Phosphorus-31 Nuclear Relaxation Rate Studies of the Nucleotides on Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: The interactions of nucleotide substrates with the enzyme phosphoenolpyruvate carboxykinase and its Mn^{2+} complex were investigated by several methods. Direct binding shows the formation of stoichiometric complexes. The presence of Mn^{2+} increases the affinity of the enzyme for nucleotide. A higher affinity for GTP (K_d < 2 μM) than for GDP (K_d = 15 μM) was determined. Solvent proton relaxation rate studies indicate no substantial difference in titration curves for free nucleotide or for Mg–nucleotide to the enzyme–Mn complex. The effect of Mn^{2+} on the ^{31}P relaxation rates of IDP and of ITP in the binary Mn–nucleotide complex indicates the formation of direct coordination complexes. The distances of the α- and β-^{31}P of IDP to Mn^{2+} are identical (3.5 Å). The Mn^{2+} distance to the β- and γ-^{31}P of ITP is also identical (3.7 Å) and is 0.2 Å further from the α-phosphorus. In the presence of P-enolpyruvate carboxykinase, the effect of Mn^{2+} on the ^{31}P relaxation rates was measured at 40.5 MHz and at 121.5 MHz. The dipolar correlation time was calculated to be 0.6–5.4 ns, depending upon assumptions made. The Mn^{2+} to phosphorus distances indicate the nucleotide substrates form a second sphere complex to the bound Mn^{2+}. From $1/T_2$ measurements, electron delocalization from Mn^{2+} to the phosphorus atoms is indicated; this effect occurs although direct coordination does not take place. The exchange rate of GTP from the enzyme–Mn complex ($k_{off} = 4 \times 10^4$ s^{-1}) is rapid compared to k_{cat} with a lower energy of activation (9.2 kcal/mol) than for catalytic turnover. The bound Mn^{2+} must exert electronic effects across long distances at the catalytic site.

Phosphoenolpyruvate (P-enolpyruvate)[1] carboxykinase [GTP/ITP oxalacetate carboxylase (transphosphorylating), EC 4.1.1.32] catalyzes the following reversible reaction:

$$\text{oxalacetate} + \text{ITP (GTP)} \overset{Mn^{2+}}{\rightleftharpoons} \text{P-enolpyruvate} + \text{IDP (GDP)} + CO_2 \quad (1)$$

The primary role of this enzyme in higher organisms appears to be the catalysis of the formation of P-enolpyruvate from oxalacetate as the first committed step in gluconeogenesis.

In addition to the physiological reaction (1), P-enolpyruvate carboxykinase catalyzes the irreversible decarboxylation of oxalacetate to form pyruvate (Noce & Utter, 1975).

$$\text{oxalacetate} \xrightarrow{\text{GDP (IDP)}} \text{pyruvate} + CO_2 \quad (2)$$

The physiological significance, if any, of reaction 2 is not known.

The results of kinetic studies have demonstrated two roles for the divalent cation (Lee et al., 1981) for the readily reversible reaction (1). The cation binds to the enzyme to form the active enzyme–metal complex; Mn^{2+} is the most effective cation to activate the enzyme. The cation can also form a metal–nucleotide complex which serves as the substrate for the reaction. The kinetic results indicate that Mg–ITP is a better substrate than is Mn–ITP. The kinetic data suggest that free nucleotide also serves as a substrate for this enzyme.

The binding of Mn^{2+} to P-enolpyruvate carboxykinase and of substrates to the P-enolpyruvate carboxykinase–Mn complex isolated from chicken liver has been studied by using EPR and water proton relaxation rate (PRR) techniques. From these studies it was proposed that IDP binding does not occur directly to the enzyme-bound Mn^{2+} of the binary enzyme–Mn complex. ITP does interact directly with the Mn^{2+} of the binary enzyme–Mn complex, however, by displacing half of the water molecules on Mn^{2+} (Hebda & Nowak, 1982b).

In the present work, we have used ^{31}P NMR in order to map the active site of P-enolpyruvate carboxykinase isolated from chicken liver mitochondria. This paper examines the effect of the bound Mn^{2+} on the nuclear relaxation rates of the nucleotides by ^{31}P NMR studies. These studies have been performed as a function of Mn^{2+} concentration and of temperature and frequency. The results of these studies have yielded values for the correlation time for the Mn–nucleotide interactions and led to a determination of the kinetic and structural parameters which describe the ternary enzyme–Mn–nucleotide complexes.

This paper also reports the binding of nucleotides and Mg–nucleotide complexes to P-enolpyruvate carboxykinase by gel separation and PRR techniques.

Experimental Procedures

Materials

The nucleotides IDP, ITP, GDP, GTP, and ADP and PEP and NADH were purchased from Sigma. [8-^{3}H]GDP and [8-^{14}C]GTP were purchased from Amersham Co. (*Z*)-Phosphoenol-α-ketobutyrate (*Z*-PEB) was a gift from Dr. Thomas H. Duffy, University of Notre Dame. The oxalacetate was obtained either from Calbiochem or Sigma. The $MnCl_2$ and $MgCl_2$ were purchased from Baker. Tris base was obtained from Schwarz/Mann, and Chelex-100 resin was purchased from Bio-Rad. The enzymes pyruvate kinase, lactate

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[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; EPR, electron paramagnetic resonance; PRR, solvent proton relaxation rates; PEB, phosphoenol-α-ketobutyrate; Tris, tris(hydroxymethyl)aminomethane.

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dehydrogenase, and malate dehydrogenase were purchased from Boehringer. P-enolpyruvate carboxykinase was purified from chicken liver mitochondria by a modification of the method described by this laboratory (Hebda & Nowak, 1982a). The calcium phosphate adsorption fractionation was replaced by column chromatography by using DEAE Bio-Gel A (no prior preparation of resin needed). The extract (1200 units, 4500 mg of protein) was loaded onto the anion-exchange column (2.5 × 40 cm) at pH 8.0 in 5 mM potassium phosphate buffer. The column was washed with 400 mL of 5 mM potassium phosphate buffer, pH 8.0. Approximately 160 units of activity came off this wash. Enzyme was eluted with 5 mM potassium phosphate buffer, pH 7.0 (700 units, 475 mg of protein). The enzyme was then loaded on an Affi-Gel Blue column (Bio-Rad) (2.5 × 10 cm) (1.5 mL/min). This column replaces the Blue Dextran column described by Hebda & Nowak (1982a). This enzyme purification was faster than the preceding procedure and gave protein >99% pure and a specific activity of 7.1–7.3 units/mg. The concentration of the enzyme solution was determined by using the extinction coefficient $\epsilon_{1\%}^{280} = 16.5 \pm 0.1$ and a molecular weight of 73 000 (Hebda & Nowak, 1982a). The specific activity (25 °C) of the enzyme was determined by measuring the carboxylation of P-enolpyruvate to yield oxalacetate using the MDH coupled assay (Lee et al., 1981). The specific activity is defined as units of enzyme activity per milligram of protein where 1 unit is the amount of enzyme catalyzing the formation of 1 μmol of product mL^{-1} min^{-1} under experimental conditions. The enzyme with specific activity of greater than 6.0 was used for experiments. The concentration of the nucleotide was determined enzymatically (Lee et al., 1981). The concentration of Mn^{2+} was determined by PRR measurements of aqueous Mn^{2+} solutions.

