Carboxylation and dephosphorylation of phosphoenol-3-fluoropyruvate by maize leaf phosphoenolpyruvate carboxylase

Daniel H. GONZALEZ and Carlos S. ANDREO*

Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Suipacha 531, 2000 Rosario, Argentina

The analogue (Z)-phosphoenol-3-fluoropyruvate [(Z)-3-fluoro-2-(phosphono-oxy)propenoic acid] was tested as substrate of maize leaf phosphoenolpyruvate carboxylase. Studies with $\text{NaH}^{14}\text{CO}_3$ indicate that the analogue is carboxylated by the enzyme. However, this reaction accounts for only one-tenth of the activity measured by P_i liberation. The rest of the analogue is merely dephosphorylated. This is the first analogue for which both carboxylation and dephosphorylation have been observed.

INTRODUCTION

Phosphoenolpyruvate (Pyr-*P*) carboxylase catalyses the reaction

This enzyme is present in plants, algae and bacteria, but its properties vary widely with the source (for reviews see Utter & Kolenbrander, 1972; O'Leary, 1982; Andreo *et al.*, 1987). Pyr-*P* carboxylase is specially important in C_4 plants, where it catalyses the initial reaction of photosynthetic carbon fixation (Hatch & Slack, 1966; Edwards *et al.*, 1985).

The carboxylase uses a bivalent cation, preferentially Mg^{2+} or Mn^{2+} , as cofactor (O'Leary *et al.*, 1981a). The mechanism for the reaction seems to be a two-step process that involves the formation of carboxy phosphate and the enolic form of pyruvate as the first step, and carboxylation of this form as the second one (O'Leary *et al.*, 1981a; Hansen & Knowles, 1982).

Studies with Pyr-*P* analogues have provided important information on the structure of the active site and the reaction mechanism of the carboxylase (O'Leary *et al.*, 1981b; Fujita *et al.*, 1984; Wirsching & O'Leary, 1985, 1988a,b; González & Andreo, 1986, 1988a; Jenkins *et al.*, 1987; Díaz *et al.*, 1988; Sikkema & O'Leary, 1988). One of them, 2-phosphoenolbutyrate, which contains an additional methyl group at C-3, is a substrate for the carboxylase; however, it is dephosphorylated rather than carboxylated by the enzyme (Fujita *et al.*, 1984; González & Andreo, 1986, 1988a,b). This reaction requires bicarbonate (Fujita *et al.*, 1984; González & Andreo, 1986) and the actual product is the enolic form of 2-oxobutyrate (González & Andreo, 1988a). Hence it is very likely that the dephosphorylation reaction is analogous to the first step of the carboxylation of Pyr-*P*, and that the second step is precluded by the existence of steric hindrance due to the additional methyl group (Fujita *et al.*, 1984; González & Andreo, 1986, 1988a,b). It has been shown that the analogues 1-carboxyallanyl phosphate (Wirsching & O'Leary, 1988b), phosphoenolthiopyruvate (Sikkema & O'Leary, 1988) and phosphoenol-3-bromopyruvate (Díaz *et al.*, 1988) are also dephosphorylated by the carboxylase. Carbamoyl

phosphate is also hydrolysed, presumably by a similar mechanism (González *et al.*, 1987).

One of the most commonly used analogues of Pyr-*P* is phosphoenol-3-fluoropyruvate (F-Pyr-*P*). It has been described as a good substrate for pyruvate kinase (Stubbe & Kenyon, 1972; Blumberg & Stubbe, 1975; Duffy & Nowak, 1984; Hoving *et al.*, 1985), enolase (Stubbe & Kenyon, 1972; Duffy & Nowak, 1984), Pyr-*P* carboxykinase (Duffy & Nowak, 1984; Hwang & Nowak, 1986), pyruvate,phosphate dikinase (Duffy & Nowak, 1984) and enzyme I of the sugar phosphotransferase system of *Escherichia coli* (Hoving *et al.*, 1985). Since fluoride is almost isosteric to hydrogen, we decided to test F-Pyr-*P* as a substrate for Pyr-*P* carboxylase from maize leaves. Our results indicate that F-Pyr-*P* is a good substrate for the carboxylase. The analogue is both carboxylated and dephosphorylated by the enzyme, indicating the existence of partition of the intermediate between the two reactions. The results show that steric factors are important for the occurrence of the second step of the reaction and that the electronic distribution around C-3 also plays a key role in the carboxylation of the intermediate.

