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Stereochemical Course of Thiophosphoryl Transfer Catalyzed by Cytosolic Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: Rat liver cytosolic phosphoenolpyruvate carboxykinase (PEPCK) utilizes inosine 5′-(3-thiotriphosphate) (ITPγS) as an excellent substrate, with K_m and V values of 0.08 mM and 37 μmol min^{-1} (mg of protein)$^{-1}$, respectively, compared with the corresponding values of 0.168 mM and 76 μmol min^{-1} (mg of protein)$^{-1}$ for ITP. Thus, the V/K_m values for the two substrates are the same. Reaction of (R_P)-[γ-$^{18}O_2$]ITPγS with oxalacetate catalyzed by cytosolic PEPCK produces (S_P)-thio[^{18}O]phosphoenolpyruvate. Therefore, thiophosphoryl transfer catalyzed by this enzyme proceeds with overall inversion of configuration at P. The reaction mechanism involves an uneven number of phosphotransfer steps, most likely a single step transfer between bound substrates. The results do not support the involvement of a phosphoryl enzyme intermediate in the mechanism.

Mammalian liver phosphoenolpyruvate carboxykinase (PEPCK)[1] is found in two cellular compartments, the mitochondria and the cytosol. Notwithstanding similarities between them, the cytosolic and mitochondrial forms are known to be different enzymes (Tilghman et al., 1976). The two forms are immunochemically distinct (Ballard & Hanson, 1969) and are encorded by different mRNA species transcribed from different genes (Hod et al., 1982).

The stereochemical course of phosphoryl transfer catalyzed by guinea pig liver mitochondrial PEPCK was previously investigated by Sheu et al. (1984) and found to proceed with inversion of configuration. This was interpreted to indicate that the phosphoryl group is probably transferred directly between the substrates by a single step, nucleophilic displacement mechanism. As for the cytosolic enzyme, it has been reported that rat liver cytosolic PEPCK catalyzes the

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[1] Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; IMP, inosine 5′-phosphate; ITPγS, inosine 5′-(3-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ADPαS, adenosine 5′-(1-thiodiphosphate); GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; NADH, reduced nicotinamide adenine dinucleotide; IDP, inosine 5′-diphosphate; 2′,3′-(methoxymethylidene)-IMP, 2′,3′-(methoxymethylidene)inosine 5′-phosphate; 2′,3′-(methoxymethylidene)-AMP, 2′,3′-(methoxymethylidene)adenosine 5′-phosphate; GC/MS, gas chromatography/mass spectrometry.

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transfer of the phosphoryl group from GTP to [^{14}C]GDP in the absence of PEP or oxalacetate at a rate 40% greater than the maximum velocity for the formation of phosphoenolpyruvate under similar conditions, suggesting that a phosphoryl enzyme may be formed under certain conditions (Jomain-Baum & Schramm, 1978). This raises the possibility that phosphoryl transfer catalyzed by the cytosolic enzyme may proceed via a mechanism that differs from that of the mitochondrial enzyme, i.e., a two-step transfer involving a phosphoryl enzyme intermediate.

This paper describes the synthesis of the compound (R_P)-inosine 5′-(3-thio[3-^{18}O]triphosphate) ([γ-$^{18}O_2$]ITPγS), its effectiveness as a substrate with rat liver cytosolic PEPCK, and its use in thedetermination of the stereochemical course of phosphoryl transfer catalyzed by the enzyme.

Materials and Methods

Materials. Rat liver cytosolic PEPCK was purified as described by Colombo et al. (1978). The purified enzyme was dialyzed vs. 20% (v/v) glycerol/H_2O in 0.1 mM EDTA, 2 mM DTT, and 10 mM triethanolamine hydrochloride buffer at pH 7.5. The final enzyme concentration and specific activity were 1.4 mg/mL and 11.4 units/mg of protein, respectively. The enzyme was stored at 4 °C under N_2 and used within a few days. 2′,3′-(Methoxymethylidene)-IMP and ^{18}O-labeled nucleotides were synthesized as described below; all other enzymes, coenzymes, and substrates were purchased from commercial vendors and used as supplied. Triethylamine used to prepare TEAB was distilled before use, and all solvents were dried and distilled before being used. Diazomethane was generated by treatment of *N*-nitrosomethylurea with KOH, extracted into diethyl ether, and redistilled before being used.

