Guanosine Thiophosphate Derivatives as Substrate Analogues for Phosphoenolpyruvate Carboxykinase†

Myoung Hee Lee,‡,§ Roger S. Goody,‖ and Thomas Nowak*,‡,⊥

Department of Chemistry, Program in Biochemistry and Biophysics, University of Notre Dame, Notre Dame, Indiana 46556, and Max-Planck-Institut für Medizinische Forschung, 6900 Heidelberg 1, West Germany

Received March 6, 1985

ABSTRACT: The interactions of nucleotides with phosphoenolpyruvate carboxykinase were studied by using the stereospecific thiophosphate analogues of GDP and GTP. The metal ion dependent stereoselectivity of these analogues was determined by using steady-state kinetics. The R_P and S_P isomers of guanosine 5′-*O*-(1-thiodiphosphate) (GDPαS) were substrates with low turnover, and a small preference for the R_P isomer was observed. Neither the enzyme–metal nor the nucleotide–metal complex elicited any substantial change in the selectivity. Guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) exhibited no substrate activity for the enzyme, regardless of the cations. This nucleotide was a competitive inhibitor against GDP, however. Both R_P and S_P diastereomers of guanosine 5′-*O*-(1-thiotriphosphate) (GTPαS) were good substrates for phosphoenolpyruvate carboxykinase; in several cases, depending upon the cation, k_{cat} and/or V_m/K_m for the R_P isomer is greater than for the substrate GTP. The enzyme–metal complex but not the nucleotide–metal complex affects the relative K_m and the V_{max} values. In contrast, guanosine 5′-*O*-(2-thiotriphosphate) (GTPβS)(S_P) is a much better substrate (>50 times) than is GTPβS(R_P). The metal ions have little effect on the selectivity. These results suggest a specific interaction of the β-phosphate of the nucleotide with the protein. The analogue guanosine 5′-*O*-(3-thiotriphosphate) (GPTγS) serves as a substrate to yield GDP and thiophosphoenolpyruvate. The latter was detected by ^{31}P NMR and was shown to slowly hydrolyze to form phosphoenolpyruvate. The relative efficiency of GTPγS as a substrate is dependent upon the enzyme cation and the nucleotide cation. The α,β-methylene or β,γ-imido and β,γ-methylene derivatives of the nucleotides all fail to elicit substrate activity or inhibition. These results suggest strict geometric specificity of the nucleotide substrate at the β-position with interaction of the β-phosphate with the enzyme and with the metal ion. The GTP–cation complex which serves as the substrate is probably the bidentate coordinate.

Phosphoenolpyruvate (P-enolpyruvate)[1] carboxykinase [GTP:oxaloacetate carboxy-lyase (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 is the catalysis of the formation of P-enolpyruvate from oxalacetate as the first committed step in gluconeogenesis. This reaction is specific for either GTP or ITP as the substrate.

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

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

This reaction requires a specific nucleotide diphosphate as a cofactor for this reaction. There is no known physiological significance of reaction 2.

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

Eckstein and his co-workers have introduced a series of nucleotide analogues where a nonbridging oxygen atom is replaced by a sulfur atom. A variety of phosphothioate analogues of adenine such as ATPαS, ATPβS, ATPγS, ADPαS, and ADPβS have been used in the investigation of nucleotide–enzyme interactions (Goody et al., 1972; Goody & Eckstein, 1971; Schlimme et al., 1973; Eckstein, 1983). The replacement of a nonbridging oxygen atom on the α-, β-, or γ-phosphorus by a sulfur atom results in the formation of chiral or prochiral phosphate groups.

Jaffe and Cohn first showed the metal ion dependence of stereospecificity for these analogues as the probe of the chelate structure of the metal–nucleotide substrate complex (Jaffe & Cohn, 1978a). These experiments were based on the preference of Mg^{2+} chelation to oxygen, Cd^{2+} to sulfur, and Co^{2+} and Mn^{2+} to both oxygen and sulfur ligands (Cotton & Wilkinson, 1972). Jaffe and Cohn reported that in the hexokinase reaction, in the presence of Mg^{2+}, only the R_P isomer

† This research was supported in part by grants from the National Institutes of Health (AM 17049 and AM 00486).

‡ University of Notre Dame.

§ Work performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at the University of Notre Dame. Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843.

‖ Max-Planck-Institut für Medizinische Forschung.

⊥ Research Career Development Awardee of the National Institutes of Health (Grant AM 00486).

[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; GDPαS, guanosine 5′-*O*-(1-thiodiphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPαS, guanosine 5′-*O*-(1-thiotriphosphate); GTPβS, guanosine 5′-*O*-(2-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

0006-2960/85/0424-7594$01.50/0

of ATPβS is utilized as a substrate. When Cd^{2+} is the divalent cation, the S_P isomer is a substrate, and when Co^{2+} is the cation, both isomers are used as substrates. The use of phosphothioate analogues and the investigation of isomeric specificity using various metal ions as cofactors have been applied to a variety of other enzyme systems (Burgers & Eckstein, 1980; Churchich & Wu, 1982; Connolly & Eckstein, 1981; Jaffe & Cohn, 1978a; Jaffe et al., 1982; Pillai et al., 1980).

Thiophosphate analogues of guanosine have been synthesized, and the absolute configuration of the diastereomers has been established (Connolly et al., 1982; Rösch et al., 1984; Goody & Leberman, 1979). In the present work, we report the use of thiophosphate analogues of the nucleotide substrate to investigate the stereospecificity of the interactions of GDP and of GTP to avian liver P-enolpyruvate carboxykinase. Kinetic studies with methylene- and imidophosphate analogues of the nucleotides are also reported.

Experimental Procedures

Materials. The nucleotides GDP, GTP, and ADP, P-enolpyruvate, and NADH were purchased from Sigma.

GDPαS (S_P and R_P), GTPαS (S_P and R_P), and GTPβS (S_P and R_P) were synthesized as previously described (Goody & Leberman, 1979; Rösch et al., 1984). GDPβS was either purchased from Boehringer or synthesized. GTPγS was purchased from Boehringer. α,β-Methylene-GTP (GPCPOP), β,γ-methylene-GTP (GPOPCP), and β,γ-imido-GTP (GPOPNP) were purchased from P-L Biochemicals. Oxalacetate was obtained from either Calbiochem or Sigma. $CoCl_2$ was purchased from Mallinkrodt; $CdCl_2$, $MnCl_2$, $MgCl_2$, and $Mg(NO_3)_2$ were purchased from Baker. "Ultrapure" $Mg(NO_3)_2$ was obtained from Ventron. Tris base was obtained from Schwarz/Mann, and DE-32 resin was purchased from Whatman. The enzymes pyruvate kinase, lactate dehydrogenase, and malate dehydrogenase were purchased from Boehringer. P-enolpyruvate carboxykinase was purified from chicken liver mitochondria either as previously described by Hebda & Nowak (1982a) or by a modification of that procedure (Lee & Nowak, 1984).

Enzyme Assays. The enzyme prepared by the modified method (Lee & Nowak, 1984) was used for all of the experiments except those performed with GTPγS. In these experiments, the enzyme prepared by the method of Hebda & Nowak (1982a) was used.

When the enzyme which was prepared by the modified method was used, it was preincubated for 10 min with 0.2 mM EDTA to remove any contaminant inhibitory metal ions which are sometimes apparently associated with the enzyme. This treatment is utilized to obtain the optimum specific activity. The final EDTA concentration in the assay never exceeded 0.5 μM.

In the carboxylation reaction of P-enolpyruvate to yield oxalacetate, the continuous assay which is coupled to malate dehydrogenase was used (Lee et al., 1981).

