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Interaction of acetyl phosphate and carbamyl phosphate with plant phosphoenolpyruvate carboxylase*

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1. Acetyl phosphate produced an increase in the maximum velocity ($V_{max.}$ for the carboxylation of phosphoenolpyruvate catalysed by phosphoenolpyruvate carboxylase. The limiting $V_{max.}$ was 22.2 μmol·min^{-1}·mg^{-1} (185% of the value without acetyl phosphate). This compound also decreased the K_m for phosphoenolpyruvate to 0.18 mM. The apparent activation constants for acetyl phosphate were 1.6 mM and 0.62 mM in the presence of 0.5 and 4 mM-phosphoenolpyruvate respectively. 2. Carbamyl phosphate produced an increase in $V_{max.}$ and K_m for phosphoenolpyruvate. The variation of $V_{max.}/K_m$ with carbamyl phosphate concentration could be described by a model in which this compound interacts with the carboxylase at two different types of sites: an allosteric activator site(s) and the substrate-binding site(s). 3. Carbamyl phosphate was hydrolysed by the action of phosphoenolpyruvate carboxylase. The hydrolysis produced P_i and NH_4^+ in a 1:1 relationship. Values of $V_{max.}$ and K_m were 0.11 ± 0.01 μmol of P_i·min^{-1}·mg^{-1} and 1.4 ± 0.1 mM, respectively, in the presence of 10 mM-$NaHCO_3$. If HCO_3^- was not added, these values were 0.075 ± 0.014 μmol of P_i·min^{-1}·mg^{-1} and 0.76 ± 0.06 mM. $V_{max.}/K_m$ showed no variation between pH 6.5 and 8.5. The reaction required Mg^{2+}; the activation constants were 0.77 and 0.31 mM at pH 6.5 and 8.5 respectively. Presumably, carbamyl phosphate is hydrolysed by phosphoenolpyruvate carboxylase by a reaction the mechanism of which is related to that of the carboxylation of phosphoenolpyruvate.

INTRODUCTION

Phosphoenolpyruvate (PEP) carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating); EC 4.1.1.31] catalyses the conversion of PEP and HCO_3^- into oxaloacetate and P_i. This enzyme occurs in plants, algae and bacteria, but its functions and properties vary widely with the source (Utter & Kolenbrander, 1972; O'Leary, 1982; Latzko & Kelly, 1983). In 'C_4' plants, the carboxylation of PEP by PEP carboxylase is the first step in the assimilation of atmospheric CO_2 (Hatch & Slack, 1966; Edwards & Huber, 1981).

PEP is carboxylated by a stepwise mechanism in which the first step is the production of carboxyphosphate and the enolate anion of pyruvate from the substrates (O'Leary *et al.*, 1981*a*; Hansen & Knowles, 1982). The reaction requires Mg^{2+} as an essential cofactor. Mn^{2+} and Co^{2+} also support the carboxylation of PEP (O'Leary *et al.*, 1981*a*).

Glucose 6-phosphate and other ester phosphates activate the enzyme by binding at an allosteric site (Coombs *et al.*, 1973; Selinioti *et al.*, 1985). Compounds with a phosphate and a carboxy group in positions similar to those of PEP are strong competitive inhibitors of the carboxylase from various sources (Miziorko *et al.*, 1974; O'Leary *et al.*, 1981*b*; O'Leary, 1982, 1983; Wirsching & O'Leary, 1985). Two PEP analogues [^{35}S]thio[^{18}O]phosphoenolpyruvate and phosphoenol-3-bromopyruvate, are carboxylated by the enzyme (Hansen & Knowles, 1982; O'Leary & Díaz, 1982). By contrast, phosphoenol-α-oxobutyrate and phosphoenol-α-oxoisovalerate are only dephosphorylated by the enzyme, presumably following a mechanism closely related to that for the carboxylation reaction (Fujita *et al.*, 1984).