Methods. The following procedure was followed in rate measurements used to evaluate K_m and V values for ITP and ITPγS: 0.8 mL of an ice-cold solution containing 100 mM HEPPS–KOH, 1.25 mM ADP, 5 mM $MgCl_2$, 0.125 mM $MnCl_2$, and 0.25 mM NADH, pH 8.0, was mixed in a quartz cuvette of 1-cm path length with 10–100 μL of a roughly 10 mM solution in water of either ITP or ITPγS, pH 10.6 (adjusted with triethylamine to prevent loss of terminal sulfur in ITPγS), and up to 90 μL of water to bring the total volume to 0.9 mL. The cuvette was temperature equilibrated in a 25 °C water bath for 2 min, after which time 0.1 mL of an ice-cold solution of 200 mM HEPPS–KOH, 10 mM oxalacetate, 0.5 mM NADH, and 10 μg/mL lactate dehydrogenase at pH 8.0 was mixed in, and the cuvette was again allowed to equilibrate to 25 °C. Lactate dehydrogenase (50 μg) and 100 μg of pyruvate kinase were then added, and the initial rate of decrease in A_{340} due to decarboxylation of oxalacetate to pyruvate was recorded. Immediately afterward, 10 μL of PEPCK [2.3 mg/mL in 70% saturated $(NH_4)_2SO_4$, 10 mM triethanolamine hydrochloride, and 1 mM DTT, pH 7.5; specific activity 2 μmol of PEP carboxylated min^{-1} (mg of protein)$^{-1}$] was added, and the initial rate of A_{340} loss again was recorded. Under these conditions, all necessary reaction components were saturating except for the nucleotide substrate. Steady-state concentrations of thiophosphoenolpyruvate were less than 10 μM as determined from the lag times of the initial velocities. The initial rates of reaction prior to the addition of PEPCK (7–24%) were subtracted from those following addition. ITPγS did not stimulate the oxalacetate decarboxylase activity of PEPCK. The rates of product formation were determined from these differences, assuming stoichiometric oxidation of NADH with formation of either phosphoenolpyruvate or thiophosphoenolpyruvate, by using an extinction coefficient for NADH of 6.2 mM^{-1} cm^{-1} at 340 nm. The actual concentrations of ITP and ITPγS stock solutions used were determined by absorbance at 249 nm, assuming an extinction coefficient of 12 mM^{-1} cm^{-1}. K_m, V_m, andd V/K_m values for ITP and ITPγS with rat liver cytosolic PEPCK were calculated by fitting the initial velocities obtained at varying concentrations of nucleotide to the equation $v = VA/(K + A)$, where v is the initial velocity, V is the maximum velocity, K is the Michaelis constant, and A is the nucleotide concentration, with a computer program similar to that previously published (Cleland, 1967).

NMR spectra were obtained with a Bruker 270-MHz spectrometer. Ultraviolet absorbance spectra were recorded with a Hitachi 100-80A spectrophotometer.