MATERIALS AND METHODS

Enzyme purification

Pyr-*P* carboxylase was extracted and purified from *Zea mays* L. leaves as described by Iglesias *et al.* (1986). The purified enzyme showed a single protein band in SDS/polyacrylamide-gel electrophoresis and did not hydrolyse β -glycerophosphate, indicating that it was free from unspecific phosphatase activity. Specific activity with Pyr-*P* as substrate was 12–15 $\mu\text{mol}/\text{min}$ per mg in the absence of thiol compounds (see Iglesias & Andreo, 1984).

Assays of enzyme activity

Pyr-*P* carboxylase activity with Pyr-*P* as substrate was determined spectrophotometrically (by monitoring NADH oxidation at 340 nm) at 30 °C by coupling the Pyr-*P* carboxylase reaction with that of malate dehydrogenase. The standard assay medium contained 50 mM-Tris/HCl buffer, pH 8, 5 mM-MgCl₂, 0.15 mM-NADH,

Abbreviations used: Pyr-*P*, phosphoenolpyruvate; F-Pyr-*P*, phosphoenol-3-fluoropyruvate [(Z)-3-fluoro-2-(phosphono-oxy)propenoic acid].

* To whom correspondence and reprint requests should be addressed.

10 mM- NaHCO_3 , 5 units of malate dehydrogenase, 14 mM-Pyr-*P* and Pyr-*P* carboxylase (2–4 μg of protein) in a total volume of 1 ml. The production of 3-fluoropyruvate from F-Pyr-*P* was monitored similarly except that 50 units of lactate dehydrogenase replaced malate dehydrogenase, F-Pyr-*P* replaced Pyr-*P* and 20–40 μg of protein were used.

The liberation of P_i from Pyr-*P* or F-Pyr-*P* was measured in 0.4 ml samples containing Tris/HCl buffer, pH 8, MgCl_2 and NaHCO_3 (at the same concentrations as described above), plus Pyr-*P* or F-Pyr-*P* and Pyr-*P* carboxylase. The reaction mixture was incubated at 30 °C for different times, and then stopped by addition of 0.4 ml of 10% (w/v) trichloroacetic acid. The samples were immediately transferred to an ice bath and centrifuged for 1 min in an Eppendorf micro-centrifuge. After centrifugation, 0.7 ml samples were taken and the P_i in them was determined by the method of Taussky & Shorr (1953). Controls were made from which the enzyme was omitted; the values of these controls were subtracted from the P_i liberated in the presence of enzyme.

The incorporation of ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into the reaction products was measured in 1 ml of a similar assay medium, except that $\text{NaH}^{14}\text{CO}_3$ (sp. radioactivity 0.4 mCi/mmol) was used. The label in the oxaloacetate or fluoro-oxaloacetate produced was trapped in two ways: (i) the reaction was stopped with 2,4-dinitrophenylhydrazine (1 mM in 0.1 M-HCl); (ii) 1 mM-NADH and 50 units of malate dehydrogenase were included in the assay medium, and the reaction was stopped with trichloroacetic acid. The samples were then incubated overnight to remove the $^{14}\text{CO}_2$ and then counted for radioactivity in a Beckman LS 8100 liquid-scintillation counter with a mixture of 0.25% 2,5-diphenyloxazole and 10% naphthalene in 1,4-dioxan as scintillation cocktail.

Paper chromatography

The 2,4-dinitrophenylhydrazones of the reaction products were chromatographed together with standards on Whatman no. 1 paper previously immersed in 0.1 M- NaHCO_3 and dried. As the solvent system butanol/ethanol/0.1 M- NaHCO_3 (10:3:10, by vol.) was used (Ariga, 1972).

H.p.l.c.

