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Reaction Mechanism of Phosphoenolpyruvate Carboxylase. Bicarbonate-Dependent Dephosphorylation of Phosphoenol-α-ketobutyrate†

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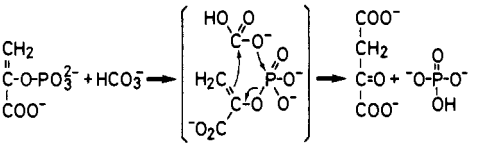
ABSTRACT: Phosphoenolpyruvate carboxylase (EC 4.1.1.31) of *Escherichia coli* was found to catalyze the cleavage reaction of phosphoenol-α-ketobutyrate, a potent competitive inhibitor with the substrate, to yield inorganic phosphate and α-ketobutyrate. The rate of phosphate liberation was about $^1/_{20}$th of that in the normal reaction with phosphoenolpyruvate. Although HCO_3^- and Mg^{2+} were the necessary components in this reaction as in the normal reaction, no CO_2 fixation could be detected. When the reaction was carried out in the presence of [^{18}O]HCO_3^-, multiple incorporations of ^{18}O atoms into the liberated phosphate molecule were observed. The molar proportions of phosphate having one, two, and three ^{18}O atoms were 70, 25, and 5%, respectively. No multiple but only one ^{18}O atom incorporation was observed when phosphoenolpyruvate was used as a substrate. These results suggest that the liberation of phosphate can proceed without CO_2 fixation, being not consistent with the concerted mechanism [Maruyama, H., Easterday, R. L., Chang, H. C., & Lane, M. D. (1966) *J. Biol. Chem. 241*, 2405–2412] but essentially consistent with the current stepwise mechanism [O'Leary, M. H., Rife, J. E., & Slater, J. D. (1981) *Biochemistry 20*, 7308–7314].

Phosphoenolpyruvate (PEP)[1] carboxylase (EC 4.1.1.31) catalyzes the following reaction in the presence of a divalent metal ion: PEP + HCO_3^- → oxaloacetate + P_i. The reaction is highly exergonic (Tchen et al., 1955), and the reverse reaction is not demonstrated. This enzyme, which is widespread in all higher plants and many kinds of bacteria (Utter & Kolenbrander, 1972), plays a role in replenishing oxaloacetate to the tricarboxylic acid cycle (Ashworth & Kornberg, 1966). The enzyme of Enterobacteriaceae such as *Escherichia* and *Salmonella* families is known to be regulated by many kinds of metabolites (Utter & Kolenbrander, 1972; Morikawa et al., 1980).

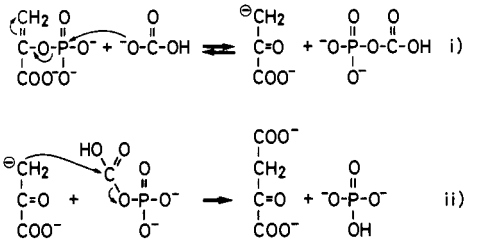
Scanty studies have been made on the mechanism of the enzyme reaction, since neither partial reaction nor exchange reaction has been found. In 1966, Maruyama et al. revealed with the peanut enzyme that ^{18}O was incorporated from [^{18}O]HCO_3^- into inorganic phosphate and oxaloacetate in a ratio of 1:2. They proposed the "concerted mechanism" shown in Scheme I. After a long blank, some studies that argue against their proposal have recently appeared. O'Leary et al. (1981) pointed out that the reaction was hardly explainable in terms of the concerted mechanism, on the basis of the results of precise determination of carbon isotope effect of bicarbonate ions in the reaction with the maize enzyme. Hansen & Knowles (1982) studied the reaction catalyzed by the wheat enzyme with [(*S*)-^{16}O,^{17}O]thiophosphoenolpyruvate as a substrate in [^{18}O]H_2O and found that inversion of the configuration at phosphorous atom occurred in the reaction. Both groups of investigators proposed an alternative reaction mechanism (shown in Scheme II) in which carbonic phosphoric anhydride and enolate anion of pyruvate were formed as an intermediate. This mechanism seems attractive because the question how the carbon atom of bicarbonate, which is inherently a poor electrophile, is activated to accomplish C–C bond formation is well explainable. However, no direct demonstration of the formation of enolate anion of pyruvate and carbonic phosphoric anhydride, which was found in other enzyme reactions (Powers & Meister, 1976; Wimmer et al., 1979), has been made.