Our previous work has been devoted to the study of the substrate-binding site of plant PEP carboxylase (Iglesias & Andreo, 1983, 1984; Iglesias *et al.*, 1984; Andreo *et al.*, 1986; González *et al.*, 1986; Podestá *et al.*, 1986). In an attempt to obtain more information on this subject, we tested the effect on the carboxylase activity of two analogues of the proposed intermediate carboxyphosphate: acetyl phosphate and carbamyl phosphate. These two compounds would presumably interact with the HCO_3^--binding site of the enzyme (O'Leary, 1983) and so inhibit the catalytic activity. Unexpectedly, the results obtained indicate that both analogues bind at an activating site. In addition, carbamyl phosphate is hydrolysed by the carboxylase, and it is proposed that it acts as an isosteric analogue of PEP in this reaction. The present work describes the experiments that led us to these conclusions.

EXPERIMENTAL

Enzyme purification

PEP carboxylase was extracted and purified from *Amaranthus viridis* L. leaves as described by Iglesias *et al.* (1986). The purified enzyme had a specific activity of 10–13 μmol·min^{-1}·mg^{-1} and migrated as a single band on polyacrylamide-gel electrophoresis in the presence of

Abbreviation used: PEP, phosphoenolpyruvate.

* Dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday (September 6, 1986).

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SDS and on isoelectric focusing, indicating that was more than 98% pure.

Assays of enzyme activity

PEP carboxylase activity was determined spectrophotometrically (monitoring NADH oxidation at 340 nm) at 30 °C by coupling the PEP carboxylase reaction with that of malate dehydrogenase. The standard assay medium contained 50 mM-Tris/HCl, pH 8, 5 mM-$MgCl_2$, 0.15 mM-NADH, 10 mM-$NaHCO_3$, 2 units of malate dehydrogenase, 4 mM-PEP and PEP carboxylase (4 μg of protein), in a total volume of 1 ml.

The liberation of P_i from carbamyl phosphate was measured as follows: 0.4 ml aliquots containing 50 mM-Tris/HCl, pH 8, 5 mM-$MgCl_2$, 10 mM-$NaHCO_3$, carbamyl phosphate and PEP carboxylase (amounts indicated in the Figure legends) were incubated at 30 °C, usually for 20 min. The reaction was started with carbamyl phosphate and stopped by addition of 0.4 ml of 10% (w/v) trichloroacetic acid. The samples were immediately transferred to an ice bath to minimize the hydrolysis of the remaining carbamyl phosphate and centrifuged for 1 min in an Eppendorf microcentrifuge. After centrifugation, 0.7 ml aliquots were taken and the P_i in them was determined by the method of Taussky & Shorr (1953). Controls were made in which the enzyme was added after the reaction was stopped with trichloroacetic acid; the values of these controls were subtracted from the P_i liberated in the presence of enzyme. The addition of different amounts of enzyme after the reaction was stopped with trichloroacetic acid produced similar values of P_i in the controls.

The determination of P_i and NH_4^+ in parallel was made by incubating aliquots containing 50 mM-Mops/NaOH, pH 7, 5 mM-$MgCl_2$, 10 mM-$NaHCO_3$, 2 mM-carbamyl phosphate and PEP carboxylase (approx. 300 μg/ml). At different times, two aliquots (200 μl each) were removed: one of them was added to a mixture of 200 μl of 50 mM-Mops/NaOH (originally pH 7) plus 400 μl of 10% (w/v) trichloroacetic acid; the other was mixed with 100 μl of reagent 1 (0.532 M-phenol/0.85 mM-sodium nitroferricyanide/0.3 mM-manganous ethylenebisdithiocarbamate) plus 10 mM-EDTA. P_i was determined in the first aliquot as described above. NH_4^+ was determined in the second one by adding 100 μl of reagent 2 (36.6 mM-NaOCl/0.12 mM-*p*-toluensulphonchloramide/0.625 M-NaOH), incubating for 15 min at 30 °C and measuring A_{540} after the addition of 1 ml of water. $(NH_4)_2SO_4$ was used as standard.

The hydrolysis of carbamyl phosphate at different pH values was measured in Mops/Hepes/borate (40 mM each) buffer at the corresponding pH. Other conditions were as specified above, except that 10% (v/v) glycerol was added to stabilize the enzyme. The addition of glycerol at pH 8 produced no effect on the hydrolysis of carbamyl phosphate.

The inactivation by specific reagents was performed by incubating the enzyme at pH 8 in the presence of these compounds before the reaction was started by the addition of carbamyl phosphate.

Protein measurement

Protein concentration was determined by the method of Lowry *et al.* (1951) or by the Coomassie Brilliant Blue G 250 dye-binding method (Sedmak & Grossberg, 1977). Bovine serum albumin was used as standard.

