Phosphoenolpyruvate carboxykinase from chicken liver mitochondria and rat liver cytosol catalyzes the phosphorylation of α-substituted carboxylic acids such as glycolate, thioglycolate, and D1-β-chlorolactate in reactions with absolute requirements for divalent cation activators. 31P NMR analysis of the reaction products indicates that phosphorylation occurs at the α-position to generate the corresponding O- or S-bridged phosphate monoesters. In addition, the enzymes catalyze the bicarbonate-dependent phosphorylation of hydroxylamine. The chicken liver enzyme also catalyzes the bimolecular-dependent phosphorylation of fluoride ion. The kcat values for these substrates are 20-1000-fold slower than the kcat for oxaloacetate. Pyruvate and β-hydroxy pyruvate are not phosphorylated, since the enzyme does not catalyze the enolization of these compounds. Oxalate, a structural analogue of the enolate of pyruvate, is a competitive inhibitor of phosphoenolpyruvate carboxykinase (Km of 5 μM) in the direction of phosphoenolpyruvate formation. Oxalate is also an inhibitor of the chicken liver enzyme in the direction of oxaloacetate formation and in the decarboxylation of oxaloacetate. The chicken liver enzyme is inhibited by β-sulfopyruvate, an isoelectronic analogue of oxaloacetate. The extensive homologies between the reactions catalyzed by phosphoenolpyruvate carboxykinase and pyruvate kinase suggest that the divalent cation activators in these reactions may have similar functions. The substrate specificity indicates that phosphoenolpyruvate carboxykinase decarboxylates oxaloacetate to form the enolate of pyruvate which is then phosphorylated by MgGTP on the enzyme.

In higher organisms, phosphoenolpyruvate carboxykinase (P-enolpyruvate carboxykinase) catalyzes the first committed step in gluconeogenesis:

\[
\text{Me}^{2+}, M^+ \cdot \text{P-enolpyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}
\]

The enzyme has been purified and characterized from a variety of vertebrate sources, including rat liver cytosol (1-3) and chicken liver mitochondria (4, 5). Considerable information concerning the geometrical constraints at the active site has been provided by kinetic studies of the substrate specificity of the chicken liver enzyme (5-7) as well as by magnetic resonance studies of complexes of the enzyme with substrates and inhibitors (8-11). P-enolpyruvate carboxykinase displays a high degree of specificity for the substrate P-enolpyruvate in the direction of oxaloacetate formation. Thus, (Z)-F-P-enolpyruvate1 is a substrate for the enzyme with a Vmax of 27% relative to P-enolpyruvate (5, 7), while other analogues of P-enolpyruvate such as (Z)-Br-P-enolpyruvate, (E)-F-P-enolpyruvate, and the E and Z diastereomers of P-enol-α-keto butyrate show no detectable substrate activity, but are inhibitors of the enzyme (7, 12). To date, no analogues of oxaloacetate which function as alternate substrates of P-enolpyruvate carboxykinase have been reported.

The reaction catalyzed by P-enolpyruvate carboxykinase is in many respects analogous to the reaction catalyzed by pyruvate kinase (Equation 2):

\[
\text{Me}^{2+}, M^+ \cdot \text{pyruvate} + \text{ATP} \rightarrow \text{P-enolpyruvate} + \text{ADP}
\]

Both P-enolpyruvate carboxykinase and pyruvate kinase catalyze phosphoryl transfer from P-enolpyruvate to a nucleoside diphosphate in reactions which have absolute requirements for divalent cation activators (13-16). Metal ion binding and kinetic studies with these enzymes have indicated that 1 equivalent of divalent cation binds directly to the free enzyme, while the second divalent cation is associated with the nucleotide substrate (8, 17-21). In addition to the physiologically significant reactions described by Equations 1 and 2, both enzymes also catalyze the decarboxylation of oxaloacetate in reactions which require either divalent cations (pyruvate kinase (22)) or nucleoside diphosphates (P-enolpyruvate carboxykinase (4)).

Oxaloacetate → pyruvate + CO2

Finally, although there is tight coupling between the phosphoryl transfer and carboxylation reactions for P-enolpyruvate carboxykinases from most sources, the enzyme from yeast has been reported to display a pyruvate kinase activity, described by Equation 2, which is independent of carboxylation (23).

In addition to the reactions described by Equations 2 and 3, rabbit muscle pyruvate kinase also catalyzes the ATP-dependent phosphorylation of α-substituted carboxylic acids such as glycolate (24), L- and D-lactate (25), and thioglycolate

1 The abbreviations used: F-P-enolpyruvate, phosphoenol-3-fluoropyruvate; Br-P-enolpyruvate, phosphoenol-3-bromopyruvate; P-enol-α-keto butyrate, phosphoenol-α-keto butyrate; thioglycolate, 2-thioglycerate; β-sulfopyruvate, 2-carboxy-2-oxoethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Alternate Substrates for Phosphoenolpyruvate Carboxykinase

(25), as well as the ATP- and bicarbonate-dependent phosphorylations of fluoride ion (26) and hydroxylamine (27–29). On the basis of the homologies between pyruvate kinase and P-enolpyruvate carboxykinase outlined above, it seemed possible that P-enolpyruvate carboxykinase might catalyze an analogous set of phosphorylation reactions. The present study characterizes the kinetic properties of a series of a-substituted carboxylic acid substrates with P-enolpyruvate carboxykinase isolated from chicken liver mitochondria and rat liver cytosol. In addition, the inhibition of the enzymes by β-sulfopyruvate, an analogue of oxaloacetate, and oxalate, a structural analogue of the enolate of pyruvate is described.