In the decarboxylation reaction of oxalacetate to form P-enolpyruvate, a fixed-time assay was normally used. This assay is performed in two steps and is a modification of an assay previously described (Seubert & Huth, 1965). In the first step, the assay contains 65 μmol of Tris–acetate, pH 7.4, 1 μmol of GTP, 2 μmol of oxalacetate, 1 μmol of Mn^{2+}, and 71.5 μmol of β-mercaptoethanol in 1 mL. Variations in metal composition or in concentrations were made as required. The assay was incubated at 25 °C and the reaction initiated by the addition of an appropriate amount of P-enolpyruvate carboxykinase. The reaction was allowed to proceed for 5 min and was terminated by the addition of 0.2 mL of 12% perchloric acid. The mixture was placed on ice for 3 min followed by the addition of 10–12 mg of KBH_4. The mixture was neutralized by the addition of 0.04 mL of 30% KOH.

The amount of P-enolpyruvate formed was determined in the second step. To an assay in 0.5 mL which contains 65 μmol of Tris–acetate, pH 7.4, 100 μmol of KCl, 1 μmol of ADP, 4 μmol of $MnCl_2$, and 0.15 μmol of NADH was added 0.5 mL of the reaction mixture from step one. The absorbance at 340 nm was recorded. The addition of 20 μg of lactate dehydrogenase (in 2 μL) gave an absorbance change due to pyruvate. Subsequent addition of 20 μg of pyruvate kinase (in 2 μL) gave an absorbance change due to P-enolpyruvate.

When GTPγS was used as an inhibitor, the continuous assay coupled to pyruvate kinase and lactate dehydrogenase was used (Lee et al., 1981). β-Mercaptoethanol was used in the enzyme assays only when Mn^{2+} was used as the divalent cation either with Mn^{2+} as a sole activating metal ion or with Mg^{2+} and Mn^{2+} as a mixed metal system. When the other metal ions (i.e., Co^{2+}, Cd^{2+}) were used, precipitation of β-mercaptoethanol–metal complexes occurred.

When GTPγS was used as a substrate, the enzyme catalyzed the transfer of a thiophosphoryl group to oxalacetate to form thiophosphoenolpyruvate. The thiophosphoenolpyruvate was not stable (vide infra); the slow decomposition of this compound yields P-enolpyruvate. Thiophosphoenolpyruvate was utilized as a substrate for pyruvate kinase which was coupled with lactate dehydrogenase for analysis.

The analysis of kinetic data to determine if free nucleotide can serve as a substrate for P-enolpyruvate carboxykinase was performed by using the equation derived from a model where MnITP and ITP are both substrates (Segel, 1975):

$$v = \frac{V_m([\mathrm{MnITP}]/K_{\mathrm{MnITP}}) + V_m'([\mathrm{ITP}]/K_{\mathrm{ITP}})}{1 + K_A/[\mathrm{Mn}^{2+}] + [\mathrm{MnITP}]/K_{\mathrm{MnITP}} + [\mathrm{ITP}]/K_{\mathrm{ITP}}} \quad (3)$$

The term V_m is the maximum velocity of the enzyme with MnITP as substrate, and V_m' is the maximum velocity with free ITP as substrate. The designated Michaelis constants are for MnITP and for free ITP. When the K_A is much smaller [K_A = 1–2 μM (Lee et al., 1981)] than the concentration of free Mn^{2+} used in the experiments, the term $K_A/[Mn^{2+}]$ becomes small, and eq 3 is simplified to

$$v = \frac{V_m([\mathrm{MnITP}]/K_{\mathrm{MnITP}}) + V_m'([\mathrm{ITP}]/K_{\mathrm{ITP}})}{1 + [\mathrm{MnITP}]/K_{\mathrm{MnITP}} + [\mathrm{ITP}]/K_{\mathrm{ITP}}} \quad (4)$$

The velocity of the reaction is measured as a function of nucleotide at fixed variable concentrations of free Mn^{2+}. A replot of the reciprocal of the apparent K_m for MnITP vs. the product of the dissociation constant for MnITP and free Mn^{2+} (Mn^{2+}_f) yields $1/K_m$ for ITP as the ordinate intercept. A replot of the slope of each line vs. $K_D[Mn^{2+}]_f$ yields an ordinate intercept which is V_m/K_{MnITP}. The slope of this replot is equal to V_m'. From the values obtained from the secondary plots, it is possible to calculate values for V_m and V_m'. Linear plots for this treatment were criteria for the suitability of this model.

Determination of Purity and of Concentration of Thiophosphate Analogues. The purity of the thiophosphate analogues of GDP and of GTP was analyzed by HPLC using a Beckman Model 421 unit. The separation was carried out on a Varian Micro-Pak AX-10 anion-exchange column (30 cm × 4 mm) equilibrated in 1 M KCl and 0.25 M KH_2PO_4, pH 4.5, buffer. Elution was performed by using the same buffer at a flow rate of 2.0 mL/min. Each of the analogues and GMP and GTP were all readily resolved from each other. The

Table I: Kinetic Parameters for the Substrate GDPαS

metal ion	nucleotide	K_m (mM)	V_m (units/mg)	rel V_m (%)	V_m/K_m [(min·mg)$^{-1}$]	X^a
4.0 mM Mg^{2+}, 0.02 mM Mn^{2+}	GDP	0.023	5.88	100	255.7	
	GDPαS(S_P)	0.033	0.024	0.41	0.74	
	GDPαS(R_P)	0.118	0.061	1.04	0.52	0.7
4.0 mM Mg^{2+}, 0.02 mM Co^{2+}	GDP	0.042	1.82	100	43.3	
	GDPαS(S_P)	0.059	0.05	2.5	0.85	
	GDPαS(R_P)	0.087	0.22	12.2	2.53	3.0
4.0 mM Mg^{2+}, 0.005 mM Cd^{2+}	GDP	0.027	2.94	100	108.9	
	GDPαS(S_P)	0.049	0.01	0.37	0.22	
	GDPαS(R_P)	0.091	0.02	0.58	0.19	0.9
10 mM Co^{2+}	GDP	0.210	3.10	100	14.8	
	GDPαS(S_P)	0.149 (0.100)[b]	0.13	4.2	0.87	
	GDPαS(R_P)	0.141 (0.170)[b]	0.62	20.0	4.40	5.1
4.0 mM Mn^{2+}	GDP	0.056	5.6	100	100.0	
	GDPαS(S_P)	0.146 (0.138)[b]	0.15	2.8	1.03	
	GDPαS(R_P)	0.204 (0.204)[b]	0.42	7.5	2.06	2.0

[a] Ratio of V_m/K_m for GDPαS(R_P) to V_m/K_m for GDPαS(S_P). [b] K_I value measured under identical experimental conditions.

diastereomeric pairs could not be resolved under these experimental conditions. The purity of diastereomeric products was independently determined by using reverse-phase HPLC and elution with 50 mM potassium phosphate, pH 6.0, at 2.0 mL/min. A contamination of less than 5% for several of the compounds and no contamination (<1%) were detected for most of the compounds used. Commercially purchased GDPβS had 10% GMP and 5% GDP present, and commercially purchased GTPγS contained 5–10% GTP and more than 20% GTP. These analogues were purified by DE-32 column chromatography. The purified GTPγS used in our experiments was >98% pure. The concentration of the solutions of thiophosphate analogues of GDP and of GTP was estimated by absorbance measurements at 260 nm (ϵ = 11 800 M^{-1}) (Boehringer, Biochemical Information I technical bulletin).