Methods

Dissociation Constants of Mn–Nucleotide Complexes. The binding of Mn^{2+} to nucleotide to determine the dissociation constants of the Mn–nucleotide complexes was measured by electron paramagnetic resonance spectroscopy under the same conditions as previously described (Hebda & Nowak, 1982b). The data were treated in the form of a Scatchard plot (Scatchard, 1949).

Binding of Nucleotide and Mg–Nucleotide to P-enolpyruvate Carboxykinase. The binding of nucleotide and Mg–nucleotide to the various enzyme complexes (enzyme, enzyme–Mn, enzyme–Mn–substrate) was determined by using a gel separation technique (Hirose & Kano, 1971). Sephadex G-50-80 (Sigma) was treated as previously described (Hirose & Kano, 1971). To 50 mg of the dried Sephadex G-50-80 was added 0.4 mL of 0.065 M Tris–acetate buffer (pH 7.4). After swelling of the gel was completed by standing overnight, 0.1 mL of the buffer solution containing the enzyme and [8-^{3}H]GDP or [8-^{14}C]GTP was added to the gel solution. A fixed concentration of metal and of substrate analogue (*Z*-PEB) were also added in experiments where stated. The solution was incubated for 2–3 min at room temperature with continuous stirring. A sample of 0.05 mL of the solution outside the gel was withdrawn with a micropipet and added to 2.0 mL of an aqueous scintillation fluid cocktail which contained 1.0 g of Omni Scint, 250 mL of toluene, and 125 mL of Triton X-100. The radioactivity was determined by using a Packard Tri-Carb liquid scintillation counter, Model 3375. The inside and outside volumes of the gel solution were determined separately by using the enzyme and GDP as controls. The data were treated in the form of a Scatchard plot (Scatchard, 1949).

For an investigation of the structure of enzyme–Mn–nucleotide complexes, PRR rates were measured as a function of the formation of the respective complexes as previously described (Hebda & Nowak, 1982b). The titrations of enzyme–Mn were performed with an identical sample which also contained the nucleotide to be titrated. Increments of the second sample were then titrated into the first so that no change in enzyme or Mn^{2+} concentration occurred, and the PRR was then measured. The dissociation constants for the ligand from the ternary enzyme–Mn–ligand complex, K_3, the dissociation constant for the enzyme–ligand complex, K_s, and the enhancements of the ternary complexes, ϵ_T, were determined by a computer fit to the PRR titration data which minimized the percent standard deviation of ϵ_T (Reed et al., 1970).

^{31}P Relaxation Rate Measurements. The spectra at 40.5 MHz were obtained with a Varian XL-100-15 spectrometer interfaced to a TTI pulse system and Nicolet 1080 computer. The spectra at 121.5 MHz were obtained with a Nicolet NTC 300 spectrometer interfaced to a 239 A pulse system and 1180 E computer. The experiments were run in 12- (XL-100) and 10-mm (NTC 300) sample tubes that contained 2.0–2.5-mL sample volume. Teflon plugs (Wilmad Co.) were used as a vortex suppressor. Both spectrometers were field frequency locked on internal ^{2}H present as ^2H_2O in the solvent. The spectra obtained with the XL-100 were proton decoupled as were the spectra obtained with the NTC-300 for the temperature-dependent studies. All the experiments were performed at 20 ± 1 °C unless otherwise stated. The $1/T_1$ values were measured by the inversion recovery method (Allerhand et al., 1971; Vold & Waugh, 1968). The $1/T_2$ values were estimated from the line widths of the resonance of the normal Fourier-transformed spectra from the following relationship:

$$1/T_2 = \pi(\nu_{1/2} - B) \quad (3)$$

where $\nu_{1/2}$ is the spectral line width at half-height in hertz and B is the artificial line broadening obtained when the spectra were processed. Values for B were usually 0.5 Hz.

In the Mn^{2+} titration experiments, microliter quantities of $MnCl_2$ solutions were titrated into buffered solutions of the specific sample, and $1/T_1$ and $1/T_2$ values for each resonance were measured at each increment of $MnCl_2$ added. In the studies of the temperature dependence of $1/T_2$, the temperature was varied by passing cooled nitrogen over a thermostated heating filament before it arrived at the sample zone. The temperature at the sample within the probe was measured directly by means of a thermocouple. In each experiment performed, the concentration of ligand and of enzyme were chosen such that virtually 100% of the Mn^{2+} added is in the enzyme–Mn–nucleotide complex. These calculations were based on dissociation constants measured in this paper and a K_D for Mn–enzyme of 50 μM (Hebda & Nowak, 1982b). The calculations were performed by using the program that was used to fit the PRR data which takes into account all possible equilibria and the binding parameters (K_3 and K_s) which gave a "best fit" for the data. Binding of Mn^{2+} to the enzyme in the presence of nucleotide is enhanced by 2 orders of magnitude. The frequency dependence of the $1/T_1$ values verify the results of our calculations (see below).

Detailed descriptions of the analysis of nuclear relaxation rate data have been previously described in detail [i.e., see Mildvan & Engle (1972) and Nowak (1981)]. The Luz–Meiboom and Swift–Connick equations (Luz & Meiboom, 1964; Swift & Connick, 1962) relate the normalized measured relaxation rates [$1/(pT_p)$] to the relaxation times of the nuclei of the ligands in the metal complex, T_{1M} and T_{2M}, and the

mean residence time, τ_m, of the ligand in the metal complex.

$$\frac{1}{pT_{1p}} = \frac{1}{T_{1M} + \tau_m} \quad (4)$$

$$\frac{1}{pT_{2p}} = \frac{1}{T_{2M} + \tau_m} \quad (5)$$

The longitudinal relaxation rate of the nucleus is described by the simplified form of the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) as

$$1/T_{1M} = \left(\frac{C}{r^6}\right) f(\tau_c) \quad (6)$$

and the transverse relaxation rate as

$$1/T_{2M} = \left(\frac{c}{2r^6}\right) f'(\tau_c) + SF \quad (7)$$

The term C is a collection of constants, r is the electron-nuclear distance, and $f(\tau_c)$ and $f'(\tau_c)$ are correlation time functions. The term SF represents a scalar function.

Calculation of τ_c and Distance r. When the longitudinal relaxation rate is in fast exchange ($\tau_m \ll T_{1M}$), the value of r can be calculated by using eq 6. Since $f(\tau_c)$ is dependent upon the Larmor frequency, a measure of $1/T_{1M}$ for the nucleus in question as a function of frequency can yield values for τ_c, thus allowing the calculation of r.

Chemical Exchange Rates. Equations 4 and 5 describe the effects of chemical exchange ($1/\tau_m$) on relaxation. If relaxation is dominated by τ_m, then Arrhenius behavior is expected when a temperature dependence of the relaxation rate is measured. Exchange rates and their energy barriers can be estimated from such a study.