In the present study with the *Escherichia coli* enzyme, we found that PEKB, a potent competitive inhibitor (Silverstein,

Scheme I: Postulated "Concerted Mechanism" for PEP Carboxylase



Scheme II: Postulated "Stepwise Mechanism" for PEP Carboxylase



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[1] Abbreviations: PEP, phosphoenolpyruvate; PEKB, phosphoenol-α-ketobutyrate; DNPH, 2,4-dinitrophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane.

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1972; Izui et al., 1983), was cleaved by the enzyme to yield inorganic phosphate and α-ketobutyrate depending on the presence of bicarbonate ions and that multiple incorporation of ^{18}O from $[^{18}O]HCO_3^-$ into phosphate molecule occurred. Discussion was made on the reaction mechanism.

Materials and Methods

Reagents. Cyclohexylammonium salt of PEP was synthesized by the method of Clark & Kirby (1963). Cyclohexylammonium salts of PEKB and phosphoenol-α-ketoisovalerate were kind gifts of Dr. A. E. Woods, Middle Tennessee State University. These compounds contained no detectable amounts of free inorganic phosphate, and their purities were estimated to be more than 90% on the basis of the amount of inorganic phosphate liberated by hydrolysis with perchloric acid. The ratio of *Z* and *E* isomers in the preparation of PEKB was not determined. $[^{14}C]KHCO_3$ was prepared from $[^{14}C]BaCO_3$ (New England Nuclear, 58 Ci/mol) and used after dilution as indicated. $[^{18}O]KHCO_3$ was prepared by equilibration of 10 mg of $KHCO_3$ with 100 μL of $[^{18}O]H_2O$ (Commissariat a l'Energie Atomique France; containing 2.3% ^{16}O, 0.4% ^{17}O, and 97.3% ^{18}O) in a sealed tube at 100 °C for 4 h, followed by lyophilization. Diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine with a MNNG-diazomethane kit [Aldrich Chemical Co.; originally designed by Fales et al. (1973)] or from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide by the usual method (Boer & Backer, 1963). Hexyl-Sepharose was prepared as described previously (Izui et al., 1982). Trimethyl ester of carbonic phosphoric anhydride was synthesized from silver dimethyl phosphate (Lossen & Kohler, 1891) and methyl chloroformate according to Powers & Meister (1978). Dioxane was purified by redistillation after being refluxed over lithium aluminum hydride and stored under nitrogen in sealed tubes until use. Swine heart malate dehydrogenase (EC 1.1.1.37) and swine heart glutamate-oxaloacetate transaminase (EC 2.6.1.1) were obtained from Boehringer-Mannheim, and AG1 X2 ion-exchange resin was from Bio-Rad. All other chemicals were obtained or prepared as described previously (Izui et al., 1982).

Preparation of PEP Carboxylase. PEP carboxylase was prepared from *E. coli* W by hexyl-Sepharose chromatography by using specific elution with L-aspartate, one of the allosteric inhibitors (Izui et al., 1982). The obtained preparation (sp act. 82 units/mg of protein) was homogeneous as judged from polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The enzyme preparation was stored at 0 °C in suspension in 100 mM Tris–H_2SO_4 buffer (pH 7.4) containing ammonium sulfate (60% saturation), 20 mM L-aspartate, and 20 mM $MgSO_4$. The enzyme, prior to use, was treated with 1 mM dithiothreitol and was passed through a Sephadex G-50 column equilibrated with 50 mM Tris–H_2SO_4 buffer (pH 7.4) to be freed from compounds of low molecular weight.

Assay of the Enzyme Activity. The activity of PEP carboxylase was spectrophotometrically determined by using the coupled reaction system with malate dehydrogenase and NADH (Yoshinaga et al., 1970). One unit of the enzyme is defined as the amount consuming 1 μmol of NADH/min. The specific activity of enzyme is expressed as units per milligram of protein. Unless otherwise indicated, the phosphate-liberation activity of the enzyme toward PEP or its homologues was determined as follows. The reaction mixture (0.5 mL) containing 25 μmol of Tris–H_2SO_4 buffer (pH 8.5), 5 μmol of $MgSO_4$, 5 μmol of $KHCO_3$, 2.5 μmol of PEP (or its homologue), the enzyme, and 7% dioxane (as an activator) was incubated at 30 °C for the indicated time. Perchloric acid was added to the mixture to terminate the reaction. After appropriate dilution with water followed by removal of protein by centrifugation, inorganic phosphate in the supernatant was determined by the molybdovanadophosphate method (Koenig & Johnson, 1942). When PEP was used as a substrate, the amount of phosphate determined by the present method was in good agreement with that of NADH decrease determined by the spectrophotometric method.