Purification of GTPγS by Chromatography on DE-32. Whatman DE-32 resin was washed first with 25 mM HCl and then with 25 mM NaOH followed by deionized water. The resin was equilibrated in 1 mM NH_4HCO_3 without pH adjustment. A 0.5 × 10 cm column was packed with DE-32 resin in 1 mM NH_4HCO_3. Approximately 20 mg of GTPγS was dissolved in 2 mL of 1 mM NH_4HCO_3 and loaded onto the column. The column was washed with 30 mM NH_4HCO_3 to remove GMP. After completion of the wash, the column was eluted with a linear gradient of 100 mL each of 30 and 150 mM NH_4HCO_3. Fractions (1.0-mL volume) were collected and monitored at 260 nm. GDP eluted at 70 mM NH_4HCO_3, and GTPγS eluted at 100 mM NH_4HCO_3. Fractions containing GTPγS were pooled and lyophilized. The purity of GTPγS was greater than 98% as determined by HPLC.

Results

Activation of the Reversible Oxalacetate Decarboxylation Reaction by Mn^{2+}. In the experiments where velocity is measured as a function of nucleotide (ITP) at fixed varying Mn^{2+} concentrations, the distributions of MnITP, ITP, and Mn^{2+}_f are calculated by using a dissociation constant of 11 x 10^{-6} M (Lee & Nowak, 1984). A Lineweaver–Burk plot of the data, treated as MnITP or as ITP, gives straight lines. These results cannot distinguish if MnITP or if ITP can serve as substrate. A similar treatment has been performed in the reverse direction using IDP (Hebda & Nowak, 1982). The kinetic data were then treated according to the model which assumes that both forms of the nucleotide serve as substrates. The approach used is outlined under Experimental Procedures, and eq 4 was used to analyze the data. This analysis of the data was previously used with ATP diphosphohydrolase to determine the form of the substrate used by that cation-requiring enzyme (Laliberte et al., 1982). All the primary and secondary plots of the data gave straight lines (Figure 1). An evaluation of the kinetic constants for both forms of ITP yields a K_m for ITP of 0.25 mM and a V_m of 14.5 units/mg, and for ITP·Mn, a K_m of 0.40 mM and a V_m of 27.2 units/mg are calculated. The results are consistent with both MnITP and free ITP serving as substrates for avian liver P-enolpyruvate carboxykinase. A replot of the kinetic data as $1/v$ vs. $1/[Mn^{2+}]_f$ shows that the apparent K_M for Mn^{2+} decreases upon increasing the total ITP concentration (data not shown). Upon extrapolation to zero concentration of ITP, a value for the K_A of Mn^{2+} of 17 μM is obtained, and at 2 mM oxalacetate and saturating ITP, the K_m is 2 μM.

Kinetic Studies of the Carboxylation Reaction. The S_P and R_P diastereomers of GDPαS were investigated as potential substrates and as inhibitors of P-enolpyruvate carboxykinase in the P-enolpyruvate carboxylation reaction. Both of these diastereomers served as substrates for the reaction. The kinetic measurements were performed with Mn^{2+} and with several other activating cations and combinations of cations. The results of these experiments are tabulated in Table I. The R_P diastereomer is a better substrate than the S_P diastereomer on the basis of the V_{max} values measured (R_P/S_P ratio of 2–5), regardless of the cations used. The stereospecific discrimination is not substantial.

Since the diastereomers of GDPαS elicit a low V_m compared to GDP, inhibition kinetics could also be studied. The inhibition kinetics were measured in the presence of Mn^{2+} and Co^{2+}. Both diastereomers of GDPαS showed linear competitive inhibition with K_I values similar to the K_m values measured for these analogues in the presence of 4 mM Mn^{2+} or 10 mM Co^{2+}, respectively, as the cation activator. The K_I values are given in Table I.

GDPβS demonstrates no substrate activity for P-enolpyruvate carboxykinase under the experimental conditions, regardless of the metal ion activators. The cation activators

Table II: Inhibition Constants for GDPβS

metal ion	K_m for GDP (mM)	K_I for GDPβS (mM)
1.0 mM Mn^{2+}	0.017	0.108
4.0 mM Mg^{2+} + 0.01 mM Mn^{2+}	0.045	0.092
4.0 mM Co^{2+}	0.079	0.099

tested were 1.0 mM Mn^{2+}, 4.0 mM Co^{2+}, and 4.0 mM Mg^{2+} with 0.01 mM Mn^{2+}. Increasing the P-enolpyruvate concentration to 20 mM in the presence of 4 mM Mn^{2+} and a high enzyme concentration did not elicit substrate activity with GDPβS (<0.01% activity). GDPβS shows linear competitive inhibition measured with the three different activating metal ions. The inhibition constants (K_I) are given in Table II.

Kinetic Studies of the GTP-Dependent Decarboxylation Reaction. Thiol analogues of GTP (αS, βS, and γS) were investigated as possible substrates for the decarboxylation reaction of oxalacetate to yield P-enolpyruvate. The reaction was monitored by the two-step assay to determine P-enolpyruvate formation. These analogues were studied by using various cations or combinations of cations as activators. Control experiments using GTP as a substrate were performed. The kinetic parameters measured for the GTPαS isomers are given in Table III. In the presence of Mg^{2+}, Co^{2+}, or 2 mM Mg^{2+} with 0.01 mM Co^{2+} as the cation activators, the R_P diastereomer of GTPαS is a better substrate than is the S_P diastereomer on the basis of the V_m/K_m and V_{max} values. However, in the presence of 2 mM Mg^{2+} and 0.05 mM Mn^{2+} or of 2 mM Mg^{2+} and 0.01 mM Cd^{2+} as the cation activators, the S_P diastereomer is a better substrate than the R_P diastereomer on the basis of the V_m/K_m and V_{max} values. In the presence of Co^{2+} or Mg^{2+}/Co^{2+}, GTPαS(R_P) is even a better substrate than is GTP.

GTPβS diastereomers show high stereospecificity regardless of the metal ions used in the assay (Table IV). The S_P diastereomer is a better substrate than the R_P diastereomer on the basis of the V_m/K_m and V_{max} values. No activity is observed with GTPβS(R_P) in the presence of Mg^{2+} or Mn^{2+}

Table III: Kinetic Parameters for the Substrate GTPαS

metal ion	nucleotide	K_m (mM)	V_m (units/mg)	rel V_m (%)	V_m/K_m [(min·mg)$^{-1}$]	X^a
2.0 mM Mg^{2+}	GTP	0.078	5.13	100	65.8	
	GTPαS(S_P)	0.049	1.25	24	27.2	
	GTPαS(R_P)	0.017	2.05	40	120.6	4.4
2.0 mM Mg^{2+}, 0.05 mM Mn^{2+}	GTP	0.135	25.0	100	185.2	
	GTPαS(S_P)	0.488	16.7	67	34.2	
	GTPαS(R_P)	0.400	6.9	27	17.2	0.5
2.0 mM Mg^{2+}, 0.01 mM Co^{2+}	GTP	0.070	11.3	100	162.9	
	GTPαS(S_P)	0.33	9.6	84	72.2	
	GTPαS(R_P)	0.036	17.2	150	477.8	6.6
2.0 mM Mg^{2+}, 0.01 mM Cd^{2+}	GTP	0.083	11.7	100	273.5	
	GTPαS(S_P)	0.050	4.17	18.4	83.4	
	GTPαS(R_P)	0.270	2.70	11.9	10.0	0.12
4.0 mM Co^{2+}	GTP	0.177	12.0	100	67.8	
	GTPαS(S_P)	0.200	9.4	78	47.0	
	GTPαS(R_P)	0.105	13.1	109	124.8	2.7
[Mn^{2+}] = $^1/_2$[nucleotide]	GTP	0.143	11.26	100	79.4	
	GTPαS(S_P)	0.200	10.0	88	50.0	
	GTPαS(R_P)	0.045	7.94	70	176.4	3.5

[a] Ratio of V_m/K_m for GTPαS(R_P) to V_m/K_m for GTPαS(S_P).

