

# Isotope Trapping and Positional Isotope Exchange with Rat and Chicken Liver Phosphoenolpyruvate Carboxykinases<sup>†</sup>

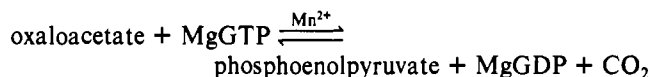
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**ABSTRACT:** Isotope-trapping studies of the enzyme·MgGTP complex were carried out with rat liver cytosolic and chicken liver mitochondrial phosphoenolpyruvate carboxykinases. For the rat liver enzyme, MgGTP was partially trapped from both E·MgGTP and E·MgGTP·OAA complexes, consistent with a steady-state random mechanism. For the chicken liver enzyme, MgGTP was 100% trapped from the E·MgGTP·OAA complex, consistent with a steady-state ordered mechanism. The rate constants for the interaction of MgGTP with the free enzymes are approximately  $10^7 \text{ M}^{-1} \text{ s}^{-1}$ , somewhat lower than the diffusion limit for association. The dissociation rate for the enzyme·MgGTP complexes is  $26\text{--}92 \text{ s}^{-1}$ , reflecting a tightly bound complex with high commitment to catalysis in the presence of oxaloacetate. Positional isotope-exchange studies were also carried out with phosphoenolpyruvate carboxykinases from rat and chicken. No exchange of the  $\beta\gamma\text{-}^{18}\text{O}$  in  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  to form  $[\beta\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  was detected in the absence of oxaloacetate. In the presence of oxaloacetate, no positional isotope exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  was detected during initial rate conditions. The results indicate that at least one of the products dissociates rapidly from the E·MgGDP·PEP·CO<sub>2</sub> complex relative to the net rate of MgGTP formation from the E·MgGDP·PEP·CO<sub>2</sub> complex. A rapid equilibrium between the central complexes in which the  $\beta$ -phosphoryl of GDP is restricted with respect to torsional rotation cannot be excluded but is unlikely on the basis of the relative rates of catalysis and torsional rotation. The addition of Mn<sup>2+</sup>, an activator of phosphoenolpyruvate carboxykinase, did not influence the positional isotope-exchange results. The  $\beta\gamma\text{-}^{18}\text{O}$  to  $\beta\text{-}^{18}\text{O}$  exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  was not induced by oxalate as a substrate analogue of oxaloacetate. The results are consistent with a mechanism of direct, unidirectional phosphoryl transfer between GTP and oxaloacetate during initial reaction rate conditions.

**P**hosphoenolpyruvate carboxykinase (P-enolpyruvate carboxykinase) (EC 4.1.1.32) catalyzes the reversible reaction



The primary function of this enzyme in vertebrates is to catalyze the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis. The enzyme exists as cytosolic and mitochondrial isoenzymes. These distinct proteins from different cellular compartments do not cross-react immunochemically (Ballard & Hanson, 1969). The enzymes are encoded by different mRNAs and have only 63% amino acid identity (Hod et al., 1982; Weldon et al., 1990). However, they share similar kinetic properties, which include the decarboxylation of oxaloacetate to pyruvate and CO<sub>2</sub> in the presence of GDP and activation by Mn<sup>2+</sup> even in the presence of excess Mg<sup>2+</sup> (Schramm et al., 1981). In rat liver, the enzyme is found exclusively in the cytosol (Cornell et al., 1986). In contrast, the enzyme is found only in the mitochondria in chicken liver (Watford et al., 1981).

In contrast to the knowledge of tissue specificity and expression of the enzymes (Beale et al., 1985; Weldon et al.,

1990), the catalytic mechanisms are poorly understood. Kinetic and isotope-exchange experiments (Jomain-Baum & Schramm, 1978) indicated a steady-state random mechanism for the rat enzyme while NMR studies of the complexes of substrates with the chicken enzyme have indicated rapid association–dissociation between substrates and enzyme on the NMR time scale (Lee & Nowak, 1984).

In the present studies, both the rat and chicken liver P-enolpyruvate carboxykinases are characterized with respect to the rate constants for interaction with MgGTP, the metabolically significant metal–nucleotide triphosphate complex. Isotope-trapping experiments (Rose et al., 1974) are used to establish the relative rates of catalysis and MgGTP dissociation. We also quantitate the rate constants for phosphoryl transfer relative to the rate of product release and the rate of re-formation of MgGTP from the E·MgGDP·PEP·CO<sub>2</sub> complex using positional isotope exchange (Midelfort & Rose, 1976; Rose, 1980a).

## MATERIALS AND METHODS

**Materials.**  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ ,  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  (triethylammonium salt), and  $[8\text{-}^3\text{H}]\text{GDP}$  (trisodium salt) were purchased from New England Nuclear.  $^{32}\text{P}]\text{P-enolpyruvate}$  was synthesized from oxaloacetate and  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  by using P-enolpyruvate carboxykinase. GTP and GDP were purchased from Pharmacia. HEPES, P-enolpyruvate, and oxaloacetate were purchased from Sigma. Triethanolamine was purchased from Fisher Scientific. Poly(ethylenimine)–cellulose F precoated plastic sheets for thin-layer chromatography were purchased from E. Merck.

**Purification of Chicken Liver and Rat Liver P-enolpyruvate Carboxykinase.** Rat liver P-enolpyruvate carboxykinase was

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purified from rat liver cytosol (Brinkworth et al., 1981) except that ITP-Sepharose was used in place of GTP-Sepharose. Chicken liver P-enolpyruvate carboxykinase was extracted and purified from chicken liver mitochondria (Ash et al., 1990).

**Enzyme Assay.** The routine assay in the direction of P-enolpyruvate formation was carried out at 30 °C in a reaction mixture containing 50 mM Hepes, pH 8.0, 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{MnCl}_2$ , 0.1 mM GTP, 4 mM L-malate, and 1 mM  $\text{NAD}^+$ . Reaction rates were based on the extinction coefficient of NADH of  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  at 340 nm. Malate dehydrogenase (11 units) was added first to generate oxaloacetate. The reaction was then initiated by adding P-enolpyruvate carboxykinase. A unit of catalytic activity forms 1  $\mu\text{mol}$  of P-enolpyruvate/min, with the routine assay described above.

The assay in the direction of oxaloacetate formation was carried out at 30 °C by using a similar procedure except the reaction mixture contained 50 mM Hepes, pH 8.0, 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{MnCl}_2$ , 0.5 mM GDP, 0.5 mM P-enolpyruvate, 50 mM  $\text{KHCO}_3$ , 0.14 mM NADH, and 11 units of malate dehydrogenase. The reaction was initiated by the addition of P-enolpyruvate carboxykinase. A unit of catalytic activity forms 1  $\mu\text{mol}$  of oxaloacetate/min with this assay.

The protein concentration was determined by the method of Bradford (1976) with reagents purchased from Bio-Rad and with bovine serum albumin as a standard.

The  $K_m$  for oxaloacetate was determined in a reaction mixture of 50 mM triethanolamine at pH 8.0, with 5 mM  $\text{MgCl}_2$ , 5 mM GTP, 50  $\mu\text{M}$   $\text{MnCl}_2$ , varied amounts of L-malate, and 1 mM  $\text{NAD}^+$ . Malate dehydrogenase (12 units) was added to establish the equilibrium with oxaloacetate. The difference of absorption at 340 nm was used to determine oxaloacetate concentration. The initial reaction rates were determined as described above.

**Isotope Trapping of E-MgGTP.** Isotope-trapping studies were carried out at room temperature as described by Rose (1980b). A 10- $\mu\text{L}$  equilibrium solution contained 50 mM triethanolamine, pH 8.0, 0.5 mM  $\text{MgCl}_2$ , 0.05 mM  $\text{MnCl}_2$ , and  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  and P-enolpyruvate carboxykinase in the indicated amounts. For chicken liver P-enolpyruvate carboxykinase, 50  $\mu\text{M}$  enzyme (based on MW = 72 000; specific activity = 14.9 units/mg) was included in the equilibrium solution. For rat liver P-enolpyruvate carboxykinase, 55  $\mu\text{M}$  enzyme (based on MW = 72 000; specific activity = 14.9 units/mg) was included. A 100- $\mu\text{L}$  chase solution contained 50 mM triethanolamine, pH 8.0, 30 mM  $\text{MgCl}_2$ , 30 mM GTP, and 50  $\mu\text{M}$   $\text{MnCl}_2$ , and oxaloacetate concentration was varied from 0.025 to 2 mM. The catalytic reaction was initiated by rapidly mixing the equilibrium mixture with the chase solution. After 2 s, the reaction was terminated by the addition of formic acid to a concentration of 1.2 M. The precipitated protein was removed by centrifugation.  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  was isolated from  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  on a PEI-cellulose plate developed with 0.75 M potassium phosphate, pH 3.5. The amount of  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  formed was determined by scintillation counting. The controls were treated identically, except the same amount of  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  used in the equilibrium solution was mixed in the chase solution to correct for the amount of  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  produced from  $\text{Mg}[\alpha\text{-}^{32}\text{P}]\text{GTP}$  following release from the E-Mg $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  complex and before stopping the reaction with formic acid. The  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  formed in these control experiments was negligible in comparison with the experimental value. The experiments were repeated with varied amounts of  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  in the equilibrium mixture to determine the dissociation constant of E-MgGTP and the amount of  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  formed

at saturating oxaloacetate and saturating  $\text{Mg}[\alpha\text{-}^{32}\text{P}]\text{GTP}$  ( $P^*_{\text{max}}$ ).

