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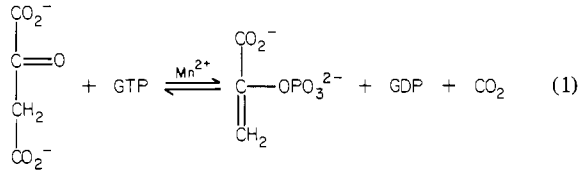
Stereochemical Course of Thiophosphoryl Group Transfer Catalyzed by Mitochondrial Phosphoenolpyruvate Carboxykinase†

Kwan-Fu Sheu,‡ Hsu-Tso Ho,§ Lynn D. Nolan, Paul Markovitz, John P. Richard, Merton F. Utter,⊥ and Perry A. Frey*

ABSTRACT: Guinea pig liver mitochondrial phosphoenolpyruvate carboxykinase catalyzes the conversion of (R_P)-guanosine 5′-(3-thio[3-^{18}O]triphosphate) and oxalacetate to (S_P)-[^{18}O]thiophosphoenolpyruvate, GDP, and CO_2 by a mechanism that involves overall *inversion* in the configuration of the chiral [^{18}O]thiophosphate group. This result is most consistent with a single displacement mechanism in which the [^{18}O]thiophosphoryl group is transferred from (R_P)-guanosine 5′-(3-thio[3-^{18}O]triphosphate) bound at the active site directly to enolpyruvate generated at the active site by the decarboxylation of oxalacetate. In particular, this result does not indicate the involvement of a covalent thiophosphoryl-enzyme on the reaction pathway.

Mitochondrial phosphoenolpyruvate carboxykinase catalyzes the reaction of oxalacetate with GTP[1] to produce phosphoenolpyruvate, GDP, and CO_2 according to eq 1. The



(1)

reaction involves both decarboxylation and phosphoryl group transfer. It is not known whether phosphoryl group transfer proceeds by a single displacement mechanism, with direct transfer of the terminal phosphoryl group of GTP to the C-2 oxygen of oxalacetate, or by a double-displacement mechanism in which the enzyme mediates this transfer by covalent catalysis via an intermediate covalent phosphoryl-enzyme. Kinetic evidence suggests a sequential binding mechanism involving a compulsory ternary complex in the reaction as written in eq 1 (Miller & Lane, 1968; Chang et al., 1966). The kinetics, therefore, provides no direct evidence of a free covalent phosphoryl-enzyme as an intermediate. However, the kinetics does not exclude the possibility that a phosphoryl-enzyme might exist as a component of a central complex on the catalytic pathway.

† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (L.D.N. and J.P.R.), and the Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106 (K.-F.S., P.M., and M.F.U.). *Received October 17, 1983.* Supported by Grant GM30480 to P.A.F. and Grant AM21859 to M.F.U.

* Author to whom correspondence should be addressed at the Institute for Enzyme Research, University of Wisconsin, Madison, WI 53705.
‡ Present address: Burke Rehabilitation Center, White Plains, NY 10605.
§ Institute for Enzyme Research, University of Wisconsin, Madison, WI 53705. Present address: Department of Chemistry, University of Lowell, Lowell, MA 01854.
⊥ Deceased Nov 28, 1980.

[1] Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS, guanosine 5′-(3-thio[2,3-^{18}O]triphosphate) with the *R* configuration about P^3; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; 2′,3′-methoxymethylidene-GMP, 2′,3′-methoxymethylideneguanosine 5′-phosphate; 2′,3′-methoxymethylidene-AMP, 2′,3′-methoxymethylideneadenosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane.

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To obtain information about whether phosphoryl transfer proceeds by a single- or double-displacement mechanism, we have carried the reaction out using (R_P)-[β,γ-^{18}O,γ-^{18}O]-GTPγS in place of GTP and then determined the configuration of the [^{18}O]thiophosphoryl group in the resulting [^{18}O]thiophosphoenolpyruvate. We found the product to be (S_P)-[^{18}O]thiophosphoenolpyruvate, demonstrating that thiophosphoryl group transfer proceeds with overall inversion of configuration at phosphorus.

