Inactivation of Chicken Mitochondrial Phosphoenolpyruvate Carboxykinase by o-Phthalaldehyde*

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Chicken liver mitochondrial phosphoenolpyruvate carboxykinase is inactivated by o-phthalaldehyde. The inactivation followed pseudo first-order kinetics, and the second-order rate constant for the inactivation process was 29 m⁻¹ s⁻¹ at pH 7.5 and 25 °C. The modified enzyme showed maximal fluorescence at 427 nm upon excitation at 337 nm, consistent with the formation of isoinodole derivatives by the cross-linking of proximal cysteine and lysine residues. Activities in the physiologic reaction and in the oxaloacetate decarboxylase reaction were lost in parallel upon modification with o-phthalaldehyde. Plots of (percent of residual activity) versus (mol of isoinodole incorporated/mol of enzyme) were biphasic, with the initial loss of enzymatic activity corresponding to the incorporation of one isoinodole derivative/enzyme molecule. Complete inactivation of the enzyme was accompanied by the incorporation of 3 mol of isoinodole/mol of enzyme. β-Sulffopyruvate, an iso-electronic analogue of oxaloacetate, completely protected the enzyme from reacting with o-phthalaldehyde. Other substrates provided protection from inactivation, in decreasing order of protection: oxaloacetate > phosphoenolpyruvate > MgGDP, MgGTP > oxalate. Cysteine 31 and lysine 39 have been identified as the rapidly reacting pair in isoinodole formation and enzymatic inactivation. Lysine 56 and cysteine 60 are also involved in isoinodole formation in the completely inactivated enzyme. These reactive cysteine residues do not correspond to the reactive cysteine residue identified in previous iodoacetate labeling studies with the chicken mitochondrial enzyme (Makinen, A. L., and Nowak, T. [1989] J. Biol. Chem. 264, 12148–12157). Protection experiments suggest that the sites of o-phthalaldehyde modification become inaccessible when the oxaloacetate/phosphoenolpyruvate binding site is saturated, and sequence analyses indicate that cysteine 31 is located in the putative phosphoenolpyruvate binding site.

Phosphoenolpyruvate carboxykinase (P-enolpyruvate carboxykinase, EC 4.1.1.32) catalyzes the reversible decarboxylation of oxaloacetate to form P-enolpyruvate in the first committed step of gluconeogenesis.

\[
\text{Mg}^{2+} + \text{oxaloacetate} + \text{MgGTP} \rightarrow \underset{\text{P-enolpyruvate} + \text{MgGDP} + \text{CO}_2}{\text{M}}
\]  

The enzyme also catalyzes the nucleoside diphosphate-dependent decarboxylation of oxaloacetate (1).

\[
\text{MgGDP} + \text{oxaloacetate} \rightarrow \text{pyruvate} + \text{CO}_2
\]  

Two structurally distinct isozymes of P-enolpyruvate carboxykinase exist, the cytosolic and mitochondrial isozymes. Both are immunodistinct (2–4) and encoded by separate mRNA species (5). Primary sequences of P-enolpyruvate carboxykinase have been derived from cDNA clones of the rat cytosolic (6), chicken cytosolic (7), chicken mitochondrial (8), and Drosophila melanogaster enzymes (9). Comparison of sequence data indicates an 83% sequence homology between the cytosolic forms of the enzyme from chicken and rat but only a 62–64% sequence homology between the mitochondrial enzyme and the cytosolic enzymes from rat or chicken (8).

Despite the sequence differences between the mitochondrial and cytosolic isozymes of P-enolpyruvate carboxykinase, the enzymes are similar in their catalytic properties. Both require two metal ions for activity, one associated with the nucleotide substrate, and a second equivalent that binds to a specific enzymic site as an activator (10–12). Both enzymes catalyze phosphoryl transfer with inversion of configuration, consistent with a single-step nucleophilic mechanism (13, 14). In addition to the reactions shown in Equations 1 and 2, the enzymes from chicken mitochondria and rat cytosol catalyze phosphoryl transfer from GTP to α-substituted carboxylic acids such as glycolate (15).