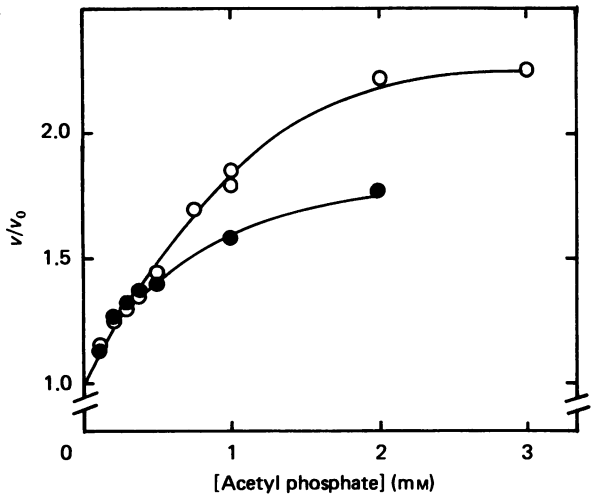


Fig. 1. Activation of PEP carboxylase by acetyl phosphate

The conditions were those described in the text, except that the indicated concentrations of acetyl phosphate were added. The concentrations of PEP were 0.5 mM (○) and 4 mM (●). v_0 represents the activity of a sample without carbamyl phosphate at the corresponding PEP concentration.

Materials

PEP (monopotassium salt), NADH, porcine heart malate dehydrogenase, carbamyl phosphate (disodium salt), acetyl phosphate (lithium potassium salt), and all the inactivating reagents, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

RESULTS

Effect of acetyl phosphate and carbamyl phosphate on the PEP carboxylase reaction

Acetyl phosphate was found to be an excellent activator of PEP carboxylase (Fig. 1). As shown, the activation was more important (as a percentage of control activity) at low PEP concentration, indicating that the activator produced an increase in the affinity towards the substrate. In fact, this was observed in double-reciprocal plots of velocity against [PEP] at different acetyl phosphate concentrations, together with an increase in maximum velocity (results not shown). A value of 0.18 mM for the Michaelis constant for PEP in the presence of saturating activator concentrations was calculated and the maximum velocity was 22.2 μmol·min^{-1}·mg^{-1} (185% of the value without acetyl phosphate). The concentrations of activator producing a half-maximal effect were 1.6 and 0.62 mM for the reactions with 0.5 and 4 mM-PEP respectively. Thus the affinity of the carboxylase for acetyl phosphate was also increased by the presence of the substrate.

The addition of carbamyl phosphate to the assay medium of PEP carboxylase produced either activation or inhibition of the enzyme, depending on the concentrations of PEP and carbamyl phosphate (Fig. 2). The inhibitory effect was observed mainly at low PEP and high carbamyl phosphate concentrations, thus indicating a competitive binding of these compounds to the enzyme. In addition, the plots of activity as a function of PEP concentration became sigmoidal in the presence of the