Table IV: Kinetic Parameters for the Substrate GTPβS

metal ion	nucleotide	K_m (mM)	V_m (units/mg)	rel V_m (%)	V_m/K_m [(min·mg)$^{-1}$]	X^a
2.0 mM Mg^{2+}	GTP	0.045	5.7	100	126.7	
	GTPβS(S_P)	0.043	0.074	1.3	1.7	
	GTPβS(R_P)			<0.01		
2.0 mM Mg^{2+}, 0.05 mM Mn^{2+}	GTP	0.123	28.6	100	232.5	
	GTPβS(S_P)	0.088	5.1	18	58.0	
	GTPβS(R_P)	0.042	0.03	0.1	0.7	0.012
2.0 mM Mg^{2+}, 0.01 mM Co^{2+}	GTP	0.089	11.2	100	125.8	
	GTPβS(S_P)	0.087	2.8	25	32.2	
	GTPβS(R_P)	0.043	0.03	0.3	0.7	0.022
2.0 mM Mg^{2+}, 0.01 mM Cd^{2+}	GTP	0.027	24.8	100	918.5	
	GTPβS(S_P)	0.034	5.5	22	161.7	
	GTPβS(R_P)	0.043	0.04	0.2	0.9	0.006
4.0 mM Co^{2+}	GTP	0.227	14.2	100	63.0	
	GTPβS(S_P)	0.084	1.2	8.4	14.3	
	GTPβS(R_P)	0.172	0.01	0.07	0.06	0.004
[Mn^{2+}] = $^1/_2$[nucleotide]	GTP	0.143	11.36	100	79.4	
	GTPβS(S_P)	0.132	2.94	26	22.3	
	GTPβS(R_P)			<0.01		

[a] Ratio of V_m/K_m for GTPβS(R_P) to V_m/K_m for GTPβS(S_P).

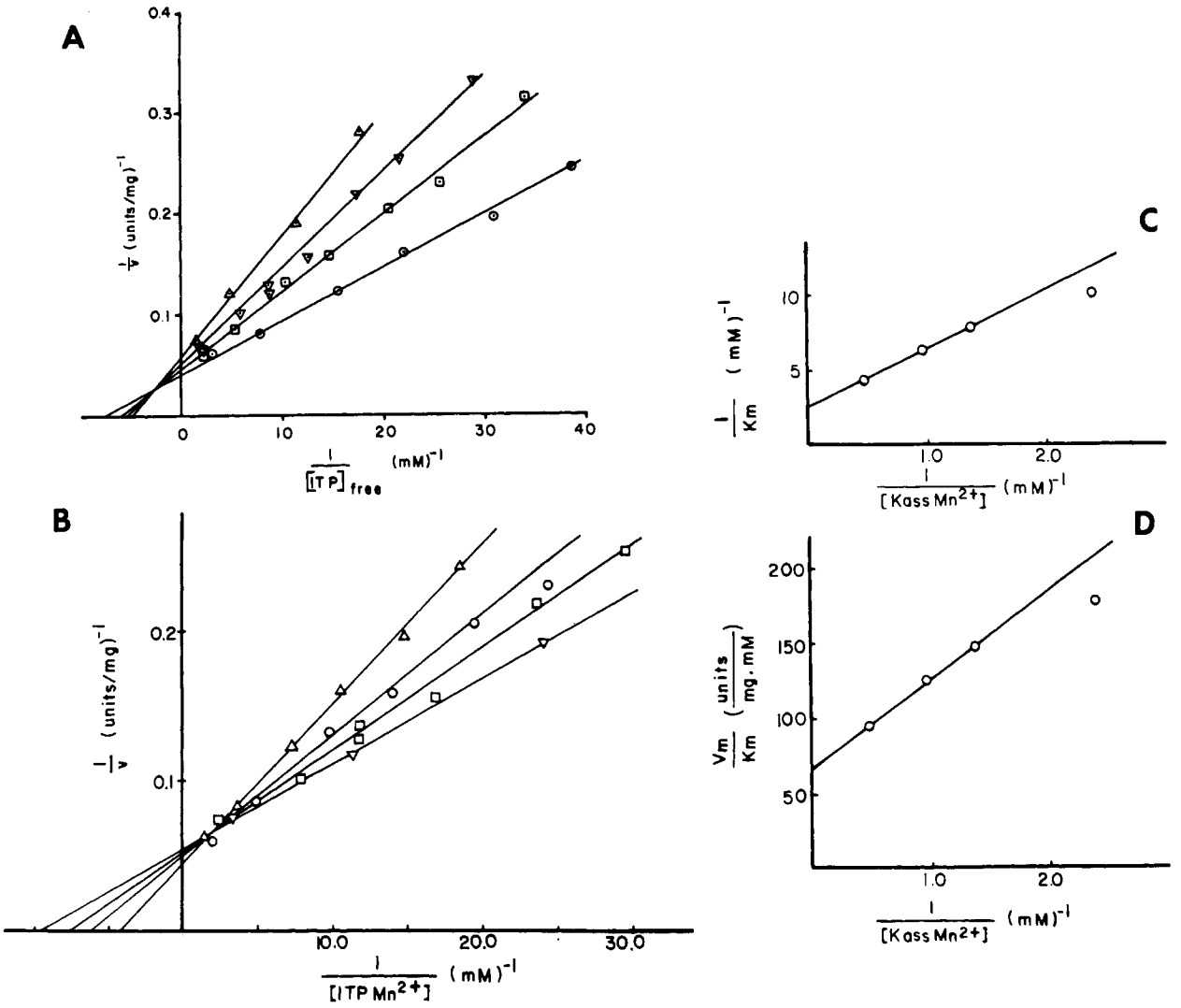


FIGURE 1: Kinetic treatment of the effect of Mn^{2+} concentration on enzymatic activity as a function of ITP and MnITP concentrations. The fixed-time assay with 2 mM oxalacetate was performed. The ITP concentration was varied at fixed varying concentrations of Mn^{2+}. The data were plotted as (A) reciprocal free ITP concentration at free Mn^{2+} concentrations of 4 (△), 7 (▽), 10 (□), and 20 μM (○) and as (B) reciprocal ITP·Mn concentration at free Mn^{2+} concentrations of 4 (△), 7 (▽), 10 (□), and 20 μM (○). In (C), the data are replotted from (A) as $1/K_m$ apparent vs. reciprocal of the product of the association constant for ITP·Mn [9.09×10^4 M^{-1} (K_A)] and free Mn^{2+}. In (D), the data are replotted from (B) as slope (V_m/K_m) vs. $K_A[Mn^{2+}]$.

as the divalent cation ($V_m < 0.01\%$).

GTPγS demonstrated substrate activity in the P-enolpyruvate carboxykinase catalyzed reaction. The substrate activity was first shown by ^{31}P NMR. The proton-decoupled ^{31}P spectra of GTP, GTPγS (contaminated with 20% GDP and 10% GTP), and GTPγS after reaction with oxalacetate in the presence of P-enolpyruvate carboxykinase are shown in Figure 2. After a reaction mixture which contained GTPγS and P-enolpyruvate carboxykinase was incubated for 2 h at room temperature, a spectrum of GDP was observed as well as a proton-decoupled singlet, 41 ppm downfield from where the singlet for P-enolpyruvate is normally observed. This large downfield shift is consistent with the magnitude of the ^{31}P shift observed when a nonbridge oxygen in a phosphate dianion is replaced by a sulfur atom. When the above sample was kept in D_2O solution for several hours at room temperature, the thiophosphoryl peak of thiophosphoenolpyruvate completely disappeared, and the resonance for P-enolpyruvate appeared (data not shown). The intensities of the singlet 41 ppm downfield from P-enolpyruvate and the peak for P-enolpyruvate initially were approximately equal when first observed.

