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Review Letter

Higher plant phospho*enol*pyruvate carboxylase

Structure and regulation

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1. INTRODUCTION

Phospho*enol*pyruvate (PEP) carboxylase is a key enzyme of photosynthesis in those plant species exhibiting the C_4 or CAM pathway for CO_2 fixation. It catalyses the β-carboxylation of PEP to yield oxaloacetate and P_i. The enzyme is present in plants, algae and bacteria. Its properties are very different depending on the source. In plants it has been described as a cytoplasmic enzyme with a variety of functions ranging from photosynthetic CO_2 fixation to nitrogen assimilation [1,2]. After an earlier report by Bandurski and Greiner [3] on the presence of PEP carboxylase in spinach, the enzyme from several sources has been studied and reviewed [1,4]. Moreover, a whole issue of *Physiologie Végétale* (vol.21, no.5, 1983) was devoted to the carboxylase.

A revision seems worthwhile, since in the last 3 years a lot of new information has been obtained including the elucidation of the primary structure of the enzyme from maize, *Anacystis nidulans* and *Escherichia coli* [5–11], studies on the biosynthesis of this protein [12–14], studies on the structure of the catalytic site using chemical modifiers [15–22] and substrate analogues [23–26], the confirmation of one of the two proposed reaction mechanisms [27,28], biophysical studies on the arrangement of its subunits [22], new studies on its quaternary structure [29,30], regulation by light of the enzyme from C_4 [31–33] and CAM plants [29,34,35] and regulation by metabolites and pH [36,37]. The purpose of this review is to summarize and discuss these recent reports on the properties and factors governing the regulation of plant PEP carboxylase.

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2. PHYSIOLOGICAL ROLE

Among the various functions of PEP carboxylase perhaps the most important is photosynthetic CO_2 fixation in C_4 and CAM plants. In C_4 plants, the enzyme is located in mesophyll cells of leaves and it catalyses the first step of a metabolic route known as the C_4 dicarboxylic acid pathway [38]. The existence of this metabolism minimizes loss of energy produced by photorespiration and explains the higher growth rates observed in C_4 plants at higher temperatures, illumination and O_2 levels as compared to C_3 plants [38].

The levels of mesophyll cell mRNA [39] and enzyme synthesis largely increase during leaf development and greening of etiolated leaves

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[12,40,41]. The biosynthesis of the carboxylase is controlled by phytochrome [42]. Furthermore, the cloning and characterization of mRNAs from different isozymic forms of the maize enzyme show that PEP carboxylase is encoded by a small gene family [13,14]. The leaf photosynthetic form is rather different in its amino acid sequence from the root form [13,14].

In CAM plants the function of PEP carboxylase is similar to that of C_4 plants, although primary CO_2 fixation by this enzyme occurs during the night, thereby accumulating malate [43]. This mechanism minimizes the losses of water in desert plants, where the stomata open only during the night [43]. The levels of PEP carboxylase are increased and the molecular and kinetic properties of the enzyme change upon induction of CAM [44–46].

Several further functions have been described for the enzyme from C_3 plants. These are, as listed by Latzko and Kelly [2]: (i) replenishment of tricarboxylic acid cycle intermediates; (ii) NADPH generation; (iii) recapture of respired CO_2; (iv) 'malate fermentation'; (v) nitrogen assimilation and amino acid synthesis; (vi) pH maintenance; (vii) maintenance of electroneutrality. These functions should also be considered important for the non-photosynthetic forms of the ezyme in C_4 and CAM plants.

Recently, some reports on the location [47] and molecular [48] and kinetic [49] properties of PEP carboxylase from species exhibiting characteristics intermediate to those of C_3 and C_4 plants (called C_3-C_4 intermediate plants) have appeared. Further studies in this field will help us to understand the development and evolution of the C_4 pathway.