Rat cytosolic and chicken mitochondrial P-enolpyruvate carboxykinases require thiol compounds such as β-mercaptoethanol or dithiothreitol for maximal activity, and both enzymes are sensitive to inhibition by thiol-modifying reagents. Alignment of the sequences for the catalytic form of the enzyme from rat (6) and chicken liver (7) indicates that all 13 cysteine residues are conserved whereas a similar comparison of sequences for the cytosolic and mitochondrial forms of the chicken enzyme shows the conservation of 12 of 13 cysteine residues (8). Chemical modification studies of the rat cytosolic and chicken mitochondrial enzymes are consistent with the presence of at least 1 reactive cysteine residue in the vicinity of the nucleotide binding site. Cysteine 288 of the rat cytosolic enzyme is specifically modified by hydrophobic mal- eimides (16) whereas 8-azidoguanosine 5′-triphosphate (17) is thought to inactivate this enzyme by promoting the formation of an intramolecular disulfide between the vicinal thiols detected previously during modifications with 5,5'-

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‡The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, N-morpholinoethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; HPLC, high performance liquid chromatography.
that contained 40 mM cysteine and 10 mM 2-mercaptoethanol. The transferring an aliquot of the mixture to an equal volume of buffer by these thiol-directed reagents is afforded by the nucleotide enzyme was then assayed in the direction of P-enolpyruvate formation. The reaction was terminated at the indicated times by addition of stop solution containing 40 mM cysteine and 10 mM 2-mercaptoethanol. The enzyme was then assayed in the direction of P-enolpyruvate formation.

**Materials—**Chicken liver mitochondrial P-enolpyruvate carboxykinase was purified as described previously (15). The specific activity of the preparation was routinely between 18 and 20 units/mg when assayed at 30 °C in the direction of P-enolpyruvate formation. P-enolpyruvate carboxykinase was obtained from Sigma. β-Sulfopyruvate was prepared by the method of Griffith and Weinstein (23) and quantitated by the spectrophotometric assay with malate dehydrogenase. All other reagents were of the highest purity commercially available.

**Enzyme Assays—**Enzyme activity in the direction of P-enolpyruvate formation was determined at 30 °C with a Cary 210 spectrophotometer using malate dehydrogenase as a coupling enzyme. The reaction mixtures contained 50 mM Hepes/KOH (pH 8.0), 10 mM MgCl₂, 0.1 mM MnCl₂, 0.1 mM GTP, 4 mM malate, and 1 mM NAD. Malate dehydrogenase (6 units/ml) was added to establish the malate oxaloacetate equilibrium rapidly. The oxaloacetate concentration was assumed to equal the NADH formed at approximately 20 s after malate dehydrogenase addition. The nonenzymatic decarboxylation of oxaloacetate caused a background rate of NADH formation which was subtracted from the rate obtained after the addition of P-enolpyruvate carboxykinase. The protein concentration was determined by the absorbance at 280 nm with ε₁₀₀ = 16.5 (24) or by the Pierce protein assay with bovine serum albumin as standard using a molecular weight of approximately 67,200 for the chicken mitochondrial enzyme (8).

Assays in the direction of oxaloacetate formation were carried out at room temperature using malate dehydrogenase as a coupling enzyme. The reaction mixtures contained 50 mM Hepes/KOH (pH 7.5), 1 mM GDP, 1 mM oxaloacetate, 0.16 mM NADH, and 20 units of lactate dehydrogenase. The reactions were initiated by the addition of P-enolpyruvate carboxykinase, and the observed rates were corrected for the background, nonenzymatic decarboxylation of oxaloacetate as described above.

**Modification of P-enolpyruvate Carboxykinase by o-Phthalaldehyde—**Solutions of o-phthalaldehyde were prepared fresh daily by dissolving o-phthalaldehyde in methanol and diluting to the desired concentration with distilled water. The modification was carried out at room temperature by incubating chicken liver mitochondrial P-enolpyruvate carboxykinase at approximately 1.0 mg/ml with o-phthalaldehyde in buffer containing 50 mM Hepes/KOH (pH 7.5), 100 mM KCl, and 1 mM dithiothreitol. The final concentration of methanol in the incubation mixture was maximally 1% (v/v) and was found to have no effect on enzyme activity. At the indicated time intervals, an aliquot of this solution was added to an equal volume of stop solution containing 40 mM cysteine and 10 mM β-mercaptoethanol in 50 mM Hepes/KOH, 100 mM KCl at pH 7.5. An aliquot of this enzyme solution was then assayed for P-enolpyruvate or oxaloacetate formation and the decarboxylation of oxaloacetate as described above. The remaining solution was dialyzed extensively against 50 mM Hepes/KOH (pH 7.5), 100 mM KCl, and 1 mM dithiothreitol to remove the isocindole derivative formed from o-phthalaldehyde and the cysteine in the stop solution. The isocindole content of the inactivated enzyme was determined from the absorption at 337 nm using an extinction coefficient of 7.66 mM⁻¹ cm⁻¹ (25). Protection experiments were performed in a similar manner except that the enzyme was preincubated with substrate(s) or substrate analogue(s) for 5 min before the modification was initiated by the addition of o-phthalaldehyde. The levels of oxaloacetate were measured with malate dehydrogenase at the conclusion of protection experiments with this substrate to determine if decarboxylation was complete.