The products of the carboxylation reaction in the coupled assay system were chromatographed in a Waters Associates h.p.l.c. system, consisting of an M-6000 A chromatography pump and an U6K model valve loop injector coupled to a Partisil 10 SAX (strong-anion-exchange) column (250 mm $\times$ 5 mm; Kontron Analytical). Elution of the products was monitored continuously at 214 nm with a Gilson Holochrome spectrophotometer. Equilibration and elution were performed at 2 ml/min.

Decarboxylation of the products by NADPH-dependent malic enzyme

The products of the reaction in the coupled assay system with either Pyr-*P* or F-Pyr-*P* and $\text{NaH}^{14}\text{CO}_3$ as substrates were incubated (after adjusting the pH of the sample to 8 with KOH) at 30 °C with NADPH-dependent malic enzyme purified from maize leaves in 50 mM-Tris/HCl buffer, pH 8, 1 mM-EDTA, 0.4 mM-NADP $^+$ and 10 mM- MgCl_2 . After 3 h, the reaction was stopped with trichloroacetic acid and radioactivity was measured after

removing $^{14}\text{CO}_2$ overnight. A control in which the enzyme was replaced by water was run in parallel.

Protein concentration

Protein concentration was determined by the colorimetric method of Lowry *et al.* (1951), or by the Coomassie Brilliant Blue dye-binding method (Sedmak & Grossberg, 1977), with bovine serum albumin as standard.

Materials

Z-F-Pyr-*P* was kindly given by Professor Thomas Nowak, Notre Dame University, Notre Dame, IN, U.S.A., who synthesized and characterized it as described by Duffy & Nowak (1984). Pyr-*P* (monocyclohexylammonium salt), NADH, 3-fluoropyruvate, pig heart malate dehydrogenase and rabbit muscle lactate dehydrogenase were purchased from Sigma Chemical Co. $\text{NaH}^{14}\text{CO}_3$ was from Comisión Nacional de Energía Atómica (Buenos Aires, Argentina). All other reagents were of analytical grade.

Stock solutions of F-Pyr-*P* were prepared each day, and their concentration was determined enzymically with Pyr-*P* carboxylase, by using malate dehydrogenase and lactate dehydrogenase as coupling enzymes and measuring total NADH oxidation when F-Pyr-*P* was the limiting factor. Solutions used for h.p.l.c. experiments were previously filtered through Millipore HA 0.45 μm -pore-size filters and degassed under vacuum.

RESULTS AND DISCUSSION

Z-Phosphenol-3-fluoropyruvate is a good substrate for Pyr-*P* carboxylase from maize. Fig. 1 shows the release of P_i from this analogue by the enzyme action. At 0.3 mM-F-Pyr-*P* the specific activity, determined from the linear portion of this curve, was 1.17 $\mu\text{mol}/\text{min}$ per mg, approximately one-tenth of the activity observed with Pyr-*P*.

To determine whether the analogue was carboxylated to fluoro-oxaloacetate, the carboxylase was incubated with F-Pyr-*P*, Mg^{2+} and $\text{H}^{14}\text{CO}_3^-$, and the incorporation of radioactivity into the reaction products was measured. When NADH and malate dehydrogenase

Fig. 1. Liberation of P_i from F-Pyr-*P* by Pyr-*P* carboxylase action as a function of time

P_i was determined as described in the Materials and methods section; 10 μg of enzyme was used.

Fig. 2. Incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into the products of the reaction of Pyr-*P* carboxylase with F-Pyr-*P* as substrate

The carboxylase reaction with $\text{H}^{14}\text{CO}_3^-$ was coupled to that of malate dehydrogenase as described in the Materials and methods section. At different times 100 μl samples were removed, the reaction was stopped with 10 μl of 100% (w/v) trichloroacetic acid, and radioactivity was measured in the samples; 10 μg of enzyme was used.

were used to convert the labelled product, if any, into fluoromalate, ^{14}C fixation was observed (Fig. 2). Chromatography on an ion-exchange h.p.l.c. column of the reaction products showed one major peak of radioactivity, which was eluted soon after the solvent front (Fig. 3a). The peak was attributed to fluoro[^{14}C]malate, since both standard L-malate and the labelled product of an analogous reaction with Pyr-*P* as substrate were eluted in this region (Fig. 3b). A second, minor, peak of radioactivity was observed at 17 min, which may be due to the presence of some fluoro-oxaloacetate that was not already reduced when the reaction was stopped.