3. KINETICS AND REACTION MECHANISM

PEP carboxylase (orthophosphate:oxaloacetate carboxylyase (phosphorylating); EC 4.1.1.31) catalyses the following reaction:

$$\text{PEP} + HCO_3^- \xrightarrow{Mg^{2+}} \text{oxaloacetate} + P_i$$

This process is highly exergonic and the reverse reaction has not been measured [1,4]. The enzyme has an absolute requirement for a divalent cation, with Mg^{2+} probably filling this requirement in vivo [1,4]. In vitro, Mn^{2+} can replace Mg^{2+} as a cofactor [50,51], while Co^{2+} is less effective as an activator [51]. Other cations such as Zn^{2+}, Hg^{2+} [51], Ca^{2+} [52], Cu^{2+} and Cd^{2+} [53] inhibit the enzyme.

The role of Mg^{2+} in catalysis is still not clear. Maruyama et al. [54] and Miziorko et al. [50] have suggested that an enzyme-metal-PEP bridge complex is formed at the active site of the carboxylase. However, from kinetic studies it has been deduced that the active substrate is the free form of PEP rather than the metal-PEP complex [50,51]. Chemical modification studies with the maize leaf enzyme indicated that Mg^{2+} is not essential for the binding of PEP to the carboxylase, although its presence increases the affinity for this substrate [20,36], probably by inducing conformational changes in the enzyme [22].

The detailed kinetic mechanism of PEP carboxylase from any plant source is unknown. A study concerning the enzyme from *Brevibacterium flavum*, reported that the mechanism is a rapid equilibrium random Bi Bi with a dead end complex enzyme-bicarbonate-P_i [55]. In view of the large differences in kinetic properties between bacterial and plant PEP carboxylase, a detailed study with the plant enzyme is required.

The current experimental evidence indicates that the carboxylation of PEP by PEP carboxylase occurs by a two-step mechanism as proposed by Walsh [56]. This evidence came first from isotope effect studies by O'Leary et al. [51] and was confirmed by studying the configuration of the chiral thiophosphate obtained when (S,^{16}O,^{17}O)thiophospho*enol*pyruvate was used as substrate in $H_2{}^{18}O$ [27]. The first step of the reaction would be the formation of carboxy phosphate and the enolate of pyruvate from the substrates. This step would be reversible and rate-limiting [51]. The second step would be the carboxylation of the enolate with the formation of products. This step is rate-limiting under some conditions [51].

More recently, it has been demonstrated that the carboxylase from *E. coli* catalyses the dephosphorylation of the analogue phosphoenol-α-ketobutyrate, and that bicarbonate, although essential for the reaction, is not incorporated into the reaction products [28]. In view of the proposed stepwise reaction mechanism, it was assumed that bicarbonate is necessary for the carboxy phosphate and enolate formation (first step) while the second

step (carboxylation of enolate) does not take place. We have obtained similar results with the enzyme from maize leaves together with evidence indicating that the rate-limiting step would be the same in the hydrolysis of phosphoenol-α-ketobutyrate and in the carboxylation of PEP [57]. We have also observed that the carboxylase can catalyse carbamyl phosphate hydrolysis, probably by a similar mechanism [58].

4. STRUCTURE

4.1. *Primary structure*

The primary structures of PEP carboxylase from *E. coli* [5], *Anabaena variabilis* [9] and maize leaves [11] have been recently deduced by a Japanese group from sequence analysis of cDNA. The alignment of the amino acid sequences shows that the enzyme molecule contains a conserved C-terminal half and a variable N-terminal one [10,11]. The DNA sequence of the carboxylase from maize, which encodes 935 amino acid residues, reveals a 43% homology with that of *E. coli* enzyme and a 33% homology with that of the cyanobacterial carboxylase [11].

While the kinetic and regulatory properties of PEP carboxylase from bacteria and higher plants are rather different [4], little is known about the cyanobacterial enzyme. The homology found in the C-terminal half suggests that the catalytic domain may be located in this region. Furthermore, the sequence between residues 603 and 616 of the *Zea mays* enzyme (FHGRGGSIGRGGAP) is highly conserved in the three proteins and seems to be unique for PEP carboxylase [10,11]. It has been proposed that this sequence may be involved in the binding of PEP [10].