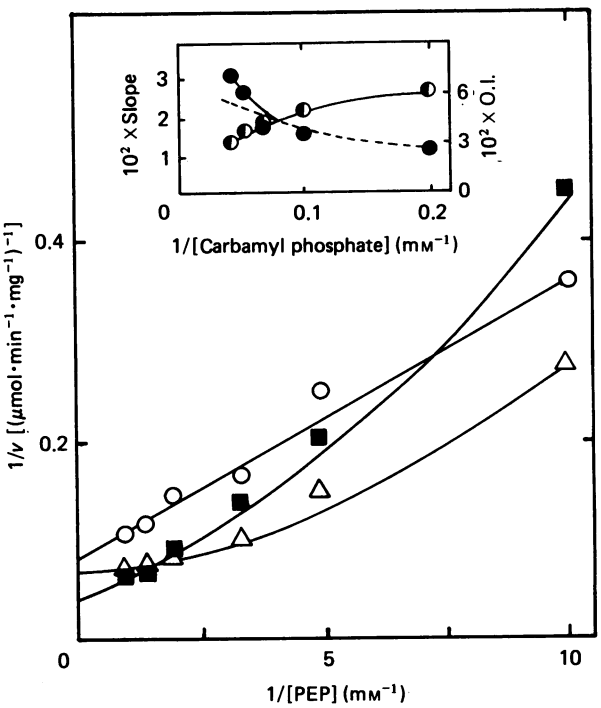


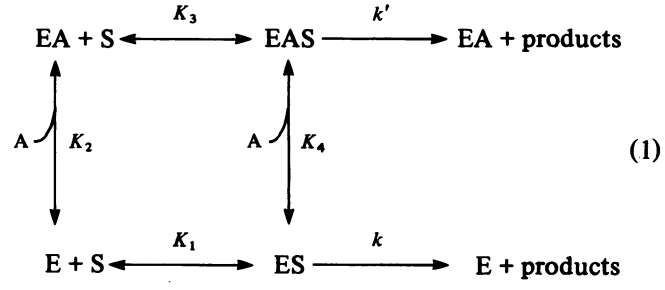
Fig. 2. Effect of carbamyl phosphate on the PEP carboxylase reaction

PEP carboxylase activity was measured in the absence (○) or presence of 5 mM (△)- or 20 mM (■)-carbamyl phosphate as described in the text, except that the indicated amounts of PEP were added. Inset: plot of the slopes (●) and ordinate intercepts (O.I.) (◐) on a double-reciprocal plot. The broken line represents the theoretical curve for eqn. (3), assuming the following values: $K_I = 1$ mM, $K_2 = 1$ mM, $K_4 = 0.1$ mM and $K_1 = 0.3$ mM (see the text). Slopes were calculated from the initial portion of the curves (i.e. at high PEP concentrations).

anhydride. The double-reciprocal plots showed an increase in $V_{max.}$ and K_m (Fig. 2).

The plots of the slopes and vertical intercepts against 1/[carbamyl phosphate] are shown in the inset to Fig. 2. These plots are not linear; the deviation is greater at high carbamyl phosphate concentrations. Besides, the increase in slope is more pronounced than the decrease in the value of the vertical intercept. This fact also indicates a competitive effect between the binding of PEP and of carbamyl phosphate to the carboxylase. The activation may be a consequence of the binding at an allosteric site, probably the same site at which acetyl phosphate binds to the enzyme.

The interaction of acetyl phosphate with PEP carboxylase can be described by the following mechanism:



(1)

where E, A and S represent the enzyme, activator and substrate (PEP) respectively. K_1–K_4 represent dissociation constants of the indicated complexes. k and k' are the rate constants for product formation from ES and EAS respectively.

According to Smith (1977), the slopes of the lines of a plot of $1/v$ versus 1/[PEP] at different activator concentrations would be equal to:

$$\frac{K_1}{V_{max.}} \cdot \frac{1+[A]/K_2}{1+[A]/K_4} \qquad (2)$$

where $V_{max.}$ will be variable if $k \neq k'$.

If it is assumed that carbamyl phosphate interacts in a similar way with the activator-binding site of the carboxylase and that the inhibition is caused by the binding at a competitive site, eqn. (2) can be rearranged as follows:

$$\text{Slope} = \frac{K_1}{V_{max.}} \cdot \frac{(1+[A]/K_I)(1+[A]/K_2)}{1+[A]/K_4} \qquad (3)$$

where K_I is the dissociation constant of the EA complex, with carbamyl phosphate bound to the inhibitory site. This equation also assumes that both type of sites are independent.

As shown in the inset to Fig. 2, this equation fits well with the observed slope values for carbamyl phosphate concentrations below 15 mM if values of 1 mM to K_2 and K_I, 0.3 mM to K_1 (Iglesias *et al.*, 1986) and 0.1 mM to K_4 are assigned. The deviation at high carbamyl phosphate concentrations is probably due to a decrease in K_I produced by the binding of the anhydride at the activator site (a similar effect to that observed with the substrate when acetyl phosphate was used as activator). A value of 0.70 mM for K_I would explain this deviation.