Results

Various Mn–Nucleotide Dissociation Constants. The binding of Mn^{2+} to guanosine and to inosine nucleotides was measured by EPR spectroscopy. The data obtained were treated according to Scatchard (1949). The formation of Mn–nucleotide complexes occurs with a 1:1 stoichiometry for each of the nucleotides studied under experimental conditions (0.065 M Tris–acetate buffer, pH 7.4; 0.1 M KCl) analogous to those that exist for kinetic studies. The results yield dissociation constants for Mn^{2+} complexes with IDP [$(8.5 \pm 0.68) \times 10^{-5}$ M], GDP [$(4.5 \pm 0.38) \times 10^{-5}$ M], ITP [$(1.1 \pm 0.27) \times 10^{-5}$ M], and GTP [$(2.2 \pm 0.27) \times 10^{-5}$ M].

Binding of Nucleotide and Mg–Nucleotide by P-enolpyruvate Carboxykinase. The binding of GDP and GTP to P-enolpyruvate carboxykinase under several experimental conditions was measured by using an equilibrium gel separation technique (Hirose & Kano, 1971). The nucleotides, GDP, and GTP were shown to bind to P-enolpyruvate carboxykinase in the absence and in the presence of Mn^{2+}. The binding data were treated in the form of a Scatchard plot; examples of two experiments are shown in Figure 1. The dissociation constants for the complexes studied are summarized in Table I. Under experimental conditions with P-enolpyruvate carboxykinase having a very weak affinity for Mg^{2+} [$K_A \simeq 2$ mM (Lee et al., 1981)] only a small fraction of the enzyme exists as the Mg–enzyme complex. The results show that the nucleotide binds to the enzyme with a 1:1 stoichiometry under the various experimental conditions. The presence of Mn^{2+} increases the affinity of the enzyme for nucleotide and for Mg–nucleotide. The presence of the P-enolpyruvate analogue *Z*-PEB, which acts as a competitive inhibitor vs. P-enolpyruvate (Duffy et al., 1982), also increases

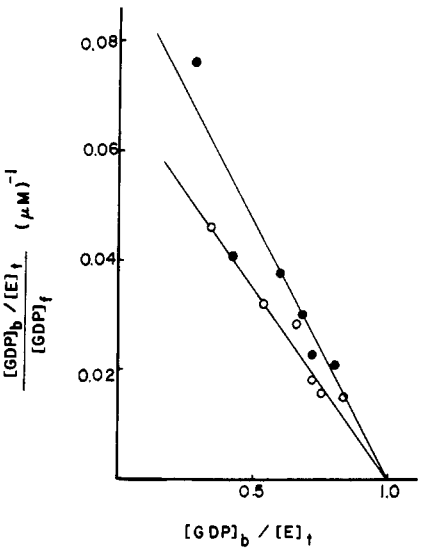

FIGURE 1: Scatchard plot of GDP binding to P-enolpyruvate carboxykinase in the presence and absence of Mn^{2+}. The binding of [8-^{3}H]GDP was measured as described under Methods. Each sample contained 3.7×10^{-5} M enzyme in 65 mM Tris, pH 7.4. The concentration of GDP was varied over a range of 8.9×10^{-6} to 7.1×10^{-5} M in the absence of Mn^{2+} (○) and over a range of 8.9×10^{-6} to 4.5×10^{-5} M in the presence of 2×10^{-4} M Mn^{2+} (●).

Table I: Binding of Nucleotide and M^{2+}–Nucleotide to P-enolpyruvate Carboxykinase

experimental conditions	complex	K_d (μM)
no metal	enzyme–GDP	14.5 ± 1.8
200 μM Mn^{2+}	enzyme–Mn–GDP	10.6 ± 1.4
$[Mg^{2+}] = 3[GDP]$	enzyme–Mg–GDP	10.4 ± 1.8
$[Mg^{2+}] = 3[GDP] + 60$ μM Mn^{2+}	enzyme–Mn–Mg–GDP	5.6 ± 1.0
no metal, 227 μM *Z*-PEB	enzyme–*Z*-PEB–GDP	10.9 ± 1.8
2 mM Mg^{2+}, 227 μM *Z*-PEB	enzyme–*Z*-PEB–Mg–GDP	11.5 ± 1.5
200 μM Mn^{2+}, 227 μM *Z*-PEB	enzyme–Mn–*Z*-PEB–GDP	2.1 ± 1.0
2 mM Mg^{2+}, 60 μM Mn^{2+}, 227 μM *Z*-PEB	enzyme–Mn–*Z*-PEB–Mg–GDP	<2.0
no metal	enzyme–GTP	<2.0
200 μM Mn^{2+}	enzyme–Mn–GTP	<2.0
2 mM Mg^{2+}, 200 μM Mn^{2+}	enzyme–Mn–Mg–GTP	<2.0

the affinity of the enzyme for GDP. The substrate GTP binds to the enzyme more than an order of magnitude tighter than does GDP. Data obtained with tight binding ligands ($K_d <$ 2 μM) were too noisy to yield reliable absolute values for K_d; therefore, upper limits to the data are reported.

PRR Measurements. The formation of the ternary P-enolpyruvate carboxykinase–Mn–nucleotide complex was investigated by PRR techniques. The titration of IDP, Mg–IDP, GDP, and Mg–GDP to the binary enzyme–Mn complex caused no significant change in the observed enhancement values. The titration of ITP, Mg–ITP, GTP, and Mg–GTP into the enzyme–Mn complex elicited a decrease in the observed enhancement (Figure 2).

The best fit to the titration data with GTP as the titrant gave a $K_3 = (0.5–2.0) \times 10^{-7}$ M with a $K_s = (5–10) \times 10^{-6}$ M and a calculated $\epsilon_t = 3.6 \pm 0.2$. The data were fit with 12% standard deviation. The titration with Mg–GTP as the ligand elicited a similar titration curve and was fit with $K_3 = (1.0–10.0) \times 10^{-7}$ M; K_s varied from 1×10^{-5} to 1×10^{-3} M, and $\epsilon_t = 6.3 \pm 0.2$. Optimal fits less than 25% standard deviation could not be obtained with these data. No further deenhancement was observed at the higher concentration of nucleotides (or Mg–nucleotides), suggesting no significant

Table II: ^{31}P Relaxation Rates of Binary Mn–IDP and Mn–ITP Complexes

complex	nucleus	$1/(pT_{1p})^a$ (s^{-1})	$1/(pT_{2p})^a$ (s^{-2})	$1/(pT_{1p})^b$ (s^{-1})
Mn–IDP	α-^{31}P	$(4.60 \pm 0.20) \times 10^4$	$(6.60 \pm 0.65) \times 10^5$	$(3.75 \pm 0.18) \times 10^4$
	β-^{31}P	$(4.76 \pm 0.22) \times 10^4$	$(6.37 \pm 0.97) \times 10^5$	$(3.94 \pm 0.17) \times 10^4$
Mn–ITP	α-^{31}P	$(2.94 \pm 0.11) \times 10^4$	$(2.16 \pm 0.24) \times 10^5$	$(2.36 \pm 0.07) \times 10^4$
	β-^{31}P	$(4.34 \pm 0.18) \times 10^4$	$(3.38 \pm 0.83) \times 10^5$	$(2.99 \pm 0.16) \times 10^4$
	γ-^{31}P	$(4.22 \pm 0.11) \times 10^4$	$(2.54 \pm 0.19) \times 10^5$	$(2.99 \pm 0.12) \times 10^4$

[a] Measured at 40.5 MHz at 20 °C. [b] Measured at 121.5 MHz at 20 °C.