During the 2-s incubation, a maximum of 81  $\mu\text{M}$  each of GDP,  $\text{HCO}_3^- + \text{CO}_2$ , and P-enolpyruvate is formed. With a kinetic constant of 30–50 mM for  $\text{HCO}_3^-$  at pH 8 (Jomain-Baum & Schramm, 1978) the reverse exchange reaction is assumed to be negligible during this period.

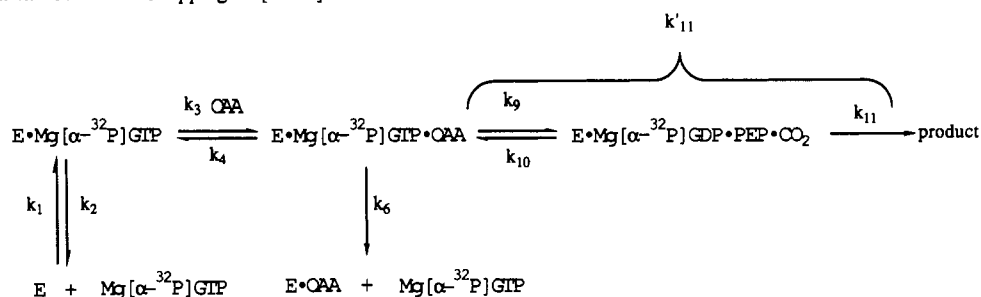
**Synthesis of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$ .**  $^{18}\text{O}$ -Enriched inorganic phosphate was synthesized with  $\text{PCl}_5$  and  $\text{H}_2^{18}\text{O}$  according to the procedure of Hackney et al. (1980) with slight modifications. The synthesis was carried out in a sealed plastic bag under  $\text{N}_2$  evacuated to a NaOH trap. After  $^{18}\text{O}$ -enriched  $\text{H}_2\text{O}$  (1.0 mL, 50 mmol) reacted with  $\text{PCl}_5$  (1.79 g, 8.6 mmol), the solution was neutralized with 4.2 mL of dry pyridine. The concentration of  $^{18}\text{O}$ -enriched inorganic phosphate was determined according to Lanzetta et al. (1979).  $^{31}\text{P}$  NMR analysis indicated that the pyridine phosphate salt was 91%  $^{18}\text{O}_4$  enriched and 9%  $^{18}\text{O}_3$  enriched.

$[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  was synthesized according to the procedure of Webb (1980).  $^{31}\text{P}$  NMR analysis demonstrated that the synthesized GTP was 90%  $^{18}\text{O}_4$  enriched in the  $\gamma$ -phosphorus and 10%  $^{18}\text{O}_3$  enriched.

**Positional Isotope Exchange with  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$ .** The positional isotope-exchange experiments were carried out with both rat and chicken P-enolpyruvate carboxykinase. For chicken liver P-enolpyruvate carboxykinase, the experiments were carried out with oxaloacetate either added directly or produced from malate by the malate dehydrogenase reaction. For rat liver P-enolpyruvate carboxykinase, the experiments were carried out with oxaloacetate added directly.

When positional isotope-exchange experiments were carried out with oxaloacetate produced from the malate dehydrogenase reaction, the oxaloacetate concentration was determined by NADH production. The activity of P-enolpyruvate carboxykinase was determined in the positional isotope-exchange reaction mixture by measuring depletion of oxaloacetate coupled to the malate dehydrogenase reaction. Typically, the positional isotope-exchange reactions were carried out at 30 °C in a mixture of 100 mM HEPES, pH 8.0, 8.5 mM  $\text{MgCl}_2$ , 3.5 mM  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$ , 1 mM  $\text{NAD}^+$ , 50 mM malate ( $\text{K}^+$ ), and 11 units/mL malate dehydrogenase. After a 10-min incubation at 30 °C, an aliquot (1 mL) was collected as a control.  $\text{NAD}^+$ , malate, and malate dehydrogenase were not included when the experiments were carried out in the absence of oxaloacetate. P-enolpyruvate carboxykinase was added last to initiate the reaction. At variable intervals, 1 mL of the reaction mixture was removed and added to 50  $\mu\text{L}$  of  $\text{CCl}_4$  and vortexed vigorously. After the precipitated protein was removed by centrifugation, the extent of the reaction was determined by measuring the increase in NADH during the reaction. The supernatant was then mixed with 25 mM EDTA, pH 9.0, and 40%  $\text{D}_2\text{O}$  (for field frequency locking), followed by  $^{31}\text{P}$  NMR measurement. The GDP produced was also quantitated by the  $^{31}\text{P}$  NMR measurement based on the chemical shift of the  $\beta$ -phosphorus of GDP (0.33 ppm upfield relative to the  $\gamma$ -phosphorus of GTP).

When the experiments were carried out with oxaloacetate added directly, the oxaloacetate was dissolved immediately before use at 0 °C and was spectrophotometrically calibrated by the depletion of NADH in a reaction mixture containing 100 mM HEPES ( $\text{K}^+$ ), pH 8.0, 0.22 mM NADH, and 6 units/mL malate dehydrogenase. The activity of P-enolpyruvate carboxykinase in the reaction mixture for the positional isotope exchange was measured by the production of P-enolpyruvate coupled to the pyruvate kinase and lactate

Scheme I: Steps in the Substrate Trapping of [ $\alpha$ - $^{32}$ P]GTP<sup>a</sup>

<sup>a</sup>The complex of E·Mg[ $\alpha$ - $^{32}$ P]GTP is formed in the equilibrium mixture before the addition of additional unlabeled MgGTP and oxaloacetate (OAA).

dehydrogenase reactions. The reaction mixture contained 100 mM HEPES (K<sup>+</sup>), pH 8.0, 18 mM MgCl<sub>2</sub>, 5.0 mM GTP, 3.0 mM oxaloacetate (K<sup>+</sup>), 1 mM ADP, 0.5 mM NADH, 100 mM KCl, 8 units/mL pyruvate kinase, and 15 units/mL lactate dehydrogenase. The results were corrected for the nonenzymatic decarboxylation of oxaloacetate. The activity of P-enolpyruvate carboxykinase was also determined in the presence of 50  $\mu$ M MnCl<sub>2</sub> in an analogous procedure.

Typical positional isotope-exchange reactions were carried out at 30 °C in reaction mixtures that contained 100 mM HEPES (K<sup>+</sup>), pH 8.0, 18 mM MgCl<sub>2</sub>, and 5.0 mM [ $\beta$ - $\gamma$ - $^{18}$ O, $\gamma$ - $^{18}$ O<sub>3</sub>]GTP. Oxaloacetate at 3 mM was added immediately before the P-enolpyruvate carboxykinase to minimize nonenzymatic decarboxylation. At indicated intervals, the reaction was terminated by vortexing 1 mL of the samples with 50  $\mu$ L of CCl<sub>4</sub>. Samples were then treated as described above. Positional isotope-exchange experiments with oxalate were the same, except that oxaloacetate was substituted by 1 mM oxalate.

**<sup>31</sup>P NMR Measurements.** The <sup>31</sup>P spectra were measured on a Varian 500-MHz multinuclear spectrometer operating at 202.3 MHz by using a VXR-4000 data station. Spectra were recorded at 25 °C with a spectral width of 8000 Hz and an acquisition time of 2 s. Zero filling to 32 000 was applied before Fourier transformation with a digital resolution of 0.489 Hz/data point. The samples were measured in a 10-mm probe, and 600–1000 scans were averaged to obtain an adequate signal-to-noise ratio. The chemical shifts were measured relative to K<sub>2</sub>HPO<sub>4</sub> as an external standard. The rate of positional isotope exchange was determined by the peak area corresponding to P[ $^{18}$ O<sub>4</sub>] and PO[ $^{18}$ O<sub>3</sub>] at the  $\gamma$ -phosphoryl group of GTP. The fraction of the original [ $\beta$ - $\gamma$ - $^{18}$ O, $\gamma$ - $^{18}$ O<sub>3</sub>]GTP pool converted into GDP during the positional isotope-exchange experiment was determined by the peak area of the  $\gamma$ -phosphorus of GTP ( $\delta$  = -8.053 ppm,  $J$  = 19.1 Hz) relative to the  $\beta$ -phosphorus of GDP ( $\delta$  = -8.386 ppm,  $J$  = 21.5 Hz).