Since the substrates of the carboxylase are anions at neutral pH, positively charged groups of the enzyme are likely to be involved in their binding. Chemical modification studies with the enzyme from maize using phenylglyoxal [20], eosin isothiocyanate [22] and pyridoxal 5′-phosphate [21] have shown that two arginine and four lysine residues per tetrameric enzyme molecule are essential for activity and that these groups are protected by PEP. Three lysines and seventeen arginines are conserved in the amino acid sequences of the carboxylase from different sources [11]. Two arginines are located in the sequence described above as unique for PEP carboxylase [10,11].

Kinetic studies at different pH have also indicated the importance of two groups with pK_a of about 7.3 for the binding of PEP and/or Mg^{2+} to the maize enzyme [18,51]. These groups are probably histidines as inferred from modification of the enzyme with diethyl pyrocarbonate [18]. Four histidines are conserved in the three proteins, one of them in the region between residues 603 and 616 of the maize enzyme [10,11]. Photooxidation studies with the pyridoxal 5′-phosphate-derivatized enzyme have shown the existence of histidine residues in the vicinity of the essential lysines mentioned above [21].

Many authors have reported that thiol groups are also essential for activity [15–17,19,26]. Four of these groups per tetramer seem to be present at the PEP-binding sites [19] and studies using bromopyruvate as affinity label suggest that they may be located near the region that interacts with the methylene group of PEP [26]. A similar observation has been made for the *E. coli* enzyme [59].

Maize PEP carboxylase contains 32 cysteine residues [11]. The analysis of the known primary structures of the enzyme from different sources indicates that none of them is conserved [11]. Only 16 residues are labeled with *N*-ethylmaleimide after treatment with SDS or urea plus dithiothreitol [32]. This observation shows the existence of regions that are not exposed to the reagent even after the described treatments. Eight groups are accessible in the native enzyme and twelve after dithiothreitol treatment [32]. Four groups are only accessible after denaturation with SDS or urea [32]. The reduction of two disulfide bonds per tetramer seems to be involved in the activation by dithiothreitol and other thiol compounds ([32]; see section 5.1). Recently, some of these results were confirmed and it was established that the native enzyme contains 8 disulfide bonds [60].

The elucidation of the primary structures of the carboxylase from C_3, C_4 and CAM plants, including the forms from different tissues, and X-ray diffraction studies are still needed to relate structure and kinetic properties. The exact location of the amino acids that participate in catalysis and/or substrate binding will only be achieved after the isolation and sequencing of the peptides that contain the residues that are essential for activity. The identification of the regions involved in the

regulatory functions (see section 5) needs more work, including the use of reactive analogues of the modulators and, convincingly, site-directed mutagenesis of the enzyme.

4.2. *Quaternary structure*

PEP carboxylase has usually been described as a homotetramer [1,61]. However, some reports show that the carboxylase may exist in different oligomeric forms in vivo [29,30,60–64].

Jones et al. [62] reported a concentration-dependent dissociation into active dimer of PEP carboxylase purified from *Bryophyllum fedtschenkoi* (a CAM plant). Furthermore, from cross-linking experiments, these authors concluded that the dimer is also an important unit of polymerization. Recently, it was observed that the carboxylase from another CAM plant (*Crassula argentea*) exists as a tetramer when purified from leaves during the night, and as a dimer during the day [29]. Although both forms show similar activity, the 'day form' is sensitive to inhibition by L-malate whereas the 'night form' is not. Both can be interconverted by incubation of the enzyme at different pH or in the presence of Mg^{2+}, PEP or malate. The physiological relevance of these observations is discussed in section 5.2.