Synthesis of 2′,3′-(Methoxymethylidene)-IMP. The synthesis of this compound was based on that of 2′,3′-(methoxymethylidene)-AMP described by Richard and Frey (1982), which in turn was based on the method of Darlix et al. (1967). The barium salt of β-cyanoethyl phosphate dihydrate (12.9 g, 4 mmol) was converted to the acid form by dissolving in a 120-mL aqueous slurry containing 48 g of AG 50W-X8 (H^+) ion-exchange resin (50–100 mesh), which was added to the top of a 5 × 15 cm column of the same resin and eluted with water. Fractions (20 mL) were collected, those exhibiting a pH below 2.5 were pooled, and the water was removed by rotary flash evaporation in vacuo at 30 °C. The resulting clear syrup was dried by flash evaporation in vacuo of two additions of 4 mL of dry DMF at 45 °C. The dried residue was redissolved in 40 mL of trimethyl orthoformate and 2.7 g (10 mmol) of dried inosine added. The suspension was stirred magnetically at 35 °C until clear (about 8 h), and thin-layer chromatography indicated complete conversion of inosine to 2′,3′-(methoxymethylidene)inosine. Trimethyl orthoformate was removed by rotary flash evaporation and residual solvent removed by successive evaporation of three additions of 25 mL of dry pyridine. The residue was dissolved in 100 mL of dry pyridine, 10.32 g of dicyclohexylcarbodiimide added, and the solution magnetically stirred for 17 h in a sealed vessel. Water (15 mL) was added and stirring continued for 45 min. The solution was then evaporated under reduced pressure to remove pyridine, and the resulting thick slurry was taken up in 50 mL of water and filtered to remove precipitated dicyclohexylurea, which was rinsed with an additional 50 mL of water. The filtrate was mixed with 200 mL of 7 M ammonium hydroxide, followed by heating in a 60 °C water bath for 2 h. Ammonium hydroxide was removed by stirring magnetically with water aspirator evacuation until the pH fell below 8.6. The solution was then filtered, diluted with water to 500 mL, and passed through a 4 × 34 cm column of DEAE-Sephadex A-25 in the HCO_3^- form equilibrated with 0.15 M TEAB at pH 8. The column was eluted at 3 mL/min with a 4-L linear gradient of TEAB, increasing in concentration from 0.15 to 0.35 M, and 20-mL fractions were collected. 2′,3′-(Methoxymethylidene)-IMP appeared as a major band in fractions 87–162, which were pooled and freed of buffer salts by evaporation to dryness. The yield was 4.2 mmol, 42% based on inosine. The P/inosine ratio was 1.01 ± 0.02 (SD, three measurements). The UV spectral parameters were λ_{max} = 249 nm (ϵ_{249} = 12 × 10^3 M^{-1}·cm^{-1}) and λ_{min} = 227 nm (ϵ_{227} = 4.5 × 10^3 M^{-1}·cm^{-1}). ^{1}H NMR spectral parameters were δ 8.372 (s, 1 H, C-8), 8.075 (s, 0.6 H, C-2, exo), 8.068 (s, 0.4 H, C-2, endo), 6.387 (d, 0.7 H, C-1′, endo, $J_{1',2'}$ = 3.2 Hz), 6.217 (d, 0.8 H, C-1′, exo, $J_{1',2'}$ = 3.0 Hz), 6.103 (s, 0.5 H, methine, exo), 5.990 (s, 0.5 H, methine, endo), 5.471 (dd, 0.6 H, C-2′, exo, $J_{1',2'}$ = 3.0 Hz, $J_{2',3'}$ = 6.2 Hz), 5.372 (dd, 0.4 H, C-2′, endo, $J_{1',2'}$ = 3.0 Hz, $J_{2',3'}$ = 7.0 Hz), 5.224 (dd, 0.6 H, C-3′,

exo, $J_{3',4'}$ = 2.4 Hz, $J_{2',3'}$ = 6.2 Hz), 5.155 (dd, 0.4 H, C-3', endo, $J_{3',4'}$ = 3.0 Hz, $J_{2',3'}$ = 7.1 Hz), 4.565 (m, 0.4 H, C-4', endo), 4.475 (m, 0.4 H, C-4', exo), 4.09 (m, 2.6 H, C-5', exo + endo), 3.440 (s, methoxyl, endo, and 3.313 (s, methoxyl, exo). Spectral assignments were facilitated by reference to related standard spectra found in the Aldrich NMR index and in several research papers (Evans & Sarma, 1974, 1975; Hruska et al., 1973).

Synthesis of (R_P)-[γ-$^{18}O_2$]*ITP*γ*S*. Bis(triethylammonium) 2',3'-(methoxymethylidene)-IMP (380 μmol) dissolved in water was passed into a 4 × 13 cm column of SP-Sephadex C-25 in the pyridinium form, eluted with water, lyophilized, dissolved in 15 mL of methanol, mixed with 0.19 mL of tri-*n*-octylamine, and evaporated to dryness. The sample was dried by twice dissolving in anhydrous DMF and evaporating to dryness. The dried sample was dissolved in 1.5 mL of dry dioxane and mixed with 0.125 mL of diphenyl phosphorochloridate and 0.19 mL of tri-*n*-butylamine for two h at 25 °C. Solvent was removed by rotary evaporation; the residue was shaken with 50 mL of ice-cold petroleum ether–diethyl ether (4:1) and allowed to stand at 0 °C for 0.5 h. The ether layer was decanted, residual ether removed by rotary evaporation, and the residue redissolved in 1.5 mL of dioxane.