**Exchange of [8-<sup>3</sup>H]GDP into GTP.** Reaction conditions were the same as the positional isotope-exchange experiment. After 40 min of reaction in the positional isotope-exchange reaction, a trace amount of [8-<sup>3</sup>H]GDP (1  $\mu$ Ci, 10<sup>-4</sup>  $\mu$ mol) was added to a 1-mL reaction mixture to follow the exchange reaction into GTP. At variable intervals, samples were terminated by adding 8  $\mu$ L of the reaction mixture to 9  $\mu$ L of 1 N formic acid. The GDP was separated from GTP on PEI-cellulose TLC, developed in 0.75 M KH<sub>2</sub>PO<sub>4</sub>, pH 3.5. The [8-<sup>3</sup>H]GDP and [8-<sup>3</sup>H]GTP spots were excised, and the radioactivity was measured.

**Exchange of [<sup>32</sup>P]P-enolpyruvate into GTP.** The experiments were similar to that for [8-<sup>3</sup>H]GDP exchange, except that [8-<sup>3</sup>H]GDP was replaced by a trace amount of [<sup>32</sup>P]P-

enolpyruvate (0.3  $\mu$ Ci) in the exchange reaction mixture. The P-enolpyruvate was separated from GTP by using PEI-cellulose TLC, developed in 0.75 M KH<sub>2</sub>PO<sub>4</sub>, pH 3.5. The  $R_f$  values for GTP and P-enolpyruvate are 0.22 and 0.79, respectively. The [<sup>32</sup>P]P-enolpyruvate exchange rate was based on the fraction of [<sup>32</sup>P]P-enolpyruvate exchanged and its concentration.

**Data Analysis.** Results of isotope trapping were fitted with the rate equation (eq 1) and the programs developed by Cleland (1979):

$$P^* = \frac{P^*_{\max}[\text{OAA}]}{K'_{\text{OAA}} + [\text{OAA}]} \quad (1)$$

where  $P^*$  is the amount of GTP\* trapped at a given concentration of oxaloacetate varied in the chase. [OAA] represents the concentration of oxaloacetate.  $P^*_{\max}$  represents the amount of [ $\alpha$ - $^{32}$ P]GTP trapped when the oxaloacetate concentration is saturating.  $K'_{\text{OAA}}$  represents the concentration of oxaloacetate to give half of  $P^*_{\max}$ . Results of  $P^*_{\max}$  at each fixed concentration of Mg[ $\alpha$ - $^{32}$ P]GTP in the enzyme-MgGTP equilibrium mixture were then analyzed by using

$$\frac{1}{P^*_{\max}} = \frac{1}{P^*_{\max,\text{sat}}} \left( 1 + \frac{K_d}{[A] - P^*_{\max}} \right) \quad (2)$$

where [A] represents the concentration of Mg[ $\alpha$ - $^{32}$ P]GTP and  $P^*_{\max}$  represents the amount of [ $\alpha$ - $^{32}$ P]GTP trapped at saturating oxaloacetate.  $P^*_{\max,\text{sat}}$  represents the  $P^*_{\max}$  when the enzyme is saturated with MgGTP in the equilibrium mixture, and  $K_d$  represents the dissociation constant for the E·MgGTP complex.

As shown in Scheme I, the ratio of the rate of dissociation of Mg[ $\alpha$ - $^{32}$ P]GTP from E·Mg[ $\alpha$ - $^{32}$ P]GTP·OAA ( $k_6$ ) relative to its conversion to [ $\alpha$ - $^{32}$ P]GDP ( $k'_{11}$ ) can be estimated from

$$\frac{k_6}{k'_{11}} = \frac{E \cdot \text{Mg}[\alpha\text{-}^{32}\text{P}]\text{GTP}}{P^*_{\max}} - 1 \quad (3)$$

A limiting value for the dissociation rate of Mg[ $\alpha$ - $^{32}$ P]GTP from E·Mg[ $\alpha$ - $^{32}$ P]GTP ( $k_2$ ) can be determined with (Rose, 1980b)

$$\frac{K'_{\text{OAA}}}{K_{\text{OAA}}} \frac{V_{\max}}{E_t} \leq k_2 \leq \frac{K'_{\text{OAA}}}{K_{\text{OAA}}} \frac{[E \cdot \text{Mg}[\alpha\text{-}^{32}\text{P}]\text{GTP}]}{P^*_{\max}} \frac{V_{\max}}{E_t} \quad (4)$$

where  $K_{\text{OAA}}$  represents the concentration of oxaloacetate to give half  $V_{\max}/E_t$ ,  $V_{\max}/E_t$  represents the turnover number of P-enolpyruvate carboxykinase in the direction of P-enolpyruvate formation, and  $K'_{\text{OAA}}$  and  $P^*_{\max}$  are the same as described above.

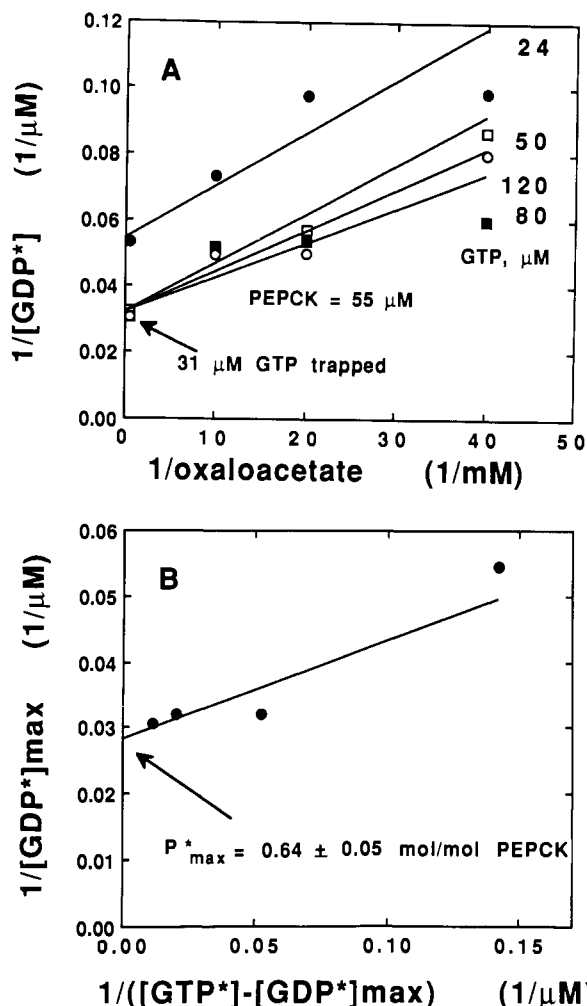


FIGURE 1: Isotope trapping from the E-Mg[ $\alpha$ - $^{32}$ P]GTP complex with rat liver P-enolpyruvate carboxykinase. (A) The [ $\alpha$ - $^{32}$ P]GDP produced at varied oxaloacetate concentrations (0.025–2.0 mM) was determined as described in the text. Each line represents experiments beginning with a different concentration of Mg[ $\alpha$ - $^{32}$ P]GTP in the equilibrium mixture. E-Mg[ $\alpha$ - $^{32}$ P]GTP at different concentrations was produced in the equilibrium solution by mixing 55  $\mu$ M enzyme and Mg[ $\alpha$ - $^{32}$ P]GTP at concentrations of (●) 24, (□) 50, (■) 80, and (○) 120  $\mu$ M. The lines are the best fit of the data to eq 1. (B) The  $P^*_{\max}$  ([ $\alpha$ - $^{32}$ P]GDP produced at a saturating concentration of oxaloacetate) determined with varied Mg[ $\alpha$ - $^{32}$ P]GTP in the equilibrium mixture was analyzed in the double-reciprocal plot of  $1/P^*_{\max}$  vs  $1/(\text{Mg}[\alpha\text{-}^{32}\text{P}]\text{GTP} - P^*_{\max})$ . The lines are the best fit of the data to eq 2.