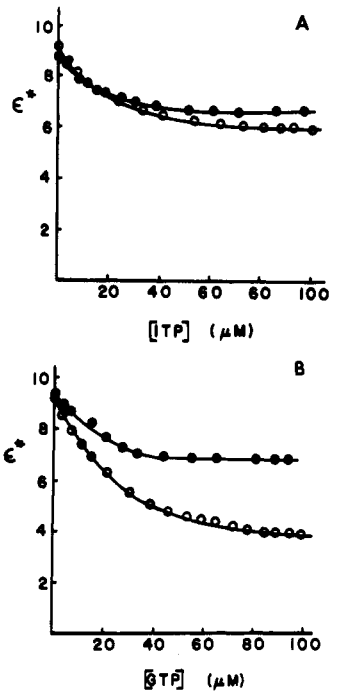

FIGURE 2: Titration of the binary enzyme–Mn^{2+} complex with nucleotide triphosphate. (A) The observed enhancement (ϵ^*) is plotted as a function of the concentration of ITP or MgITP. A solution containing 1.4×10^{-4} M enzyme and 5.0×10^{-5} M Mn^{2+} was titrated with an identical solution which also contained 2.0×10^{-4} M ITP (○) or 2.0×10^{-4} M ITP and 3.0×10^{-4} M Mg^{2+} (●). (B) The observed enhancement (ϵ^*) is plotted as a function of the concentration of GTP or MgGTP. A solution containing 1.3×10^{-4} M enzyme and 5.0×10^{-5} M Mn^{2+} was titrated with an identical solution which also contained 2.0×10^{-4} M GTP (○) or 2.0×10^{-4} M GTP and 3.0×10^{-4} M Mg^{2+} (●).

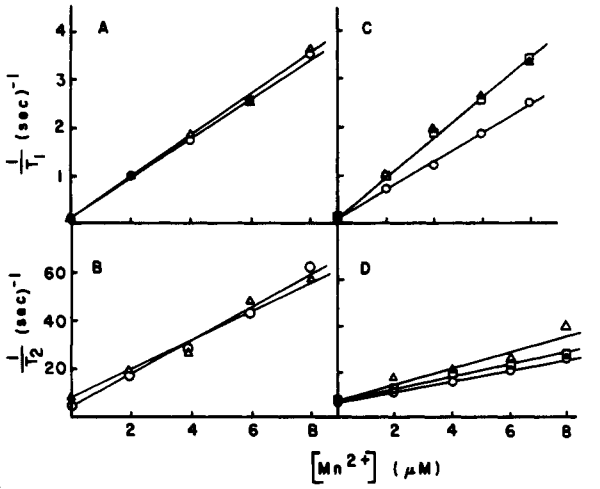

FIGURE 3: Effect of Mn^{2+} on the $1/T_1$ and $1/T_2$ values of the phosphorus atoms of IDP and ITP at 40.5 MHz. The values of $1/T_1$ (A) and of $1/T_2$ (B) for the α-phosphorus (○) and for the β-phosphorus (△) atoms of IDP (109 mM) are shown as a function of added Mn^{2+}. The values of $1/T_1$ (C) and of $1/T_2$ (D) for the α-phosphorus (○), β-phosphorus (△), and γ-phosphorus (□) atoms of ITP (100 mM) are shown as a function of Mn^{2+}. The samples were in 65 mM Tris–acetate buffer containing 100 mM KCl at pD 7.4. The 180° pulse was 79 μs, and the 90° pulse was 39.5 μs. The recycle time was 10–20 times the null point, and the number of transients collected varied from 2 to 30 as the concentration of Mn^{2+} increased. Temperature = ±20 °C.

competitive formation of the Mn–nucleotide complex under the experimental conditions.

Relaxation Parameters for ^{31}P in the Binary MnIDP and MnITP Complexes. The effects of Mn^{2+} on $1/T_1$ and $1/T_2$ of the ^{31}P nuclei of IDP and of ITP were measured. These relaxation effects for IDP and for ITP were determined both at 40.5 MHz and at 121.5 MHz. These control experiments were performed at nucleotide concentrations higher than that used in the presence of enzyme. The results of experiments performed at 40.5 MHz are plotted in Figure 3 as $1/T_1$ and $1/T_2$ vs. $[Mn^{2+}]$. From the slopes, the normalized paramagnetic effect on the relaxation rates, $1/(pT_{1p})$ and $1/(pT_{2p})$, were calculated. The normalized relaxation rate values calculated from Figure 3 and from the analogous experiments at 121.5 MHz are given in Table II.

Calculations of τ_c and of r in the Binary Complex. The value of τ_c for the binary Mn–nucleotide complexes was estimated from the frequency dependence of $1/(pT_{1p})$ for the ^{31}P resonances in the MnIDP and in the MnITP complexes at 40.5 and 121.5 MHz. In these calculations the term in the correlation function (eq 4) that contains ω_s is much smaller than the term that contains ω_I. Assuming τ_c for these complexes is frequency independent over this frequency range, the value of $\tau_c = (6.6 \pm 0.3) \times 10^{-10}$ s is calculated for the MnIDP complex and a value of $\tau_c = (8.6 \pm 0.8) \times 10^{-10}$ s is calculated for the MnITP complex.

Table III: Mn^{2+}–^{31}P Distances Calculated for the Mn–IDP and the Mn–ITP Complexes

complex	nucleus	r^c (Å)	r^d (Å)
Mn–IDP[a]	α-^{31}P	3.54 ± 0.05	3.55 ± 0.05
	β-^{31}P	3.52 ± 0.07	3.52 ± 0.05
Mn–ITP[b]	α-^{31}P	3.94 ± 0.05	3.90 ± 0.05
	β-^{31}P	3.69 ± 0.05	3.75 ± 0.05
	γ-^{31}P	3.71 ± 0.04	3.75 ± 0.05

[a] Values calculated by using $\tau_c = (6.6 \pm 0.3) \times 10^{-10}$ s. [b] Values calculated by using $\tau_c = (8.6 \pm 0.8) \times 10^{-10}$ s. [c] Values calculated by using $1/(pT_{1p})$ measured at 40.5 MHz. [d] Values calculated by using $1/(pT_{1p})$ measured at 121.5 MHz.