Tris(triethylammonium) (S_P)-[α-$^{18}O_2$]ADPαS (190 μmol), prepared as described by Richard and Frey (1982), was converted to the bis(tri-*n*-octylammonium) salt by the procedure described above for 2',3'-(methoxymethylidene)-IMP. The dried sample was redissolved in 1 mL of dry pyridine and the solution of activated 2',3'-(methoxymethylidene)-IMP added dropwise with stirring. After being stirred for 6.5 h at 25 °C the solution was placed in a desiccator at −20 °C overnight. The solvent was removed by rotary evaporation and the residue dissolved and shaken with 5 mL of diethyl ether and 30 mL of water. The pH of the aqueous layer was adjusted to 7.5 with NaOH and mixed with 2.3 mL of 100 mM $NaIO_4$ (1.5 equiv). The pH was maintained at pH 7.5 (NaOH) while the sample was stirred for 20 min, after which time 0.38 g of DTT was added. The solution was adjusted to pH 2.0 with 1 M HCl, maintained for 30 min at 25 °C, adjusted to pH 10.5 with 6 M NaOH, and maintained at 50 °C for 30 min. The reaction mixture was diluted with ice-cold water to 150 mL and applied to a 4 × 32 cm column of DEAE-Sephadex A-25 in the HCO_3^- form equilibrated with 0.3 M TEAB at pH 8. The column was eluted with a 4-L linear gradient of TEAB, increasing in concentration from 0.3 to 0.7 M, and 20-mL fractions were collected at a flow rate of 3 mL/min. (R_P)-[γ-$^{18}O_2$]ITPγS emerged in fractions 154–180, which were pooled and freed of salts by rotary evaporation. The yield was 64 μmol (34%). ITPγS synthesized as described above from ADPαS was characterized by chemical and spectral analysis. The P/inosine ratio was 3.01 ± 0.05 (SD, three measurements). The ultraviolet spectra parameters were λ_{max} = 249 nm (ϵ_{249} = 12 × 10^3 M^{-1}·cm^{-1}) and λ_{min} = 227 nm (ϵ_{227} = 7.53 × 10^3 M^{-1}·cm^{-1}). ^{1}H NMR spectral parameters were δ 8.528 (s, 1 H, C-8), 8.235 (s, 1 H, C-2), 6.151 (d, 1 H, C-1', $J_{1',2'}$ = 5.9 Hz), 4.668 (m), 4.588 (m), 4.419 (m), and 4.305 (m, 5 H, ribose C-2' to C-5').

Enzymatic Synthesis of Thio[^{18}O]*phosphoenolpyruvate*. The 156-mL reaction mixture consisted of 0.5 mM (R_P)-[γ-$^{18}O_2$]ITPγS, 52 mM K-HEPPS buffer at pH 8.1, 1.9 mM $MgCl_2$, 0.19 $MnCl_2$, 1 mM DTT, 0.6 mM oxalacetate, 0.5 unit/mL alkaline phosphatase, and 0.1 unit/mL PEPCK at 30 °C. Thiophosphoenolpyruvate formation was monitored by assaying aliquots of the reaction mixture for phosphoenolpyruvate, with pyruvate kinase, ADP, NADH, and lactate dehydrogenase. The reaction mixture was replenished with an additional 0.6 mM oxalacetate after 11 min. Assays showed the reaction to be complete after a total of 15 min, at which time the pH was raised to 8.6 with KOH. The solution was chilled on ice for 15 min and then chromatographed at 4 °C through a 2.6 × 10 cm column of DEAE-Sephadex A-25 in the HCO_3^- form equilibrated with 0.15 M TEAB. The column was washed with 50 mL of 0.15 M TEAB at pH 8.5, eluted with a 700-mL linear gradient of TEAB increasing from 0.15 to 0.6 M, and finally washed with 200 mL of 0.7 M TEAB. Fractions (10 mL) were collected and assayed for thiophosphoenolpyruvate and reactivity toward DTNB. Thio-[^{18}O]phosphoenolpyruvate was found in fractions 54–66, which were pooled and immediately prepared for transfer of the [^{18}O]thiophosphoryl group to ADP. To the pooled fractions were added 2 mM ADP, 4 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, and finally 20 units/mL pyruvate kinase. Assays of aliquots withdrawn for pyruvate determination using lactate dehydrogenase showed the reaction to be complete within 10 min. The solution was diluted to 350 mL with water and loaded on a 2.6 × 10 cm column of DEAE-Sephadex A-25 in the HCO_3^- form equilibrated with 0.3 M TEAB. The column was washed with 50 mL of 0.3 M TEAB and eluted with a linear gradient of TEAB 1 L in volume increasing from 0.3 to 0.7 M. The recovery of [γ-^{18}O]ATPγS was 66 μmol, for an 85% yield from (R_P)-[γ-^{18}O]ITPγS.