**Experimental Procedures**

**Chemical Modifications of Phosphoenolpyruvate Carboxykinase**

**A** and B, replots of the pseudo first-order rate constants versus the concentration of o-phthalaldehyde.

**FIG. 1. Inactivation of chicken liver mitochondrial P-enolpyruvate carboxykinase by o-phthalaldehyde.** The enzyme was incubated with the indicated concentrations of o-phthalaldehyde (o-PA) in 50 mM Hepes/KOH (pH 7.5), 100 mM KCl, and 1 mM dithiothreitol. The reaction was terminated at the indicated times by transferring an aliquot of the mixture to an equal volume of buffer that contained 40 mM cysteine and 10 mM β-mercaptoethanol. The enzyme was then assayed in the direction of P-enolpyruvate formation. A, data are plotted as the natural logarithm of percent of activity remaining versus time. B, replot of the pseudo first-order rate constant versus the concentration of o-phthalaldehyde.
**Chemical Modifications of Phosphoenolpyruvate Carboxykinase**

**FIG. 2.** Fluorescence spectra of o-phthalaldehyde-modified P-enolpyruvate carboxykinase. The enzyme (0.25 mg/ml) was treated with 0.029 mM o-phthalaldehyde until 30% of the original activity remained. The sample was then dialyzed versus 50 mM Hepes/KOH (pH 7.5), 0.1 mM dithiothreitol, 1 mM EDTA, and 100 mM KCl. A, excitation spectrum with emission monitored at 427 nm. B, emission spectrum with excitation at 337 nm.

**FIG. 3.** Stoichiometry of o-phthalaldehyde inactivation. The inactivation was carried out at 25 °C in a 2.9-ml solution containing 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 1 mM dithiothreitol, and 3 mg/ml P-enolpyruvate carboxykinase. The reaction was initiated by the addition of 0.5 mM o-phthalaldehyde. At various times, 350-µl aliquots of the mixture were withdrawn and mixed with stop solution to give final concentrations of 50 mM Hepes/KOH (pH 7.5), 100 mM KCl, 20 mM cysteine, and 5 mM β-mercaptoethanol. After extensive dialysis versus 50 mM Hepes/KOH (pH 7.5), 100 mM KCl, and 1 mM dithiothreitol, residual enzyme activity was determined in the direction of P-enolpyruvate formation (O), oxaloacetate formation (V), or in the oxaloacetate decarboxylase reaction (W). Isoindole formation was determined from the absorbance at 337 nm as described under “Experimental Procedures.”

**Table 1**
Pseudo first-order rate constants for the inactivation of P-enolpyruvate carboxykinase in the presence of substrates and substrate analogues

<table>
<thead>
<tr>
<th>Added component</th>
<th>$k_{obs}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.79</td>
</tr>
<tr>
<td>10 mM MgCl₂ + 1 mM GTP</td>
<td>0.37</td>
</tr>
<tr>
<td>10 mM MgCl₂ + 1 mM GDP</td>
<td>0.34</td>
</tr>
<tr>
<td>2 mM oxalate</td>
<td>0.45</td>
</tr>
<tr>
<td>2 mM P-enolpyruvate</td>
<td>0.21</td>
</tr>
<tr>
<td>10 mM oxaloacetate*</td>
<td>0.06</td>
</tr>
<tr>
<td>2 mM β-sulfopyruvate</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*The concentration of oxaloacetate present at the end of the incubation was 9 mM as determined using malate dehydrogenase.*

**Fluorescence Measurements**—Fluorescence spectra were recorded at room temperature with a Perkin-Elmer 650-105 fluorescence spectrometer using 5 nm excitation and emission slit widths. P-enolpyruvate carboxykinase was modified with o-phthalaldehyde and dialyzed as described above. Fluorescence emission spectra were recorded with an excitation wavelength of 337 nm. The molar transition energy was calculated using Equation 3 (26), where $\lambda_{max}$ is the wavelength of the maximum fluorescence

$$E_V = 2.985\lambda_{max} - 1087.28$$

emission for the isoindole derivative.

