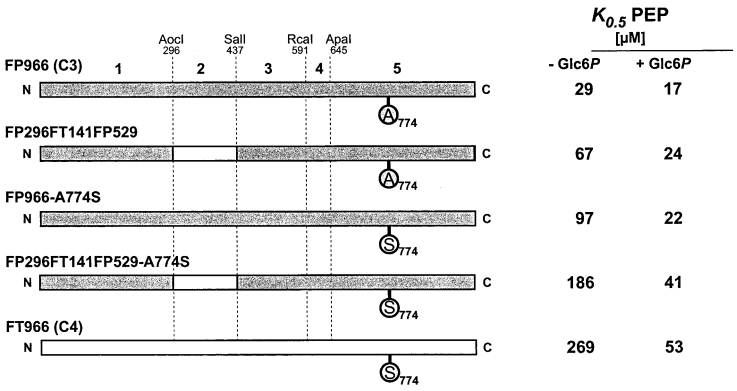

Fig. 1. Converting a C3 enzyme into a C4-like enzyme. The sequences that originate from the C3 enzyme are shown in gray while white regions represent the C4 enzyme. The numbers at the top designate regions of the *ppcA* PEPCases as divided by common restriction sites (also shown above). The amino acid residue at position 774 is highlighted.

3. Results

3.1. The experimental strategy

We wanted to analyze the influence of C4-type region 2 and the presence of serine at position 774 on PEP saturation kinetics in the context of an otherwise C3 *ppcA* PEPCase. To accomplish this a swapping strategy was adopted as illustrated in Fig. 1. Region 2 (amino acids 296–437) of the *F. pringlei* (C3) *ppcA* enzyme (FP966) was interchanged for the corresponding region of the *F. trinervia* (C4) *ppcA* PEPCase (FT966) creating the chimeric enzyme FP296FT141FP529. In a second experiment the C3 conserved Ala774 of FP966 was substituted for the C4 invariant serine (FP966-A774S). Finally, we performed the double swap where both region 2 and position 774 of the C3 enzyme were interchanged for their corresponding C4 counterparts (FP296FT141FP529-A774S).

In a complementary series of swapping experiments the C4 enzyme was interchanged with C3 sequences (Fig. 2). The chimeric enzymes created, as well as the original C4 and C3 PEPCases, were investigated by PEP saturation kinetics in the presence and absence of the activator Glc6P. The changes in the $K_{0.5}$-PEP as a result of interchanging enzyme segments are presented in the following as the percentage of the difference between the C3 and C4 values.

3.2. Converting a C3 PEPCase into a C4-like enzyme

A swap of region 2 from C3 to C4 sequence led to a minor but significant rise in the $K_{0.5}$-PEP both in its non-activated state and when activated with 5 mM Glc6P. The non-activated chimeric enzyme increased by 15% (67 μM) of the difference between the C3 and C4 values while the activated enzyme with 24 μM went 20% in the direction towards the C4 value (Fig. 1). As shown before [1] the alanine/serine substitution at position 774 shows a similar pattern. The $K_{0.5}$-PEP increased by 30% of the difference between the enzymes, to 97 μM for the non-activated enzyme, while the Glc6P-activated chimeric PEPCase only increased by 15% due to this amino acid exchange (compare FP966-A774S with FP966 in Fig. 1). It follows from these two single changes of C4 sequences for their C3 counterparts that neither of them alone gives rise to values at, or even close to, the C4 enzyme. The resulting chimeric enzyme is still C3-like. In contrast, the double swap, where both region 2 and position 774 were changed from C3 to C4 sequences (FP296FT141FP529-A774S), had a dramatic impact on PEP saturation kinetics. The $K_{0.5}$-PEP increased by 70% for both the activated and the non-activated chimeric enzyme, thereby displaying characteristics of a C4-like enzyme.

3.3. Converting a C4 PEPCase into a C3-like enzyme

The importance of these two regions was confirmed by the reciprocal experiment. When region 2 of the C4 enzyme was replaced by its C3 counterpart the resulting chimeric enzyme FT296FP141FT529 showed a substantial shift in $K_{0.5}$-PEP. This was especially obvious for the Glc6P-activated enzyme where the $K_{0.5}$-PEP decreased by about 80% of the difference

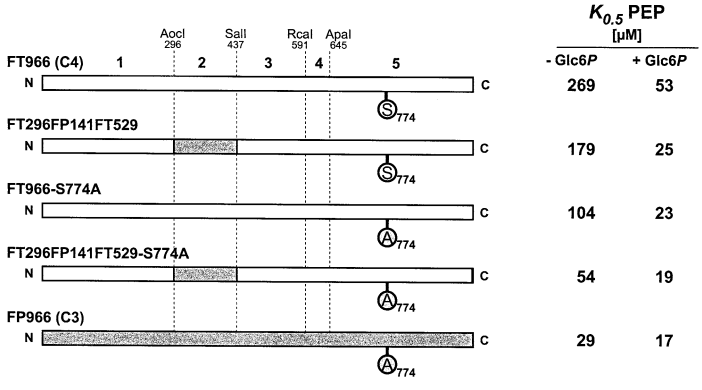

Fig. 2. Converting a C4 PEPCase into a C3-like enzyme. See legend of Fig. 1 for explanations.