Incubation of the labelled product with NADPH-dependent malic enzyme produced a significant decrease in the amount of label (from 4900 to 2100 c.p.m.). This result indicates that the ^{14}C label was on C-4 of fluoro-oxaloacetate, as would be expected for the β -carboxylation of F-Pyr-*P* catalysed by Pyr-*P* carboxylase.

In a second approach, the products of the Pyr-*P* carboxylase reaction were trapped as their 2,4-dinitrophenylhydrazone derivatives. This method also showed a time-dependent incorporation of radioactivity when $\text{H}^{14}\text{CO}_3^-$ was used as substrate (Fig. 4a). Paper chromatography of the extracted yellow derivatives, followed by

Fig. 3. H.p.l.c. profiles of the products of the reaction of Pyr-*P* carboxylase with F-Pyr-*P* (a) or Pyr-*P* (b) as substrate

The reaction was performed as described in Fig. 2 legend for 10 min. After the reaction was stopped with trichloroacetic acid, a sample was injected into the h.p.l.c. system described in the Materials and methods section previously equilibrated with 10 mM- H_3PO_4 . Elution was accomplished with a 1 h linear gradient between this solution and 100 mM-potassium phosphate (pH 3); 2 ml samples were collected and radioactivity (shown by the histogram) was measured in them as described in the Materials and methods section.

Fig. 4. Radioactive 2,4-dinitrophenylhydrazones of the reaction products of Pyr-*P* carboxylase

The reaction was performed with $\text{H}^{14}\text{CO}_3^-$ as described in the Materials and methods section. At different times 100 μl samples were removed, the reaction was stopped with an equal volume of 2,4-dinitrophenylhydrazine, and radioactivity was measured in the samples. (a) Incorporation of ^{14}C as a function of time. (b) The hydrazones formed after 10 min of reaction were extracted with ethyl acetate (Kawano *et al.*, 1962), the organic solvent was removed *in vacuo*, and the remaining solid was dissolved in a minimum volume of ethyl acetate and chromatographed on paper as described in the Materials and methods section. When chromatography was completed, the paper was dried and autoradiographed with an X-ray film in the presence of an intensifying screen; lanes a and b are different amounts of the products of the reaction with F-Pyr-*P* as substrate; lanes c-e are the products when Pyr-*P* was used as substrate.

autoradiography, showed labelled spots with similar R_F values for the reactions with either Pyr-*P* or F-Pyr-*P* (Fig. 4b).

Analysis of the yellow spots in the paper indicated that, apart from the radioactive one, another hydrazone that co-migrated with the authentic dinitrophenylhydra-

Fig. 5. Absorption-spectral changes during the reaction of Pyr-*P* carboxylase with F-Pyr-*P* as substrate

Sample and reference cuvettes contained 20 mM-potassium phosphate, buffer, pH 7, 10 mM- NaHCO_3 , 100 μM -F-Pyr-*P* and enzyme in a volume of 1 ml. Reaction was started by the addition of 2.5 μl of 1 M- MgCl_2 , and the spectra were recorded every 30 s from curve b (30 s) to curve j (270 s); curve a is the baseline. The scan speed was 200 nm/min.

zone of fluoropyruvate (prepared by us from 3-fluoropyruvate and 2,4-dinitrophenylhydrazine) was present in the reaction with F-Pyr-*P* (results not shown). This observation was correlated with the fact that the specific activity measured by ^{14}C fixation was about 0.1 $\mu\text{mol}/\text{min}$ per mg, less than one-tenth of that determined by P_i liberation. The discrepancy could not be due to decarboxylation of fluoro-oxaloacetate. Efficient reduction of this keto acid by malate dehydrogenase has been previously reported (Hoving *et al.*, 1985; Hwang & Nowak, 1986). The amount of this enzyme added to the assay medium is similar to that used by those authors. So, if fluoro-oxaloacetate was the only product, most of it must have been converted into fluoromalate. Instead of this, it is likely that during the experiments some of the analogue was carboxylated and the rest was merely dephosphorylated by Pyr-*P* carboxylase.