The steady-state kinetic studies using GTPγS as the substrate and various divalent cations as the activator were performed with the two-step assay (Table V). The kinetics of the reaction were metal ion dependent. The highest activity was obtained when Mn^{2+} was used as a sole cation activator. GTPγS was also shown to be a potent competitive inhibitor

Table V: Kinetic Parameters for the Substrate GTPγS

metal ion	nucleotide	K_m (mM)	V_m (units/mg)	rel V_m (%)	V_m/K_m [(min·mg)$^{-1}$]
2.0 mM Mg^{2+}	GTP	0.27	6.1	100	22.6
	GTPγS	0.29	0.19	3.1	0.66
2.0 mM Mg^{2+}, 0.05 mM Mn^{2+}	GTP	0.53	33.3	100	62.8
	GTPγS	0.40	5.4	16.2	13.5
2.0 mM Mg^{2+}, 0.02 mM Co^{2+}	GTP	0.33	6.6	100	20.0
	GTPγS	0.56	0.77	12.8	1.4
2.0 mM Mg^{2+}, 0.01 mM Cd^{2+}	GTP	0.47	11.8	100	25.1
	GTPγS	0.50	0.50	4.2	1.0
[Mn^{2+}] = $^1/_2$[nucleotide]	GTP	0.40	10.5	100	26.3
	GTPγS	0.30	3.5	33.3	11.7

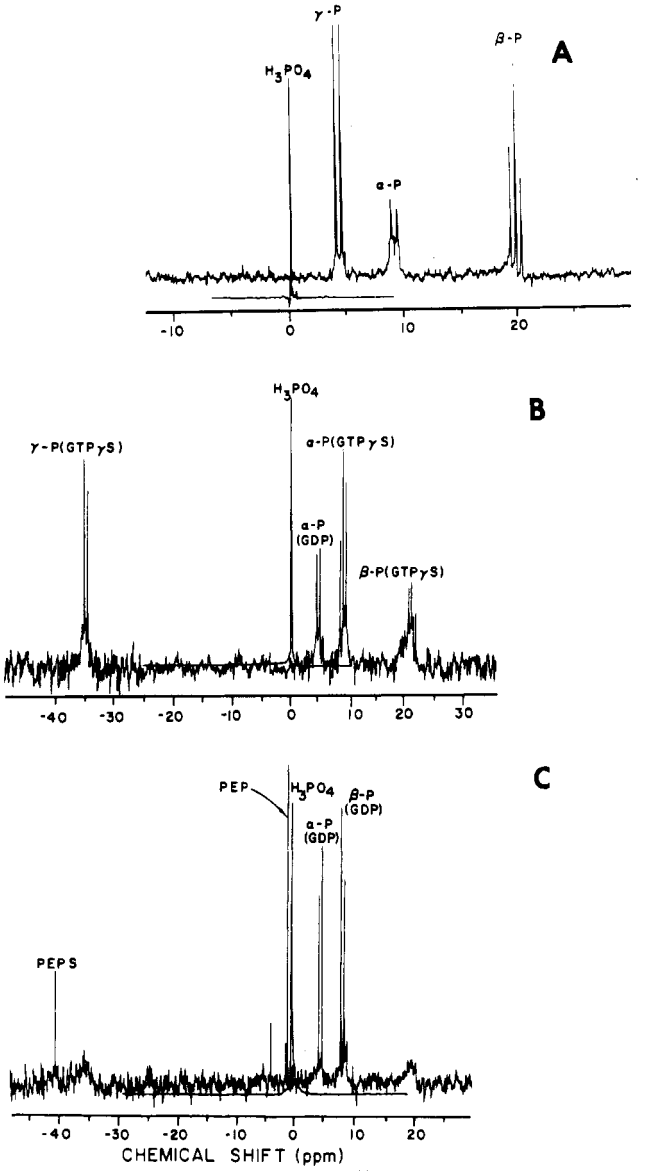


FIGURE 2: Proton-decoupled ^{31}P NMR spectra of GTP and GTPγS. Sample volumes were 2.0 mL and contained ~50% D_2O. The ^{31}P chemical shifts are expressed relative to 50% H_3PO_4 as an external standard, positive values being assigned to shifts at higher field than that of the reference substance. The spectra were obtained at 40.5 MHz. (A) Spectrum of 28 mM GTP in 65 mM Tris–acetate buffer, pH 7.4. NMR parameters: pulse delay, 1 s; sweep width, 2.5 kHz; memory size, 4K; number of acquisitions, 50; line broadening, 1 Hz. (B) Spectrum of 6 mM GTPγS in 64 mM Tris–acetate buffer, pH 7.4, which contained 6.5 mM oxalacetate, 71.5 mM β-mercaptoethanol, 0.5 mM EDTA, and 84 μg of P-enolpyruvate carboxykinase. NMR parameters: pulse delay, 1 s; sweep width, 5 kHz; memory size, 4K; number of scans, 1000; line broadening, 1 Hz. (C) Spectrum of 6 mM GTPγS in Tris–acetate buffer, pH 7.4, containing 71.5 mM β-mercaptoethanol, 2 mM Mg^{2+}, 0.05 mM Mn^{2+}, 10 mM oxalacetate, and 0.17 mg of P-enolpyruvate carboxykinase. The spectrum was obtained after the sample was incubated at room temperature for 2 h. NMR parameters were the same as in (B).

in the presence of 1 mM Mg^{2+} and 0.05 mM Mn^{2+}. These inhibition studies were performed by using the continuous assay. The inhibition constant (K_I = 0.005 mM) was 6 times lower than the apparent Michaelis constant for GTP (K_m = 0.032 mM), measured under identical experimental conditions.

The substrate analogues of GTP, α,β-methylene-GTP (GPCPOP), β,γ-methylene-GTP (GPOPCP), and β,γ-imido-GTP (GPOPNP), were also investigated as possible substrates for the reversible decarboxylation of oxalacetate to form P-enolpyruvate. None of these analogues showed any substrate activity in the P-enolpyruvate carboxykinase catalyzed reaction. These analogues also failed to demonstrate any significant inhibition of activity as measured in the presence of 2 mM Mg^{2+} and 0.05 mM Mn^{2+} as cation activators. The analogues did appear to increase the initial velocity slightly at subsaturating GTP concentrations. This slight "activation" was not due to a contamination by substrate (GTP) since the purity of these analogues, determined by HPLC, >98%. These results suggest that the P–O–P bond length and/or bond angle of the nucleotide at the β-position is absolutely critical for the nucleotide interaction with P-enolpyruvate carboxykinase.

Discussion

Kinetic studies have demonstrated (Lee et al., 1981) a dual cation function for avian liver P-enolpyruvate carboxykinase catalysis. One cation is required to activate the enzyme while the second cation serves as part of the metal–nucleotide complex which is a substrate. The cation which binds to the enzyme is involved in the binding of the substrates P-enolpyruvate and nucleotide (Hebda & Nowak, 1982b). For simplicity in this discussion, M_1 and M_2 are designed as the enzyme-activating cation and as the cation which serves as part of the substrate (the metal–nucleotide complex), respectively.