Preparation of Bicarbonate-Free Stock Solutions. Bicarbonate-free Tris–H_2SO_4 buffer was prepared as follows. Tris base (50–75% of the necessary amount) was dissolved in water, and the pH of the solution was adjusted to 2 with 3 M H_2SO_4. After the solution was bubbled with nitrogen, which had been purified by passage through a column of soda lime, it was brought to the desired pH by the addition of another part of solid Tris base. The enzyme solution was made free from bicarbonate by passage of the solution through a Sephadex G-50 column equilibrated with the buffer prepared as above. Bicarbonate-free stock solutions of other reagents were prepared by dissolution of solid reagents in water that had been made bicarbonate-free by boiling after slightly acidified with H_2SO_4 (about pH 4), and they were used without neutralization.

Thin-Layer Chromatography of Keto Acid DNPH's. Keto acid DNPH's were chromatographed as their sodium salts according to the method of Ariga (1972). As the solvent system, ethyl acetate (saturated with 0.1 M $NaHCO_3$)–methanol (5:1 v/v) or butanol–ethanol–0.1 M $NaHCO_3$ (10:3:10 v/v) was used. Silica gel plates (Merck, silica gel 60) were immersed in 0.1 M $NaHCO_3$, reactivated at 110 °C for 40 min, and stored in a desiccator until use.

^{18}O Incorporation Experiment. Inorganic phosphate was isolated from the reaction mixture and was measured for its ^{18}O content according to the methods of Hackney et al. (1980) and Midelfort & Rose (1976) as follows. Reaction was terminated by the addition of 2 mL of 0.63 M perchloric acid to the reaction mixture (0.5 mL). after removal of protein, a 0.5-mL aliquot of the supernatant was used for the determination of inorganic phosphate. Inorganic phosphate in the remainder of the supernatant, after conversion to phosphomolybdate followed by extraction with butyl acetate, was extracted into 0.2 M Tris solution and then separated by AG1 ion-exchange chromatography. After the eluate containing phosphoric acid was lyophilized, it was redissolved in 100 μl of 90% methanol and then methylated with a fresh solution of diazomethane in ether. The methylated sample was concentrated to 50 μL, and an aliquot was analyzed by gas chromatography–mass spectrometry. Separation of trimethyl phosphate by gas chromatography was well achieved on a column of 10% PEG-20M (2 m, 125 °C). For the ionization of trimethyl phosphate, the usual electron-impact ionization method (70 eV) was used. Mass spectrum was measured with a Shimadzu-LKB Model 9000 gas chromatograph–mass spectrometer (Institute for Biological Science, Sumitomo Chemical Co., Ltd., Osaka), and data analysis was made with a GC-MS PAC model 300D.

Other Determinations. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. α-Ketobutyrate was determined by the DNPH method (Katsuki et al., 1971). Radioactivity was determined with a Beckman LS-230 liquid scintillation spectrometer in toluene–Triton X-100 scintillator (2:1).

Results

Enzymatic Dephosphorylation of PEKB. In the course of our study on the protecting effect of substrate analogues