Hydrolysis of carbamyl phosphate by PEP carboxylase

When carbamyl phosphate was incubated in the presence of purified PEP carboxylase and 5 mM-$MgCl_2$ at pH 8, the production of P_i in a time-dependent manner was observed (results not shown). The amount of P_i produced at a given time was proportional to the amount of enzyme that was added to the mixture (results not shown). Similar results were obtained with two different preparations. When ATP or β-glycerophosphate was included in the incubation mixture instead of carbamyl phosphate, no phosphate liberation was observed, indicating the absence of an unspecific phosphatase activity. When the assay medium containing enzyme was heated for 5 min at 90 °C before the addition of carbamyl phosphate, the rate of hydrolysis was markedly decreased. Moreover, the preincubation of the enzyme with 1.1 mM-diethyl pyrocarbonate, 5 mM-*N*-ethylmaleimide, 2.5 mM-phenylglyoxal, 5 mM-bromopyruvate or 0.9 mM-pyridoxal 5′-phosphate at pH 8 and 30 °C for 30 min decreased the rate of hydrolysis to values that were 43, 44, 55, 59 and 27% respectively of that of a control in which the enzyme was incubated in the absence of modifiers.

The other products of the hydrolysis of carbamyl phosphate should be NH_4^+ and HCO_3^- in a 1:1 relationship with P_i. When the production of NH_4^+ from carbamyl phosphate was monitored in parallel with that of P_i, similar amounts of both products were measured at different times (e.g. 0.32 μmol of P_i/ml and 0.33 μmol of NH_4^+/ml at 10 min when 100 μg of enzyme and 2 mM-carbamyl phosphate were used).

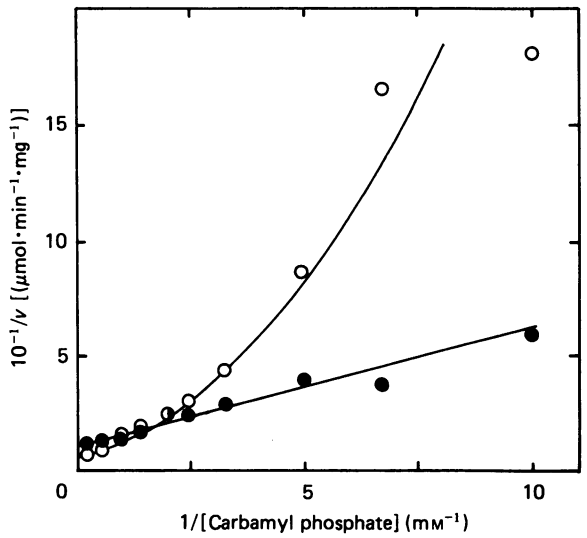


Fig. 3. Effect of several carbamyl phosphate concentrations on the hydrolysis of carbamyl phosphate by PEP carboxylase

Double-reciprocal plots, in the presence of 10 mM-$NaHCO_3$ (○) or without the addition of HCO_3^- (●) are shown; 100 μg of enzyme was used.

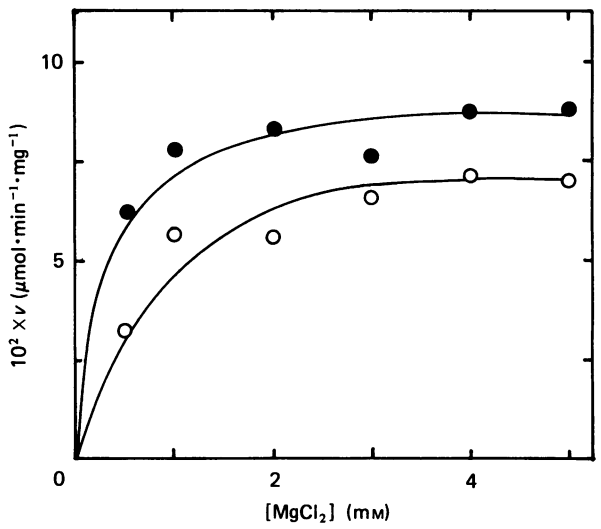


Fig. 4. Effect of $MgCl_2$ concentration on the hydrolysis of carbamyl phosphate by PEP carboxylase

The reaction was performed in Mops/Hepes/borate buffer, as indicated in the text, at pH 6.5 (○) or 8.5 (●).

Acetyl phosphate was also tested as a substrate of the carboxylase, but, owing to its low stability, we could not obtain reliable results.