In C_4 plants other conditions could be employed to observe dissociation. Walker et al. [30,63] reported that modification of thiol groups by *p*-chloromercuribenzoate and histidines by diethyl pyrocarbonate causes dissociation into dimers and monomers. It is of interest that the final product after incubation with the thiol reagent alone was a mixture of dimers and monomers, while modification by both reagents produced a complete conversion into monomers. These results indicate that cysteine and histidine residues are involved in the maintenance of the tetrameric structure. The enzyme modified by diethyl pyrocarbonate could be reactivated and reassociated only when it was present as a dimer/tetramer mixture, but not when it was in the monomeric form. These authors also concluded that dimers and monomers are inactive, but this assertion would only be valid for the modified carboxylase.

We have observed that incubation with NaCl also produces dissociation of the maize carboxylase in a time- and protein-concentration-dependent manner [64]. Dimers and monomers are obtained at pH 8 or above, and only dimers at pH 7. The dissociation was faster at lower pH. The changes in the quaternary structure could be correlated with a time-dependent decay in activity which was protected by substrates and by the allosteric activator glucose 6-phosphate. The different responses to NaCl observed at pH 7 and 8 suggest that histidines may be involved in the process of dissociation in agreement with the results of Walker et al. [30]. Furthermore, the NaCl-treated enzyme showed a shift in the pK_a of the histidines involved in catalysis and/or substrate binding (Wagner, R., González, D.H., Podestá, F.E. and Andreo, C.S., unpublished). The dissociation by NaCl produces active dimers and inactive monomers [64]. The enzyme can also be dissociated by incubation at pH 6 or 9 [60].

The spatial arrangement of the subunits in the tetramer has been determined by measuring the rotational diffusion of the maize leaf carboxylase labeled with eosin isothiocyanate [22]. The hydrodynamic structure of the enzyme in the absence and presence of all substrates could be described by a tetrahedral arrangement, while with bicarbonate and Mg^{2+}, or PEP and Mg^{2+}, it was preferentially described by a distorted centered triangle arrangement. It was speculated that these structures may represent conformational transitions during the catalytic cycle [22]. The assumption that the enzyme may have a square arrangement based on its number of disulfide bonds is not supported by experimental data [60].

The interaction between subunits seems to play an important role in the kinetic and regulatory properties of PEP carboxylase. Some evidence from cross-linking experiments [62], titration of sulfhydryl groups [32] and dissociation by chemical modification [30] indicates that an asymmetric arrangement of the subunits could exist under some conditions. Resonance energy transfer measurements in the enzyme modified with fluorescent reagents seem to support this hypothesis (Wagner, R., González, D.H., Podestá, F.E. and Andreo, C.S., unpublished).

4.3. *Active site*

The structure of the active site has been studied by the use of competitive inhibitors which exhibit some analogy with the substrate PEP [1,23–25,50,57,65]. The general conclusion of

these studies is that the phosphate and carboxyl groups are very important for the binding, with an increase in dissociation constants by 2 orders of magnitude when one of them is absent. The angle at C-2 (around 120°) and the C-O-P bridging oxygen are also important [24] as well as any substitution at the methylene group (C-3). For example, the substitution of a hydrogen of this group by one bromine [23] or one methyl group [57] produces a decrease by 1 order of magnitude in the dissociation constant. However, the replacement of both hydrogens by methyl groups produces a decrease in the affinity [57], indicating the existence of steric hindrance at one of the two positions. Interestingly, (*E*)-cyanophospho*enol*pyruvate (which contains a cyano group *trans* to the phosphate) shows a high dissociation constant (1.36 mM) in the presence of Mg^{2+} [25]. Probably substitutions at the (*E*) position are responsible for the steric hindrance and substitutions at the (*Z*) position (*cis* to the phosphate) produce the observed higher affinity. This, however, remains to be demonstrated.