**pH Dependence of o-Phthalaldehyde Inactivation**—The inactivation of P-enolpyruvate carboxykinase by o-phthalaldehyde was quantitated over the pH range 6.0–9.2 in a buffer system containing 30 mM each of Mes, Hepes, and Bicine. Because of the marked pH dependence of the inactivation process, a single concentration of o-phthalaldehyde could not be used over the entire pH range. Rather, the concentration of o-phthalaldehyde was varied over the range 17–100 µM to obtain readily measurable rates of inactivation. At various times, aliquots of the reaction mixtures were removed and diluted 4-fold into the stop solution to give a final solution composition of 80 mM Hepes/KOH (pH 7.2), 80 mM KCl, 19 mM L-cysteine, and 4.8 mM β-mercaptoethanol. The activity of the enzyme was then determined in the direction of P-enolpyruvate formation with the standard assay. Control samples which were prepared in an identical fashion in the absence of o-phthalaldehyde showed no significant loss of activity after incubations at pH values of 6.0–9.2. The pseudo first-order rate constants for the inactivation were calculated at each pH value and o-phthalaldehyde concentration from plots of In (percent residual activity) versus time and then divided by the o-phthalaldehyde concentration to give the apparent second-order rate constant.

The pH dependence of the apparent second-order rate constants was analyzed according to Equation 4, where $k_{calc}$ represents the calculated second-order rate constant

$$
\frac{1}{k_{calc}} = \frac{1}{k_{max}} + \frac{[H^+]}{K}
$$

at the indicated hydrogen ion concentration, [H⁺], $k_{max}$ represents the
maximal second-order rate constant, and $K$ is the proton dissociation constant for the ionizing residue.

**Preparation and Purification of Tryptic Peptides—**Chicken liver P-enolpyruvate carboxykinase (5 mg/ml, 69 μM) was treated with 1.38 mM o-phthalaldehyde at room temperature until the residual activity was < 1% of control samples. The o-phthalaldehyde-modified enzyme was denatured with 7 M guanidine HCl at 37 °C for 30 min and dialyzed against 100 mM NH₄HCO₃ at pH 7.6. The sample was then subjected to proteolysis with 1:10-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (5:1, substrate:trypsin (w/w)) at 37 °C for 2 h. After a 5-fold dilution with distilled water, the peptides from the tryptic digests were isolated by reverse-phase HPLC on a Vydac C-18 column (0.45 × 25 cm, 5-μm particulate size, 300 Å pore size) using a Hitachi D-2000 liquid chromatograph equipped with an L-6200 pump and a Linear UVIS-204 dual wavelength detector. Separation was first carried out in a programmed gradient of buffer A (10 mM sodium phosphate with 0.1% trifluoroacetic acid at pH 6.5) and buffer B (75% acetonitrile in buffer A): 7% buffer B in the first 3 min, 7-64% buffer B from 3 to 140 min, 64-100% buffer B from 140 to 150 min with a flow rate of 1 ml/min. The elution of peptides and the isoindole derivatives was monitored by absorption measurements at 215 and 337 nm, respectively. The isoindole-containing peptides were collected and dried with a Savant SpeedVac concentrator. The dried samples were stored at ~70 °C between chromatography steps. Each isoindole-containing peptide was rechromatographed on the Vydac C-18 column in a programmed gradient of buffer A (10 mM ammonium acetate at pH 6.5) and buffer B (methanol): 0-95% buffer B from 0 to 60 min with a flow rate 1 ml/min. The purified, isoindole-containing peptides were dried as described above and stored at ~70 °C for sequence analysis.

**Amino Acid Sequence Determination—**Automated sequencing of the purified peptides was carried out on an Applied Biosystems 477A protein sequenator under the supervision of Dr. J. E. Labdon in the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine.

![Graph A](image1.png)

**Fig. 4.** pH dependence of o-phthalaldehyde inactivation. A, the apparent second-order rate constants were determined as described under "Experimental Procedures." The solid line represents the fit of the data to Equation 4 with $k_{\text{inact}}$ of 158 M⁻¹s⁻¹ and a $K$ of 2.15 × 10⁻⁸ M. B, replot of data from A as $1/k_{\text{inact}}$ versus $[H^+]$.