MATERIALS AND METHODS

Enzymes—Chicken liver mitochondrial P-enolpyruvate carboxykinase was prepared by a modification of previously published procedures (6). Fresh chicken livers were obtained from Weaver Chicken, New Holland, PA. The livers were removed from the animals approximately 15 min after death and placed in 0.3 M sucrose at 0 °C. The mitochondrial fraction from the liver was prepared, lyophilized, and extracted by Heidsieck and Nelles (5) a lyophyshate gel absorption step and chromatography on Sepharose 4B Blue Dextran utilizing these authors were replaced by chromatography on columns of Amicon Orange A Matrix Gel and ITP-Sepharose. The Amicon Orange A column was equilibrated with 10 mM Hepes-KOH, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 2 mM MgCl₂ at pH 7.2, and the enzyme was eluted with a linear gradient of 0–0.2 M KCl in the equilibration buffer. The peak of enzymatic activity eluted from the Orange A column at 0.04 M KCl. The ITP-Sepharose column was equilibrated with 10 mM Hepes-KOH, 10% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM EDTA at pH 7.2. The enzyme was eluted at 0.2 mM ITP with a linear gradient of 0–1 mM ITP in the elution buffer. The enzymatic activity of the enzyme preparations was routinely between 10 and 13 units/mg when assayed at room temperature in the direction of P-enolpyruvate formation and 20 units/mg at 30 °C. The standard assay (Assay I) consisted of 10 mM MgCl₂, 0.1 mM MnCl₂, 0.1 mM GTP, 4 mM malate, and 1 mM NAD in 50 mM Hepes-KOH at pH 8.0. Malate dehydrogenase (6 units/ml) was added to rapidly establish the equilibrium: malate + NADH + H⁺. The oxaloacetate concentration was assumed to equal the NADH formed at approximately 20 s following malate dehydrogenase addition. The nonenzymatic decarboxylation of oxaloacetate caused a background rate of NADH formation which was subtracted from the rate obtained following the addition of P-enolpyruvate carboxykinase. Protein was determined with the Pierce protein assay reagent using bovine serum albumin as the standard. The concentration of enzyme in purified preparations was determined using the extinction coefficient of 16.5 for a 1% solution measured at 280 nm (5).

P-enolpyruvate carboxykinase from rat liver cytosol was prepared as described previously (30), except that ITP-Sepharose was used instead of GTP-agarose. The specific activity of the purified enzyme was between 9 and 12 units/mg at 30 °C using Assay I (see above). During storage the specific activity of the enzyme decreases. Enzyme of specific activities below 5 units/mg was not used. Chicken mitochondrial and rat cytosolic P-enolpyruvate carboxykinases were near homogeneous (>95% purity) as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Rabbit muscle pyruvate kinase was obtained as a lyophilized, salt-free solid from United States Biochemical Corp. The enzyme was dissolved in 50 mM Hepes-KOH, 0.1 M KCl at pH 7.5 prior to use.

Chemicals—4-Hydroxy-1-cyclopropane carboxylic acid was obtained from Aldrich, and 1-hydroxy-1-cyclopropane carboxylic acid phosphate was provided by Dr. Marion O’Leary, University of Wisconsin. β-Sulfopyruvate was prepared by the method of Griffith and Weinstein (31) and quantitated by the spectrophotometric assay with malate dehydrogenase. All other reagents were of the highest purity commercially available.

Substrate Activity of a-Substituted Carboxylic Acids—Preliminary screening of a-substituted carboxylic acids for substrate activity employed a chromatographic assay for nucleotide products similar to that previously reported (25). Reaction mixtures contained 0.2 mM GTP, 2 mM MgCl₂, 0.75 mM MnCl₂, in 150 mM Hepes-KOH at pH 7.5. The concentration of substrate analogue was 10 mM and the reactions were initiated by the addition of 5–20 μg of P-enolpyruvate carboxykinase to 100 μl of reaction mixture. At various times aliquots of the mixtures and of control samples lacking enzyme were spotted on polyethyleneimine thin-layer chromatography plates (EM Reagents). The plates were developed in 0.75 M potassium phosphate at pH 3.5 and the GTP and GDP spots were visualized with 254 nm light (Assay IV).

The activity of pyruvate kinase with 1-hydroxy-1-cyclopropane carboxylic acid was monitored in a similar manner. Reaction mixtures contained 2 mM ATP, 5 mM MnCl₂, 10 mM MgCl₂, 1 mM EDTA, and 0.03 μCi of carrier-free [α-32P]GTP, and 0.1 mM carrier GTP in a total volume of 20 μl. Reactions (30 °C) were initiated by the addition of the enzyme to a final concentration of 50 mM. D₂O (20%, v/v) was added to the samples for field-frequency lock. The conditions for 32P NMR analyses of the fluorokinase and hydroxylamine kinase reactions are provided in the legend to Fig. 1.

Kinetic constants for the alternate substrates were determined by quantitating the amount of GDP produced in the phosphorylation reaction (Assay III). For these experiments the reaction mixtures contained 50 mM Hepes-KOH, pH 8.0, 5 mM MgCl₂, 0.1 mM MnCl₂, 0.03 μg of carrier free [α-32P]GTP, and 0.1 mM carrier GTP in a total volume of 20 μl. Reactions (30 °C) were initiated by the addition of P-enolpyruvate carboxykinase. Aliquots (2 μl) were removed from duplicate samples at various times and applied to polyethyleneimine thin-layer chromatography plates which had been pre-spotted with 2 μl of 10 mM EDTA to stop the reaction. The plates were developed in 4 M ammonium formate at pH 3.1. The spots corresponding to GDP and the unreacted GTP were cut from the strips and counted in 4 ml of scintillation fluid in small glass vials. Control experiments included incubations lacking enzyme or substrate. The fraction of GTP converted to GDP was used to measure reaction rates. Two or more points were used to establish initial rate conditions.