Effect of HCO_3^- and of different carbamyl phosphate concentrations

The effects of HCO_3^- and of several carbamyl phosphate concentrations on the rate of hydrolysis of carbamyl phosphate by PEP carboxylase are observed in Fig. 3. As shown, HCO_3^- inhibited the hydrolysis of the anhydride at low carbamyl phosphate concentrations and produced a slight, but significant, activation at high carbamyl phosphate concentrations. From double-reciprocal plots, values of 1.4 ± 0.1 mM for K_m and 0.11 ± 0.01 μmol·min^{-1}·mg^{-1} for $V_{max.}$ in the presence of 10 mM-$NaHCO_3$ were calculated; the same values in the absence of added HCO_3^- were 0.76 ± 0.06 mM and 0.075 ± 0.014 μmol·min^{-1}·mg^{-1} respectively. It should be noted that, in the absence of HCO_3^- added at pH 8, the amount of this compound dissolved in the assay medium would be around 0.8–1 mM. This means that if a Michaelis constant of 0.17 mM for HCO_3^- as reported by Iglesias *et al.* (1986) is assumed, then the observed velocity at saturating carbamyl phosphate concentration in the absence of added HCO_3^- would be 84–87% of that in the presence of 10 mM-HCO_3^-. Then the results of $V_{max.}$ can be explained if HCO_3^- is a substrate for the reaction of hydrolysis of carbamyl phosphate by PEP carboxylase.

Metal-ion-dependence

In the absence of $MgCl_2$, no difference was observed in the rate of hydrolysis between the mixture with and without PEP carboxylase. $MnCl_2$ at a concentration of 5 mM could replace $MgCl_2$, yielding similar rates of hydrolysis. In the presence of 5 mM-$CoCl_2$ values of less than 50% of those with a similar amount of $MgCl_2$ were observed.

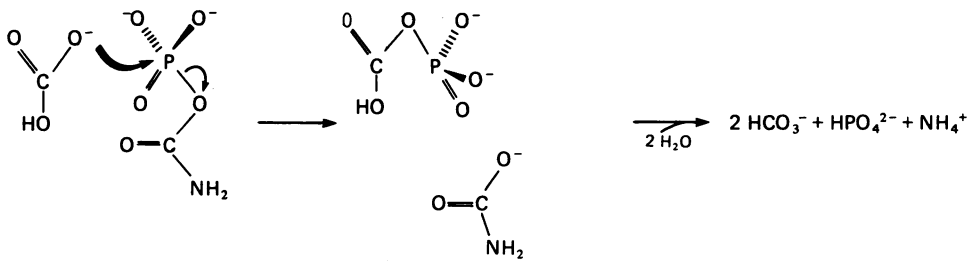
Fig. 4 shows the effect of several concentrations of $MgCl_2$ on the rate of production of P_i from carbamyl phosphate at pH 6.5 and 8.5. The rate of hydrolysis seems to follow Michaelis–Menten kinetics at both pH values, and from double-reciprocal plots K_m values of 0.77 and 0.35 mM respectively were calculated. These values are 2–3-fold lower than those observed for the carboxylation of PEP, but show a similar variation with pH.

Effect of pH

The effect of H^+ concentration on $V_{max.}$ and $V_{max.}/K_m$ for the hydrolysis of carbamyl phosphate was also tested. The plot of $V_{max.}$ versus pH is similar to that observed for the carboxylation of PEP. However, $V_{max.}/K_m$ seems to be completely pH-independent over the range pH 6.5–8.5. Thus the slight increase in $V_{max.}$ observed over pH 6.5 is followed by a similar increase in K_m for carbamyl phosphate.

DISCUSSION

In the present study, two anhydrides of phosphoric acid have been found to behave as activators of PEP carboxylase from *Amaranthus viridis*. Since some phosphate esters such as glucose 6-phosphate, fructose 1,6-bisphosphate and 3-phosphoglycerate have been described as allosteric activators of PEP carboxylase from C_4 plants (Coombs *et al.*, 1973; Wong & Davies, 1973; Selinioti *et al.*, 1985), it is proposed that both anhydrides interact with the carboxylase at the same site at which the abovementioned esters do. Furthermore, acetyl phosphate produces an increase in maximum velocity and in the affinity of the carboxylase for PEP, a similar effect to that reported for glucose 6-phosphate (Coombs *et al.*, 1973; Ting & Osmond, 1973; Mukerji, 1977; González *et al.*, 1984). It is noteworthy that all the activators contain a phosphate group linked through an oxygen to a carbon atom, indicating that this structure could be responsible for their effects.