![Graph B](image2.png)

**Fig. 5.** Reverse-phase HPLC of the tryptic peptides generated from o-phthalaldehyde-inactivated P-enolpyruvate carboxykinase. A, the peptides were eluted with the gradient of acetonitrile in 10 mM sodium phosphate (pH 6.5) as described under "Experimental Procedures." The absorbance at 215 nm was used to monitor the peptide elution. B, elution profile shown in A monitored at 337 nm.

### Table II

<table>
<thead>
<tr>
<th>Peptide A</th>
<th>Peptide B</th>
</tr>
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<tbody>
<tr>
<td>Cycle</td>
<td>Residue</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Glu (Glu)</td>
</tr>
<tr>
<td>2</td>
<td>Val (Val)</td>
</tr>
<tr>
<td>3</td>
<td>Leu (Leu)</td>
</tr>
<tr>
<td>4</td>
<td>Leu (Leu)</td>
</tr>
<tr>
<td>5</td>
<td>Cys (Cys)</td>
</tr>
<tr>
<td>6</td>
<td>Asp (Asp)</td>
</tr>
<tr>
<td>7</td>
<td>Gly (Gly)</td>
</tr>
<tr>
<td>8</td>
<td>Ser (Ser)</td>
</tr>
<tr>
<td>9</td>
<td>Glu (Glu)</td>
</tr>
<tr>
<td>10</td>
<td>Glu (Glu)</td>
</tr>
<tr>
<td>11</td>
<td>Glu (Glu)</td>
</tr>
<tr>
<td>12</td>
<td>Gly (Gly)</td>
</tr>
<tr>
<td>13</td>
<td>Lys (Lys)</td>
</tr>
<tr>
<td>14</td>
<td>Glu (Glu)</td>
</tr>
<tr>
<td>15</td>
<td>Leu (Leu)</td>
</tr>
<tr>
<td>16</td>
<td>Leu (Leu)</td>
</tr>
<tr>
<td>17</td>
<td>Arg (Arg)</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>
RESULTS

Inactivation of P-enolpyruvate Carboxykinase by o-Phthalaldehyde—When P-enolpyruvate carboxykinase was incubated at pH 7.5 with o-phthalaldehyde at concentrations of 0.029–0.23 mM, a time-dependent decrease in catalytic activity, measured in the direction of P-enolpyruvate formation, was observed as shown in Fig. 1A. The pseudo first-order rate constants, \( k_{\text{obs}} \), obtained at each o-phthalaldehyde concentration, are replotted as a function of o-phthalaldehyde concentration in Fig. 1B. The second-order rate constant for the inactivation of the enzyme by o-phthalaldehyde was \( 29 \text{ M}^{-1} \text{s}^{-1} \) as determined from the slope of this plot. A double-reciprocal plot of these data indicates that o-phthalaldehyde binds weakly, if at all, to the enzyme before reaction.

Characterization of the o-Phthalaldehyde-inactivated Enzyme—The UV-visible spectrum for o-phthalaldehyde-modified P-enolpyruvate carboxykinase is characterized by an absorption at 337 nm which is not present in the spectrum of the unmodified enzyme. The protein-bound chromophore has a fluorescence excitation maximum of 337 nm (Fig. 2A) and an emission maximum at 427 nm (Fig. 2B), consistent with the formation of an isoidole derivative (27).

The correlation between isoidole formation and enzymatic activity is shown in Fig. 3. All three of the reactions catalyzed by the enzyme are inactivated in parallel. During the first 60% of the inactivation process there is a linear relationship between isoidole formation and the loss of enzymatic activity which extrapolates to a stoichiometry of 1 mol of isoidole/mol of enzyme. Beyond 60% inactivation, however, additional isoidole formation occurs. Extrapolation of this portion of the curve to 100% inactivation indicates that enzyme completely inactivated by o-phthalaldehyde has a stoichiometry of three isoidole derivatives/enzyme molecule.

Native and completely modified P-enolpyruvate carboxykinase showed identical mobilities when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No higher molecular weight species appeared from cross-linking of enzyme monomers. Thus, the modification of the enzyme by o-phthalaldehyde results in the formation of cross-links between cysteine and lysine residues within the same polypeptide chain.