Enolization Reactions—The enolization of pyruvate catalyzed by P-enolpyruvate carboxykinase or pyruvate kinase was quantitated by measuring the loss of the H NMR resonance for the methyl protons of pyruvate (25), as well as the ATP- and bicarbonate-dependent phosphorylations of fluoride ion (26) and hydroxylamine (27–29). The solutions contained 20 mM Tris·HCl, pH 7.5, 0.1 mM KC1, 5 mM MgCl₂, 0.1 mM MnCl₂, and either 40 mM potassium phosphate or 2 mM GTP for the assay with P-enolpyruvate carboxykinase or 2 mM ADP for the assay with pyruvate kinase. The solutions were repeatedly lyophilized and redissolved in D₂O. To initiate the reaction 1 mM malate dehydrogenase was added to 0.5 ml of the D₂O-exchanged solutions to give a final concentration of 50 mM, and the reactions were initiated by the addition of 5 units of either P-enolpyruvate carboxykinase or pyruvate kinase. The methylene protons of the Tris buffer served as an internal integration standard, and plots of ln(3H NMR resonance for the methyl protons of pyruvate. The carbon protons of malate were used to quantitate the nonenzymatic exchange of the methyl protons of pyruvate.

Inhibition Experiments—The inhibition of P-enolpyruvate carboxykinase by oxalate was determined in the direction of P-enolpyruvate formation with 1-hydroxy-1-cyclopropane carboxylic acid phosphorylated to the production of NADH from the malate dehydrogenase reaction (Assay IV). The reaction mixtures contained 50 mM Hepes-KOH, 1 mM MgCl₂, 50 mM MnCl₂, 50 μM GTP, 1 mM NAD, and 6 units/ml malate dehydrogenase at pH 8. The concentration of malate was varied to produce the desired concentrations of oxaloacetate. With 5 mM malate as substrate, malate dehydrogenase was used to quantitate the formation of oxaloacetate (Assay V). These reaction mixtures contained 50 mM Hepes-KOH at pH 8.1, 1 mM GDP, 100 mM KHCO₃, 2 mM MgCl₂, 0.1 mM MnCl₂, 0.2 mM NADH, and 6 units/ml malate dehydrogenase.

Inhibition of P-enolpyruvate carboxykinase by β-sulfopyruvate in the direction of P-enolpyruvate formation was determined in a coupled assay with pyruvate kinase and lactate dehydrogenase (Assay...
Alternate Substrates for Phosphoenolpyruvate Carboxykinase

RESULTS

Survey of Alternate Substrates—The substrate activity of \( \alpha \)-substituted carboxylic acids with P-enolpyruvate carboxykinase was examined using the thin-layer chromatographic assay for nucleotides (Assay II) as described under "Materials and Methods." The results of these experiments with the P-enolpyruvate carboxykinases from chicken and rat liver are presented in Table I along with a comparison of the substrate activity of these compounds with rabbit muscle pyruvate kinase. With the exceptions of pyruvate, \( \beta \)-hydroxy pyruvate, and D-lactate, the alternate substrates for pyruvate kinase are also substrates for phosphorylation reactions catalyzed by chicken liver P-enolpyruvate carboxykinase. Although D-lactate is not a substrate for the chicken liver enzyme, this compound is phosphorylated at a slow rate by the rat liver enzyme. The phosphorylation of hydroxylamine by P-enolpyruvate carboxykinase has a requirement for bicarbonate similar to that for the pyruvate kinase-catalyzed reaction, suggesting that N-hydroxycarbamate may be the true substrate in this reaction (34). The phosphorylation of fluoride ion by the chicken mitochondrial enzyme also required the addition of bicarbonate, as was no GDP formation above control levels in those samples which did not contain added bicarbonate. Bicarbonate alone did not support the hydrolysis of GTP by P-enolpyruvate carboxykinase. The enzyme from rat liver did not phosphorylate fluoride ion. These results demonstrate that decarboxylation is not required for phosphoryl transfer by the enzyme.

1-Hydroxy-1-cyclopropane carboxylic acid was a substrate for both P-enolpyruvate carboxykinase and pyruvate kinase. Neither of these substrate activities has been previously reported.

Characterization of Phosphorylated Alternate Substrates by \( \textsuperscript{31} \)P NMR—Formation of GDP in the presence of substrate analogues does not distinguish GTP hydrolysis from analogue phosphoryl transfer by the enzyme.

Table I

<table>
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<td>Glycolate</td>
<td>Yes</td>
<td>Yes(^b)</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>Yes</td>
<td>Yes(^c)</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>No</td>
<td>Slow(^c)</td>
</tr>
<tr>
<td>DL-( \beta )-Chlorolactate</td>
<td>Yes</td>
<td>Yes(^e)</td>
</tr>
<tr>
<td>L-Glycerate</td>
<td>Yes</td>
<td>Yes(^c)</td>
</tr>
<tr>
<td>L-Hydroxy-1-cyclopropane carboxylate</td>
<td>Yes</td>
<td>Yes(^e)</td>
</tr>
<tr>
<td>Thiglycolate</td>
<td>No</td>
<td>Slow(^e)</td>
</tr>
<tr>
<td>Hydroxylamine(^d)</td>
<td>Yes</td>
<td>Yes(^d)</td>
</tr>
<tr>
<td>Fluoride(^d)</td>
<td>No</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>( \beta )-Hydroxy pyruvate</td>
<td>No</td>
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</tr>
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<td>L-Malate</td>
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\(^a\) Assay method II. The sensitivity of the assays was sufficient to detect 20 \( \mu \)M GDP after a 5-h incubation with 50 \( \mu \)g/ml enzyme. Background levels of GDP in solutions lacking enzyme or substrate were less than 5 \( \mu \)M.