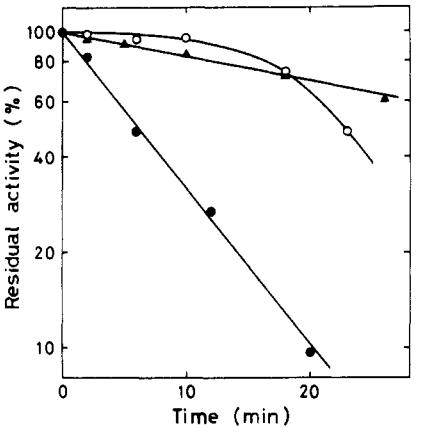

FIGURE 1: Protecting effect of the substrate analogues against the enzyme inactivation by *N*-ethylmaleimide. A partially purified preparation of the enzyme (sp act. 30 units/mg of protein) was used, as an exceptional case, in this experiment. The reaction mixture (1 mL) contained 1 μmol of *N*-ethylmaleimide, the enzyme (70 μg of protein), 100 μmol of *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (Hepes)–NaOH buffer (pH 8.0), 10 μmol of $MgSO_4$, 0.5 μmol of acetyl-CoA, and the substrate analogue as indicated. The mixture was incubated at 30 °C. At appropriate time intervals, 10-μL aliquots were withdrawn and added to the assay mixture for NADH consumption, and their residual enzyme activities were determined. (○) 1 μmol of PEKB; (▲) 10 μmol of DL-phospholactate; (●) without analogue as a control.

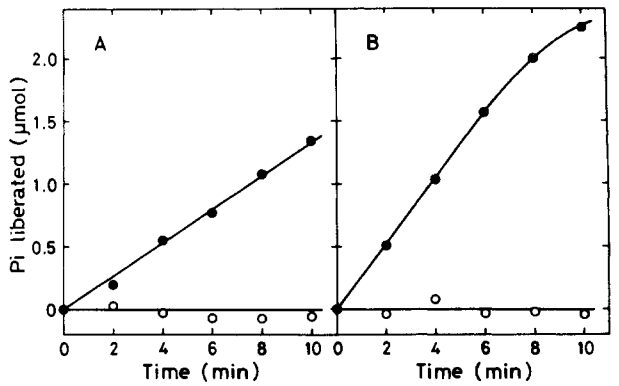

FIGURE 2: Liberation of inorganic phosphate from PEKB and PEP by enzyme action. (A) PEKB was incubated with the enzyme, and the phosphate-liberation activity was measured. A total of 30 μg each of the native (●) or heat-inactivated (○) enzyme was used. The heat-inactivated enzyme was prepared by heating the native enzyme solution at 90 °C for 5 min. The ordinate represents the total amount of phosphate liberated. (B) Reaction was carried out with PEP instead of PEKB as a control experiment. A total of 3 μg each of the native (●) or heat-inactivated (○) enzyme was used.

against inactivation of *E. coli* PEP carboxylase by chemical modification, we made, by chance, an observation suggesting that PEKB was degraded by the enzyme. As shown in Figure 1, the enzyme was inactivated by *N*-ethylmaleimide following pseudo-first-order kinetics (Teraoka et al., 1974) in the reaction mixture containing Mg^{2+} and acetyl-CoA, one of the allosteric activators. The inactivation rate was markedly decreased when phospholactate (Teraoka et al., 1974) was added to the reaction mixture as a protector of the catalytic site of the enzyme. When PEKB was used instead of phospholactate, a more marked protection was observed at the beginning of the incubation. However, the effect of PEKB almost completely disappeared after 20-min incubation. On the basis of the presumption that this phenomenon was due to the degradation of PEKB by the enzyme, PEKB was incubated with pure preparation of the enzyme to see if inorganic phosphate was liberated (Figure 2). In order to increase the affinity of the enzyme for PEKB (Izui et al., 1983) (or PEP), dioxane, one

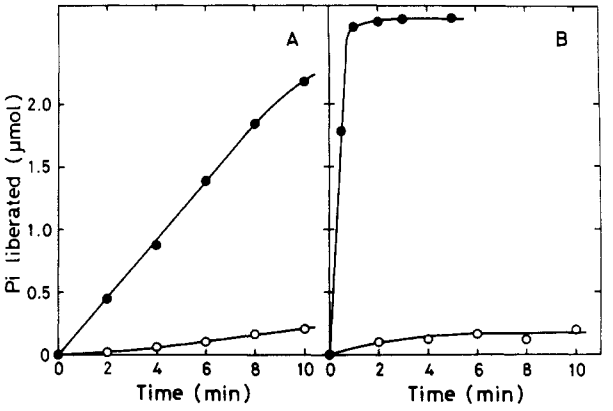

FIGURE 3: Bicarbonate requirement of the phosphate liberation from PEKB and PEP. (A) PEKB was incubated with the enzyme in the presence (●) and absence (○) of 10 mM $KHCO_3$, and the phosphate-liberation activity was measured. The reaction mixture was prepared from bicarbonate-free stock solutions. Other reaction conditions were the same as described under Materials and Methods except for the use of 25 mM Tris–H_2SO_4 (pH 8.0) as buffer. The amount of enzyme added was 54 μg. The ordinate represents the total amount of phosphate liberated. (B) A control experiment was carried out with PEP instead of PEKB. The reaction mixture and the amount of enzyme added were the same as in (A).