Experimental Procedures

Materials

Phosphoenolpyruvate carboxykinase was purified from guinea pig liver mitochondria by the procedure of Duffy et al. (1981). Pyruvate kinase, adenylate kinase, lactate dehydrogenase, alkaline phosphatase, hexokinase, guanosine, ADP, AMP, NAD, NADH, dithiothreitol, sodium pyruvate, acetyl phosphate, tris(hydroxymethyl)aminomethane, and DEAE-Sephadex A-25, were purchased from Sigma Chemical Co. Acetate kinase and adenosine were purchased from Boehringer Mannheim. Reagent grade inorganic salts were purchased from commercial suppliers and used without further purification. Triethylamine was purchased from Fisher Chemical Co. and redistilled before use. Diphenyl phosphorochloridate, thiophosphoryl chloride, tri-*n*-octylamine, and tri-*n*-butylamine, used in the synthesis of (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS, were purchased from Aldrich Chemical Co. Thiophosphoryl chloride was redistilled just before being used. Triethyl phosphate, dimethylformamide, tri-*n*-alkylamines, and pyridine from commercial suppliers were carefully dried, redistilled, and stored in the presence of dehydrating agents before being used in the synthesis of (R_P)-[β,γ-^{18}O,γ-^{18}O]-GTPγS. $H_2{}^{18}O$ used in this synthesis was purchased from Monsanto Research Corp., Mound Laboratory.

Methods

Phosphoenolpyruvate carboxykinase was assayed in the direction of oxalacetate formation as described by Noce & Utter (1975). The progress of the reaction between (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS and oxalacetate catalyzed by phosphoenolpyruvate carboxykinase was monitored by measuring the [^{18}O]thiophosphoenolpyruvate in aliquots withdrawn from the reaction solution. The aliquots were added to solutions containing excess ADP, Mg^{2+}, NADH, pyruvate kinase, and lactate dehydrogenase. The decrease in A_{340} due to consumption of NADH was used to calculate the amount of [^{18}O]thiophosphoenolpyruvate in the aliquot. [^{18}O]Thiophosphoenolpyruvate eluted from DEAE-Sephadex columns was measured by the same method.

Adenine nucleotide concentrations were calculated from measurements of A_{260} using the extinction coefficient 15×10^3 $M^{-1}\cdot cm^{-1}$. Nucleotides eluted from DEAE-Sephadex columns by gradients of triethylammonium bicarbonate were desalted by rotary evaporation (in vacuo, bath temperature below 30 °C). Residues were twice dissolved in ethanol and again evaporated to remove the last traces of salt. The desalted nucleotides were dissolved in water, adjusted to pH 10 by addition of triethylamine, and stored at −15 °C.

Preparation of Diazoethane. Diazoethane was obtained as an ethereal solution by adding 0.5 g of *N*-ethyl-*N*-nitrosourea to a small flask containing 5 mL of 40% KOH and 20 mL of diethyl ether. The suspension was stirred carefully until all the solid dissolved and diazoethane appeared in the ethereal layer (orange-yellow color). The ethereal solution was distilled into an ice–water-cooled receiver by gently heating the reaction flask at 40 °C.

Synthesis of (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS. This synthesis was carried out as described for the synthesis of (R_P)-[β,γ-^{18}O,γ-^{18}O]ATPγS substituting 2′,3′-methoxymethylidene-GMP for 2′,3′-methoxymethylidene-AMP (Richard & Frey, 1978, 1982). 2′,3′-Methoxymethylidene-GMP was synthesized as described for 2′,3′-methoxymethylidene-AMP, substituting guanosine for adenosine (Richard & Frey, 1982). This method has also been adapted to the synthesis of (R_P)-[γ-^{17}O,^{18}O]-ATPγS (Webb, 1982).