\(^b\) Ref. 24.

\(^c\) Ref. 25.

\(^d\) Ref. 26.

\(^e\) Substrate activity was detected only in the presence of bicarbonate.

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</tr>
<tr>
<td>Hydroxylamine(^d)</td>
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</tr>
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<td>No</td>
<td>No(^d)</td>
</tr>
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<td>L-Malate</td>
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\(^a\) Assay method II. The sensitivity of the assays was sufficient to detect 20 \( \mu \)M GDP after a 5-h incubation with 50 \( \mu \)g/ml enzyme. Background levels of GDP in solutions lacking enzyme or substrate were less than 5 \( \mu \)M. A yes, slow, or no in the activity column corresponds to initial velocities of >0.05, <0.001, or <0.0001 \( \mu \)mol/min/mg of protein, respectively.

\(^b\) Ref. 24.

\(^c\) Ref. 25.

\(^d\) Ref. 26.

\(^e\) Not determined.

VI). This assay avoids complications due to the substrate activity of \( \beta \)-sulfopyruvate with malate dehydrogenase. Assay mixtures contained 50 mM Hepes-KOH, 150 mM KCl, 0.1 mM GTP, 0.2 mM ADP, 2 mM MgCl\(_2\), 50 \( \mu \)M MnCl\(_2\), 0.1 mM NADH, 10 units/ml pyruvate kinase, and 10 units/ml lactate dehydrogenase at pH 7.5. The effects of oxalate and \( \beta \)-sulfopyruvate on the oxaloacetate decarboxylase activity of P-enolpyruvate carboxykinase were determined in a coupled assay with lactate dehydrogenase (Assay VII) as described by Noce and Uter (4). The assay mixtures contained 50 mM Hepes-KOH, 1 mM GDP, 0.16 mM NADH, and 30 units/ml lactate dehydrogenase at pH 7.5. Background rates of NADH oxidation due to the nonenzymatic decarboxylation of oxaloacetate were subtracted from the rate obtained after the addition of P-enolpyruvate carboxykinase. The kinetic data for oxalate and \( \beta \)-sulfopyruvate were fit to the equation for competitive inhibition using the computer program of Cleland (33).

Inhibition constants for the weak inhibitors (\( K_i \geq 5 \) mM) were determined by titrating standard reaction mixtures for Assay I with increasing inhibitor concentration. The \( K_i \) was estimated from the equation for competitive inhibition, even though complete inhibition patterns were not determined.

NMR Spectra—\( \textsuperscript{1} \)H NMR (300 MHz) and \( \textsuperscript{31} \)P NMR (121.5 MHz) spectra were recorded with a Bruker WM 300 wide bore spectrometer. Proton coupled \( \textsuperscript{31} \)P NMR spectra were recorded at ambient temperature with a 45° flip angle, a 0.8-2.7-s acquisition time, and a 2-s relaxation delay. 31P NMR chemical shifts are reported relative to external 85% phosphoric acid.

Fig. 1. \( \textsuperscript{31} \)P NMR spectra for reaction mixtures with chicken liver P-enolpyruvate carboxykinase. Solutions contained 100 mM Hepes-KOH at pH 7.5, 5 mM GTP, 5 mM MgCl\(_2\), 0.5 mM MnCl\(_2\), and 5 units of P-enolpyruvate carboxykinase. Reactions were terminated after a 24-h incubation at room temperature by the addition of EDTA to a final concentration of 50 mM. \( \text{D}_2\)O was added to 20% (v/v). Spectra were recorded at 121.5 MHz and represent 1000 transients taken with a 0.8-s acquisition time, a 2-s relaxation delay, and a 45° flip angle. Resonances due to the phosphate groups of the nucleotides are: GDP \( \beta P\) and GTP \( \gamma P\), -6 ppm; GDP \( \alpha P\) and GTP \( \alpha P\), -10 ppm; and GTP \( \beta P\), -21 ppm. A, no additions. B, addition of 100 mM KHCO\(_3\), and 50 mM hydroxylamine. C, addition of 100 mM KHCO\(_3\), and 100 mM NaF.
phosphorylation. Thus, the products of the reactions were characterized by \(^{31}\)P NMR. The chemical shift values and the \(^1\)H,\(^{31}\)P coupling constants for the products of the reactions of chicken liver P-enolpyruvate carboxykinase with glycolate, L-lactate, L-glycerate, thioglycolate, and DL-\(\beta\)-chloroacetate agree with those previously reported (results not shown) (25).

The product 1-hydroxy-1-cyclopropane carboxylic acid phosphate (delta = 2.0 ppm) was identified by the addition of authentic compound to the NMR sample. Incubation of P-enolpyruvate carboxykinase with hydroxylamine, bicarbonate, GTP, and divalent cations resulted in a product with a \(^{31}\)P resonance at 8.3 ppm (Fig. 1B). O-Phosphorylhydroxylamine was identified by comparison with the \(^{31}\)P NMR spectrum for o-phosphorylhydroxylamine formed by pyruvate kinase (27-29). Fluorophosphate (Fig. 1C) was identified on the basis of the \(^{31}\)P chemical shift values and the \(^{19}\)F coupling constant of 869 Hz (35). \(^{31}\)P NMR analysis of the reaction products established that phosphoryl transfer occurred to alternate substrates without inducing a GTP hydrolytic activity. Thus, no \(^{31}\)P NMR resonance for phosphate was detected except where subsequent hydrolysis of unstable phosphorylated products occurred. The structures of the products formed in the reaction of the rat liver enzyme with glycolate, L-lactate, and L-glycerate were also confirmed by \(^{31}\)P NMR.