Enzymatic Syntheses of [β-^{18}O]*ADP*β*S*, (R_P)-[β-^{18}O]-*ATP*β*S, and* (S_P)-[β,γ-^{18}O]*ATP*β*S*. Transfer of the thiol-[^{18}O]phosphoryl group from [γ-^{18}O]ATPγS to AMP catalyzed by adenylate kinase and stereoselective phosphorylations of [β-^{18}O]ADPβS to R_P and S_P epimers of ATPβS catalyzed by acetate kinase and pyruvate kinase were carried out essentially as described earlier (Sheu et al., 1984).

Chemical Degradation of ATPβ*S*. Samples of ^{18}O-enriched R_P and S_P epimers were chemically degraded to trimethyl phosphate and trimethyl phosphorothiolate as follows: to 2 μmol of ATPβS in 2 mL of water at pH 8 was added 30 μL of 100 mM $NaIO_4$. The pH was readjusted to 8 with NaOH and the solution stirred at 25 °C for 30 min, after which time 2.5 μL of 2-mercaptoethanol was added. The pH was adjusted to 10.7 with 1 N NaOH and the solution heated at 50 °C for 30 min. The cooled solution was chromatographed through a 0.7 × 11 cm column of DEAE-Sephadex A-25 in the HCO_3^- form equilibrated with 0.3 M TEAB at pH 8. After being washed with 30 mL of 0.3 M TEAB, the column was eluted with 0.7 M TEAB. Fractions were analyzed for total phosphate after washing. 2-Thiotriphosphate-containing fractions were pooled, freed of water and buffer salt by rotary evaporation, and dissolved with 200 μL of methanol and 10 μL of water. The solution was acidified with 10 μL of 2 N HCl and treated with diazomethane in diethyl ether added dropwise at 0 °C with stirring until the color persisted. After the solvent was removed by rotary evaporation, the residue was dissolved in 0.2 mL of water and heated at 100 °C for 1 h. The solvent was again removed and the residue dissolved in 0.2 mL of methanol and again methylated as described above. Excess ether and diazomethane were removed in a stream of N_2, which was also used to reduce the volume to 50 μL in preparation for GC/MS analysis. This method was similar to one briefly described in a communication (Richard et al., 1978).

Results

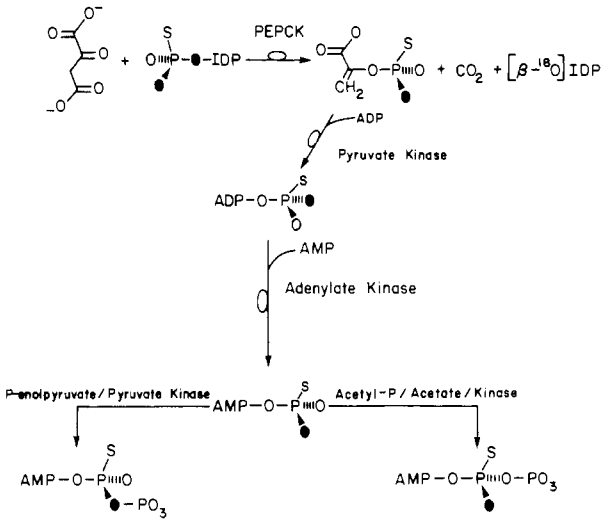
ITPγ*S as a Substrate for PEPCK*. ITPγS is an excellent substrate for rat liver cytosolic PEPCK. K_m and V values for ITP and ITPγS as PEPCK substrates are given in Table I. Both K_m and V for ITPγS are approximately half the values

Table I: Kinetic Parameters for ITP and ITPγS as PEPCK Substrates

substrate[a]	K_m (mM)[b]	V (μmol min^{-1} mg^{-1})[b]	V/K_m
ITP	0.168 ± 0.009	75.9 ± 1.4	453 ± 16
ITPγS	0.080 ± 0.004	37.0 ± 0.4	464 ± 18

[a] [ITP] and [ITPγS] in the rate measurements ranged from 0.1 to 1 mM. [b] Quoted uncertainties in kinetic parameters are standard errors.