of the nonphysiological activators (Katsuki et al., 1967), was included in the reaction mixture. The competitive inhibitor constant (K_i) of PEKB, which was determined in the presence of dioxane at lower concentrations of the enzyme, was 0.024 mM, a value that was about 1 order of magnitude lower than the K_i value for DL-phospholactate (0.47 mM) or the K_m value for PEP (0.25 mM). As seen in Figure 2, inorganic phosphate was liberated when PEKB as well as PEP was incubated with the enzyme. The phosphate-liberation activity per milligram of the enzyme in the reaction with 5 mM (large excess to the K_i value) PEKB was about $^1/_{20}$th of that in the normal reaction with PEP. Phosphoenol-α-ketoisovalerate, in which two methyl groups are substituted for two hydrogen atoms attached to the β-carbon of PEP (K_i value 0.89 mM), was also cleaved to yield inorganic phosphate when incubated with the enzyme. The phosphate-liberation activity in this case was about one-fourth of that in the reaction with PEKB. The phosphate liberation was not observed when the heat-inactivated enzyme was used instead of the enzyme (Figure 2). In addition, the enzyme preparation used here was electrophoretically pure and absolutely free from phosphatases as evidenced by the lack of the reactivity toward phospholactate or *p*-nitrophenyl phosphate. These results clearly indicate that the phosphate-liberation reaction observed here is catalyzed by PEP carboxylase.

The enzymatic liberation of phosphate from PEKB led us to investigate the requirement of HCO_3^- and Mg^{2+}, which were the necessary components of the normal reaction, for the reaction. As shown in Figure 3, the liberation of phosphate was markedly depressed by the omission of HCO_3^- from the reaction mixture, indicating that HCO_3^- was necessary for the cleavage of PEKB. The slight liberation of phosphate in the absence of exogenous HCO_3^- (Figure 3) was probably due to trace amounts of HCO_3^- originating from other reagents or the atmosphere. Similarly, the liberation of phosphate from PEKB was completely depressed when Mg^{2+} was omitted from the reaction mixture as in the case of the reaction with PEP (data not shown).

Identification of Reaction Products. In order to identify the reaction product, the enzyme reaction was carried out in the presence of $[^{14}C]HCO_3^-$, and the product, after conversion to its DNPH derivative followed by extraction, was analyzed by thin-layer chromatography (Figure 4). When reaction was

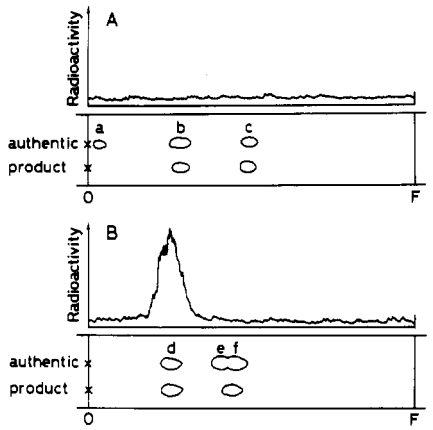

FIGURE 4: Analysis of DNPH derivatives of the reaction products by thin-layer chromatography. The enzyme reaction mixture (0.5 mL) contained 50 μmol of Tris–H_2SO_4 buffer (pH 8.5), 5 μmol of $MgSO_4$, 5 μmol of [^{14}C]$KHCO_3$ (680000 dpm), 7% dioxane, 2.5 μmol each of PEKB (A) or PEP (B), and 57 (A) or 3 μg (B) of the enzyme. After 5-min incubation at 30 °C, 5.8 μmol of 2,4-dinitrophenylhydrazine dissolved in 3 mL of 0.5 M HCl was added, and the mixture was allowed to stand at room temperature for 1 h. The DNPH derivatives thus obtained were extracted (Kawano et al., 1962) and analyzed by thin-layer chromatography. (A) Chromatogram of the products from PEKB, which were developed in ethyl acetate (saturated with 0.1 M $NaHCO_3$)–methanol (5:1 v/v). (B) Chromatogram of the products from PEP, which were developed in butanol–ethanol–0.1 M $NaHCO_3$ (10:3:10 v/v). Radioactivity on the plate was detected with a Packard Model 7201 radiochromatogram scanner. The authentic DNPH's of keto acids used were (a) methyloxaloacetate, (b) α-ketobutyrate (trans), (c) α-ketobutyrate (cis), (d) oxaloacetate, (e) pyruvate (trans), and (f) pyruvate (cis).