Enzymatic Conversion of (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS and Oxalacetate to (S_P)-[^{18}O]Thiophosphoenolpyruvate. [^{18}O]-Thiophosphoenolpyruvate was produced by the action of phosphoenolpyruvate carboxykinase on (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS and oxalacetate in a 30-mL reaction mixture consisting of 0.65 mM (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS, 0.6 mM oxalacetate, 1.5 mM $MnCl_2$, 24 mM Tris-HCl buffer at pH 8.0, 37 units of phosphoenolpyruvate carboxykinase, and 30 units of alkaline phosphatase at room temperature. The concentration of oxalacetate was monitored throughout the course of the reaction by the use of malate dehydrogenase and maintained at approximately 1 mM by periodic supplementation as required. Production of [^{18}O]thiophosphoenolpyruvate was also monitored with pyruvate kinase and lactate dehydrogenase. After 70 min, 43% of the theoretical yield of [^{18}O]thiophosphoenolpyruvate had been produced. After 90 min, 5.7 mg of oxalacetic acid were added. After 140 min, the theoretical yield of [^{18}O]thiophosphoenolpyruvate, based on (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS had been produced, whereupon the reaction mixture was passed directly into a 1.5 × 25 cm column of DEAE-Sephadex A-25 that had been equilibrated with 0.15 M triethylammonium bicarbonate at 4 °C. The column was eluted in the cold with a linear gradient of the same buffer formed from 350 mL of 0.15 M and 350 mL of 0.6 M buffer, and 14-mL fractions were collected. [^{18}O]Thiophosphoenolpyruvate was detected in fractions 42–50 by enzymatic assay. Fractions 43–47 were pooled, and buffer was removed by rotary flash evaporation in vacuo at a bath temperature below 30 °C. The residue was twice redissolved in small volumes of ethanol and evaporated to ensure that all the buffer had evaporated. The recovery of thiophosphoenolpyruvate by enzymatic assay was 15 μmol.

Enzymatic Transformation of [^{18}O]Thiophosphoenolpyruvate to [γ-^{18}O]ATPγS. [^{18}O]Thiophosphoenolpyruvate isolated above was immediately converted with ADP to pyruvate and [γ-^{18}O]ATPγS by the action of pyruvate kinase in a reaction mixture that contained 15 μmol of each reactant in 20 mL of a buffer consisting of 20 mM potassium phosphate, 5 mM $MgCl_2$, and 0.5 M dithiothreitol at pH 8.0. The reaction was initiated by the addition of 80 units of rabbit muscle pyruvate kinase and was complete in less than 1 h, as judged by monitoring pyruvate production using lactate dehydrogenase and NADH. [γ-^{18}O]ATPγS was purified by chromatography through a column (2 × 60 cm) of DEAE-Sephadex A-25. The column was eluted with a 1500-mL linear gradient of triethylammonium bicarbonate changing in concentration from 0.15 to 0.7 M at pH 8.0, and 16-mL fractions were collected. [γ-^{18}O]ATPγS appeared in fractions 63–71, which contained 8.5 μmol of [γ-^{18}O]ATPγS.

Enzymatic Transfer of the [^{18}O]Thiophosphoryl Group to AMP. The ^{18}O]thiophosphoryl group of [γ-^{18}O]ATPγS was transferred to AMP in a 20-mL reaction mixture consisting of 50 mM Tris-HCl buffer at pH 8.0, 10 mM $MgCl_2$, 2 mM AMP, 1 mM dithioerythritol, 0.2 mM [γ-^{18}O]ATPγS, and 4.5 units·mL^{-1} adenylate kinase. The reaction proceeded for 100 min at 25 °C. [β-^{18}O]ADPβS was purified from the

solution by chromatography through a 1 × 20 cm column of DEAE-Sephadex eluted with a linear gradient of increasing ionic strength formed from 500 mL each of 0.1 and 0.4 M triethylammonium bicarbonate. Fractions containing [β-^{18}O]ADPβS were pooled, desalted, and stored as described above. The yield of [β-^{18}O]ADPβS was 89.5%.

Stereoselective Enzymatic Phosphorylation of [β-^{18}O]-ADPβS. To determine the configuration of the [^{18}O]thiophosphoryl group in [β-^{18}O]ADPβS, the sample was split into two portions, one of which was converted to (R_P)-[β-^{18}O]-ATPβS and the other to (S_P)-[β-^{18}O]ATPβS. The R_P epimer was produced in a 0.4-mL reaction mixture consisting of 75 mM Tris-HCl buffer at 7.8, 7.5 mM $MgCl_2$, 0.6 mM dithiothreitol, 30 mM acetyl phosphate, 3 mM [β-^{18}O]ADPβS, and 120 units·mL^{-1} acetate kinase. The reaction proceeded for 8 h at 28 °C. (R_P)-[β-^{18}O]ATPβS was purified by chromatography through a 0.7 × 1.5 cm column of DEAE-Sephadex eluted by a linear salt gradient of increasing ionic strength formed from 250 mL each of 0.25 and 0.45 M triethylammonium bicarbonate. Fractions containing (R_P)-[β-^{18}O]ATPβS were pooled, desalted, and stored as described above. The overall yield from 1.2 μmol of [β-^{18}O]ADPβS was 0.85 μmol of (R_P)-[β-^{18}O]ATPβS.