The spectral changes in light absorption between 240 and 320 nm during the reaction of F-Pyr-*P* with Pyr-*P* carboxylase indicate the formation of a compound with an absorption maximum at around 270 nm (Fig. 5). This compound is almost probably fluoro-oxaloacetate. Together with this, a transient increase, followed by a constant decrease, in absorbance at 220 nm was observed (results not shown). As previously reported (González & Andreo, 1988a), these changes can be attributed to the release from the active site of the enolic form of the pyruvate analogue, which is then ketonized in solution.

The formation of fluoropyruvate from F-Pyr-*P* by the action of Pyr-*P* carboxylase was monitored spectrophotometrically by reduction with NADH and lactate dehydrogenase. From activity measurements at different analogue concentrations, a K_m of 14 μM and a V_{max} of 0.97 $\mu\text{mol}/\text{min}$ per mg were calculated. When tested as

Scheme 1. Carboxylation and dephosphorylation of F-Pyr-*P* by Pyr-*P* carboxylase

Carboxy phosphate and the enolate of 3-fluoropyruvate are intermediates for the two reactions. The enolate is represented as its carbanion form as postulated by O'Leary *et al.* (1981a). About one-tenth of the molecules of intermediates react to produce carboxylation. The rest are probably released from the active site, producing HCO_3^- , P_i and 3-fluoroenolpyruvate, which is ketonized in solution, as demonstrated for the analogue 2-phosphoenolbutyrate (González & Andreo, 1988a).

inhibitor, competitive inhibition with respect to Pyr-*P* was observed; K_i was 3–4 μM . Activity measurements with F-Pyr-*P* as substrate and malate dehydrogenase showed a slow decrease in absorbance at 340 nm, in accordance with the experiments reported above. Because of the low activity observed, the kinetic parameters for this reaction were not determined. The parameters determined by us are different from those reported by Díaz *et al.* (1988). We do not know the reason for this discrepancy.

Conclusive evidence indicating that F-Pyr-*P* is carboxylated and dephosphorylated by Pyr-*P* carboxylase has been presented by us in this paper. Although the characterization of the radioactive product is not complete, it is sufficient to postulate that it is 3-fluoro-oxaloacetate with a ^{14}C label at C-4. Provided that the substrate is F-Pyr-*P* and that incorporation of radioactivity from $\text{H}^{14}\text{CO}_3^-$ into a single product was observed with both methods used, it can be concluded that the radioactive compound arises from the β -carboxylation of the analogue catalysed by Pyr-*P* carboxylase. The fact that the product, after reduction with malate dehydrogenase, is decarboxylated by NADPH-dependent malic enzyme is also consistent with this conclusion.

Our results, summarized in Scheme 1, together with others obtained with a set of different analogues with substitutions at C-3 (Fujita *et al.*, 1984; González & Andreo, 1986, 1988a,b; Díaz *et al.*, 1988; Sikkema & O'Leary, 1988; Wirsching & O'Leary, 1988b), indicate that steric factors are important for the occurrence of the second step (i.e. fluorine, the smallest substituent used, is the only one that allows carboxylation of the intermediate). However, other factors, such as the electronic distribution around C-3, seem to play a key role in the attack of carboxy phosphate to produce carboxylation.

These strict requirements, which are not observed for the first step of the reaction, lead to the conclusion that the only substrate suitable for carboxylation is one that shows neither steric nor electronic constraints. This substrate is, obviously, Pyr-*P*.

While this manuscript was in preparation, we received a copy of the paper by Díaz *et al.* (1988), which reports the existence of NADH oxidation when the reaction of F-Pyr-*P* with Pyr-*P* carboxylase is coupled either to lactate dehydrogenase or to malate dehydrogenase. From these experiments the authors suggest that F-Pyr-*P* is both carboxylated and dephosphorylated by the enzyme. These conclusions are in agreement with the results of the experiments described in our study.