It has been shown by ^{31}P NMR studies (Lee & Nowak, 1984) that the enzyme-bound Mn^{2+} interacts with the α- and β-phosphoryl groups of GDP and the α-, β-, and γ-phosphoryl groups of GTP in second-sphere coordination complexes. The Mn^{2+}–phosphate distances in the respective ternary complexes are nearly identical (6.0–6.3 Å). The mode of interactions of M_2 to the nucleotide in the ternary P-enolpyruvate carboxykinase–M_1–nucleotide complex has yet to be established. If M_2 interacts directly with the polyphosphoryl groups of the nucleotide, one might expect that the nucleotide orientation would be more dependent upon M_2 than on M_1. This expectation is based on the premise that a specific orientation of the polyphosphate group is required by the enzyme. The kinetic results presented in Figure 1 offer evidence that M_2 is not obligatory for this reaction however.

In the experiments performed with the nucleotide thiophosphate ligands reported in this paper, nearly all of the nucleotide is as the M_2–nucleotide complex. Experimental conditions were such that M_2 concentrations were usually 2–10 mM except when Mn^{2+} was used as a sole cation activator where the Mn^{2+} to nucleotide concentration ratio was kept constant (1:2) and the concentrations of nucleotide were varied from 0.05 to 1.0 mM. Even under these conditions, Lineweaver–Burk plots were linear. Kinetic parameters were measured, and substrate specificity was compared from V_{max} and V_m/K_m values. The V_m/K_m term combines rate and binding terms and has the form of the second-order rate constant (k_1) for the formation of the enzyme–substrate complex. In considering this term, the better the substrate for the reaction, the closer the V_m/K_m value approaches the actual rate constant (k_1) for formation of the enzyme–substrate complex.

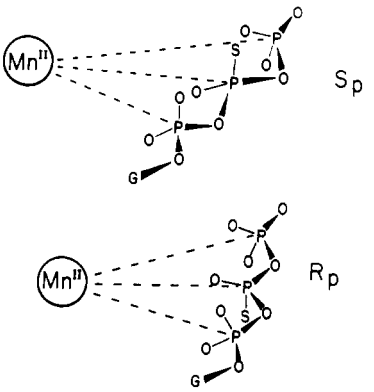
Both the R_P and S_P diastereomers of GDPαS which serve as substrates for P-enolpyruvate carboxykinase undergo slow turnover (0.4–20%) compared to GDP. The metal ions elicit only a small effect on the relative specificity of these pseudosubstrates. The R_P isomer is a somewhat better substrate than the S_P isomer regardless of the activating metal ions used in the assay. The Michaelis constants (K_m) for S_P and R_P isomers are nearly the same. The substitution of sulfur for an oxygen in the α-position is not detrimental to nucleotide interaction. The inhibition constants (K_I) for the diastereomers of GDPαS are the same as the K_m values when these ligands

are substrates. This correlation indicates that the K_m and K_I values are both dissociation constants for the analogues and that the formation of the enzyme–M–ligand inhibitory complex is the same as the enzyme–M–ligand active complex which undergoes catalysis. The replacement of the M_2 cation Mg^{2+} by Co^{2+} or by Mn^{2+} did not show any reversal or relaxation of stereospecificity which might be expected if M_2 is specifically liganded to the α-phosphorus of GDPαS or if the orientation of the metal–substrate complex on the enzyme is specific. One explanation for these results is that M_2 is not directly liganded to the α-phosphorus of GDP in the enzyme complex. Any plausible interaction of the α-P with M_2 is a weak one. There is a favored, but not an absolute, conformation of a M_2–GDP complex which is utilized in oxalacetate formation. The interaction of the α-phosphorus of GDP with the enzyme may be more important than its interaction with M_2 to elicit efficient catalytic activity.

Both the R_P and S_P diastereomers of GTPαS were found to be good substrates for P-enolpyruvate carboxykinase. The stereospecificity is M_1 dependent. The differences in stereospecificity seen with GTPαS are small ($0.4 < R_P/S_P < 2$, based on the relative V_m) when compared to the large differences in stereoselectivity obtained with GTPβS (Table IV). The lack of change in stereoselectivity for GTPαS with change in M_2 is consistent with the lack of coordination to the α-phosphate group of GTP in the enzyme–M_1–GTP–M_2 complex.

The analogue GDPβS did not show any substrate activity (<0.001%) with P-enolpyruvate carboxykinase using either Mn^{2+} (4 mM), Co^{2+} (10 mM), or Mg^{2+} (4 mM) and Co^{2+} (0.02 mM) as the cation activators. GDPβS is a competitive inhibitor against GDP, indicating that this analogue binds to the active site although with a somewhat lower affinity than GDP ($K_I > K_m$). Since GTPβS serves as a substrate for the enzyme, one would expect that GDPβS would also act as a substrate in the reverse direction. The detection of this reaction is made more difficult by the anticipated shift in the equilibrium constant of this reaction by a factor of 60, as shown to occur for ATP-utilizing kinases (Lerman & Cohn, 1980). This effect does not totally explain our lack of observation of enzymatic turnover, however. The substitution of one oxygen atom with a sulfur atom in the β-position of GDP yields a prochiral phosphate. If the interaction between the β-phosphate of the nucleotide and M_1 occurs via hydrogen bonding through an intervening water molecule, an oxygen rather than the sulfur atom in the β-position of GDP will be preferred as the hydrogen-bond acceptor. In this case, the β-phosphate of GDPβS in the enzyme–M_1–GDPβS complex becomes chiral. If an additional interaction between the β-phosphate of GDP and M_2 (or an acidic amino acid on the enzyme) occurs with a strong preference for binding to the oxygen atom, then the sulfur atom would remain as the nucleophile which potentially reacts with P-enolpyruvate. Such a complex might be favorable for binding but unfavorable for the chemical reaction where the sulfur atom would be a poor nucleophile for attack at P-enolpyruvate. It can be demonstrated that such a competitive "incorrect" substrate binding can increase the K_m and decrease the V_m observed for the substrate. Such an incorrect orientation has previously been postulated for GDPβS binding at the active site of elongation factor from *Bacillus stearothermophilus* (Wittinghofer et al., 1982). An alternate possibility is that steric hindrance of GDPβS by the sulfur atom could prevent either of the two prochiral oxygen atoms from being in a favorable conformation to elicit nucleophilic attack on the phosphoryl group of P-enolpyruvate. This would require very selective binding of nucleotides by the enzyme. The lack of any interaction of nucleotide analogues modified at the bridging oxygen atoms around the β-phosphorus with this enzyme is consistent with high selectivity.

Scheme I: Proposed Model of the E–M_1–GTPβS Complex



The GTPβS diastereomers show a high stereospecificity for P-enolpyruvate carboxykinase. Substrate reactivity of the S_P isomer is about 50–200 times greater than that of the R_P isomer on the basis of the V_m/K_m or the relative V_m values (Table IV). The difference in the Michaelis constants between the R_P and S_P isomers is not significant in most cases and implies that the R_P isomer interacts with the enzyme to the same degree as does the S_P isomer. The stereoselectivity is metal ion (M_1 or M_2) independent, however. Since M_1 interacts with the β-phosphate as the second-sphere coordination complex (Lee & Nowak), 1984), M_1-dependent stereospecificity was not expected. However, the relative V_{max} with GTPβS(S_P) increases with M_1 in the order Mg^{2+} (1.2%) < Mn^{2+} (18%) < Cd^{2+} (22%) < Co^{2+} (25%). The electronegativity of these metal ions are in the same order: Mg^{2+} (1.2) < Mn^{2+} (1.5) < Cd^{2+} (1.7) < Co^{2+} (1.8). This correlation suggests that the interaction of M_1 with the β-phosphorus of GTPβS is important even though it is modulated by a bridging ligand. The interaction of M_1 with the β-phosphorus of GTPβS is likely to be via hydrogen bonding of a water ligand. The strength of hydrogen bonding between a water molecule on M_1 and the β-phosphate is proportional to the electronegativity of M_1. The oxygen atom rather than the sulfur atom in the β-position of GTPβS is the preferred hydrogen-bond acceptor. The interaction between the oxygen atom in the β-position of GTPβS and M_1 via a hydrogen bond could yield constraints of conformation of the nucleotide at the active site of the enzyme. These constraints could make the β-sulfur atom in the enzyme–M_1–GTPβS–M_2 complex bind to M_2 without a preference of "hard" vs. "soft" metal ions. The conformation of the γ-phosphate is different for the two diastereomers when these constraints are invoked.