The rate constants of the positional isotope-exchange reaction ( $V_{\text{ex}}$ ) were estimated according to (Litwin & Wimmer, 1979)

$$V_{\text{ex}} = \frac{X}{\ln(1-X)} \frac{A_0}{t} \ln(1-F) \quad (5)$$

where  $X$  = fraction of the initial [ $\beta$ - $^{18}\text{O}$ ,  $\gamma$ - $^{18}\text{O}_3$ ]GTP converted to GDP,  $F$  = fraction of positional isotope-exchange equilibrium attained in the [ $\beta$ - $^{18}\text{O}$ ,  $\gamma$ - $^{18}\text{O}_3$ ]GTP pool at time  $t$ , and  $A_0$  = the concentration of the initial [ $\beta$ - $^{18}\text{O}$ ,  $\gamma$ - $^{18}\text{O}_3$ ]GTP pool.

## RESULTS

**Isotope Trapping of E-MgGTP by Rat Liver P-enolpyruvate Carboxykinase.** The amount of [ $\alpha$ - $^{32}$ P]GTP trapped as a function of oxaloacetate concentration in the chase was determined at each fixed concentration of [ $\alpha$ - $^{32}$ P]GTP in the equilibrium mixture and analyzed as shown in Figure 1A. The upper line in Figure 1A has excess enzyme (55  $\mu$ M) relative

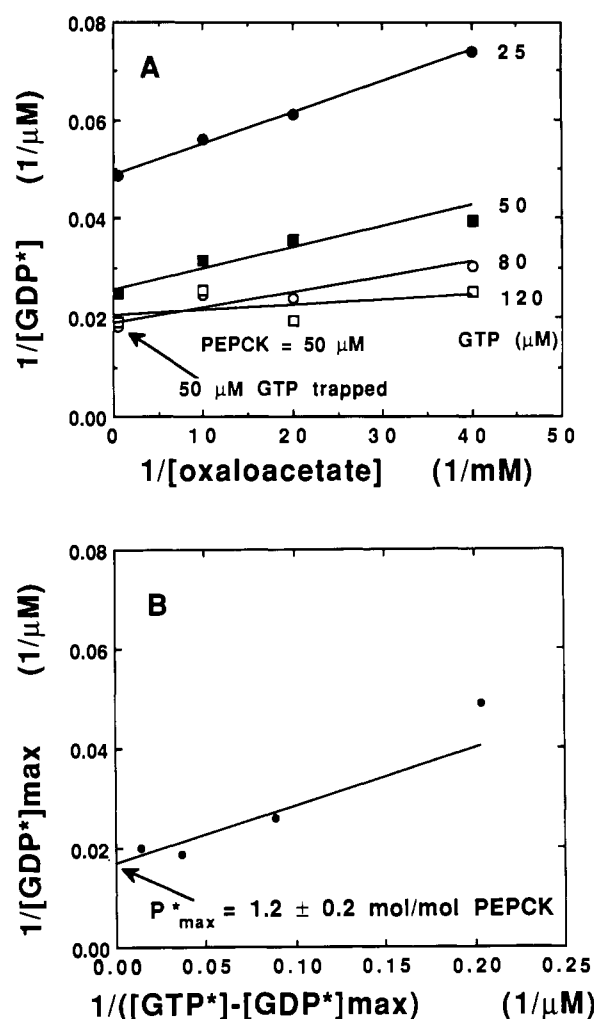


FIGURE 2: Isotope trapping from the E-Mg[ $\alpha$ - $^{32}$ P]GTP complex with chicken liver P-enolpyruvate carboxykinase. The experimental conditions are the same as in panels A and B in Figure 1 except that the chicken liver enzyme was present at 50  $\mu$ M.

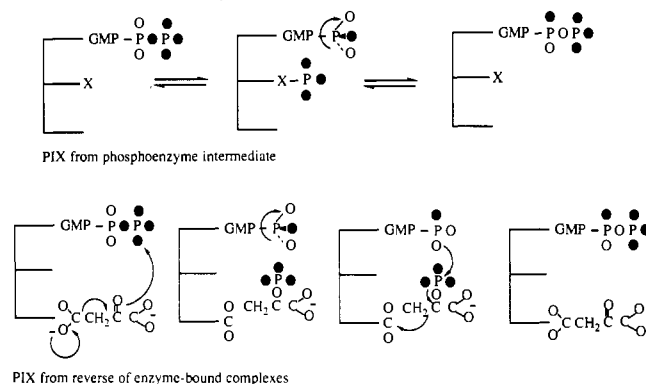
to the total MgGTP concentration of 24  $\mu$ M. Under these conditions, most of the MgGTP should be enzyme bound before addition of the chase solution containing oxaloacetate. This experiment therefore has an upper limit of 24  $\mu$ M for GTP trapping. The values for  $K'_{\text{OAA}}$  determined at each [ $\alpha$ - $^{32}$ P]GTP concentration are the same within experimental error and are  $40 \pm 10$   $\mu$ M. The  $P^*_{\max}$  ([ $\alpha$ - $^{32}$ P]GTP trapped at saturating oxaloacetate concentration) determined at each [ $\alpha$ - $^{32}$ P]GTP concentration was then analyzed in a double-reciprocal plot (Figure 1B). The dissociation constant for the E-MgGTP complex was determined to be  $5 \pm 2$   $\mu$ M. The  $P^*_{\max}$  obtained when the enzyme is saturated with Mg[ $\alpha$ - $^{32}$ P]GTP prior to oxaloacetate addition is equal to  $35 \pm 3$   $\mu$ M, which corresponds to  $0.6 \pm 0.1$  mol of GTP trapped per enzyme subunit. As shown in Scheme I, the relative rate of Mg[ $\alpha$ - $^{32}$ P]GTP dissociation from E-Mg[ $\alpha$ - $^{32}$ P]GTP-OAA ( $k_6$ ) to conversion to [ $\alpha$ - $^{32}$ P]GDP ( $k'_{11}$ ) is  $0.64 \pm 0.05$ . With the kinetic constants from Table I, the rate of Mg[ $\alpha$ - $^{32}$ P]GTP dissociation from E-Mg[ $\alpha$ - $^{32}$ P]GTP ( $k_2$ ) can be determined to be within the limits of  $42 \pm 16$  to  $66 \pm 26$   $\text{s}^{-1}$ .

**Isotope Trapping of E-MgGTP by Chicken Liver P-enolpyruvate Carboxykinase.** The amount of [ $\alpha$ - $^{32}$ P]GTP trapped with varied concentrations of oxaloacetate was determined and analyzed as shown in Figure 2A. Each line represents trapping with a fixed amount of [ $\alpha$ - $^{32}$ P]GTP in the equilibrium mixture. The  $K'_{\text{OAA}}$  values determined at each [ $\alpha$ - $^{32}$ P]GTP concentration are the same within the experimental errors and

Table I: Summary of Kinetic Constants from  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ -Trapping Experiments with P-enolpyruvate Carboxykinases

kinetic constants	rat liver P-enolpyruvate carboxykinase	chicken liver P-enolpyruvate carboxykinase
$P^*_{\max}$ (mol/mol of E)	$0.6 \pm 0.1$	$1.2 \pm 0.2$
$K_{\text{OAA}}^a$ ( $\mu\text{M}$ )	$17 \pm 5$	$11 \pm 2$
$K_{\text{OAA}}^b$ ( $\mu\text{M}$ )	$40 \pm 10$	$16 \pm 5$
$V_1^c$ ( $\text{s}^{-1}$ )	18	18
$V_2^d$ ( $\text{s}^{-1}$ )	22	13
$K_d^e$ ( $\mu\text{M}$ )	$5 \pm 2$	$7 \pm 3$
$k_6/k_{11}^f$	$0.6 \pm 0.1$	$\sim 0$
$k_2^g$ ( $\text{s}^{-1}$ )	$42 \pm 16$ to $66 \pm 26$	$26 \pm 9$

<sup>a</sup> The concentration of oxaloacetate to give half-maximum  $V/E_t$ , which is determined as described in the text. <sup>b</sup> The concentration of oxaloacetate to give half-maximum trapping of GTP. <sup>c</sup>  $V_1$  represents the turnover number in the direction of P-enolpyruvate formation under conditions of the experiment. <sup>d</sup>  $V_2$  represents the turnover number in the direction of oxaloacetate formation under conditions of the experiment. <sup>e</sup>  $K_d$  is the dissociation constant for the E-MgGTP complex. <sup>f</sup> See Scheme I for these rate constants.