Table I: Stoichiometric Formation of Inorganic Phosphate and α-Ketobutyrate from PEKB[a]

time (min)	P_i (μmol)[b]	α-keto-butyrate (μmol)[b]	ratio[c]
5	2.6	2.4	0.92
10	4.3	4.0	0.92
15	4.5	4.1	0.91

[a] The reaction mixture (1 mL) contained 100 μmol of Tris–H_2SO_4 buffer (pH 8.5), 10 μmol of $MgSO_4$, 10 μmol of $KHCO_3$, 5 μmol of *n*-octyl glucoside, 4.5 μmol of PEKB, and 100 μg of the enzyme. *n*-Octyl glucoside was included as the activator (Izui et al., 1982) instead of dioxane since the latter (probably due to the presence of trace amounts of peroxide) was found to interfere with the determination of keto acids. Aliquots (300 μL) were withdrawn after 5-, 10-, and 15-min incubations at 30 °C, and the reaction was terminated by the addition of perchloric acid. After centrifugation at 4000*g* for 10 min, aliquots of the supernatant were subjected to the determination of inorganic phosphate and α-ketobutyrate. [b] The values represent the total amounts per 1-mL reaction mixture. [c] The value represents the ratio of the amount of α-ketobutyrate to that of P_i.

performed with PEP as a control, formation of DNPH of radioactive oxaloacetate was observed. Small amounts of DNPH's of pyruvate, which probably resulted from decarboxylation of oxaloacetate, were also found. When PEKB was used as a substrate, in contrast, no radioactive compound was observed, and only DNPH's of nonradioactive α-ketobutyrate were the products. The molar amount of α-ketobutyrate produced in the reaction was almost equal to that of inorganic phosphate liberated (Table I). These results suggest that CO_2 fixation does not occur on PEKB. However, the possibility that CO_2 fixation occurred on PEKB and the resulting methyloxaloacetate, the expected fixation product, underwent a decarboxylation to yield α-ketobutyrate owing to its lability (Sakkab & Martell, 1976) in the course of the reaction or in

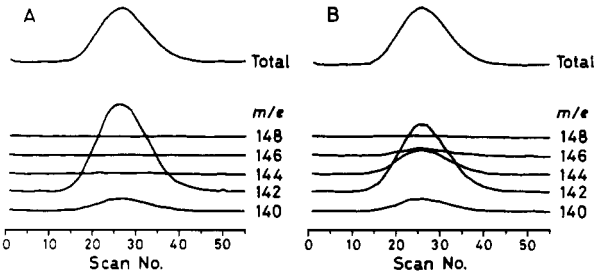

FIGURE 5: Mass chromatographic analysis of inorganic phosphate liberated in the reaction with [^{18}O]HCO_3^-. The reaction mixture (0.5 mL) contained 25 μmol of Tris–H_2SO_4 buffer (pH 8.5), 5 μmol of $MgSO_4$, 10 μmol of [^{18}O]$KHCO_3$, 7% dioxane, 2.5 μmol of PEP or PEKB, and the enzyme. The amounts of enzyme used were 25 μg in the reaction with PEP and 400 μg in the reaction with PEKB, respectively. The reaction mixture not containing [^{18}O]$KHCO_3$ was first prepared from bicarbonate-free stock solutions, and the reaction was started by the addition of solid [^{18}O]$KHCO_3$ to prevent the decrease of ^{18}O content of [^{18}O]HCO_3^- due to the equilibration between [^{18}O]HCO_3^- and H_2O. The reaction was carried out at 30 °C for 1 min, and termination of the reaction, isolation of inorganic phosphate, methylation, and analysis by gas chromatography–mass spectrometry were performed, successively, as described under Materials and Methods. The mass spectra in the range of *m/e* 14–166 were repeatedly monitored at 5.5-s intervals, and five continuous curves (mass chromatograms) at *m/e* 140, 142, 144, 146, and 148 were obtained. The curve that is referred to as "Total" shows the result of total ion monitoring. (A and B) Results with PEP and PEKB as a substrate, respectively.