Scheme I



for ITP, and V/K_m values are the same within experimental error for the two substrates. The values for ITP in Table I are the same as those previously measured under slightly different conditions (Colombo & Lardy, 1981).

Stereochemical Course of PEPCK-Catalyzed Thiophosphoryl Transfer. Scheme I illustrates the stereochemical course of the cytosolic PEPCK reaction, which proceeds with inversion of configuration at P. Thus, reaction of (R_P)-[γ-$^{18}O_2$]ITPγS produces (*S*)-thio[^{18}O]phosphoenolpyruvate. Also illustrated is the method by which the configuration about P in the product was established.

Thio[^{18}O]phosphoenolpyruvate produced by action of PEPCK on (R_P)-[γ-$^{18}O_2$]ITPγS and oxalacetate was immediately purified by ion-exchange chromatography. Since thiophosphoenolpyruvate is unstable, the pooled chromatography fractions were immediately combined with ADP and pyruvate kinase, which transferred the [^{18}O]thiophosphoryl group to ADP. The resultant [γ-^{18}O]ATPγS was purified and used as an adenylate kinase substrate with AMP, producing [β-^{18}O]ADPβS. Since the pyruvate kinase and adenylate kinase reactions both proceed with inversion of configuration at P (Blatter & Knowles, 1979; Richard & Frey, 1978), the P configuration of [β-^{18}O]ADPβS must have been the same as that in thio[^{18}O]phosphoenolpyruvate. Stereospecific phosphorylation of [β-^{18}O]ADPβS by the acetyl-P/acetate kinase system (Richard et al., 1978) produced (R_P)-[β-^{18}O]-ATPβS, whereas stereoselective phosphorylation by the phosphoenolpyruvate/pyruvate kinase system produced (S_P)-[β,γ-bridging-^{18}O]ATPβS. This allowed the assignment of the *S* configuration to Pβ of [β-^{18}O]ADPβS. Therefore, the PEPCK product from (R_P)-[γ-$^{18}O_2$]ITPγS must have been (*S*)-thio[^{18}O]phosphoenolpyruvate, the product of configurational inversion at P.

Analysis of ATPβS in Scheme I for bridging and nonbridging ^{18}O was carried out by chemical degradation to trimethyl phosphate, derived from Pα and Pγ, and trimethyl phosphorothiolate, derived from Pβ (Sheu et al., 1984; Richard et al., 1978). The procedure partitioned bridging ^{18}O between trimethyl phosphate and trimethyl phosphorothiolate but allowed nonbridging ^{18}O to be isolated exclusively with trimethyl phosphorothiolate. These products were subjected to GC/MS analysis for ^{18}O.

Table II: Analysis of ATPβS for Bridging and Nonbridging ^{18}O[a]

	% ^{18}O[b]	
nucleotide	trimethyl phosphate	trimethyl phosphorothiolate
(R_P)-[β-^{18}O]ATPβS[c]	1.2 ± 0.7	88.1 ± 1.0
(S_P)-[β,γ-^{18}O]ATPβS[d]	16.7 ± 0.5	44.3 ± 1.0

[a] The nucleotide samples were chemically degraded as described under Materials and Methods to trimethyl phosphate and trimethyl phosphorothiolate, which were subjected to analysis for ^{18}O by GC/MS. Samples were analyzed with a Kratos GC/MS spectrometer by using an Alltech RSL-150 column (0.33 mm × 100 cm) with temperature programmed to increase from 40 to 80 °C at a rate of 10 °C/min after injection of a 1-μL sample at 40 °C. [b] ^{18}O enrichments were determined from the ratios of parent ions, *m/e* 142 and 140 for trimethyl phosphate and *m/e* 158 and 156 for trimethyl phosphorothiolate. The tabulated data are corrected for natural abundances of heavy isotopes of S and O, partial desulfurization during chemical degradation of nucleotides, and 86% stereoselectivity in the pyruvate kinase catalyzed phosphorylation of [β-^{18}O]ADPβS to (S_P)-[β,γ-^{18}O]-ATPβS. The quoted enrichments are mean ± SD. [c] Total ^{18}O enrichment 90.4 ± 2.2%. [d] Total ^{18}O enrichment 77.7 ± 1.9%.