Effects of Substrates and Substrate Analogues on Inactivation by o-Phthalaldehyde—The inactivation studies were carried out in the presence of substrate(s) or substrate analogue(s) to define the sites of o-phthalaldehyde modification. The corresponding pseudo first-order rate constants for the inactivation in these protection experiments are summarized in Table I. The enzyme was completely protected from inactivation in the presence of \( \beta \)-sulfoenolate and partially protected in the presence of oxaloacetate, P-enolpyruvate, MgGTP, MgGDP, and oxalate. The protection by oxaloacetate was essentially complete at a concentration of 10 mM whereas at a concentration of 4 mM oxaloacetate provided only moderate protection (\( k_{\text{obs}} = 0.36 \text{ min}^{-1} \)). The addition of 0.1 mM MnCl\(_2\) had no effect on the level of protection provided by 4 mM oxaloacetate. P-enolpyruvate provided better protection than did MgGDP or MgGTP. Oxalate, a structural analogue of the enolate of pyruvate, provided little protection against inactivation by o-phthalaldehyde. Neither Mn(II) or bicarbonate provided any protection (data not shown).

Incubation of P-enolpyruvate carboxykinase with o-phthalaldehyde in the presence and absence of \( \beta \)-sulfoenolate demonstrated that formation of the isoidole derivative is prevented by this inhibitor. The activity of the enzyme samples and the extent of isoidole formation were determined

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**Table III**

Comparison of amino acid sequence of chicken liver mitochondrial P-enolpyruvate carboxykinase (PEPCK-M (8)) around Cys-31, Lys-39, Lys-66, and Cys-60 with the sequence of the chicken cytosolic PEPCK (PEPCK-C (7)), rat cytosolic PEPCK (PEPCK-C (6)), and Drosophila (9) enzymes. The arrows indicate the residues modified by o-phthalaldehyde.

---

**Table IV**

Sequence of putative P-enolpyruvate binding sites

The consensus sequence of the P-enolpyruvate binding site was proposed previously by Cook et al. (7) by comparing the homologous sequences of the P-enolpyruvate-utilizing enzymes, 3-deoxysulbin-7-heptulosonate 7-phosphate (DAHP) synthase (36), 3-enolpyruvylshikimate-3-phosphate (EPSP) synthase (37), rat cytosolic P-enolpyruvate carboxykinase (PEPCK) (6), chicken cytosolic PEPCK (7), and chicken mitochondrial PEPCK (8). The sequence is presented in single-letter amino acid nomenclature.

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**Fig. 6.** Comparison of the amino acid sequence of chicken liver mitochondrial P-enolpyruvate carboxykinase (PEPCK-M (8)) around Cys-31, Lys-39, Lys-66, and Cys-60 with the sequences of the chicken cytosolic (PEPCK-C (7)), rat cytosolic (PEPCK-C (6)), and Drosophila (9) enzymes. The arrows indicate the residues modified by o-phthalaldehyde.

---

**Table III**

Comparison of isoidole incorporation into native and iodoacetate-treated P-enolpyruvate carboxykinase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme + o-phthalaldehyde</td>
<td>0.76</td>
</tr>
<tr>
<td>Thio-carboxymethylated enzyme</td>
<td>0.79</td>
</tr>
</tbody>
</table>

---

**Table IV**

Sequence of putative P-enolpyruvate binding sites

<table>
<thead>
<tr>
<th>Residues</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DAHP synthase</td>
<td>228-255</td>
</tr>
<tr>
<td>E. coli EPSP synthase</td>
<td>85-112</td>
</tr>
<tr>
<td>Rat cytosolic PEPCK</td>
<td>24-51</td>
</tr>
<tr>
<td>Chicken cytosolic PEPCK</td>
<td>24-51</td>
</tr>
<tr>
<td>Chicken mitochondrial PEPCK</td>
<td>9-36</td>
</tr>
</tbody>
</table>

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after a 35-min incubation at 25 °C of enzyme with 0.3 mM o-
phthalaldehyde with and without 2 mM β-sulfopyruvate. The
enzyme sample incubated in the presence of β-sulfopyruvate
retained approximately 100% of the original enzymatic activity
and was modified to < 0.1 mol of isoindole/mol of enzyme.
The sample which had been incubated in the absence of β-
sulfopyruvate was completely inactivated (<1% residual ac-
tivity) and contained 2.9 mol of isoindole/mol of enzyme.

pH Dependence of o-Phthalaldehyde Inactivation—The
inactivation of P-enolpyruvate carboxykinase by o-phthalalde-
hyde was examined over the pH range 6.0–9.2. Measurements
of residual enzymatic activity were restricted to that portion
of the inactivation process corresponding to 0–60% inactiva-
tion. The apparent second-order rate constants for the inac-
tivation are plotted as a function of pH in Fig. 4A. A replot of
these data according to Equation 4 (Fig. 4B) yields a pK of
7.7 for the ionizing group and a kmax value of 185 M−1 s−1.