The distances of Mn–^{31}P for both complexes were calculated by using the values of τ_c which were calculated for each complex, the relaxation rates measured at both frequencies, and $f(\tau_c)$ calculated for each frequency. Solving eq 6 for r yields

$$r = C[T_{1M} f(\tau_c)]^{1/6} \quad (8)$$

when seconds are used as units of τ_c and T_{1M} and angstrom units for r. The constant C has the value of 601 for the Mn–^{31}P interaction (Mildvan & Engle, 1971). The distances from the α,β-phosphorus of IDP and the α,β,γ-phosphorus of ITP to Mn^{2+} in the binary complexes are given in Table III. The Mn–^{31}P distances in the MnIDP complex are identical. In the MnITP complex, however, the α-phosphorus is ap-

Table IV: ^{31}P Relaxation Rates of Ternary Enzyme–Mn–Nucleotide Complexes

complex	nucleus	$1/(pT_{1p})^a$	$1/(pT_{1p})^b$ (s^{-1})	$1/(pT_{2p})^b$
E–Mn–IDP	α-^{31}P	$(3.35 \pm 0.22) \times 10^3$	$(1.65 \pm 0.13) \times 10^3$	
	β-^{31}P	$(3.93 \pm 0.58) \times 10^3$	$(2.02 \pm 0.27) \times 10^3$	
E–Mn–GTP	α-^{31}P		$(1.41 \pm 0.13) \times 10^3$	$(3.0 \pm 0.1) \times 10^4$
	β-^{31}P		$(1.76 \pm 0.21) \times 10^3$	$(3.4 \pm 0.4) \times 10^4$
	γ-^{31}P		$(1.77 \pm 0.09) \times 10^3$	$(3.0 \pm 0.5) \times 10^4$

[a] Measured at 40.5 MHz at 20 °C. [b] Measured at 121.5 MHz at 20 °C.

proximately 0.2 Å further from the Mn^{2+} than are the β- and γ-phosphoryl groups.

Relaxation Parameters for ^{31}P in the Ternary Enzyme–Mn–IDP Complex. The effect of Mn^{2+} on $1/T_1$ of the ^{31}P nucleus of IDP in the ternary enzyme–Mn–IDP was measured. Under experimental conditions (130 μM enzyme, 0–3 μM Mn^{2+}, 7.3 mM IDP) all the Mn^{2+} added is bound to the enzyme–Mn–IDP complex. The distribution of Mn^{2+} was calculated by using the binding constants reported herein and K_d for Mn–enzyme = 50 μM which was previously determined (Hebda & Nowak, 1982b). The calculations were performed by using the program used to fit PRR binding data. These calculations take into account all possible equilibria present in solution including the large increase in Mn^{2+} binding by the enzyme in the presence of IDP. The relaxation parameters were determined at two different frequencies: 40.5 and 121.5 MHz. The diamagnetic corrections to the relaxation rates were measured with the enzyme–IDP complex. The results are plotted in Figure 4 as $1/T_1$ vs. $[Mn^{2+}]$. From the slopes, the normalized paramagnetic effect on the relaxation rates, $1/(pT_{1p})$, was calculated, and the values are given in Table IV. From the strong frequency dependence of the value of $1/(pT_{1p})$ it is clear that the relaxation rate is in the fast-exchange region and $1/(pT_{1p}) = 1/T_{1M}$. The $1/T_2$ values were not tabulated due to the significant scatter because of a lower signal-to-noise. Estimates for $1/(pT_{2p})$ give values that are approximately an order of magnitude greater than values for $1/(pT_{1p})$, also indicating that $1/(pT_{1p}) = 1/T_{1M}$.

Relaxation Parameters for ^{31}P in the Ternary Enzyme–Mn–GTP Complex. The effects of Mn^{2+} on $1/T_1$ of the ^{31}P nuclei of GTP in the ternary enzyme–Mn–GTP complex were measured at 121.5 MHz. Under experimental conditions (131 μM enzyme, 0–4 μM Mn^{2+}, 6.4 mM GTP), all the Mn^{2+} added is bound to the enzyme–Mn–GTP complex as determined by calculations using known values for dissociation constants (Hebda & Nowak, 1982b). These calculations were performed by using the program used to fit the PRR binding data as described above. A solution which was identical except for the lack of Mn^{2+} served as a diamagnetic control. The results are plotted in Figure 4 as $1/T_1$ vs. $[Mn^{2+}]$. From the slopes, the normalized paramagnetic effects on the relaxation rates, $1/(pT_{1p})$, were calculated for each resonance of ^{31}P of GTP. These results are shown in Table IV. The values for $1/(pT_{2p})$ at 20 °C were obtained from the temperature dependence of $1/T_2$ (Figure 5) and were an order of magnitude greater than the values for $1/(pT_{1p})$ indicating $1/(pT_{1p}) = 1/T_{1M}$.

Calculations of τ_c and of r in the Ternary Complexes. The value for τ_c was calculated by using the frequency dependence of $1/(pT_{1p})$ for the two ^{31}P resonances of IDP in the enzyme–Mn–IDP complex at 40.5 MHz and at 121.5 MHz and assuming no frequency dependence of τ_c. The value of $\tau_c = (1.5 \pm 0.2) \times 10^{-9}$ s was obtained from these calculations. Values for τ_c were also calculated by assuming a maximal frequency dependence of τ_c. These calculations gave τ_c values of $(0.61 \pm 0.2) \times 10^{-9}$ s at 40.5 MHz and $(5.4 \pm 0.2) \times 10^{-9}$ s at 121.5 MHz. The value for τ_c, calculated for the ternary enzyme–Mn–IDP complex, is assumed to be the same for the ternary enzyme–Mn–GTP complex. The distances of Mn–^{31}P in the ternary enzyme–Mn–IDP and enzyme–Mn–GTP complexes were calculated by using eq 8 and are given in Table V.

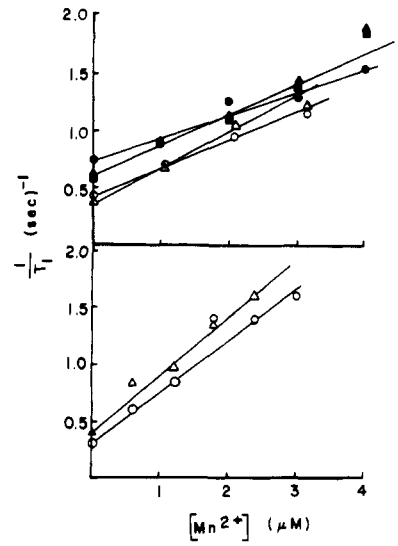

FIGURE 4: Effect of Mn^{2+} on the $1/T_1$ values of the phosphorus atoms of IDP and of GTP in the presence of P-enolpyruvate carboxykinase. (Top) The values of $1/T_1$, measured at 121.5 MHz, for α-phosphorus (○) and for β-phosphorus (△) atoms of IDP (6.5 mM) and for the α-phosphorus (●), β-phosphorus (▲), and γ-phosphorus (■) atoms of GTP (6.4 mM) in the presence of P-enolpyruvate carboxykinase (130 μM in each experiment), are shown as a function of Mn^{2+}. The experiments were performed in the presence of 65 mM Tris–acetate buffer, pD 7.4, which contained 100 mM KCl. The 180° pulse was 36 μs, and the 90° pulse was 18 μs. The number of acquisitions varied at each level of Mn^{2+} (80–640) to maintain a high signal-to-noise for each determination and the recycle time was at least 10–20 times the null point. Temperature 20 ± 1 °C. (Bottom) The values of $1/T_1$, measured at 40.5 MHz for α-phosphorus (○) and for β-phosphorus (△) atoms of IDP (7.3 mM) in the presence of P-enolpyruvate carboxykinase (130 mM), are shown as a function of Mn^{2+}. Experimental conditions are analogous to those described in the top panel except for longer pulse widths (see legend to Figure 3) and a larger number of acquisitions due to a lower signal-to-noise at the lower frequency.