Characteristics of Alternate Substrate Phosphorylation—With glycolate as an example of the alternate substrates, several properties of the phosphorylation reactions were determined from the chromatographic assay for GDP. The reaction displayed maximal GDP formation near pH 8.0, with lower rates at pH values of 7.5 or 8.5. In contrast to the reactions catalyzed by pyruvate kinase, there is no requirement for monovalent cations such as K\(^+\) in the phosphorylation reaction. Thus, the same amount of GDP was formed when tetramethylammonium ion, Na\(^+\), or K\(^+\) were used in the reaction mixtures. The phosphorylation of glycolate was preferentially stimulated by Mn(II) versus Mg(II). Similar divalent cation specificities have been reported for the normal reaction of P-enolpyruvate carboxykinase (14-16) and for the phosphorylation of \(\alpha\)-hydroxycarboxylic acids catalyzed by pyruvate kinase (25).

Kinetic Measurements—Kinetic constants for the alternate substrates were determined for the chicken liver enzyme, and are presented in Tables II and III, along with the K\(_i\) values for pyruvate, \(\beta\)-hydroxypyruvate, malate, D-lactate, and oxaloacetate. Thioglycolate, glycolate, and DL-\(\beta\)-chloroacetate are the preferred alternate substrates in terms of V\(_{max}\)/K\(_i\) for chicken liver P-enolpyruvate carboxykinase. The L-isomer of lactate is phosphorylated by the enzyme; however, the D-isomer reacts poorly or is inert.

Rat liver P-enolpyruvate carboxykinase has a substrate specificity similar to the enzyme from chicken liver, with several exceptions. The relative V\(_{max}\)/K\(_i\) values were similar for oxaloacetate, DL-\(\beta\)-chloroacetate, glycolate, and 1-hydroxy-1-cyclopropane carboxylic acid. Thioglycolate is a relatively poor substrate for the rat liver enzyme, with a V\(_{max}\)/K\(_i\) of less than 0.01 of that for the chicken liver enzyme. The stereocchemical preference for the L-isomer of lactate exhibited by the chicken liver enzyme was also observed with the rat liver enzyme. However, whereas no substrate activity was observed with D-lactate and the chicken liver enzyme, the rat liver enzyme does catalyze the phosphorylation of this compound.

Enolization of Pyruvate—Pyruvate kinase catalyzes the exchange of the methyl protons of pyruvate with solvent-derived protons in the presence of inorganic cofactors and "P-like" dianions (36, 37). The rates of this exchange (enolization)
when compared to control samples (data not shown). In contrast, the rates of pyruvate enolization for samples containing P-enolpyruvate carboxykinase and 40 mM potassium phosphate or 2 mM GDP were indistinguishable from those for control samples lacking enzyme.

Inhibition of P-enolpyruvate Carboxykinase by Oxalate—Oxalate is a competitive inhibitor of P-enolpyruvate carboxykinase from chicken liver with respect to P-enolpyruvate with a $K_i$ of 0.34 mM (Fig. 2). When either chicken or rat liver P-enolpyruvate carboxykinase is assayed in the direction of P-enolpyruvate formation, oxalate is a potent, competitive inhibitor with respect to oxaloacetate (Fig. 3). $K_i$ values of 4.6 and 5 $\mu M$, respectively, were obtained for the chicken and rat liver enzymes. These inhibition constants for oxalate are near the $K_M$ values of 2 and 5 $\mu M$ for oxaloacetate determined under the same conditions with the chicken and rat liver enzymes, respectively.

Oxalate is also a competitive inhibitor ($K_i = 12.8 \mu M$) with respect to oxaloacetate in the decarboxylation reaction (Fig. 4). In contrast to the other reactions catalyzed by P-enolpyruvate carboxykinase, the oxaloacetate decarboxylase activity of the enzyme has been reported to be independent of added divalent cation activators (4). However, the inhibition of the oxaloacetate decarboxylase activity by metal chelators such as EDTA and 2-picolinoline (4) strongly suggests a requirement for divalent cations in this reaction. The kinetic constants for oxalate in the reactions catalyzed by the enzymes are summarized in Table IV.

### Inhibition of Chicken Mitochondrial P-enolpyruvate Carboxykinase by Oxalate

![Graph showing inhibition of P-enolpyruvate carboxykinase by oxalate](image)

**FIG. 2.** Effect of oxalate on the initial velocity of chicken liver P-enolpyruvate carboxykinase with P-enolpyruvate as the variable substrate. Velocities are in units of micromoles of oxaloacetate formed per min/mg of protein. Other conditions are given under "Materials and Methods" (Assay V). The lines are drawn from the best fit of the experimental data to the equation for competitive inhibition.

![Graph showing inhibition of P-enolpyruvate carboxykinase by oxalate](image)

**FIG. 3.** Effect of oxalate on the initial velocity of chicken liver P-enolpyruvate carboxykinase with oxaloacetate as the variable substrate. Velocities are in units of micromoles of oxaloacetate utilized per min/mg of protein. Other conditions are given under "Materials and Methods" (Assay IV). The lines are drawn from the best fit of the data to the equation for competitive inhibition.