(S_P)-[β-^{18}O]ATPβS were produced in a reaction mixture containing 2 mM [β-^{18}O]ADPβS, 40 mM Tris-HCl buffer at pH 8.0, 4 mM $MgCl_2$, 380 mM KCl, 0.8 mM dithiothreitol, 2.5 mM phosphoenolpyruvate, and 40 units·mL^{-1} pyruvate kinase. The reaction proceeded for 6 h at 25 °C. (S_P)-[β-^{18}O]ATPβS was purified as described above for the R_P epimer. The yield was 85%.

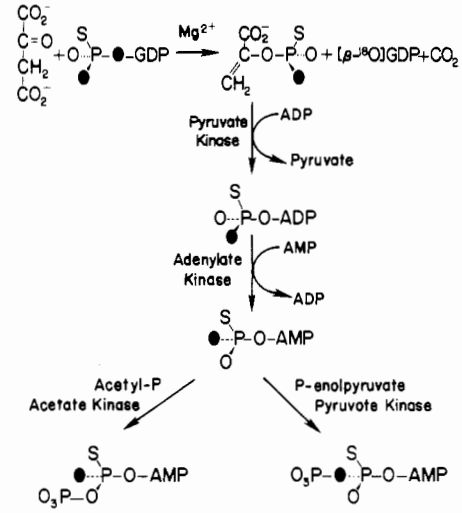
Chemical Transformation of (R_P)- and (S_P)-[β-^{18}O]ATPβS to [^{18}O]Triethyl Phosphate and [^{18}O]Triethyl Phosphorothioate. Samples of (R_P)-[β-^{18}O]ATPβS and (S_P)-[β-^{18}O]-ATPβS (0.9 μmol each) were individually treated with 1.2 μmol of $NaIO_4$ at pH 7 and 25 °C for 15 min. Excess (10 μmol) of 2-mercaptoethanol was added to reduce iodate and unreacted periodate. The solutions were adjusted with NaOH to pH 10.5 and heated at 50 °C for 30 min, conditions known to lead to essentially quantitative alkaline β elimination of phosphates from periodate-cleaved ribonucleosides (Brown et al., 1953; Whitfield & Markham, 1953). The resulting [^{18}O]-2-thiotriphosphates were purified by chromatography through 0.7 × 12 cm columns of DEAE-Sephadex. The cleaved nucleotides and excess 2-mercaptoethanol were washed through the columns with 30 mL of 0.3 M triethylammonium bicarbonate, and the [^{18}O]-2-thiotriphosphates were eluted with 30 mL of the 0.7 M triethylammonium bicarbonate. After desalting as described above for the nucleotides, the samples were dissolved in ethanol, acidified with HCl, and alkylated with excess diazoethane dissolved in diethyl ether. After solvents were removed by rotary evaporation in vacuo, the samples were disolved in water and heated at 100 °C for 1 h. They were then again ethylated as described above and analyzed for [^{18}O]triethyl phosphorothioate by gas chromatographic mass spectroscopy using a Finnigan 4021 GC–mass spectrometer system equipped with a glass column packed with 10% SE-30 on Chromosorb G.

Results

The stereochemical course of [^{18}O]thiophosphoryl group transfer in the mitochondrial phosphoenolpyruvate carboxykinase reaction is shown in Scheme I together with the enzymatic transformations of [^{18}O]thiophosphoenolpyruvate employed to establish its configuration at phosphorus.

Reaction of (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS with oxalacetate to produce (S_P)-[^{18}O]thiophosphoenolpyruvate proceeded

Scheme I



smoothly in the presence of phosphoenolpyruvate carboxykinase and alkaline phosphatase. The inclusion of alkaline phosphatase faciliated the reaction by removing GDP, a potent product inhibitor, as it was produced. While (S_P)-[^{18}O]thiophosphoenolpyruvate could be isolated by ion-exchange chromatography, it was not stable enough to hold in storage. It was, therefore, promptly converted to (R_P)-[γ-^{18}O]ATPγS by pyruvate kinase catalyzed reaction with ADP, a process known to proceed with inversion of configuration (Orr et al., 1978; Blättler & Knowles, 1979a,b). Of course, if the carboxykinase product had been (R_P)-[^{18}O]thiophosphoenolpyruvate, the pyruvate kinase product would have been (S_P)-[γ-^{18}O]ATPγS.