the subsequent experimental process could not be excluded. In order to examine this possibility, attempts were made to detect the carboxylated product that may be produced, if any, in the reaction that was carried out in the presence of [^{14}C]HCO_3^-. After treatment of the product for conversion to some stable derivatives, radioactivity was measured on the acid-nonvolatile fraction. When the mixture after the reaction with PEKB was treated with $NaBH_4$ or 2,4-dinitrophenylhydrazine, no radioactivity higher than a level of background could be detected in the fraction. This was also the case when the reaction with PEKB was carried out in the presence of excess amounts of glutamate-oxaloacetate transaminase and L-glutamate as the trapping system. In a separate experiment, it was confirmed that methyloxaloacetate worked as a good substrate of the transaminase [see Galegov (1961) and Abramsky & Shemin (1965)].

Incorporation of ^{18}O from [^{18}O]HCO_3^- into Inorganic Phosphate. In order to elucidate the molecular mechanism by which HCO_3^- promoted the cleavage of PEKB, the enzyme reaction was carried out in the presence of [^{18}O]HCO_3^-. The inorganic phosphate liberated was isolated, methylated, and analyzed by gas chromatography–mass spectrometry to examine whether ^{18}O was incorporated into the inorganic phosphate or not. Figure 5 shows the plots of signal intensities corresponding to the molecular ions of trimethyl phosphate against retention times. Mass numbers (*m/e*) of 140, 142, 144, 146, and 148 correspond to the molecular ions of trimethyl phosphate species containing zero, one, two, three, and four ^{18}O atoms, respectively. Figure 5A indicates that only one ^{18}O atom was incorporated into the phosphate in the reaction with PEP, being consistent with the result of Maruyama et al. (1966) on the peanut enzyme. Incorporation of one ^{18}O atom was also found in the reaction with PEKB (Figure 5B). Unexpectedly, however, the formation of molecular species containing two and three ^{18}O atoms was observed in addition. From the peaks in Figure 5B, after integration and correction for the initial ^{18}O-enrichment of [^{18}O]HCO_3^- (90%), the molar ratio of phosphate species that contained one, two, and three oxygen atoms originating from HCO_3^- was estimated to be

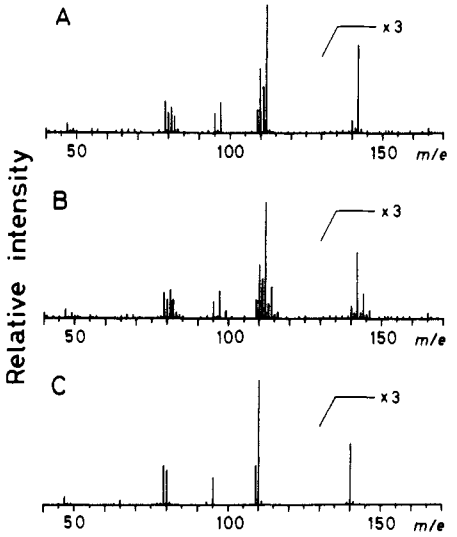

FIGURE 6: Mass spectra of trimethyl phosphate. The figures show mass spectra measured at the peaks of trimethyl phosphate on mass chromatograms (Figure 5). (A and B) Spectra at scan 26 in Figure 5A and at scan 24 in Figure 5B, respectively. (C) Result of control experiment with PEP and unenriched $KHCO_3$ as a substrate. The relative intensity on the ordinate is shown expanded 3 times at m/e larger than 130 in each case.

70:25:5. The multiple incorporation of ^{18}O in the reaction with PEKB was also demonstrated by some fragment ions (m/e 110, 109, 95, etc.) in the mass spectra (Figure 6).

Discussion

PEKB, in which methyl group is substituted for the hydrogen atom attached to β-carbon (the site for carboxylation) of PEP, is the most potent competitive inhibitor of *E. coli* PEP carboxylase among the analogues and homologues of PEP so far examined (Izui et al., 1983). The present study showed that this compound was cleaved by the enzyme to yield inorganic phosphate and α-ketobutyrate. Although Mg^{2+} and HCO_3^- were necessary for the reaction with PEKB as in the normal reaction with PEP, no CO_2 fixation could be detected. This observation seems to be the first one that enabled separation of the phosphate liberation from CO_2 fixation, being in conflict with the concerted mechanism (Scheme I).