### Discussion

Substrate Specificity—P-enolpyruvate carboxykinase from both chicken liver mitochondria and rat liver cytosol catalyzes the phosphorylation of hydroxylamine and several $\alpha$-substituted carboxylic acids. In addition, the chicken mitochondrial enzyme catalyzes the phosphorylation of fluoride ion. These findings extend the catalytic homologies between these enzymes and rabbit muscle pyruvate kinase. $^31$P NMR studies of the products of the reactions with the $\alpha$-substituted carboxylic acids indicate that phosphorylation of these compounds by P-enolpyruvate carboxykinase occurs at the $\alpha$-carbon to form the corresponding $\alpha$- or $\beta$-bridged phosphate monoesters. These products are identical to those formed in the analogous reactions catalyzed by pyruvate kinase. The cofactor requirement for these phosphorylation reactions parallels that for the physiological reaction catalyzed by P-enolpyruvate carboxykinase (Equation 1). Thus, divalent cations are required for activity with alternate substrates, and preferential stimulation of activity occurs with Mn(II). The phosphorylation of the alternate substrates by P-enolpyruvate carboxykinase occurs equally well in the presence of tetramethylammonium ion, Na+, or K+, in contrast to the reactions catalyzed by pyruvate kinase (24, 25).

Pyruvate and $\beta$-hydroxypyruvate are substrates for pyruvate kinase, but are not substrates for P-enolpyruvate carboxykinase under conditions which would have detected $10^{-4}$ the rate with oxaloacetate. The lack of substrate activity with $\beta$-hydroxypyruvate and pyruvate indicates that P-enolpyruvate carboxykinase is incapable of generating the enolates required for phospho-xyl transfer. Pyruvate kinase catalyzes the enolization of pyruvate in the presence of inorganic cofactors and $P_i$-like dianions (36, 37). From the crystal structure of cat muscle pyruvate kinase, lysine 269 has been proposed to be the enzymic base which is responsible for removal of a
Alternate Substrates for Phosphoenolpyruvate Carboxykinase

**TABLE IV**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Varied substrate</th>
<th>Source of enzyme</th>
<th>$K_m$ (substrate)</th>
<th>$K_i$ Oxalate</th>
<th>$K_i$ $\beta$-Sulfopyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxaloacetate + GTP $\rightarrow$ Oxaloacetate</td>
<td>Chicken</td>
<td>$2.0 \pm 0.3$</td>
<td>$4.6 \pm 0.6$</td>
<td>$18.6 \pm 2.4$</td>
<td></td>
</tr>
<tr>
<td>P-enolpyruvate + GDP + CO$_2$</td>
<td>Rat</td>
<td>$5.0 \pm 0.6$</td>
<td>$5^b$</td>
<td>ND$^b$</td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate $\rightarrow$ pyruvate + CO$_2$</td>
<td>Oxaloacetate</td>
<td>Chicken</td>
<td>$42 \pm 4$</td>
<td>$12.8 \pm 0.7$</td>
<td>$138 \pm 9$</td>
</tr>
<tr>
<td>P-enolpyruvate + GDP + CO$_2$ $\rightarrow$ P-enolpyruvate</td>
<td>Chicken</td>
<td>$123 \pm 17$</td>
<td>$335 \pm 41$</td>
<td>ND$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Assays IV and VI were used to quantitate inhibition by oxalate and $\beta$-sulfopyruvate, respectively.

$^b$ Amount to double the slope of the double-reciprocal plot. The slopes are a nonlinear function (hyperbolic) of oxalate concentrations.

$^d$ Assay VII.

$^c$ Assay V.

proton from the methyl group of pyruvate in the enolization reaction (38). Since P-enolpyruvate carboxykinase does not enolize pyruvate, an enzymic base corresponding to Lys-269 in pyruvate kinase is not functional. The reactive enolate intermediate is therefore likely to be formed in the decarboxylation reaction shown in Scheme I.

The phosphorylation of pyruvate to P-enolpyruvate is thermodynamically unfavorable ($\Delta G^{\circ'} = 6$ kcal/mol), making activity as a poor substrate difficult to detect. However, the phosphorylation of $\beta$-hydroxypyruvate is energetically favored ($\Delta G^{\circ'} < -2$ kcal/mol, calculated from the data in Ref. 25). The initial product of $\beta$-hydroxypyruvate phosphorylation by pyruvate kinase has been proposed to be phosphoenol-$\beta$-hydroxypyruvate. This compound undergoes a nonenzymatic rearrangement to form tartronate-1-semialdehyde phosphate, which displaces the equilibrium to favor the formation of ADP and the phosphorylated product (25). Characterization of the reaction products of the phosphorylation of $\beta$-hydroxypyruvate by pyruvate kinase has recently been reported (39).

Substrate activity of the chicken liver enzyme with L-lactate, but not D-lactate and the stereochemical preference of the rat liver enzyme for L-lactate indicate that the attacking group from the carboxylate substrate must favor the L-configuration. The $\alpha$-oxygen atom on the enolate of pyruvate is located at a position halfway between the $\alpha$-oxygen of D- and L-lactate. Thus, in the catalytic sites, the $\gamma$-phosphoryl of GTP must be located nearer the position occupied by the hydroxyl from L-lactate. The stereoselectivity exhibited by P-enolpyruvate carboxykinase also applies to the inhibition of the chicken liver enzyme by the D- and L-isomers of phospho-lactate, since the L-isomer is bound with a 10-fold greater affinity than the D-isomer (6).

The results of this study demonstrate the substrate activity of L-hydroxy-1-cyclopropane carboxylic acid with both P-enolpyruvate carboxykinase and pyruvate kinase. In addition to establishing substrate activity, this reaction provides a convenient enzymatic route to the preparation of 1-hydroxy-1-cyclopropane carboxylic acid phosphate, a potent inhibitor of maize P-enolpyruvate carboxylase and yeast enolase (40).