The ^{18}O enrichments are set forth in Table II. The data for (R_P)-[β-^{18}O]ATPβS show that 97.3 ± 2.6% of the ^{18}O is in the nonbridging Pβ position. Therefore, the configuration of its precursor [β-^{18}O]ADPβS at Pβ must have been *S*, as shown in Scheme I. As explained above and in Scheme I, the configuration of thio[^{18}O]phosphoenolpyruvate must also have been *S*, and the reaction proceeds with *inversion* of configuration at P.

Discussion

The synthesis of ITPγS and (R_P)-[γ-$^{18}O_2$]ITPγS described herein is based on that used to synthesize (R_P)-[γ-$^{18}O_2$]ATPγS (Richard & Frey, 1978, 1982). This method has also been applied to the synthesis of (R_P)-[γ-$^{18}O_2$]GTPγS and (R_P)-[γ-^{17}O,$^{18}O_2$]ATPγS (Sheu et al., 1984; Webb, 1982) and is generally applicable to the synthesis of nucleoside 5′-(3-thiotriphosphates).

Thiophosphoenolpyruvate, produced by the action of cytosolic PEPCK on ITPγS and oxalacetate, could be isolated in solution by chromatography but proved to be too unstable to isolate as a solid salt by using conventional procedures. It also decomposed within a few days in solution at 4 °C. Therefore, thio[^{18}O]phosphoenolpyruvate enzymatically generated and chromatographically isolated was converted to [γ-^{18}O]ATPγS as soon as column fractions could be identified and pooled. With this procedure, the overall yield of [γ-^{18}O]ATPγS from [γ-$^{18}O_2$]ITPγS via thiol[^{18}O]phosphoenolpyruvate was 85%.

The marked hydrolytic instability of thiophosphoenolpyruvate can be understood on the basis that there is a large degree of P–O bond cleavage in the transition state for hydrolysis of phosphoenolpyruvate and a small degree of bond formation to the solvent (Benkovic & Schray, 1971). The incipient metaphosphate in such a transition state lies at a lower energy when generated from a thiophosphoester than from a phosphoester owing to charge stabilization by S (Frey & Sammons, 1985); therefore, thiophosphomonoester anions are in general more labile to hydrolysis than phosphomonoesters.

The principal conclusions from this work are that cytosolic PEPCK catalyzes thiophosphoenolpyruvate formation from ITPγS as efficiently as it catalyzes phosphoenolpyruvate

formation from ITP and, inasmuch as the reaction proceeds with inversion of configuration at P, the mechanism is one that involves an uneven number of phospho transfer steps, most likely a single step. Similar results have been reported for the mitochondrial PEPCK with GTPγS as the substrate (Sheu et al., 1984). Therefore, notwithstanding the genetic differences, the two enzymes act by similar mechanisms.

PEPCK is unique among phosphotransferases in its ability to utilize ITP and ITPγS as substrates with equal efficiencies. Mitochondrial PEPCK also utilizes GTPγS somewhat less efficiently than GTP (Lee et al., 1985). The molecular basis for the very slow rates with which other phosphotransferases catalyze thiophospho transfer relative to phospho transfer is unknown. However, since the intrinsic chemical reactivities of thiophosphomonoesters and thiophosphoanhydrides are higher than those of phosphomonoesters and phosphoanhydrides, slow enzymatic thiophospho transfers may result from physical factors in catalysis. These would include orientational effects arising from the presence of S in the metal-chelated substrate, effects of S on rates of product release, and effects of S on rates of compulsory conformational transitions in the catalytic mechanism. It is conceivable, albeit unlikely, that in the cases of PEPCK's S has no such effect on the phospho transfer mechanism.

The high efficiencies with which PEPCK's utilize GTPγS and ITPγS may reflect a more fundamental difference between these enzymes and other phosphotransferases. PEPCK's catalyze both decarboxylation/carboxylation and phospho transfer, chemically distinct processes that almost certainly proceed as discrete steps of the overall reaction mechanism. Should the overall rate be largely determined by the decarboxylation rate constant, the observed rate would be similar for ITPγS and ITP even if the rate constant governing thiophospho transfer is smaller than that governing phospho transfer. Similar rates for ITPγS and ITP would also result from the overall rate being governed largely by rate constants for dissociation of products.

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