To establish the phosphorus configuration in [γ-^{18}O]ATPγS, we subjected it to further enzymatic transformations, beginning with adenylate kinase catalyzed [^{18}O]thiophosphorylation of AMP to [β-^{18}O]ADPβS, which is known to proceed with inversion of configuration (Richard & Frey, 1978). To determine the configuration at P_β, we subjected [β-^{18}O]ADPβS to stereoselective enzymatic phosphorylation of the chiral phosphorus in two samples, one at the *R* oxygen using phosphoenolpyruvate, Mg^{2+}, and pyruvate kinase and the other at the *S* oxygen using acetyl phosphate, Mg^{2+}, and acetate kinase. The stereochemical orientations of these phosphorylations had been established (Richard et al., 1978). Analysis for bridging and nonbridging ^{18}O in these products showed that ^{18}O was bridging in (S_P)-[β-^{18}O]ATPβS and nonbridging in (R_P)-[β-^{18}O]ATPβS (see Scheme I). We, therefore, concluded that the [β-^{18}O]ADPβS must have been the S_P epimer, and from the known stereochemistries of the pyruvate kinase and adenylate kinase reactions, both of which involve inversion of the [^{18}O]thiophosphoryl group, the precursor [^{18}O]thiophosphoenolpyruvate must have been the S_p enantiomer. Therefore, the [^{18}O]thiophosphoryl group transfer must have proceeded with inversion of configuration at phosphorus.

A number of procedures are available for determining bridging and nonbridging ^{18}O in [β-^{18}O]ATPβS. The one used in this work is that described by Richard et al. (1978) in which [β-^{18}O]ATPβS is first cleaved by periodate and base treatment to [2-^{18}O]-2-thiotriphosphate. This product is then converted, as illustrated in Scheme II, to a mixture of triethyl phosphate and triethyl phosphorothioate by alkylation with diazoethane in ethanol, hydrolysis, and repeated alkylation with diazoethane. The ^{18}O enrichment in triethyl phosphorothioate is then measured by gas chromatographic mass spectroscopy. In the ethanolysis and hydrolysis of pentaethyl [2-^{18}O]-2-thio-

Scheme II

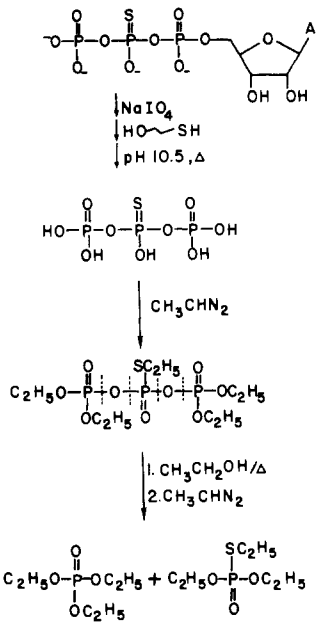


Table I: Gas Chromatographic Mass Spectroscopic Analysis of ^{18}O in (R_P)- and (S_P)-[β-^{18}O]ATPβS[a]

sample	% $m'+2$[b] triethyl phosphate	% $m'+2$[b] triethyl phosphorothioate
(R_P)-[β-^{18}O]ATPβS	1.4 ± 1.2	84.3 ± 3.2[c]
(S_P)-[β-^{18}O]ATPβS	16.8 ± 1.0	53.4 ± 4.9[c]

[a] (R_P)- and (S_P)-[β-^{18}O]ATPβS were prepared and converted to [^{18}O]triethyl phosphate and triethyl phosphorothioate as described under Experimental Procedures. [b] The base peak m' in these determinations was m/e = 99 for triethyl phosphate and m/e = 170 for triethyl phosphorothioate. [c] The ^{18}O enrichment in the R position of the [^{18}O]thiophosphoryl group of (R_P)-[β,γ-^{18}O,γ-^{18}O]-GTPγS was 87%. The ^{18}O enrichments detected in triethyl phosphate and triethyl phosphorothioate in these experiments are within error of this value.

triphosphate, bridging ^{18}O is partitioned between triethyl phosphate and triethyl phosphorothioate, whereas nonbridging ^{18}O is retained exclusively in triethyl phosphorothioate.