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) D.H.G. is a Predoctoral Fellow of CONICET, and C.S.A. is an Established Investigator of CONICET. We are indebted to Professor Thomas Nowak (Department of Chemistry, University of Notre Dame, Notre Dame, IN, U.S.A.) for the gift of phosphoenol-3-fluoropyruvate and to Professor Marion O'Leary (Department of Chemistry, University of Wisconsin, Madison, WI, U.S.A.) for kindly sending us copies of manuscripts of papers in the press.

REFERENCES

- Andreo, C. S., González, D. H. & Iglesias, A. A. (1987) *FEBS Lett.* **213**, 1–8
- Ariga, N. (1972) *Anal. Biochem.* **49**, 436–441
- Blumberg, K. & Stubbe, J. A. (1975) *Biochim. Biophys. Acta* **384**, 120–126
- Díaz, E., O'Laughlin, J. T. & O'Leary, M. H. (1988) *Biochemistry* **27**, 1336–1341
- Duffy, T. H. & Nowak, T. (1984) *Biochemistry* **23**, 661–670
- Edwards, G. E., Ku, M. S. B. & Monson, R. K. (1985) in *Photosynthetic Mechanisms and the Environment* (Barber, J. & Baker, N. K., eds.), pp. 287–327, Elsevier, Amsterdam
- Fujita, N., Izui, K., Nishino, T. & Katsuki, H. (1984) *Biochemistry* **23**, 1774–1779
- González, D. H. & Andreo, C. S. (1986) *Z. Naturforsch. C* **41**, 1004–1010
- González, D. H. & Andreo, C. S. (1988a) *Biochemistry* **27**, 177–183
- González, D. H. & Andreo, C. S. (1988b) *Eur. J. Biochem.*, in the press
- González, D. H., Iglesias, A. A. & Andreo, C. S. (1987) *Biochem. J.* **241**, 543–548
- Hansen, D. E. & Knowles, J. R. (1982) *J. Biol. Chem.* **257**, 14795–14798
- Hatch, M. D. & Slack, C. R. (1966) *Biochem. J.* **101**, 103–111
- Hoving, H., Crysell, B. & Leadlay, P. F. (1985) *Biochemistry* **24**, 6163–6169
- Hwang, S. H. & Nowak, T. (1986) *Biochemistry* **25**, 5590–5595
- Iglesias, A. A. & Andreo, C. S. (1984) *Plant Physiol.* **75**, 983–987
- Iglesias, A. A., González, D. H. & Andreo, C. S. (1986) *Planta* **168**, 239–244
- Jenkins, C. L. D., Harris, R. L. N. & McFadden, H. G. (1987) *Biochem. Int.* **14**, 219–226
- Kawano, C., Katsuki, H., Yoshida, T. & Yanaka, S. (1962) *Anal. Biochem.* **3**, 361–368
- Lowry, O. H., Rosebrough, N. I., Farr, A. L. & Randall, R. I. (1951) *J. Biol. Chem.* **193**, 265–275
- O'Leary, M. H. (1982) *Annu. Rev. Plant Physiol.* **33**, 297–315
- O'Leary, M. H., Rife, J. E. & Slater, J. D. (1981a) *Biochemistry* **20**, 7308–7314

- O'Leary, M. H., DeGooyer, W. J., Dougherty, T. M. & Anderson, V. (1981*b*) *Biochem. Biophys. Res. Commun.* **100**, 1320–1325
- Sedmak, J. & Grossberg, S. (1977) *Anal. Biochem.* **79**, 544–552
- Sikkema, K. D. & O'Leary, M. H. (1988) *Biochemistry* **27**, 1342–1347
- Stubbe, J. A. & Kenyon, G. L. (1972) *Biochemistry* **11**, 338–345
- Taussky, H. & Shorr, E. (1953) *J. Biol. Chem.* **202**, 675–685
- Utter, M. F. & Kolenbrander, H. M. (1972) *Enzymes* 3rd Ed. **6**, 117–168
- Wirsching, P. & O'Leary, M. H. (1985) *Biochemistry* **24**, 7602–7606
- Wirsching, P. & O'Leary, M. H. (1988*a*) *Biochemistry* **27**, 1348–1355
- Wirsching, P. & O'Leary, M. H. (1988*b*) *Biochemistry* **27**, 1355–1360

Received 4 December 1987/18 January 1988; accepted 14 March 1988