Two compounds, phospho*enol*-3-bromopyruvate [23] and 3-bromopyruvate [26] were shown to be useful as affinity labels of the active site of the carboxylase from maize and could then be used to determine the amino acid sequences that are involved in the binding of the substrate. The PEP-binding domain has also been studied by chemical modification experiments using specific site-directed modifiers. The results (see section 4.1 for details) indicate the presence of histidine, cysteine, arginine and lysine residues, which are essential for the catalytic activity and are involved in the binding of PEP. These data allow us to draw a hypothetical distribution of these amino acids along the carboxylase active site (fig.1). Positively charged lysine and arginine residues are shown as interacting with the substrate phosphate and carboxylate moieties; the respective interactions were assigned arbitrarily. Imidazole groups are likely to be involved in the binding of Mg^{2+} and could also bind the phosphate moiety through electrostatic interactions between the P and N atoms. This assumption could explain the partial protection afforded by the divalent cation against inactivation by diethyl pyrocarbonate [18] and the decrease in the affinity for PEP and Mg^{2+} when histidines become protonated [18,51]. The cation has been

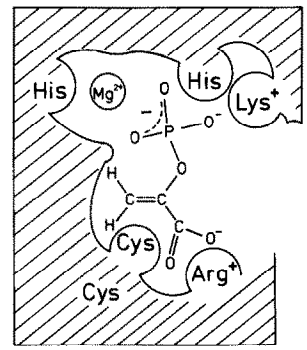

Fig.1. Schematic model for the active site of higher plant PEP carboxylase.

shown to interact with the PEP phosphate group by Miller et al. [66] and Miziorko et al. [50]. One cysteine is postulated to be located near the binding region of the methylene moiety based on data showing alkylation by the substrate analogues phospho*enol*-3-bromopyruvate [23] and bromopyruvate [26]. Since it has been demonstrated that the enzyme can be inactivated by vicinal dithiol oxidation [16,19] another proximal thiol group has been included in fig.1.

It should be noted that the scheme is largely based on results obtained by chemical modification. We consider it merely as a tentative model for future investigations using alternative methods like peptide sequencing and X-ray crystallography which will either confirm or modify this arrangement. Bicarbonate was not included in fig.1 since its binding site at the protein is unknown.

5. PHYSIOLOGICAL REGULATION

5.1. *C_4 plants*

PEP carboxylase from C_4 plants is activated by glucose 6-phosphate, a final product of CO_2 fixation [67]. This compound produces an increase in V_m and in the affinity for the binding of PEP [36,67]. Another metabolite, L-malate, which is an immediate product of the carboxylation reaction, is an inhibitor of the carboxylase [68]. It shows a cooperative effect and seems to interact with PEP carboxylase at different sites, producing non-competitive or competitive inhibition depending on pH and concentration [36]. Glucose 6-phosphate produces a decrease in the inhibitory effect of malate [36,68]. Aspartate, another impor-

tant product of the carboxylation in some C_4 plants, also inhibits the enzyme [68,69].

pH variations could also operate as a fine control of the carboxylase activity. The enzyme affinity for PEP and Mg^{2+} increases sharply between pH 7 and 8 [18,51]. The effects of glucose 6-phosphate and L-malate are more pronounced at pH 7, decreasing with increasing pH [36]. It is of interest that malate, which accumulates after the carboxylation reaction, tends to decrease pH, thereby decreasing the activity of the carboxylase by two different mechanisms: inhibition by malate itself and enhancement of its inhibitory effect by decrease in pH. For this hypothesis to be valid, the actual pH in the cytoplasm of the mesophyll cells should be around 7–7.5. Values of pH 7.1–7.25 have been measured in the cytoplasm in maize root tips [70], but nothing is known about the leaves of C_4 plants.