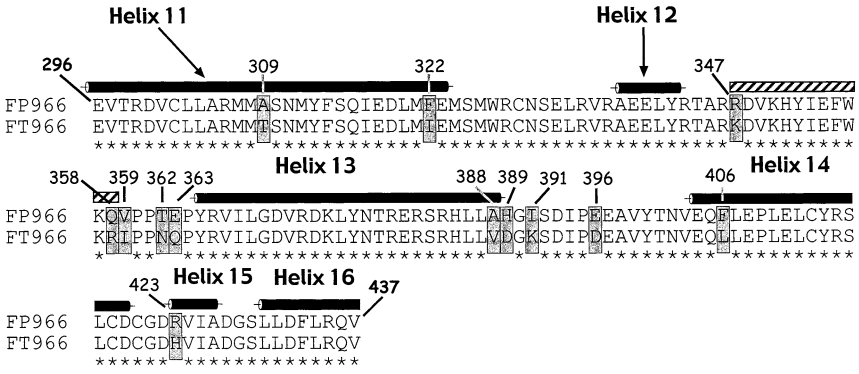

Fig. 3. The amino acid sequences of positions 296–437 (region 2) of the *ppcA* PEPCases of the C4 species *F. trinervia* (Ftr966), the C3 plant *F. pringlei* (Fbr966). Black cylinders above the sequences designate α-helices derived by comparison with the *E. coli* PEPCase. Gray boxes indicate the positions that differ between the C4 enzyme (Ftr966) and the C3 PEPCase (Fpr966). The pattern above positions 347–359 indicates a plant-specific sequence not found in the *E. coli* enzyme. Note the clustering of differing positions around helix 13.

between the two PEPCases while the non-activated chimeric enzyme showed a reduction of 40% (compare FT296FP141-FT529 with FT966 of Fig. 2). As shown before [1] the substitution of serine by alanine in the C4 enzyme (FT966-S774A) gave rise to a dramatic decrease in $K_{0.5}$-PEP. The activated and non-activated enzyme displayed a drop towards the C3 characteristics in this kinetic parameter of 70% and 80%, respectively. The double swap, where both region 2 and position 774 were changed from C4 to C3 sequence (FT296FP141FT529-S774A), increased the C3-like properties. The $K_{0.5}$-PEP decreased by 90% and 95% for the non-activated and activated chimeric PEPCase, respectively, thereby practically reaching the values of the C3 PEPCase.

4. Discussion

We are interested in resolving the steps at the molecular level that led to the evolution of C4 PEPCases and are using a pair of orthologous C3/C4 PEPCases as our experimental material [15]. By constructing reciprocal C3–C4 chimeric enzymes and analyzing their PEP saturation kinetics ($K_{0.5}$-PEP) we concluded that the serine at position 774 and amino acid residues in region 2 (positions 296–437) should contain important determinants for a C4-specific $K_{0.5}$-PEP [1]. In the present work, these experiments were extended by analyzing the effects of these two determinants on $K_{0.5}$-PEP separated from the influence of the other regions. A serine at position 774 in combination with region 2 of the C4 *ppcA* PEPCase was found to be responsible for two thirds of C4-specific properties with respect to PEP saturation kinetics. In other words, when these two regions of the C3 *ppcA* PEPCase were replaced by their C4 equivalents a C4-like enzyme was created. We conclude that these two regions must have been of prime importance for the evolution of a C4 PEPCase.

It is interesting to note that the swapping of either region 2 or position 774 from the C4 to the C3 type has a substantially larger effect than when performing the opposite experiment. The difference is especially evident when comparing the results from the activated enzymes (Figs. 1 and 2). Here the C3 to C4 substitutions increase the $K_{0.5}$-PEP by 15% and 20%, respectively, while either interchange in the C4 to C3 direction leads to a decrease by 80% of this parameter. This means that either region has little effect in gaining C4 properties but a strong effect in losing C4 properties for the kinetic parameter investigated. One explanation for this behavior would be some kind of interaction between region 2 and position 774. These results fit well with earlier observations that the C4 PEPCase of *F. trinervia* shows a cooperative behavior [1,13].

Which amino acid residues are responsible for the C4 characteristics harbored in region 2? Among the 141 amino acids of this segment 13 differ between the C3 and C4 enzymes (boxed in Fig. 3). They are not evenly distributed but eight of them between residues 358 and 396 show a distinct grouping. The differing positions are clustered in two groups of four amino acid residues each, with a stretch of 24 identical amino acids in between. Unfortunately there is no three-dimensional structure available for any plant PEPCase. However, the structure of the *Escherichia coli* PEPCase [16] is claimed to be so similar to the plant enzyme that it can be used as a reference. This would mean that the two differing clusters of amino acid residues in region 2 surround helix 13. In addition, a segment of 12 amino acids upstream of the clusters is missing in the bacterial enzyme and may be considered plant-specific. Amino acid 347 in this segment shows an additional differing position.

However, it is hard to decide at the present stage of investigation which of the differing positions in region 2 reflect key changes and which ones are only modifications of minor importance. Nevertheless, a more comprehensive survey of C3–C4 intermediate PEPCases in *Flaveria* combined with site-directed mutagenesis should allow identifying the prime C4 determinants in this domain of C4 PEPCases.

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