Scheme II: Steps in the  $^{18}\text{O}$ -Positional Isotope Exchange for P-enolpyruvate Carboxykinase<sup>a</sup>

<sup>a</sup> The upper reactions demonstrate the exchange that would occur if a phosphoenzyme intermediate could be formed. The upper arm of the E represents the guanine nucleotide binding site. X is the putative enzyme nucleophile that accepts the  $\gamma$ -phosphoryl. The lower arm of the E represents the oxaloacetate binding site. The filled oxygens represent  $^{18}\text{O}$ . The lower reactions demonstrate the positional isotope exchange that would be expected if a series of reversible steps links with E-MgGDP-PEP- $\text{CO}_2$  complex with free MgGTP.

are equal to  $16 \pm 5 \mu\text{M}$ . The amount of  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  trapped at saturating oxaloacetate concentration for each  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  concentration ( $P^*_{\max}$ ) was analyzed as shown in Figure 2B. The dissociation constant for the E-MgGTP complex equals  $7 \pm 3 \mu\text{M}$ . The  $P^*_{\max}$  obtained when the enzyme is saturated with Mg $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  and oxaloacetate is  $59 \pm 8 \mu\text{M}$ , which corresponds to  $1.2 \pm 0.2$  mol of GTP trapped per enzyme subunit. As shown in Scheme I, the dissociation of Mg $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  ( $k_6$ ) from E-Mg $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ -OAA is insignificant compared to its rate of conversion to  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  ( $k_{11}$ ). With the kinetic constants in Table I, the rate of dissociation of Mg $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  from the E-Mg $[\alpha\text{-}^{32}\text{P}]$  complex ( $k_2$ ) can be estimated to be  $26 \pm 9 \text{ s}^{-1}$ .

**Positional Isotope Exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  in the Absence of Oxaloacetate.** In order to determine whether P-enolpyruvate carboxykinase is capable of forming a reversible phosphoenzyme intermediate in the absence of oxaloacetate,  $^{18}\text{O}$  positional isotope exchange was measured in the absence of the carboxylic acid. With this condition, the positional isotope exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  can reveal any significant reversible  $\gamma$ -phosphoryl transfer from GTP to a phosphoenzyme intermediate as shown in the upper portion of Scheme II.

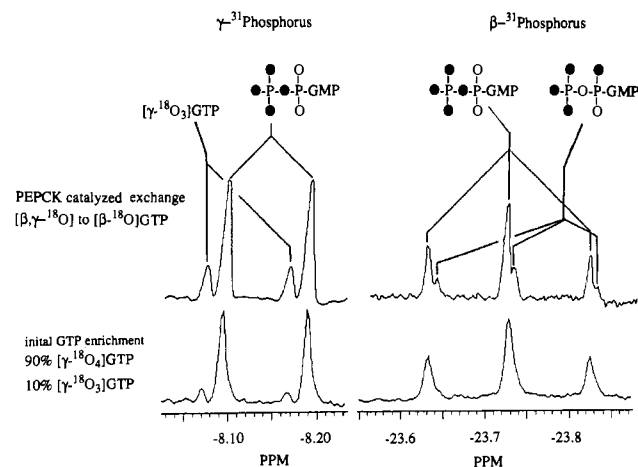


FIGURE 3:  $^{31}\text{P}$  NMR spectra for  $^{18}\text{O}$ -labeled GTP taken at 202.3 MHz in 40%  $\text{D}_2\text{O}$  at pH 9.0. The chemical shift was scaled relative to inorganic phosphate as an internal standard. The upper spectrum was determined after 90 min of the chicken liver P-enolpyruvate carboxykinase reaction in a solution containing  $46 \mu\text{M}$  oxaloacetate generated from excess malate and  $3.5 \text{ mM}$   $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  at pH 8.0 as described in the text. The lower spectrum is a control of  $3.5 \text{ mM}$   $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  from the same reaction mixture used for the upper scan, but before the reaction was initiated by the addition of enzyme.

Table II: Positional Isotope-Exchange Analysis of Chicken Liver P-enolpyruvate Carboxykinase

[OAA] ( $\mu\text{M}$ )	[MnCl <sub>2</sub> ] ( $\mu\text{M}$ )	time (min)	enzyme <sup>a</sup> (units)	fraction of GTP converted to GDP <sup>b</sup>	$\gamma\text{-P}$ of GTP PO <sup>18</sup> O <sub>3</sub> :P-[ <sup>18</sup> O <sub>4</sub> ] <sup>c</sup>
0	0	0	0	0	10:90
0	0	40	0.18	0	10:90
0	0	90	0.18	0	10:90
0	0	180	0.18	0	10:90
0	50	120	0.46	0	10:90
46	0	0	0	0	10:90
46	0	40	0.18	0.13	12:88
46	0	90	0.18	0.15	21:79
46	0	180	0.18	0.16	25:75
46	50	120	0.46	0.18	33:69
[oxalate] (mM)					
0.1	0	120	0.18	0	10:90
0.1	50	120	0.46	0	10:90

<sup>a</sup> The activity of P-enolpyruvate carboxykinase was determined in the positional isotope-exchange reaction mixture with oxaloacetate generated from malate and  $\text{NAD}^+$  by the malate dehydrogenase reaction. <sup>b</sup> In the absence of oxaloacetate the fraction of GTP converted into GDP was determined in  $^{31}\text{P}$  NMR spectra by measuring the peak corresponding to the  $\beta$ -phosphorus of GDP as described in the text. In the presence of oxaloacetate the fraction of GTP converted into GDP was determined by coupling to malate dehydrogenase as described in the text. <sup>c</sup> The fraction was determined by measuring the peak area corresponding to the  $\gamma$ -phosphorus of GTP in PO<sup>18</sup>O<sub>3</sub> and P<sup>18</sup>O<sub>4</sub> by using  $^{31}\text{P}$  NMR at 202.3 MHz as described in the text.

The positional exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  into  $[\beta\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  was determined with  $^{31}\text{P}$  NMR at 202.3 MHz based on an  $^{18}\text{O}$ -induced  $^{31}\text{P}$  chemical shift of  $-0.022 \text{ ppm}$  (upfield) in the  $\gamma$ -phosphorus and a difference of  $0.012 \text{ ppm}$  (downfield) in the  $\beta$ -phosphorus as shown in Figure 3. The experiments were carried out at  $30^\circ\text{C}$ , pH 8.0, in both the presence and absence of the  $V_{\max}$  activator  $\text{Mn}^{2+}$ , at  $50 \mu\text{M}$ . As shown in Tables II and III, when oxaloacetate is absent, no change in the  $^{31}\text{P}$  signal of the  $\gamma$ -phosphorus of GTP was observed for either chicken or rat liver P-enolpyruvate carboxykinases. Likewise, no changes were observed in the nonbridge- $^{18}\text{O}$  distribution in the  $\beta$ -phosphorus of GTP (data not shown). The results indicated no positional isotope exchange of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  to  $[\beta\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$  for

Table III: Positional Isotope-Exchange Analysis of Rat Liver P-enolpyruvate Carboxykinase with [ $\beta\gamma$ - $^{18}\text{O}$ , $\gamma$ - $^{18}\text{O}_3$ ]GTP and Excess Oxaloacetate

[OAA] (mM)	[MnCl <sub>2</sub> ] ( $\mu\text{M}$ )	time (min)	enzyme <sup>a</sup> (units)	fraction of GTP converted into GDP <sup>b</sup>	$\gamma$ -P of GTP PO[ $^{18}\text{O}_3$ ]:P-[ $^{18}\text{O}_4$ ] <sup>c</sup>
0	0	0	0	0	10:90
0	0	40	0.1	0	10:90
0	0	90	0.1	0	10:90
0	0	180	0.1	0	10:90
0	50	120	0.12	0	10:90
3	0	0	0	0	10:90
3	0	3	0.1	0.07	10:90
3	0	6	0.1	0.14	10:90
3	0	9	0.1	0.20	10:90
3	0	20	0.1	0.34	10:90
3	50	20	0.12	0.60	10:90
[oxalate] (mM)					
0.1	0	180	0.3	0	10:90
0.1	50	180	0.3	0	10:90

<sup>a</sup> The activity of P-enolpyruvate carboxykinase was determined in the positional isotope-exchange reaction mixture by using the coupled pyruvate kinase/lactate dehydrogenase assay as described in the text. <sup>b</sup> The fraction of GTP converted into GDP was determined in <sup>31</sup>P NMR spectra by measuring the peak corresponding to the  $\beta$ -phosphorus of GDP as described in the text. <sup>c</sup> The extent of  $^{18}\text{O}$  positional isotope exchange was determined by measuring the area of the peaks corresponding to the  $\gamma$ -phosphorus of GTP in PO[ $^{18}\text{O}_3$ ] and P[ $^{18}\text{O}_4$ ] by using <sup>31</sup>P NMR at 202.3 MHz as described in the text.