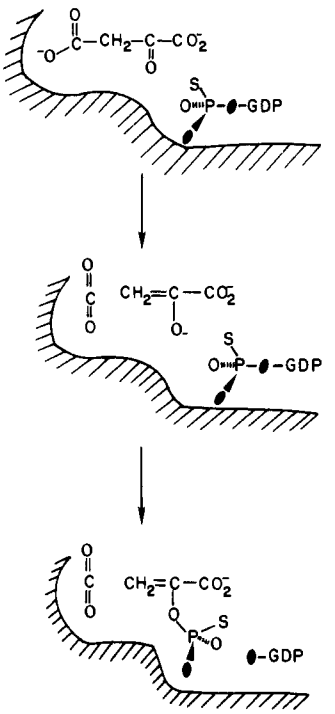
The data in Table I were obtained in the analysis of (R_P)-[β-^{18}O]ATPβS and (S_P)-[β-^{18}O]ATPβS. The ^{18}O enrichment in triethyl phosphorothioate derived from (S_P)-[β-^{18}O]ATPβS was substantially lower than that in the [^{18}O]-thiophosphoryl group in (R_P)-[γ-^{18}O]GTPγS (87% ^{18}O in the R position) showing that ^{18}O was bridging in that isomer. The balance of ^{18}O was accounted for in triethyl phosphate. The enrichment in triethyl phosphorothioate derived from (R_P)-[β-^{18}O]ATPβS was essentially the same as that in the R position of (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS, showing that it must have been nonbridging in (R_P)-[β-^{18}O]ATPβS. The configuration at P_β in [β-^{18}O]ADPβS must, therefore, have been S.

The configuration of the [^{18}O]thiophosphoryl group in [^{18}O]thiophosphoenolpyruvate must also have been S, since the two enzymatic transfers catalyzed by pyruvate kinase and adenylate kinase proceed with inversion of stereochemical configuration, i.e., overall retention between [^{18}O]thiophosphoenolpyruvate and [β-^{18}O]ADPβS (see Scheme I).

Discussion

Our observation that mitochondrial phosphoenolpyruvate carboxykinase catalyzes thiophosphoryl group transfer with overall inversion of stereochemical configuration is inconsistent with a double-displacement reaction mechanism in which the thiophosphoryl group is first transferred to an enzymic nucleophile and in a subsequent step to the 2-oxo group of oxalacetate or enolpyruvate. Thiophosphoryl and phosphoryl transfer by such mechanisms proceed with overall retention of configuration at phosphorus (Frey, 1982a,b; Sheu et al., 1979; Blättler & Knowles, 1979a,b). In the case of mitochondrial phosphoenolpyruvate carboxykinase, it is most likely that GTPγS and oxalacetate are simultaneously bound to the enzyme at adjacent sites and the thiophosphoryl group transferred directly from GTPγS to the 2-oxo group of either oxalacetate or its immediate decarboxylation product enolpyruvate. Such a mechanism is outlined in Scheme III for a two-step reaction pathway involving the initial decarboxylaation of oxalacetate followed closely by thiophosphorylation of enolpyruvate formed in the decarboxylation step. The thiophosphoryl group transfer proceeds with inversion. A mechanism in which the two processes are concerted cannot be excluded by available evidence, although it is, for lack of chemical precedent, less favored.

Scheme III



Scheme III does not include a role for the divalent metal ions required for the reaction. They presumably play a crucial role in the mechanism of phosphoryl group transfer and possibly also in the decarboxylation step. Our results do not bear on this aspect of the mechanism, however, so it is not included in Scheme III.

Other more circuitous pathways that do not violate known chemical principles and are in accord with the stereochemistry can be written. These involve additional steps whose relevance to the overall reaction and the associated mechanistic problems is questionable. In the absence of evidence implicating them, more complex catalytic pathways are less favored than that in Scheme III.