Phosphorylation of both fluoride ion and hydroxylamine by chicken liver P-enolpyruvate carboxykinase indicates that the enzyme stabilizes a configuration of the $\gamma$-phosphoryl which is susceptible to attack by good nucleophiles. The large $K_M$ of $>500$ mM for fluoride indicates that the ion is bound weakly, or more likely, that it attacks directly from solution. The substrate activity of hydroxylamine also has a high $K_M$, however in this case the requirement for both bicarbonate and hydroxylamine suggests that they may combine to form N-hydroxycarbamate, a compound with strong structural similarities with glycolate (see Table II). Pyruvate kinase has been shown to catalyze the phosphorylation of this carbamate (34).

The alternate substrate activities reported in this work are due to P-enolpyruvate carboxykinase and are not due to a trace impurity of pyruvate kinase, since $\beta$-hydroxypyruvate is a good substrate for pyruvate kinase, but not for P-enolpyruvate carboxykinase. The $V_{max}/K_M$ values for $\beta$-hydroxypyruvate and pyruvate are approximately equal for pyruvate kinase. The corresponding $V_{max}/K_M$ values for $\beta$-hydroxypyruvate were determined from the data in Ref. 25, and that for pyruvate was estimated from the data in Ref. 44. The kinetic data for these two substrates were determined under different experimental conditions, and thus the comparison of the $V_{max}/K_M$ ratios for $\beta$-hydroxypyruvate and pyruvate is an approximation.

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$^2$ The $V_{max}/K_M$ ratio for $\beta$-hydroxypyruvate was determined from the data in Ref. 25, and that for pyruvate was estimated from the data in Ref. 44. The kinetic data for these two substrates were determined under different experimental conditions, and thus the comparison of the $V_{max}/K_M$ ratios for $\beta$-hydroxypyruvate and pyruvate is an approximation.
Catalytic Efficiency—The turnover number ($k_{\text{cat}}$) for the conversion of oxaloacetate to P-enolpyruvate by P-enolpyruvate carboxykinase is 24 s$^{-1}$. By comparison, the better alternate substrates such as thio glycolate, glyceraldehyde-3-phosphate dikinase, and 2,3-dihydroxy-6-phosphogluconate are characterized by $k_{\text{cat}}$ values of 0.1–1.0 s$^{-1}$. Although these values are 20–200-fold slower than the $k_{\text{cat}}$ for oxaloacetate, the phosphorylation reactions occur at a sufficient rate to permit the quantitative conversion of GTP to GDP with a 2-fold molar excess of the alternate substrate, as observed by the direct $^{31}$P NMR experiments. Since the enzyme cannot abstract protons $\beta$ to the carboxyl, the reaction rate is dictated in part by the pH of the nucleophilic group at the $\alpha$-position. Thus, $\beta$-chloroacetate, thio glycolate, and glyceraldehyde have lower $k_{\text{cat}}$ values at this position than do poorer substrates. The relatively high $V_{\text{max}}/K_M$ for oxaloacetate comes primarily from the tight interaction of this substrate with P-enolpyruvate carboxykinase. The poorer substrates, like fluoride ion, have a $V_{\text{max}}/K_M$ only $10^{-7}$ that for oxaloacetate. However, this value reflects the very large $K_M$ for fluoride, and reaction rates are easily measured because of the relatively good turnover number at high substrate concentrations.

Inhibitors of P-enolpyruvate Carboxykinase—Oxalate is a potent inhibitor of a number of enzymes such as pyruvate kinase (32), pyruvate carboxylase (41), and pyruvate, phosphatase dikinase (42) which utilize pyruvate as a substrate. Inhibition is thought to result from the structural similarities between the diaxon of oxalic acid and the enolate of pyruvate, a proposed intermediate in the reactions catalyzed by these enzymes. Previous studies of chicken mitochondrial P-enolpyruvate carboxykinase have reported that oxalate is a weak, linear noncompetitive inhibitor ($K_I = 3.2$ mM) with respect to P-enolpyruvate when the enzyme is assayed in the direction of oxaloacetate formation (5). However, the present study has shown that oxalate is a competitive inhibitor ($K_I = 0.34$ mM) under similar experimental conditions, and that oxalate is a potent competitive inhibitor with respect to oxaloacetate in the direction of P-enolpyruvate formation. The inhibition of P-enolpyruvate carboxykinase by oxalate is consistent with the intermediate formation of the enolate of pyruvate during the normal catalytic cycle, even though the enzyme cannot generate the enolate directly from pyruvate by proton abstraction. In the "forward" direction (i.e. oxaloacetate $\rightarrow$ P-enolpyruvate) the enolate of pyruvate is generated via decarboxylation of oxaloacetate, while the production of this intermediate in the reverse reaction is a direct consequence of phosphoryl transfer from P-enolpyruvate. Oxalate is also a good competitive inhibitor with respect to oxaloacetate of P-enolpyruvate carboxykinase in the decarboxylation of oxaloacetate. As the ratio $K_O/\text{oxaloacetate}$ for these reactions (0.3–2.7) differs significantly from the value of $<10^{-2}$ anticipated for a transition state analogue, oxalate is best classified as a reaction intermediate analogue. These intermediate forms are illustrated in Scheme I.