Temperature Effect on the Observed Values for $1/(pT_{2p})$ of the Ternary Enzyme–Mn–GTP Complex. The $1/T_2$ relaxation rates of α-, β-, and γ-^{31}P resonances in the ternary complex of enzyme–Mn–GTP were measured as a function of temperature over the range 5–25 °C. The $1/T_2$ values were measured by line-width measurements using a single pulse experiment at a fixed concentration of Mn^{2+}. The identical solution except for the lack of Mn^{2+} served as a diamagnetic control. The results are plotted as ln $(1/T_{2p})$ in Arrhenius fashion and shown in Figure 5. The negative slope is characteristic of slow chemical exchange. The values of $1/T_{2p}$ for the α-, β-, and γ-phosphorus nuclei are identical. In the ternary enzyme–Mn–GTP complex apparently τ_m dominates the T_2 relaxation rates of α-, β-, and γ-^{31}P. Thus, it is apparent that the T_2 relaxation rates measured are a reflection of ligand

Table V: Mn^{2+}–^{31}P Distances Calculated for the Enzyme–Mn–IDP and the Enzyme–Mn–GTP Complexes

complex	nucleus	r^a (Å)	r^b (Å)	r^c (Å)	r^d (Å)
E–Mn–IDP	α-^{31}P	6.16 ± 0.08	6.18 ± 0.09	5.40 ± 0.07	5.42 ± 0.07
	β-^{31}P	6.00 ± 0.09	5.97 ± 0.09	5.26 ± 0.07	5.25 ± 0.07
E–Mn–GTP	α-^{31}P		6.34 ± 0.09		5.57 ± 0.08
	β-^{31}P		6.11 ± 0.09		5.37 ± 0.08
	γ-^{31}P		6.11 ± 0.08		5.37 ± 0.07

[a] Calculated by using $1/(pT_{1p})$ measured at 40.5 MHz, $\tau_c = (1.5 \pm 0.2) \times 10^{-9}$ s, and $f(\tau_c) = 3.9 \times 10^{-9}$ s. [b] Calculated by using $1/(pT_{1p})$ measured at 121.5 MHz, $\tau_c = (1.5 \pm 0.2) \times 10^{-9}$ s, and $f(\tau_c) = 1.9 \times 10^{-9}$ s. [c] Calculated by using $1/(pT_{1p})$ measured at 40.5 MHz, $\tau_c = (0.61 \pm 0.06) \times 10^{-9}$ s, and $f(\tau_c) = 1.76 \times 10^{-9}$ s. [d] Calculated by using $1/(pT_{1p})$ measured at 121.5 MHz, $\tau_c = (5.5 \pm 0.6) \times 10^{-9}$ s, and $f(\tau_c) = 0.90 \times 10^{-9}$ s.

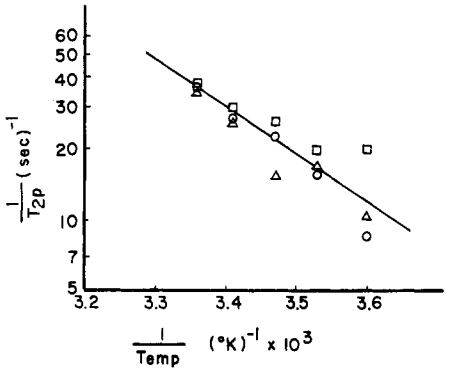

FIGURE 5: Temperature dependence of the $1/T_{2p}$ values of phosphorus atoms of GTP in the presence of Mn^{2+} and of P-enolpyruvate carboxykinase. The values of ln $(1/T_{2p})$ for α-phosphorus (○), for β-phosphorus (△), and for γ-phosphorus (□) atoms of GTP are plotted as a function of 1/temperature. The sample, in 2.0 mL, contained 5.8 mM GTP, 120 μM P-enolpyruvate carboxykinase, and 5.0 μM Mn^{2+} in 65 mM Tris–acetate, pH 7.4. The paramagnetic effect was calculated by subtraction of $1/T_2$ values of a diamagnetic control measured under identical conditions in the absence of Mn^{2+}. Experimental conditions were analogous to those described in Figure 4 (top) except a 90° pulse (18 μs) was used in a single pulse experiment and 80 acquisitions were obtained for each experiment. Temperature was varied as described under Methods.

exchange rates $(1/\tau_m)$ rather than relaxation rates $(1/T_{2M})$; $1/(pT_{2p}) = k_{off}$. At 25 °C, k_{off} for the α-, β-, and γ-^{31}P of GTP from the ternary enzyme–Mn–GTP complex is $(4.2 \pm 0.13) \times 10^4$ s^{-1}. This value is substantially faster than k_{cat} (8.5–10 s^{-1}) for the GDP-dependent carboxylation of P-enolpyruvate reaction. Also, assuming the simplest model for Mn–ligand formation, k_{on} ($k_{on} = k_{off}/K_d$) can be calculated. The K_d for GTP from the enzyme–Mn–GTP complex was determined to be $<2.0 \times 10^{-6}$ M. At 25 °C, the k_{on} calculated for GTP is $>2.1 \times 10^{10}$ s^{-1} M^{-1} for the ternary enzyme–Mn–GTP complex. From the temperature dependence of k_{off}, the activation energy (E_A) for dissociation can be calculated for GTP from the Arrhenius equation:

$$k_{off} = Ae^{-E_A/(kT)}$$

The activation energy for GTP dissociation from the ternary enzyme–Mn–GTP complex was calculated to be 9.2 kcal/mol.

Discussion

The dissociation constants for Mn^{2+} binding to IDP, GDP, ITP, and GTP are comparable to those values for Mn–ADP ($K_d = 10.0 \times 10^{-5}$ M) and Mn–ATP ($K_d = 1.4 \times 10^{-5}$ M) reported in the literature which were measured under similar experimental conditions (0.05 M Tris-HCl buffer at pH 7.5, 0.1 M KCl) (Mildvan & Cohn, 1966). There are small differences in affinities for Mn^{2+} depending upon the bases which are used.

The nucleotides GDP, Mg–GDP, and GTP interact specifically at a single site on the enzyme in the absence of Mn^{2+} to form an enzyme–nucleotide complex with a high affinity (K_s = 10–15 μM for GDP and <2 μM for GTP) as measured by the equilibrium gel separation technique. A single binding site suggests that nucleotide which serves as the substrate in the reversible reaction (eq 1) and nucleotide diphosphate which serves as the activator of the irreversible reaction (eq 2) bind at the same site. These results indicate that both of these reactions occur at a single catalytic site on P-enolpyruvate carboxykinase.

The presence of Mn^{2+} increases the binding of GDP and of Mg–GDP to the enzyme. This observation demonstrates that the binding of Mn^{2+} to the enzyme elicits a change on the enzyme which facilitates the binding of GDP or Mg–GDP to the enzyme. Such an effect of Mn^{2+} on nucleotide binding was also suggested kinetically (Hebda & Nowak, 1982a). In the presence of both Mn^{2+} and *Z*-PEB, the binding of GDP and of Mg–GDP to the enzyme increases significantly; the K_d of the enzyme–GDP complex decreases from 10–15 μM to less than 2.0 μM. The substrate analogue *Z*-PEB interacts specifically to P-enolpyruvate carboxykinase with high affinity (K_d = 31.4 μM) in the presence of Mn^{2+} (Duffy et al., 1982). The results of direct binding studies suggest that the presence of the second substrate (as an analogue) in the enzyme–Mn complex also increases the affinity of GDP or of Mg–GDP to the enzyme.