Acknowledgments

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Registry No. (R_P)-[β,γ-^{18}O,γ-^{18}O]GTPγS, 89232-33-7; (S_P)-[^{18}O]thiophosphoenolpyruvate, 89232-34-8; 2′,3′-methoxy-

methylidene-GMP, 16628-88-9; phosphoenolpyruvate carboxykinase, 9013-08-5.

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Some Aspects of the Mechanism of Complexation of Red Kidney Bean α-Amylase Inhibitor and α-Amylase†

Edward R. Wilcox‡ and John R. Whitaker*

ABSTRACT: Bovine pancreatic α-amylase binds 1 mol of acarbose (a carbohydrate α-amylase inhibitor) per mol at the active site and also binds acarbose nonspecifically. The red kidney bean α-amylase inhibitor–bovine pancreatic α-amylase complex retained nonspecific binding for acarbose only. Binding of *p*-nitrophenyl α-D-maltoside to the final complex of red kidney bean α-amylase inhibitor and bovine pancreatic α-amylase has a βK_s (K_s') value that is 3.4-fold greater than the K_s (16 mM) of α-amylase for *p*-nitrophenyl α-D-maltoside alone. The initial complex of α-amylase and inhibitor apparently hydrolyzes this substrate as rapidly as α-amylase alone. The complex retains affinity for substrates and competitive inhibitors, which, when present in high concentrations, cause dissociation of the complex. Maltose (0.5 M), a competitive inhibitor of α-amylase, caused dissociation of the red kidney bean α-amylase inhibitor–α-amylase complex. Interaction between red kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor and porcine pancreatic α-amylase proceeds through two steps. The first step has a K_{eq} of 3.1×10^{-5} M. The second step (unimolecular; first order) has a forward rate constant of 3.05 min^{-1} at pH 6.9 and 30 °C. α-Amylase inhibitor combines with α-amylase, in the presence of *p*-nitrophenyl α-D-maltoside, noncompetitively. On the basis of the data presented, it is likely that α-amylase is inactivated by the α-amylase inhibitor through a conformational change. A kinetic model, in the presence and absence of substrate, is described for noncompetitive, slow, tight-binding inhibitors that proceed through two steps.

The proteinaceous α-amylase inhibitor of red kidney bean (*Phaseolus vulgaris*) has been purified to homogeneity (E. R. Wilcox and J. R. Whitaker, unpublished results). This inhibitor is slow in inactivating α-amylase, but it is tight binding. Depending on pH and concentrations of reactants, the red kidney bean α-amylase inhibitor can take several hours to inactivate porcine pancreatic α-amylase (Powers & Whitaker, 1977b). The slowness of the inactivation could be caused by a requirement for a conformational change in either the inhibitor or α-amylase or both (Powers & Whitaker, 1977b). The time required to inhibit α-amylase by other amylase inhibitors appears to be dependent on the source of α-amylase and α-amylase inhibitor as well as on the concentrations of inhibitor and enzyme and the pH. O'Connor & McGeeney (1981a,b) found a 60 000-dalton wheat α-amylase inhibitor that inhibited human salivary α-amylase much quicker than human pancreatic α-amylase. After a 0.5-h preincubation step to permit complexation, the ratio of salivary to pancreatic α-amylase inhibition was 140.

Powers & Whitaker (1977b) showed that the porcine pancreatic α-amylase–red kidney bean α-amylase inhibitor complex, when treated with maltose, has a concentration-dependent absorption change, suggesting that the complex can still bind the competitive inhibitor, maltose. The K_i calculated for maltose binding to the complex was 13 mM. Elodi et al. (1972) reported that maltose causes a concentration-dependent absorption change at 291 nm when it binds to porcine pancreatic α-amylase. The K_i (13 mM) of maltose, on the basis of this absorbance change, is in agreement with the K_i of 25 mM for maltose binding to α-amylase as determined from activity assays (inhibitor). This is indicative that the active site of α-amylase is still partially or fully available when the red kidney bean inhibitor is bound to the enzyme.

Proteinaceous α-amylase inhibitors bind tightly to mammalian α-amylases. Red kidney bean inhibitor has a K_i of 3

† From the Department of Food Science and Technology, University of California, Davis, Davis, California 95616. *Received August 1, 1983.*
‡ Present address: Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, CA 92093.

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