Table IV: Rates of [ $\beta\gamma$ - $^{18}\text{O}$ , $\gamma$ - $^{18}\text{O}_3$ ]GTP Positional Isotope Exchange (PIX) Relative to [ $8\text{-}^3\text{H}$ ]GDP and [ $^{32}\text{P}$ ]P-enolpyruvate Exchange

[Mn] ( $\mu\text{M}$ )	$V_{\text{pix}}^a/V_{\text{cat}}^b$	$V_{\text{GDP}\rightarrow\text{GTP}}^c/V_{\text{cat}}$	$V_{\text{PEP}\rightarrow\text{GTP}}^d/V_{\text{cat}}$
0	$0.040 \pm 0.016$	$0.050 \pm 0.011$	$0.024 \pm 0.003$
50	$0.035^e$	$0.052 \pm 0.004$	$0.033 \pm 0.001$

<sup>a</sup>  $V_{\text{pix}}$  is the average value of the observed positional isotope-exchange rate, determined according to Litwin and Wimmer (1979) as described under Materials and Methods. <sup>b</sup>  $V_{\text{cat}}$  is the catalytic activity of P-enolpyruvate carboxykinase in the reaction mixture for positional isotope exchange, determined as described in the text. <sup>c</sup>  $V_{\text{GDP}\rightarrow\text{GTP}}$  is the initial rate of GDP to GTP exchange determined from Figure 4A. <sup>d</sup>  $V_{\text{PEP}\rightarrow\text{GTP}}$  is the initial rate of P-enolpyruvate to GTP exchange determined from Figure 4B. <sup>e</sup> The value is from a single experimental determination.

chicken or rat liver P-enolpyruvate carboxykinases without oxaloacetate.

**Positional Isotope Exchange of [ $\beta\gamma$ - $^{18}\text{O}$ , $\gamma$ - $^{18}\text{O}_3$ ]GTP in the Presence of Oxaloacetate.** Positional isotope-exchange experiments with chicken liver P-enolpyruvate carboxykinase were performed with the concentration of oxaloacetate maintained at  $46\text{ }\mu\text{M}$  by the equilibrium of the malate dehydrogenase reaction. Exchange from the  $\beta\gamma$ - $^{18}\text{O}$  bridge oxygen to the  $\beta$ - $^{18}\text{O}$  position of GTP increased, beginning at 40 min, and this was accompanied with an increase in the amount of nonbridge  $^{18}\text{O}$  in the  $\beta$ -phosphorus of GTP (Table II). However, control experiments demonstrated the exchange of trace amounts of [ $8\text{-}^3\text{H}$ ]GDP and [ $^{32}\text{P}$ ]P-enolpyruvate into GTP coincident with positional isotope exchange. Positional isotope exchange was detected only under conditions where both [ $8\text{-}^3\text{H}$ ]GDP and [ $^{32}\text{P}$ ]P-enolpyruvate exchanged into GTP (Figure 4). The initial rates of the product-exchange reactions are within experimental error of the observed positional isotope exchange (Table IV). Thus, the reverse reaction, after product accumulation, is responsible for the observed positional isotope exchange.

These results were further confirmed in experiments with excess oxaloacetate in the reaction mixture. In these experiments, the oxaloacetate concentration was >20-fold above its  $K_m$  during the entire time course of the incubation, including loss by nonenzymatic decarboxylation. As shown in Table V,

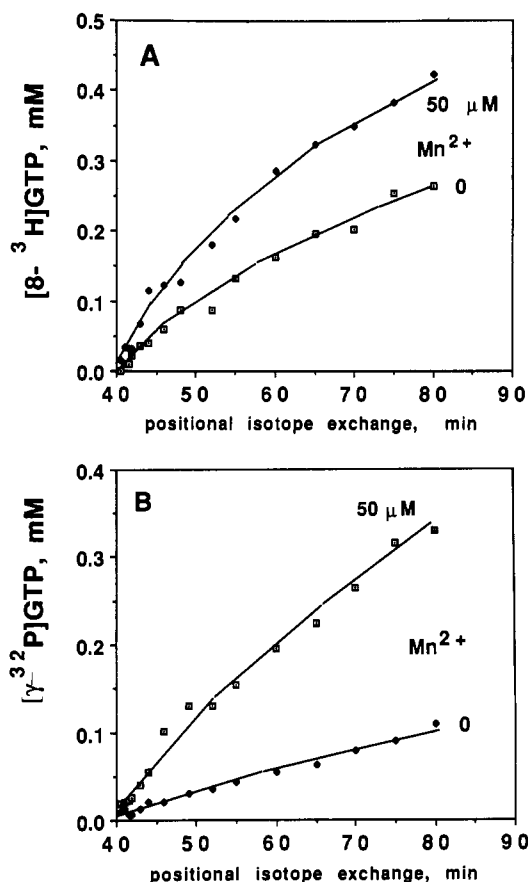


FIGURE 4: Equilibrium isotopic exchange of [ $8\text{-}^3\text{H}$ ]GDP and [ $^{32}\text{P}$ ]P-enolpyruvate into GTP with chicken liver P-enolpyruvate carboxykinase and oxaloacetate at  $46\text{ }\mu\text{M}$ . Trace amounts of (A) [ $8\text{-}^3\text{H}$ ]GDP ( $1\text{ }\mu\text{Ci}$ ) or (B) [ $^{32}\text{P}$ ]P-enolpyruvate ( $0.3\text{ }\mu\text{Ci}$ ) were added 40 min after initiation of the positional isotope-exchange reaction. This time corresponds to the period in which  $^{18}\text{O}$  positional isotope exchange begins (Table II). The concentration of [ $8\text{-}^3\text{H}$ ]GTP and [ $\gamma$ - $^{32}\text{P}$ ]GTP represents the exchange of labeled GDP and P-enolpyruvate into the pool of GTP, which was approximately  $3.0\text{ mM}$  under these conditions. The reactant product concentrations were determined as described in the text.

Table V: Positional Isotope-Exchange Analysis of Chicken Liver P-enolpyruvate Carboxykinase with [ $\beta\gamma$ - $^{18}\text{O}$ , $\gamma$ - $^{18}\text{O}_3$ ]GTP and Excess Oxaloacetate

[OAA] (mM)	[MnCl <sub>2</sub> ] ( $\mu\text{M}$ )	time (min)	enzyme <sup>a</sup> (units)	fraction of GTP converted to GDP <sup>b</sup>	$\gamma$ -P of GTP PO[ $^{18}\text{O}_3$ ]:P-[ $^{18}\text{O}_4$ ] <sup>c</sup>
3	0	0	0	0	10:90
3	0	3	0.1	0.37	10:90
3	0	6	0.1	0.41	10:90
3	0	9.5	0.1	0.41	10:90
3	0	20	0.1	0.48	10:90
3	50	5	0.15	0.37	10:90
3	50	10	0.15	0.41	10:90

<sup>a</sup> The activity of P-enolpyruvate carboxykinase in the positional isotope-exchange reaction mixture was determined with the pyruvate kinase/lactate dehydrogenase coupling enzyme assay as described in the text. <sup>b</sup> The fraction of GTP converted into GDP was determined from the <sup>31</sup>P NMR spectra by measuring the peak corresponding to the  $\beta$ -phosphorus of GDP as described in the text. <sup>c</sup> The fraction was determined by measuring the area of peaks corresponding to the  $\gamma$ -phosphorus of GTP in PO[ $^{18}\text{O}_3$ ] and P[ $^{18}\text{O}_4$ ] by using <sup>31</sup>P NMR at 202.3 MHz as described in the text and Figure 3.

no positional isotope exchange was observed even when 48% of GTP was converted to GDP in the absence of  $\text{Mn}^{2+}$ . Similar results were obtained in the presence of  $50\text{ }\mu\text{M}$   $\text{Mn}^{2+}$  after 41% of the GTP was converted to products.

Positional isotope exchange with rat liver P-enolpyruvate carboxykinase was also determined in experiments with the

direct addition of excess oxaloacetate. The amount of oxaloacetate added was also sufficient to maintain the concentration >20-fold above its  $K_m$  during the time course of the reaction (Table III). No positional isotope exchange was observed after 34% of GTP was converted to products in the absence of  $Mn^{2+}$  or when 60% of GTP was converted to products in the presence of 50  $\mu M$   $Mn^{2+}$ .