The titration of the binary enzyme–Mn complex with IDP, GDP, or their respective Mg complexes (Mg–IDP and Mg–GDP) caused no change in the observed enhancement. Since the formation of enzyme–Mn–GDP and enzyme–Mn–Mg–GDP complexes were detected by direct binding studies, the PRR results suggest that the lack of change in enhancement upon GDP (Mg–GDP) addition occurs because the ligand interacts in an outer-sphere complex with the enzyme-bound Mn^{2+} and does not affect the environment of the bound Mn^{2+}. The small differences in results reported here and those previously reported by our laboratory (Hebda & Nowak, 1982b) can be due to small variations in experimental conditions (i.e., ionic strength). However, the identical conclusions are reached from either experiment.

GTP or Mg–GTP (ITP or Mg–ITP) interacts with the binary enzyme–Mn complex and causes a decrease in the observed enhancement (Figure 2). High-resolution ^{31}P NMR experiments show that the α-, β-, and γ-phosphoryl groups of GTP are in an outer-sphere complex to the enzyme-bound Mn^{2+} (Table V). One explanation for the observed deenhancement upon the addition of GTP (or ITP) is that the γ-P interacts with the Mn^{2+} on the enzyme via a molecule of water, and this molecule of water is "frozen" or immobilized in the ternary complex. The quantitative differences between GTP and Mg–GTP (Figure 2B) suggest some diversity in the environment of bound Mn^{2+} in the respective ternary complexes. The consistency of the enhancement values at concentrations of GTP in excess of enzyme (data not shown) demonstrates the lack of formation of competitive binary Mn–GTP complexes.

From the observation of the deenhancement with GTP, but not with GDP, it is proposed that the Mn^{2+}-bound water molecule might polarize the γ-phosphoryl group of GTP by hydrogen bonding or by protonation in order to facilitate the phosphoryl transfer during the reaction catalyzed by chicken liver P-enolpyruvate carboxykinase. Duffy has recently reported that the substrate P-enolpyruvate, a phosphoryl group donor in the reverse reaction, apparently immobilizes an inner-sphere water molecule of the enzyme–Mn complex. The activation of this substrate by bound Mn^{2+} may also be mediated via bound water (Duffy, 1982). The interaction of the inner-sphere water molecule to activate the phosphoryl group of the substrate has been suggested for a number of enzymatic reactions (Melamud & Mildvan, 1975; Ray & Mildvan, 1973; Sloan et al., 1975; Sloan & Mildvan, 1976).

The τ_c values determined by the frequency dependence of $1/(pT_{1p})$ for the Mn–IDP complex, $\tau_c = 6.6 \times 10^{-10}$ s, and for the Mn–ITP complex, $\tau_c = 8.1 \times 10^{-10}$ s, were in good agreement with the value of 6×10^{-10} s reported for the Mn–ATP complex determined by Brown et al. (1973) by water proton relaxation rate measurements but larger than the values calculated for τ_c [$(1.0–1.2) \times 10^{-10}$ s] assuming τ_c increases in proportion to the increase in molecular weight of the complex.

All of the Mn^{2+} to phosphorus distances (3.5–3.9 Å, Table III) of the binary Mn–IDP and Mn–ITP complexes indicate direct phosphoryl coordination in the binary complex. In the binary Mn–IDP complex, the α- and β-phosphorus atoms are equidistant from Mn^{2+} ($r = 3.53 \pm 0.07$ Å). In the binary Mn–ITP complex, the α-phosphorus atom is slightly further away from the Mn^{2+} than are the β- or γ-phosphorus atoms ($r_\alpha = 3.92 \pm 0.05$ Å; $r_\beta = r_\gamma = 3.73 \pm 0.05$ Å). Brown et al. (1973) reported somewhat shorter values for the Mn–ATP complex ($r_\alpha = 3.7$ Å; $r_\beta = r_\gamma = 3.3$ Å). Sloan & Mildvan (1976) reported values for Mn–ATP about 0.7 Å shorter ($r_\alpha = 3.2$ Å; $r_\beta = r_\gamma = 3.1$ Å). The experiments performed here and those reported by Brown et al. (1973) were performed at high concentrations of nucleotide compared to the concentrations of ATP (<5 mM) used by Sloan & Mildvan (1976). Possible nucleotide stacking in solution at higher concentrations may account for the larger values of $1/(pT_{1p})$ and $1/(pT_{2p})$ (Table II) and the larger value for τ_c. The possible differences in structure of the complexes, reflected in larger Mn–P distances, may be a result of such nucleotide stacking.

For the ternary enzyme–Mn–nucleotide complex, the measured values of $1/(pT_{1p})$ for ^{31}P in the enzyme–Mn–IDP and in the enzyme–Mn–GTP complexes were in the region of fast exchange, and $1/(pT_{1p}) = 1/T_{1M}$. This could be verified by the inequality of $1/(pT_{1p})$ and $1/(pT_{2p})$ and the frequency dependence of $1/(pT_{1p})$. The value of $\tau_c = (1.5 \pm 0.2) \times 10^{-9}$ s, calculated from the frequency dependence of $1/(pT_{1p})$ of ^{31}P in the enzyme–Mn–IDP complex at 40.5 MHz and at 121.5 MHz, is in agreement with the values determined by Duffy (1982). He reported values for $\tau_c = (0.5–3) \times 10^{-9}$ s for several P-enolpyruvate carboxykinase–Mn–ligand complexes. Those reported values for τ_c were determined by various methods; T_{1M}/T_{2M} ratios for ^1H, frequency dependence of $1/T_{1M}$, and a frequency dependence of PRR. The agreement between the τ_c measured (1.5×10^{-9} s) and the τ_c reported by Duffy (1982) for enzyme–Mn–ligand complexes and the large differences between the relaxation rates for the binary (Table II) and ternary (Table IV) complexes also demonstrate that the complexes under investigation in the presence of enzyme are virtually all ternary complexes. These results corroborate our calculations concerning Mn^{2+} distribution.[2]