The requirement of HCO_3^- and the occurrence of ^{18}O incorporation from $[^{18}O]HCO_3^-$ into inorganic phosphate in both reactions with PEP and PEKB strongly suggest the direct involvement of HCO_3^- in the phosphate liberation. The nucleophilic attack of HCO_3^- toward the phosphorous atom of the substrate seems to be an outset of the reaction. This means that the initial step of reaction—the process of HCO_3^- activation coupled with the cleavage of the enolphosphate bond—is common to both reactions with PEP and PEKB. In the case of reaction with PEKB, however, it is presumed that PEKB cannot undergo a carboxylation owing to steric hindrance by its methyl group and that the reaction proceeds away from the normal course to yield α-ketobutyrate.

The intermediate that is expected to form first in the reaction being driven by the nucleophilic attack of HCO_3^- toward the phosphorous atom of the substrate accompanied by the cleavage of the P–O bond is carbonic–phosphoric anhydride (and enolate anion of keto acid) (see Scheme II). The anhydride is known to form as an intermediate in the carbamoyl phosphate synthetase reaction of *E. coli* (Powers & Meister, 1976; Wimmer et al., 1979). It is also expected to form in the enzymatic carboxylation of biotin on the basis of indirect evidence (Ashman & Keech, 1975; Polakis et al., 1972). In consideration of the possibility that such an anhydride with longer lifetime exists in the reaction with PEKB, we attempted to detect it as its trimethyl ester according to Powers & Meister (1976). Reaction was carried out in the presence of $[^{14}C]HCO_3^-$ with high specific radioactivity, and the mixture after reaction was methylated with diazomethane. The resulting product was analyzed by thin-layer chromatography along with the synthetic trimethyl ester, but a negative result was obtained. Phosphonoacetic acid, which is regarded as an analogue of carbonic-phosphoric anhydride, inhibited the reaction with PEP competitively, but its K_i value (5.2 mM) was not so low compared with those of other PEP analogues (Izui et al., 1983).

The multiple ^{18}O incorporation from $[^{18}O]HCO_3^-$ into inorganic phosphate suggests the involvement of an unknown reversible process in the reaction with PEKB. Presumably, lingering of the intermediate on the enzyme may cause such a microscopic reversibility. However, it must be further clarified whether the reversible process that is responsible for the multiple incorporation is inherent in the normal reaction and the difference in the isotope distribution is merely due to the difference in the lifetime of the intermediate or the process is unique to PEKB owing to its "abnormality" as a substrate.

The incorporation of one ^{18}O atom from $[^{18}O]HCO_3^-$ into the liberated inorganic phosphate is widely observed in enzymatic reactions of CO_2 fixation with HCO_3^- as a substrate (Jones & Spector, 1960; Kaziro et al., 1962; Maruyama et al., 1966). However, there has been no report of multiple incorporation of more than two ^{18}O atoms as observed here. It is of interest to examine whether such a phenomenon—a kind of isotope-scrambling phenomenon (Rose, 1979)—can be observed also with other enzymes.

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Registry No. PEKB, 25956-55-2; PEP, 138-08-9; DL-phospholactate, 32450-46-7; phosphoenol-α-ketoisovalerate, 28749-60-2; bicarbonate, 71-52-3; EC 4.1.1.31, 9067-77-0.

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

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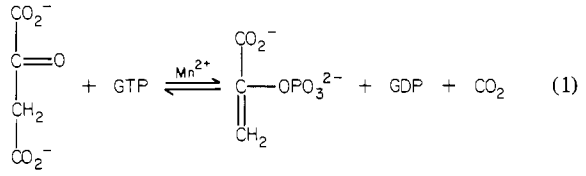
ABSTRACT: Guinea pig liver mitochondrial phosphoenolpyruvate carboxykinase catalyzes the conversion of (R_P)-guanosine 5′-(3-thio[3-^{18}O]triphosphate) and oxalacetate to (S_P)-[^{18}O]thiophosphoenolpyruvate, GDP, and CO_2 by a mechanism that involves overall *inversion* in the configuration of the 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.

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[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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