**Positional Isotope Exchange of  $[\beta\gamma\text{-}^{18}O, \gamma\text{-}^{18}O_3]GTP$  with Oxalate.** Positional isotope-exchange experiments with oxalate as a nonreactive analogue of oxaloacetate were carried out with both chicken and rat liver P-enolpyruvate carboxykinases. No positional isotope exchange was observed with 1 mM oxalate for either enzyme in the presence or absence of 50  $\mu M$   $Mn^{2+}$  after extensive incubation (Tables II and III).

## DISCUSSION

**MgGTP Trapping.** Chicken and rat liver P-enolpyruvate carboxykinases trap labeled MgGTP with stoichiometries of 1.0 and 0.6, respectively. These results establish that (a) bound MgGTP (or at least 60% in the case of the rat liver enzyme) is catalytically competent, (b) MgGTP can bind prior to oxaloacetate in both enzymes, since oxaloacetate is absent during the MgGTP-binding step, (c) MgGTP is released slowly from the E-MgGTP-OAA complex compared to the catalytic rate, and (d) MgGTP cannot dissociate at a kinetically significant rate from the E-MgGTP-OAA complex with chicken liver enzyme. On the basis of the complete trapping and high commitment to catalysis of MgGTP bound to the chicken enzyme, it is likely that the 60% trapping by the rat enzyme represents relative catalytic and desorption rates, rather than a mixture of 60% catalytically competent and 40% catalytically unreactive species of MgGTP.

The MgGTP-trapping experiments allow determination of the partition ratio  $k_6/k'_{11}$  in Scheme I. The constant  $k'_{11}$  includes catalysis and product release and therefore must be equal to or greater than the turnover number of 18  $s^{-1}$ . Since approximately 60% of the enzyme-bound MgGTP is trapped with the rat enzyme, release of MgGTP from the E-MgGTP-OAA complex ( $k_6$  in Scheme I) is in the range of 9–12  $s^{-1}$ . The limits on the rate constant for the release of MgGTP from E-MgGTP ( $k_2$  in Scheme I) are 26–92  $s^{-1}$ , and the dissociation constant for the E-MgGTP complex,  $k_2/k_1$ , is  $5 \pm 2 \mu M$ . From these values, the association rate of MgGTP with free enzyme ( $k_1$  in Scheme I) can be determined to be in the range of  $(3\text{--}20) \times 10^6 M^{-1} s^{-1}$ . These constants are summarized with other kinetic constants for rat liver P-enolpyruvate carboxykinase in Scheme III. The slow release of MgGTP from the ternary complex and the resultant partial trapping of the nucleotide are consistent with the steady-state random kinetic mechanism proposed previously (Jomain-Baum & Schramm, 1978). The rate constant of  $(3\text{--}20) \times 10^6 M^{-1} s^{-1}$  for E-MgGTP formation is somewhat smaller than the diffusion limit of  $\sim 10^8 M^{-1} s^{-1}$  for a protein and substrate the sizes of P-enolpyruvate carboxykinase and MgGTP. Thus, rapid structural changes are likely to occur in concert with MgGTP binding. Limits on the release rate of MgGTP from the E-MgGTP complex indicate that release is slow, accounting for the relatively tight dissociation constant of 5  $\mu M$ . The ability to trap 60% of the enzyme-bound MgGTP from the E-MgGTP-OAA complex in the presence of a saturating concentration of oxaloacetate and the slow release of MgGTP from this complex establish that the presence of oxaloacetate hinders MgGTP release.

Trapping of MgGTP with the enzyme from chicken liver is consistent with an ordered mechanism with MgGTP binding first. The nucleotide is unable to dissociate from the enzyme

in the presence of bound oxaloacetate. Thus, all bound MgGTP is converted to product when excess oxaloacetate is present. MgGTP binds free enzyme with a rate constant lower than the diffusion limit, and dissociation is slow, resulting in a dissociation constant of 7  $\mu M$ . Although a very slow rate of MgGTP release from the E-MgGTP-OAA complex would not have been detected in these experiments, a rate of 1  $s^{-1}$  would have been observed. Thus, at saturating oxaloacetate, enzyme-bound MgGTP is completely committed to catalysis. These results are consistent with the steady-state kinetic proposal of Felicioli et al. (1970) for a steady-state ordered mechanism for the chicken enzyme with ITP being added prior to oxaloacetate. Product release was proposed to occur in the order  $CO_2$ , P-enolpyruvate, and IDP. Apart from the extent of catalytic commitment of MgGTP, the kinetic and rate constants of the chicken and rat enzymes are similar (Scheme III). Thus, release of MgGTP from the E-MgGTP complex for the chicken enzyme is also slow at 26  $s^{-1}$ .

NMR studies with the chicken enzyme and MgGTP in the presence of enzyme-bound  $Mn^{2+}$  have provided values of greater than  $2.1 \times 10^{10} M^{-1} s^{-1}$  for association of the enzyme- $Mn^{2+}$  complex with MgGTP and  $4 \times 10^4 s^{-1}$  for dissociation of MgGTP from the complex (Lee & Nowak, 1984). These values differ substantially from the results reported here and the normal assumptions for diffusion-controlled association rates of  $10^8\text{--}10^9 M^{-1} s^{-1}$  (Eigen & Hammes, 1963). The distances between the enzyme-bound  $Mn^{2+}$  and the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -phosphates of MgGTP were reported to be 5–6 Å on the basis of the rapid-exchange assumption for MgGTP. These values may need to be reevaluated on the basis of the relatively slow release of MgGTP from the enzyme- $Mn^{2+}$  complex shown in Scheme III.

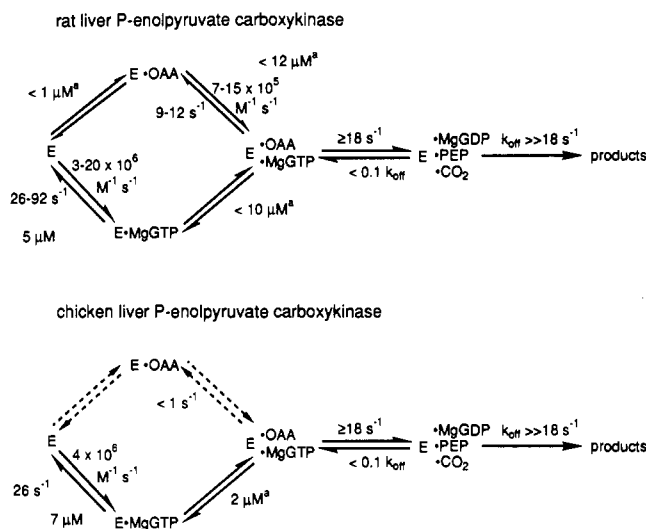
**$[\beta, \gamma\text{-}^{18}O]GTP$  Positional Isotope Exchange in the Absence of Oxaloacetate.** In the absence of oxaloacetate, neither the chicken nor the rat liver P-enolpyruvate carboxykinases were capable of causing exchange from the  $\beta, \gamma\text{-}^{18}O$  bridge oxygen to the  $\beta\text{-}^{18}O$  position. This exchange should occur if the enzyme catalyzes a  $GTP \rightleftharpoons GDP$  exchange as previously reported for the rat liver enzyme with  $Mn^{2+}$  as the sole divalent cation (Jomain-Baum & Schramm, 1978; see Scheme II). Attempts to repeat the  $GTP \rightleftharpoons GDP$  exchange with the highly purified P-enolpyruvate carboxykinases used in this study gave no exchange with MgGTP as substrate. Thus, the previous result is likely due to an enzymatic impurity that catalyzes  $GTP \rightleftharpoons GDP$  exchange or is catalyzed by P-enolpyruvate carboxykinase when both required divalent metals are  $Mn^{2+}$ .

The results of the positional isotope-exchange experiments reported here establish that any such enzymatic phosphorylation is not part of the required catalytic cycle for P-enolpyruvate carboxykinase when MgGTP is used as a substrate with  $Mn^{2+}$  as activator. The transfer of the  $\gamma$ -phosphoryl group of nucleotide triphosphates occurs with stereochemical inversion for both the guinea pig mitochondrial and the rat cytosolic P-enolpyruvate carboxykinases (Sheu et al., 1984; Konopka et al., 1986). These reports are consistent with the lack of positional isotope exchange reported here in the absence of oxaloacetate.

A requirement for phosphoryl transfer could be occupation of the binding site for the carboxylic acid. Addition of oxalate, a competitive inhibitor with respect to oxaloacetate with a  $K_i$  value of <20  $\mu M$  (Ash et al., 1990), is unable to support positional isotope exchange. The sum of these results indicates that phosphoryl transfer to enzyme does not occur in the normal catalytic cycle of mitochondrial or cytosolic P-enolpyruvate carboxykinases.