Scheme 1. Hydrolysis of carbamyl phosphate by PEP carboxylase, assuming that HCO_3^- is a substrate for the reaction

The increase in the apparent K_m for PEP produced by carbamyl phosphate may be provoked by its binding at a competitive site, as indicated by eqn. (3) and Fig. 2. This site may be the PEP-binding site itself, and the interaction would be mainly due to the presence of the phosphate group in its molecule. However, other important factors would also be involved. These are the C–O–P bridging oxygen (see Miziorko *et al.*, 1974; O'Leary, 1983) and the bond angle at the carbon atom of carbamyl phosphate (which resembles the bond angle at C-2 of PEP).

The inhibition constant calculated for carbamyl phosphate (approx. 1 mM) is two orders of magnitude greater than those observed for some PEP analogues such as phosphoenol-3-bromopyruvate (O'Leary & Díaz, 1982) and phosphoenol-α-oxobutyrate (D. H. González & C. S. Andreo, unpublished work) and one order of magnitude greater than the K_I for phospholactate and phosphoglycollate (O'Leary, 1983). The difference may be assigned to the lack of the carboxy group, which has been established to be important in the binding of several compounds to the active site (Miziorko *et al.*, 1974; O'Leary, 1983).

The hydrolysis of carbamyl phosphate produced in the presence of purified PEP carboxylase was markedly decreased by diethyl pyrocarbonate, *N*-ethylmaleimide, phenylglyoxal, bromopyruvate and pyridoxal 5'-phosphate, compounds that inactivate the carboxylase by modifying amino acid residues at the PEP-binding site of the enzyme (Iglesias & Andreo, 1983, 1984; Iglesias *et al.*, 1984; González *et al.*, 1986; Podestá *et al.*, 1986). These results suggest that the hydrolysis of the anhydride is a consequence of the catalytic activity of PEP carboxylase.

To our knowledge, carbamyl phosphate is the first compound not related to PEP that is known to act as a substrate of PEP carboxylase. Two possible mechanisms must be considered for the reaction. One is a simple hydrolytic reaction yielding P_i and carbamate, and then NH_4^+ and HCO_3^- from the latter. The other one is a mechanism related to that for the carboxylation of PEP, which involves carboxyphosphate as intermediate (Scheme 1). When considering this mechanism it should be noted that carbamyl phosphate is an isosteric analogue of PEP.

The mechanism by which the hydrolysis of carbamyl phosphate occurs is not evident from the data presented here. However, several observations favour the second hypothesis.

The hydrolysis of carbamyl phosphate showed similar cation requirements to the carboxylation of PEP (O'Leary *et al.*, 1981*a*; O'Leary, 1982), and the activation constant of Mg^{2+} showed a similar order of magnitude and a similar variation with pH (Maruyama *et al.*, 1966; Iglesias & Andreo, 1983).

The maximum velocity for the hydrolysis of carbamyl phosphate showed little variation with pH, just as occurs with the same parameter for the carboxylation of PEP (O'Leary *et al.*, 1981*a*; Iglesias & Andreo, 1983). However, the pH-dependence of $V_{max.}/K_m$ *was* different. This means that the histidine residues that are involved in the binding of PEP to the enzyme (O'Leary *et al.*, 1981*a*; Iglesias & Andreo, 1983) do not participate in the binding of carbamyl phosphate. These residues may interact with a group of PEP other than the phosphate (i.e. the carboxy group). It is of interest that the affinity for the binding of bromopyruvate (which lacks the phosphate group) changes with pH in a similar way to the affinity for PEP (González *et al.*, 1986).

Fujita *et al.* (1984) observed that HCO_3^- was required for the dephosphorylation of two PEP analogues by PEP carboxylase. From that evidence they proposed that the dephosphorylation occurs by a mechanism which involves carboxyphosphate as intermediate. The effect of HCO_3^- on the hydrolysis of carbamyl phosphate is not clear. At low [carbamyl phosphate], an inhibition was observed that could arise from the interaction of carbamyl phosphate at the HCO_3^--binding site (see O'Leary, 1983). The increase in $V_{max.}$ may indicate the participation of HCO_3^- as a substrate for the reaction, although other explanations cannot be ruled out.

As an additional point, the fact that carbamyl phosphate acts as a substrate and as an activator at similar concentrations indicates the existence of great similarities between the active site and the activating site at which many phosphate esters bind.

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