Taking into account the distances between M_1 and the α-, β-, and γ-phosphorus atoms of GTP in the enzyme–M_1–GTP complex, calculated by ^{31}P relaxation rate measurements, and the assumption that an interaction of an oxygen atom from the β-phosphate of GTP to M_1 by hydrogen bonding occurs via a water molecule, a model of the enzyme–M_1–GTPβS complex for both the R_P and S_P isomers can be made. If the sulfur atom on the β-phosphate and the oxygen atom on the γ-phosphate do not differentiate soft vs. hard metal ions within the constraints of the enzyme active site, both the R_P and S_P isomers can form a β,γ-bidentate chelate with M_2 in the enzyme–M_1–GTPβS–M_2 complex. These models are shown in Scheme I. The extended configuration of GTPβS in the enzyme–M_1–GTPβS complex is unfavorable for α,β,γ-tri-

dentate chelation with M_2. The M_2-independent stereospecificity of GTPαS also argues against a tridentate nucleotide–M_2 complex in the enzyme–M_1–GTPβS–M_2 complex. The question then arises why the enzyme shows high stereospecificity for GTPβS. If there is an interaction between the β-phosphate and an amino acid on the enzyme (as postulated for the GDPβS interaction) which could affect the M_2 interaction with the β-phosphate of GTP, the enzyme is highly stereospecific for the nucleotide interaction. The conformations about the β–γ-positions clearly differ for the two diastereomers. The proposal that the polyphosphate chain of the nucleotide interacts with an amino acid residue (or residues) of the enzyme is supported by the low K_s values for GDP (14.5 μM) and for GTP (<2 μM) determined with the apoenzyme (Lee & Nowak, 1984). Similar results have been reported for pyridoxal kinase (Churchich & Wu, 1982), acetate kinase (Romanniuk & Eckstein, 1981), and myosin ATPase (Connolly & Eckstein, 1981). It has been suggested (Connolly & Eckstein, 1981) that the Cd·ATPβS complex can form the same configuration as Mg·ATPβS if the unfavored Cd^{2+} to oxygen coordination takes place at the enzyme active site. This coordination can occur if the β-phosphate oxygen in the metal nucleotide chelate ring undergoes an additional interaction with an amino acid residue of the enzyme.

The enzyme which was used for kinetic studies with GTPγS was prepared by the modified method of Hebda & Nowak (1982a) and shows minor differences in kinetic parameters. In the enzyme-catalyzed reaction with GTPγS as the substrate, a change in M_1 elicits a change in the relative V_m. The relative V_{max} does not exactly follow the order of electronegativity of M_1 which was shown with GTPβS. However, Mg^{2+} as M_1, which has the lowest electronegativity (probably the poorest metal to facilitate hydrogen bonding between the water molecule on the Mg^{2+} and an oxygen atom in the γ-position of GTPγS), shows the lowest substrate activity. When Mg^{2+} is replaced by Mn^{2+} as M_2 (when Mn^{2+} serves as M_1), a greater stimulation is obtained, suggesting that the interaction between Mn^{2+} and the γ-phosphate of GTPγS is favorable. Mn^{2+} can chelate both the sulfur and oxygen atoms while Mg^{2+} prefers an oxygen ligand. Thus, it is proposed that M_2 is liganded directly to the γ-phosphate of GTP on the enzyme for the P-enolpyruvate carboxykinase reaction.

The GTPγS analogue was also shown to be a substrate for P-enolpyruvate carboxykinase from guinea pig liver. The stereospecifically labeled (R_P)-[3-^{18}O]GTPγS gave (S_P)-thio[^{18}O]phosphoenolpyruvate (Shen et al., 1984). This observation demonstrated that the enzyme catalyzes a direct phosphoryl transfer from GTP to oxalacetate and is consistent with the mechanism previously proposed for this enzyme (Lee & Nowak, 1984).

A comparison of the rate of the reaction (V_m) as a function of the position of substitution of oxygen with sulfur shows approximately the order GTPαS > GTPβS > GTPγS. According to the generalization of Ngoc et al. (1979), if the catalytic cleavage step is the rate-determining step in the reaction, the reactivity of the thiol analogues of GTP increases progressively with the distance from the site of sulfur substitution. The rate-determining step with P-enolpyruvate carboxykinase may thus be the catalytic cleavage step. Ligand exchange rates for P-enolpyruvate, GDP, and GTP from the enzyme–Mn–ligand complex are much faster than the catalytic constant (Duffy & Nowak, 1985; Lee & Nowak, 1984).

In conclusion, the interaction of the enzyme-bound metal ion M_1 with the α-, β-, and γ-phosphates of GTP is important in binding and in catalysis by P-enolpyruvate carboxykinase. This interaction appears to elicit the proper configuration of GDP and of GTP even though these ligands are in the second coordination sphere of M_1. The enzyme shows a high stereospecificity at the β-phosphate of GTP for catalysis. The results suggest an additional amino acid residue of the enzyme is involved in the interaction of the β-phosphate to form a chiral active site of the enzyme. Since GTP has an extended configuration in the enzyme–M_1–GTP complex, β,γ-bidentate coordination of the nucleotide with M_2 is more favorable than a α,β,γ-tridentate complex. There is no significant M_2 dependence of the stereospecificity of GDPαS or of GTPαS.

The α-phosphate of GDP is also unlikely to be liganded to M_2. Thus, it is proposed that avian liver P-enolpyruvate carboxykinase releases monodentate M_2·GDP after cleavage of the γ-phosphate bond of the β,γ-bidentate M_2·GTP with the acceptor oxalacetate.

Registry No. 5′-GDP, 146-91-8; GDPαS (S_P), 71481-45-3; GDPαS (R_P), 71481-44-2; GDPβS, 71376-97-1; GTP, 86-01-1; GTPαS (S_P), 81570-51-6; GTPαS (R_P), 81570-50-5; GTPβS (S_P), 81570-53-8; GTPβS (R_P), 81570-52-7; GTPγS, 37589-80-3; ITP, 132-06-9; ITP·Mn, 79724-82-6; Mg, 7439-95-4; Mn, 7439-96-5; Co, 7440-48-4; Cd, 7440-43-9; P-enolpyruvate carboxykinase, 9013-08-5.

References

Burgers, P. M. J., & Eckstein, F. (1980) *J. Biol. Chem. 255*, 8229–8233.

Churchich, J. E., & Wu, C. (1982) *J. Biol. Chem. 257*, 12136–12140.

Connolly, B. A., & Eckstein, F. (1981) *J. Biol. Chem. 256*, 9450–9456.

Connolly, B. A., Romaniuk, P. J., & Eckstein, F. (1982) *Biochemistry 21*, 1983–1989.

Cotton, F. A., & Wilkinson, G. (1972) *Advanced Inorganic Chemistry*, pp 510–515, Wiley, New York.

Duffy, T. H., & Nowak, T. (1985) *Biochemistry 24*, 1152–1160.

Eckstein, F. (1983) *Angew. Chem., Int. Ed. Engl. 22*, 423–439.

Goody, R. S., & Eckstein, F. (1971) *J. Am. Chem. Soc. 93*, 6252–6257.