Although the inhibition of P-enolpyruvate carboxykinase by analogues of P-enolpyruvate is well documented (5−7, 11), there are few data available concerning inhibition of the enzyme by analogues of oxaloacetate. Malate, succinate, $\alpha$-ketobutyrate, citrate, and isocitrate display only weak ($K_I > 5$ mM) inhibition of the enzyme (5). Similarly, the $\alpha$-hydroxy acid substrates described in this work and the $\alpha$-keto acids pyruvate and $\beta$-hydroxy pyruvate are relatively poor ($K_I$ of 1–20 mM) inhibitors of the conversion of oxaloacetate to P-enolpyruvate. In contrast, $\beta$-sulfopyruvate, a stable and isoelectronic analogue of oxaloacetate, inhibits P-enolpyruvate carboxykinase in the direction of P-enolpyruvate formation and in the decarboxylation of oxaloacetate. The ratios of $K_I(\beta$-sulfopyruvate) to $K_I(\text{oxaloacetate})$ in these reactions are 9.3 and 3.3, respectively. The lower affinity of the enzyme for this inhibitor compared to oxalate may be a consequence of the introduction of the tetrahedrally bonded sulfur atom at the position normally occupied by an $sp^2$-hybridized carbon atom. A more conservative substitution at this position might result in a tighter binding inhibitor. Preliminary experiments with the corresponding nitro analogue $\beta$-nitro pyruvate have indicated that this compound binds more tightly to the enzyme than $\beta$-sulfopyruvate.3 Despite the dissimilarity in structure resulting from sulfur substitution, $\beta$-sulfopyruvate binds to the enzyme-Mn(II)-MgGTP complex with a dissociation constant of 19 $\mu$M and should be a particularly useful analogue of oxaloacetate for spectroscopic studies.

Comparison of the kinetic constants for oxaloacetate in the forward and reverse directions indicates that the observed $K_M$ for oxaloacetate decreases 20-fold in response to near saturating MgGTP (Table IV). Likewise, the $K_I$ for $\beta$-sulfopyruvate decreases by nearly an order of magnitude in response to MgGTP. The similar responses of oxaloacetate and $\beta$-sulfopyruvate to MgGTP suggests that they are binding to the same site, as also suggested by the competitive relationship between oxaloacetate and $\beta$-sulfopyruvate. Oxalate also competes for the P-enolpyruvate site in the presence of near saturating MgGDP. However, the affinity of oxalate for the enzyme-MgGTP complex is approximately 100-fold greater than for the enzyme-MgGDP complex. This result suggests that binding of MgGTP favors formation of the enolate of pyruvate which is mimicked by oxalate. The presence of MgGDP favors a polarized P-enolpyruvate phosphoryl structure which is susceptible to attack by the $\beta$-phosphoryl of MgGDP. These considerations could explain, in part, why oxalate binds more tightly to one complex than to the other (see Scheme I).

Summary—P-enolpyruvate carboxykinase and rabbit muscle pyruvate kinase are catalytically similar in terms of: (i) the nature of the physiological phosphoryl transfer; (ii) the oxaloacetate decarboxylase activities; (iii) the dual-divalent cation requirements; (iv) the inhibition by oxalate; and (v) the substrate activities of fluoride ion, hydroxylamine, and the $\alpha$-substituted carboxylic acids. These similarities suggest that the divalent cations required for P-enolpyruvate carboxykinase activity may play similar or identical roles as the metals associated with pyruvate kinase. In the inhibitory complex of pyruvate kinase with oxalate, MgATP, and Mn(II), oxalate is a bidentate ligand to the enzyme bound Mn(II), and ATP serves as a bridging ligand between the two divalent cations (43). Based on the similarities of these enzymes and the demonstration that P-enolpyruvate carboxykinase catalyzes the direct transfer of the phosphoryl group with inversion of configuration (45, 46), a structure analogous to that determined for pyruvate kinase can be proposed for P-enolpyruvate carboxykinase (Scheme I). Although the decarboxylation of oxaloacetate by P-enolpyruvate carboxykinase occurs in the absence of added metal ions, several lines of evidence suggest that this activity is divalent cation dependent. The inhibition of the oxaloacetate decarboxylase activity by EDTA and o-phenanthroline is readily reversed by the addition of divalent cations such as Mn$^{2+}$ and Zn$^{2+}$ (4). Makinen and Nowak have recently reported that contaminating levels of Zn$^{2+}$ bind tightly to the enzyme, inhibit

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Mn(II) binding, and reduce enzymatic activity in the physiological reaction (47). This tightly bound Zn\(^{2+}\) is released upon treatment of the protein with thiols such as \(\beta\)-mercaptoethanol or diethiothreitol. The effect of Zn\(^{2+}\) on the oxaloacetate decarboxylase activity was not reported, and this aspect is currently under investigation in this laboratory. Thus in Scheme I, enzymatic groups as well as the enzyme bound divalent cation play important roles in the initial steps of the reaction and an additional role of the metal ions is to provide the template for the phosphoryl transfer to the nucleophile of the carboxylated substrate. Although Scheme I illustrates a stepwise mechanism, a concerted mechanism is also consistent with all of the experimental evidence.

Note Added in Proof—Guidinger and Nowak (Guidinger, P. F., and Nowak, T. (1990) Arch. Biochem. Biophys., in press) have reported that glycolate, L-lactate, and L-glycerate are phosphorylated by the chicken liver enzyme. Nowak, T. (1990) Arch. Biochem. Biophys., in press) have reported that glycolate, L-lactate, and L-glycerate are phosphorylated by chicken liver P-enolpyruvate carboxykinase in the presence of 15 mM MgCl\(_2\), and 0.1 mM MnCl\(_2\) at pH 7.4. We have shown that under these experimental conditions of pH and excess nucleotide:metal the accumulation of phosphoglycolate can be detected only after long (24 h) incubations. Under the conditions reported in this paper, however, \(^1^H\) NMR analysis clearly indicates that glycolate, L-lactate, and L-glycerate are phosphorylated by the chicken liver enzyme.

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