Finally, the activity of PEP carboxylase is higher when extracted from leaves that have been previously illuminated [31,32]. The measured changes in the activity are larger when the enzyme is assayed at pH 7 [33] and the enzyme extracted after illumination is less sensitive to inhibition by malate and is more efficiently activated by glucose 6-phosphate [71]. Activation of the enzyme extracted from leaves maintained in the dark was also observed after incubation with dithiothreitol or other thiol reducing agents [32]. This observation shows that the activation by light would be mediated by effectors which change the redox state of the carboxylase sulfhydryl groups (see section 4.1). Since the carboxylase is a cytoplasmic enzyme it is difficult to assess how this 'redox signal' would travel from the chloroplasts. The phosphorylation of the maize enzyme recently reported by Budde and Chollet [72] probably is responsible for the observed light-dark transitions.

Dissociation of the enzyme by salt could also be involved in changes in the activity in vivo (see section 4.2). It is noteworthy that the substrates and the activator glucose 6-phosphate prevent the decay in activity that follows dissociation and that the salt effect is also affected by pH [64]. A report by Angelopoulos et al. [73] on the stabilization of PEP carboxylase from C_4 plants at high pH by non-ionic cosolvents also suggests that dissociation may occur when the enzyme is diluted at high pH. In this case, the decay in activity is protected by substrate and activators [73].

The regulation of PEP carboxylase in C_4 plants has been considered for several years to be not very important in view of the drastic changes in activity upon illumination observed with other enzymes of the C_4 pathway: pyruvate, P_i dikinase and NADP-malate dehydrogenase [74]. However, it should be kept in mind that PEP can be generated by other metabolic pathways (e.g. glycolysis). Moreover, regulation of the carboxylase activity may play an important role in controlling the flux of metabolites across the chloroplast envelope in mesophyll cells, as postulated by Stitt and Heldt [75]. The current experimental evidence indicates that although sharp changes in the activity of PEP carboxylase are not obtained as a result of the action of any isolated effector, a co-ordinated effect of several of them could keep the carboxylase activity well under suboptimal conditions. Therefore, the carboxylation of PEP should be considered as another important point for the regulation of the C_4 plants.

5.2. *CAM plants*

The fact that PEP carboxylase in CAM plants is active during the night and almost inactive during the day is generally accepted [43]. However, the mechanism of this regulation is still not clear. CAM PEP carboxylase is also activated by glucose 6-phosphate [43] and inhibited by L-malate [37,43,62,76]. The inhibition by L-malate is competitive and shows a cooperative effect [37,62].

The carboxylase extracted from night leaves has similar specific activity but is less sensitive to L-malate than the enzyme extracted from day leaves [37,76]. Recently, Wu and Wedding [29] showed that in *C. argentea* the night form is a tetramer while the day form is a dimer. Both can be interconverted by incubation with malate or substrates [29]. However, other authors did not observe different oligomeric forms as a consequence of dark/light transitions in *B. fedtschenkoi* [77] or *K. daigremontiana* [78].

Another mechanism that seems to be involved in the interconversion of the day and night forms of PEP carboxylase is phosphorylation of the enzyme. This covalent modification at serine residues during the night (but not day) was reported by Nimmo et al. [34,77]. These results were confirmed by other authors [35] who also showed that the

kinetic properties of the day form could be obtained after incubation of the phosphorylated night form with an unspecific acid phosphatase. The signal(s) and enzymes (kinases/phosphatases) that are involved in this process in vivo remain elusive and their study will probably help to clarify the circadian rhythms inherent to CAM.

6. CONCLUDING REMARKS

Excellent progress has been achieved in the last years in understanding the structure, mechanism and regulation of this carboxylase. Data are now available on the primary structure of the enzyme from different sources including the C_4 plant *Z. mays*. Chemical modification studies have provided information about the structure of the active site as well as on the arrangement of the carboxylase subunits. Multiple regulatory mechanisms of PEP carboxylase activity reported recently (particularly those involving post-translational modification) could be important for the control of photosynthetic carbon assimilation. Further experimental work is needed in order to achieve a complete correlation between structure and function of this enzyme. These studies will improve the understanding of relevant steps in the photosynthetic process in higher plants.

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