By use of the value of $\tau_c = 1.5 \times 10^{-9}$ calculated from these experiments, and the values for $1/(pT_{1p})$, the distance between the enzyme-bound Mn^{2+} and the phosphorus atoms of the ligands in the ternary complexes show that the nucleotide forms a second coordination sphere complex to the bound Mn^{2+}. In the ternary enzyme–Mn–IDP complex, the α-phosphorus atom is nearly 0.2 Å more distant from the Mn^{2+} than the β-phosphorus atom ($r_\alpha = 6.17 \pm 0.09$ Å; $r_\beta = 5.99 \pm 0.09$ Å). In the ternary enzyme–Mn–GTP complex, the α-phosphorus atom is also approximately 0.2 Å further from the Mn^{2+} than are the β- or γ-phosphorus atoms, which are equidistant from the Mn^{2+} ($r_\alpha = 6.34 \pm 0.09$ Å; $r_\beta = r_\gamma = 6.11 \pm 0.09$ Å). In the calculation of τ_c, if a maximum frequency dependence of τ_c is assumed, values of 0.6 and 5.4 ns are calculated for 40.5 and 121.5 MHz, respectively. By use of these values, the calculated Mn^{2+}–P distances are approximately 0.7 Å shorter in the ternary complexes (Table V). These can be considered minimum values; however, these results are still consistent with the formation of second sphere complexes. The calculated values for r give a metal-to-phosphorus distance of up to 1 Å longer than the values for the pyruvate kinase–Mn–ATP complex (4.9–5.1 Å) reported by Sloan & Mildvan (1976). Metal-to-phosphorus distances of phosphate ligands $\simeq$ 5 Å have also been reported with other enzymes (Ray & Mildvan, 1973; Sloan et al., 1975). The Mn-to-phosphorus distance in the P-enolpyruvate carboxykinase–Mn–P-enolpyruvate complex has been determined to be 7.1 Å (Duffy, 1982). This observation is consistent with the idea that the Mn-to-phosphorus distances in P-enolpyruvate carboxykinase–Mn–GTP are longer than those calculated for the pyruvate kinase–Mn–ATP complex (Sloan & Mildvan, 1976) and that the γ-phosphate of GTP overlaps with the phosphoryl group of P-enolpyruvate in the enzyme complex.

By use of the Mn^{2+} to phosphorus distance, 6.34 Å, in the ternary enzyme–Mn–GTP complex, $1/T_{2M}$ was calculated by ignoring any scalar contribution to $1/T_2$ relaxation. The value for $1/T_{2M}$ calculated (3.3×10^3 s^{-1}) was an order of magnitude smaller than the value measured for $1/(pT_{2p})$ (4.2×10^4 s^{-1}). Since $1/(pT_{2p}) = 1/\tau_m$, the actual value of $1/T_{2M}$ is greater than 4.2×10^4 s^{-1}. Thus, the difference between the calculated dipolar term of $1/T_{2M}$ and the actual value of $1/T_{2M}$ becomes greater. This suggests that although GTP forms a second coordination sphere complex to the enzyme-bound Mn^{2+}, a significant scalar interaction is present. These results suggest a significant electron delocalization between the bound Mn^{2+} and the ^{31}P of the nucleotide. This effect must be transmitted by an intervening ligand, presumably a molecule of water.

The model built on the basis of the Mn-to-phosphorus distances in the ternary enzyme–Mn–GTP complex shows that the triphosphate chain of the GTP has an extended configu-

[2] One reviewer has suggested that a significant fraction of Mn may be distributed in the binary Mn–GTP complex under experimental conditions. This complex would therefore contribute to the observed relaxation rate. In the presence of enzyme, the $1/T_{1p}$ values are 6% the values for the binary complex. Therefore, in the extreme case if the ternary complex makes no contribution to $1/(pT_{1p})$ (observed) (because of slow exchange or a large Mn–P distance), a maximum of 6% binary complex is formed. An identical analysis of the complex which contains IDP suggests a maximum of 5–8% binary Mn–IDP complex present. These values represent maximum values of binary complexes present. If any significant amount of binary complex is present and contributes to the observed relaxation rates, then the $1/(pT_{1p})$ values from the ternary complexes are much smaller and reflect slow exchange or a much longer Mn–P distance. The latter explanation is inconsistent with studies of the P-enolpyruvate complex of this enzyme (T. Duffy and T. Nowak, unpublished observations) and spin-label studies of the enzyme–GTP complex (A. Makinen and T. Nowak, unpublished observations).

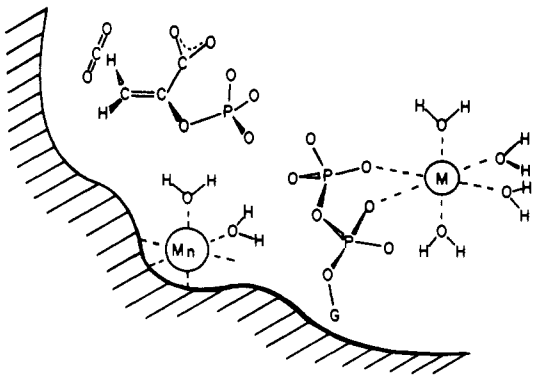

FIGURE 6: Proposed scheme for the mechanism of P-enolpyruvate carboxykinase based on the kinetics and the ^{31}P NMR studies.

ration (Figure 6). Thus, a role of the P-enolpyruvate carboxykinase bound divalent cation appears to be to adjust the enzyme to an active conformation and possibly to activate the transferring phosphoryl group through an intervening water ligand. The number of Mn^{2+}-bound water molecules in the P-enolpyruvate carboxykinase–Mn complex was calculated to be $\simeq 1.5$ from the frequency dependence of the PRR measurements of the enzyme–Mn complex (Duffy, 1982). Half of these water molecules is immobilized in the presence of GTP. The exchange rate of GTP in the active enzyme–Mn–GTP complex ($k_{off} = 1/\tau_m = 4.2 \times 10^4\ s^{-1}$) is substantially faster than the catalytic rate of the GDP-dependent carboxylation reaction ($k_{cat} = 8.5–10\ s^{-1}$). Thus, the complex detected by NMR forms and dissociates sufficiently rapidly to participate in catalysis. The rate of formation of the ternary enzyme–Mn–GTP complex ($k_{on} > 2.1 \times 10^{10}\ s^{-1}\ M^{-1}$) is calculated by using the values of k_{off} and K_s ($<2.0 \times 10^{-6}$ M). This large value may be due to diffusion control for the interaction of two charged species and is larger than the values estimated for diffusion limit of neutral species (Eigen & Hammes, 1963). This estimation of k_{on} is based on the simple assumptions for complex formation ($K_D = k_{off}/k_{on}$ and $1/\tau_m = k_{off}$). Both assumptions may be simplifications of multistep processes.

The activation energy for GTP dissociation from the ternary enzyme–Mn–GTP complex (9.2 kcal/mol) is lower than that for the P-enolpyruvate carboxykinase catalyzed carboxylation (Hebda & Nowak, 1982a) and the decarboxylation reaction (unpublished data) ($E_A = 17.6$ kcal/mol). This indicates that the rate-determining step in the P-enolpyruvate carboxykinase reaction is not the release of GTP from the enzyme–Mn–GTP complex.

The location of the second divalent cation, which serves as the metal–nucleotide complex and acts as the substrate, appears to be liganded to the β- and γ-phosphorus atoms of GTP in the enzyme–Mn–GTP complex (M. H. Lee and T. Nowak, unpublished observations). The second divalent cation may activate the transferring phosphoryl group directly and facilitate the nucleophilic attack by the substrate oxalacetate. The site of ligation and coordination geometry of this second cation has yet to be elucidated.

Acknowledgments

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Registry No. IDP, 86-04-4; ITP, 132-06-9; GDP, 146-91-8; GTP, 86-01-1; Mn-IDP, 80133-94-4; Mn-ITP, 79724-82-6; Mn-GDP, 82200-76-8; Mn-GTP, 56444-94-1; Mg-IDP, 7219-40-1; Mg-ITP, 22139-67-9; Mg-GDP, 7277-99-8; Mg-GTP, 28141-84-6; *Z*-PEB, 31302-64-4; Mn, 7439-96-5; Mg, 7439-95-4; EC 4.1.1.32, 9013-08-5.

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