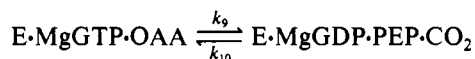
Scheme III: Kinetic Reaction Mechanisms for Rat and Chicken Liver P-enolpyruvate Carboxykinases<sup>a</sup>



<sup>a</sup> The kinetic constants obtained from trapping and positional isotope-exchange experiments are summarized together with several kinetic constants obtained from steady-state kinetic studies. The superscript a represents constants based on steady-state kinetic parameters for the enzyme with MgGTP and 20  $\mu$ M free  $\text{MnCl}_2$  (Schramm et al., 1981). The kinetic constant of 2  $\mu$ M for the chicken liver enzyme is based on experiments with MgGTP fixed at 50 and 100  $\mu$ M in the presence of 50  $\mu$ M  $\text{MnCl}_2$  (Ash et al., 1990). The dashed arrows for the chicken enzyme indicate that these steps are kinetically insignificant.

$[\beta\gamma\text{-}^{18}\text{O}]\text{GTP}$  Positional Isotope Exchange during Catalysis. Positional isotope exchange occurs during initial reaction rate conditions when (a) phosphoryl transfer in the central complexes is rapidly reversible, (b) the release rate of substrates is similar or slower than that for products, and (c) the torsional rotation of the  $\beta$ -phosphoryl group can occur following transfer of the  $\gamma$ -phosphoryl group [e.g., Raushel and Villafranca (1988)]. The chemical steps that make this exchange possible are shown in Scheme II. No such exchange was observed with either the rat or the chicken P-enolpyruvate carboxykinase in these studies.

Determination of the equilibrium constants for a number of enzyme-bound phosphoryl-transfer complexes has indicated that this constant is often near unity (Cohn & Reed, 1982). For P-enolpyruvate carboxykinase this equilibrium is for the complexes



as shown in Scheme I. The turnover numbers for P-enolpyruvate carboxykinases are approximately the same for forward and reverse reactions. Thus it is reasonable to expect that  $k_9$  and  $k_{10}$  have similar rates. Under these conditions, positional isotope exchange from the  $\beta\gamma\text{-}^{18}\text{O}$  of GTP to the  $\beta\text{-}^{18}\text{O}$  of GTP would be expected unless the  $\text{E}\cdot\text{MgGDP}\cdot\text{PEP}\cdot\text{CO}_2$  complex were too short-lived to reverse via  $k_{10}$ . This situation is illustrated in Scheme III where the rapid loss of any one of the products prevents the quaternary complex from reacting to re-form MgGTP at the catalytic site. It should be noted that the same result would be seen if a rapid equilibrium prevailed between  $\text{E}\cdot\text{MgGTP}\cdot\text{OAA}$  and  $\text{E}\cdot\text{MgGDP}\cdot\text{PEP}\cdot\text{CO}_2$  where the  $\beta$ -phosphoryl oxygens were not free to undergo torsional rotation and the  $\beta\text{-}^{18}\text{O}$  of GDP that originated as the  $\beta\gamma$ -bridge oxygen always reacted to regenerate the original  $^{18}\text{O}$ -label pattern of  $[\beta\gamma\text{-}^{18}\text{O}, \gamma\text{-}^{18}\text{O}_3]\text{GTP}$ . The rate constant for torsional rotation of a phosphoryl group

in a metal-nucleotide complex can be estimated from the dissociation constant for the complex and is at least 4 orders of magnitude more rapid than the turnover number of P-enolpyruvate carboxykinase. The most likely explanation for the lack of positional isotope exchange is therefore the release of any of the products at a rate  $>180\text{ s}^{-1}$ . Carbon dioxide is a good candidate for rapid release because of its relatively large product inhibition constant (Jomain-Baum & Schramm, 1978).

In summary, the P-enolpyruvate carboxykinases demonstrate high commitment factors for conversion of enzyme-bound MgGTP to products. The release of bound MgGTP is slow, causing the enzyme to bind the nucleotide tightly. Product release appears to be rapid compared to the rate of MgGTP release and the rate of the reverse reaction. The enzymes from rat and chicken are similar in overall rate and dissociation constants except that the chicken enzyme has a higher preference for the ordered binding of MgGTP before oxaloacetate. The large commitment factors for the P-enolpyruvate carboxykinases ensure that most molecules of MgGTP which encounter the enzyme will be efficiently converted to products.

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## Engineering Subtilisin and Its Substrates for Efficient Ligation of Peptide Bonds in Aqueous Solution

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**ABSTRACT:** Protein engineering techniques were used to construct a derivative of the serine protease subtilisin that ligates peptides efficiently in water. The subtilisin double mutant in which the catalytic Ser221 was converted to Cys (S221C) and Pro225 converted to Ala (P225A) has 10-fold higher peptide ligase activity and at least 100-fold lower amidase activity than the singly mutated thiolsubtilisin (S221C) that was previously shown to have some peptide ligase activity [Nakatsuka, T., Sasaki, T., & Kaiser, E. T. (1987) *J. Am. Chem. Soc.* 109, 3808-3810]. A 1.5-Å X-ray crystal structure of an oxidized derivative of the double mutant (S221C/P225A) supports the protein design strategy in showing that the P225A mutation partly relieves the steric crowding expected from the S221C substitution, thus accounting for its improved catalytic efficiency. Stable and synthetically reasonable alkyl ester peptide substrates were prepared that rapidly acylate the S221C/P225A enzyme, and aminolysis of the resulting thioacyl-enzyme intermediate by various peptides is strongly preferred over hydrolysis. The efficiency of aminolysis is relatively insensitive to the sequence of the first two residues in the acyl acceptor peptide whose  $\alpha$ -amino group attacks the thioacyl-enzyme. To obtain greater flexibility in the choice of coupling sites, a set of three additional peptide ligases were engineered by introducing mutations into the parent ligase (S221C/P225A) that were previously shown to change the specificity of subtilisin for the residue nearest the acyl bond (the P<sub>1</sub> residue). The specificity properties of the parent ligase and derivatives of it paralleled those of wild type and corresponding specificity variants. The set of specific peptide ligases should be useful for blockwise synthesis or semisynthesis of proteins in aqueous solution.

Chemical approaches for synthesis and engineering of proteins offer many advantages to recombinant methods in that one can incorporate nonnatural or selectively labeled amino acids. However, peptide synthesis is practically limited to small proteins (typically <50 residues) due to the accumulation of side products and racemization that complicate product purification and decrease yields [for recent reviews see Kaiser (1989) and Offord (1987)].

Proteolytic enzymes, in particular serine proteases, have been used as alternatives to synthetic peptide chemistry because of their stereoselective properties and mild reaction conditions [for reviews see Kullmann (1987) and Chaiken (1981)]. Such enzymes have also been used to complement chemical coupling methods and allow larger proteins to be synthesized by blockwise enzymatic coupling of synthetic fragments [Inouye et al., 1979; for review see Chaiken (1981)]. However, the narrow substrate specificities and hydrolytic activities of serine proteases have limited their use in peptide synthesis.

A central problem in the use of serine proteases for peptide synthesis is that hydrolysis of the acyl-enzyme intermediate is strongly favored over aminolysis (Figure 1). The partitioning may be effectively shifted to favor aminolysis by

catalyzing the reaction in mixed or pure organic solvents (Coletti-Previero et al., 1969; Barbas et al., 1988). However, enzymes are insoluble and often less stable in organic solvents (Wong et al., 1990), and kinetic activation barriers are higher for the charged transition states involved. An alternative approach was presented by Kaiser and co-workers (Nakatsuka et al., 1987) who showed that thiolsubtilisin, a derivative of the bacterial serine protease in which the active-site Ser221 was chemically converted to a Cys (S221C), shifted the preference for aminolysis relative to hydrolysis by >1000-fold. This is likely due to the inherently greater kinetic lability of thioesters toward amines as opposed to water (Chu & Mautner, 1966). On the basis of similar principles, Wu and Hilvert (1989) showed that selenolsubtilisin had a 14000-fold shift in preference for aminolysis over hydrolysis. However, the catalytic efficiencies by either thiol- or selenolsubtilisin for aminolysis of chemically activated esters are about 10<sup>2</sup>-10<sup>4</sup>-fold below the esterase activity of wild-type subtilisin. Thus, to promote acylation of thiolsubtilisin a chemically active *p*-chlorophenyl ester was used to activate an octapeptide for ligation with a tetrapeptide in 50% DMF. However, such activated esters present synthetic difficulties as well as creating substrates prone to uncatalyzed hydrolysis in aqueous solvents (Nakatsuka et al., 1987).

Here we describe the design and high-resolution structure of a variant of thiolsubtilisin whose ligase activity is enhanced

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