Goody, R., & Leberman, R. (1979) *FEBS Lett. 102*, 269–272.

Goody, R. S., Eckstein, F., & Schirmer, R. H. (1972) *Biochim. Biophys. Acta 276*, 155–161.

Hebda, C. A., & Nowak, T. (1982a) *J. Biol. Chem. 257*, 5503–5514.

Hebda, C. A., & Nowak, T. (1982b) *J. Biol. Chem. 257*, 5515–5522.

Jaffe, E. K., & Cohn, M. (1978a) *J. Biol. Chem. 253*, 4823–4825.

Jaffe, E. K., & Cohn, M. (1978b) *Biochemistry 17*, 652–657.

Jaffe, E. K., Nick, J., & Cohn, M. (1982) *J. Biol. Chem. 257*, 7650–7656.

Laliberte, J. F., St. Jean, P., & Beaudoin, A. R. (1982) *J. Biol. Chem. 257*, 3869–3879.

Lee, M. H., & Nowak, T. (1984) *Biochemistry 23*, 6506–6513.

Lee, M. H., Hebda, C. A., & Nowak, T. (1981) *J. Biol. Chem. 256*, 12793–12801.

Lerman, C. L., & Cohn, M. (1980) *J. Biol. Chem. 255*, 8756–8760.

Ngoc, L. D., Jebeleanu, G., & Bargu, O. (1979) *FEBS Lett. 97*, 65–68.

Noce, P. S., & Utter, M. F. (1975) *J. Biol. Chem. 250*, 9099–9105.

Pillai, R. P., Raushel, F. M., & Villafranca, J. J. (1980) *Arch. Biochem. Biophys. 199*, 7–15.

Romanniuk, P. J., & Eckstein, F. J. (1981) *J. Biol. Chem. 256*, 7322–7328.

Rösch, P., Goody, R. S., & Isakov, M. (1984) *Phosphorus Sulfur 21*, 9–16.
Schlimme, E., Lamprecht, W., Eckstein, F., & Goody, R. S. (1973) *Eur. J. Biochem. 40*, 485–491.
Segal, I. H. (1975) *Enzyme Kinetics*, pp 242–272, Wiley-Interscience, New York.
Seubert, W., & Huth, W. (1965) *Biochem. Z. 343*, 176–191.
Sheu, K.-F., Ho, H.-T., Nolan, L. D., Markovitz, P. J., Richard, J. P., Utter, M. F., & Frey, P. A. (1984) *Biochemistry 23*, 1779–1783.
Whittinghofer, A., Goody, R. S., Rösch, P., & Kalbitzer, H. R. (1982) *Eur. J. Biochem. 124*, 109–115.

(*E*)-3-Cyanophosphoenolpyruvate, a New Inhibitor of Phosphoenolpyruvate-Dependent Enzymes†

Peter Wirsching and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received September 4, 1984

ABSTRACT: (*E*)-3-Cyanophosphoenolpyruvate has been synthesized by reacting dimethyl chlorophosphate with the potassium enolate of ethyl cyanopyruvate. The resulting trialkyl ester was deesterified with bromotrimethylsilane followed by potassium hydroxide. Subsequent treatment with Dowex-50-H^+ resin and cyclohexylamine afforded the tricyclohexylammonium salt; only the *E* geometric isomer was obtained. This compound can be photoisomerized to a 70:30 *E*:*Z* mixture. (*E*)-3-Cyanophosphoenolpyruvate is an excellent competitive inhibitor of phosphoenolpyruvate carboxylase [$K_I(Mn^{2+})$ = 16 μM, $K_I(Mg^{2+})$ = 1360 μM], pyruvate kinase [$K_I(Mn^{2+})$ = 0.085 μM, $K_I(Mg^{2+})$ = 0.76 μM], and enolase [$K_I(Mn^{2+})$ = 360 μM, $K_I(Mg^{2+})$ = 280 μM]. The compound is a substrate for pyruvate kinase (V_{max} ~ 1% of phosphoenolpyruvate rate), but not for the other two enzymes. No irreversible inactivation is observed with phosphoenolpyruvate carboxylase or pyruvate kinase.

Phosphoenolpyruvate is a key intermediate in a variety of metabolic processes in all living organisms (Davies, 1979). Its chemistry is mediated by enzymes that utilize either the high-energy enol phosphate bond, for example, in glycolysis and in the C-4 pathway of photosynthesis, or the ability to act as an enol pyruvyl donor, for example, in the shikimate pathway and in bacterial peptidoglycan synthesis.

In view of the central role of phosphoenolpyruvate in metabolism, enzymes that metabolize phosphoenolpyruvate are logical targets for active-site-directed (specific) and mechanism-based (suicide) inhibitors. The principal question in such studies is whether specificity of inhibition can be obtained among the variety of phosphoenolpyruvate-metabolizing enzymes that exist. Studies of the antibiotic fosfomycin indicate that such selectivity may be possible. The enzymes pyruvate kinase, phosphoenolpyruvate carboxykinase, enolase, and phosphoenolpyruvate–shikimate-5-phosphate enolpyruvoyltransferase show only competitive inhibition with this compound. However, pyruvoyltransferase is irreversibly inactivated (Cassidy & Kahan, 1973). Such specificity is not predictable at this point.

Among the other phosphoenolpyruvate analogues that have been studied, the most notable are perhaps 3-bromophosphoenolpyruvate and 3-fluorophosphoenolpyruvate. The *Z* isomer of the former compound shows very strong competitive inhibition with phosphoenolpyruvate carboxylase at short times and irreversible (suicide) inactivation at longer times (O'Leary & Diaž, 1982). Thus, the compound is presumably a substrate for phosphoenolpyruvate carboxylase. This compound is also a substrate for pyruvate kinase (Nowak & Duffy, 1984), but the enzyme is not inactivated. By contrast, this same substance is neither a substrate nor an inhibitor for enolase.

(*Z*)-3-Fluorophosphoenolpyruvate is a substrate for pyruvate kinase (Nowak & Duffy, 1984; Stubbe & Kenyon, 1972) and for phosphoenolpyruvate carboxylase (E. Diaž, unpublished results). The compound is an inhibitor and a substrate for enolase (Nowak & Duffy, 1984). The *E* isomer of this same compound is an inhibitor of pyruvate kinase and enolase and a substrate for pyruvate kinase (Nowak & Duffy, 1984).

(*E*)-3-Cyanophosphoenolpyruvate was conceived as an inhibitor of phosphoenolpyruvate-dependent enzymes. Mechanism-based inactivation by this compound would presumably proceed by way of a ketene imine intermediate (Maycock et al., 1975; Miles, 1975; Villafranca & Baldori, 1980). Here we report the synthesis of this compound and studies of its inhibitory properties against phosphoenolpyruvate carboxylase, pyruvate kinase, and enolase.

Experimental Procedures

Materials

Phosphoenolpyruvate carboxylase was isolated from maize (Zea mays, strain W64A) by a slight modification of the method of Uedan and Sugiyama (Uedan & Sugiyama, 1976; O'Leary et al., 1981). Pyruvate kinase (type II from rabbit muscle), enolase (type III from baker's yeast), lactate dehydrogenase, malate dehydrogenase, hexokinase (from yeast), glucose-6-phosphate dehydrogenase (from *Leuconstoc mesenteroides*), phosphoenolpyruvate, ADP, ATP, NAD,[1] NADH, and Tris-HCl were purchased from Sigma Chemical Co. HEPES[1] buffer (free acid) was purchased from United States

† This work was supported by Grant 83-CRCR-1-1297 from the Competitive Grants Program, U.S. Department of Agriculture.

[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; TMSP, sodium 3-(trimethylsilyl)propionate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-7602$01.50/0