Tryptic Peptide Mapping of Modified P-enolpyruvate Car-
boxykinase—Tryptic digests were prepared from enzyme modi-

**DISCUSSION**

Chicken mitochondrial P-enolpyruvate carboxykinase is
inactivated irreversibly by o-phthalaldehyde in a pseudo first-
order process. Replots of the kinetic data yield a second-order
rate constant of 29 M−1 s−1 for the inactivation process at 25 °C and pH 7.5. The kinetic analysis provides no evidence for the reversible formation of a binary enzyme-(o-phthalal-
dehyde) complex prior to inactivation. The spectroscopic
properties of the inactivated enzyme are consistent with the
formation of isoindole derivatives via the cross-linking of
proximal cysteine and lysine residues. There is a direct cor-
relation between isoindole formation and the loss of enzyme-
activity in either the physiologic reaction (Equation 1) or in
the decarboxylation of oxaloacetate (Equation 2). In the
early stages of inactivation there is a linear relationship
between the percent of residual activity and isoindole forma-
tion, which extrapolates to a stoichiometry of 1 mol of isoind-
ole/mol of enzyme. Analysis of tryptic digests from partially
inactivated enzyme has implicated cysteine 31 and lysine 39
as the rapidly reacting residues. Complete inactivation of the
enzyme by o-phthalaldehyde is accompanied by the incorpo-
ration of 3 mol of isoindole/mol of enzyme.

There is considerable evidence to suggest that at least one of
the cysteine-lysine pairs modified by o-phthalaldehyde is
near the P-enolpyruvate/oxaloacetate binding site. Thus,
complete protection from inactivation and isoindole forma-
tion was afforded by β-sulfopyruvate, an isoelectronic ana-
logue of oxaloacetate (15), and greater than 90% protection
was observed in the presence of 10 mM oxaloacetate. Signifi-
cantly less protection was provided by P-enolpyruvate or
oxalate, a reaction intermediate analogue of the enolate of
pyruvate (15). These results and the lack of protection by
HCO3− would suggest that occupation of the oxaloacetate
binding site results in a protein conformation change that
shields all three cysteine-lysine pairs from reaction with o-
phthalaldehyde.

The pH dependence of the inactivation process indicates that
a group with a pK of 7.7 must be deprotonated to permit
reaction with o-phthalaldehyde. This pK is most likely due to
the rapidly reacting cysteine 31-lysine 39 pair identified in
Fig. 3. Although the group responsible for this ionization
cannot be unambiguously assigned, it is likely the lysine
residue of this pair, which must exist in a deprotonated state
according to the proposed mechanisms of isoindole formation
(28, 29).

o-Phthalaldehyde has been employed in chemical modifi-
cation studies aldolase (26), CAMP- and cGMP-dependent
protein kinases (30, 31), hexokinase (32), 6-phosphofructo-2-
kinase/fructose-2,6-bisphosphatase (33), and 5-enolpyruvyl-
shikimate-3-phosphate synthase (34). Although the isoindole
derivatives of proteins are relatively stable (35), the deriva-
tives of the resulting peptides are subject to reactions that

Different Cysteines Are Modified by o-Phthalaldehyde and
Iodoacetate—Previous chemical modification studies of

Chicken mitochondrial P-enolpyruvate carboxykinase have
shown that only 1 of the 13 cysteine residues is highly reactive
to iodoacetate (19). The formation of isoindole derivatives
was compared with both the native enzyme and o-phthalaldehyde-
inactivated enzyme. No difference in the extent of isoindole
formation was observed (Table III). In a second experiment
the incorporation of 14C label from 14C iodoacetate was com-
pared using both the native enzyme and o-phthalaldehyde-
inactivated enzyme. The extent of 14C incorporation into both
enzyme samples was identical (data not shown). These results
indicate that the cysteine residue identified by iodoacetate
labeling does not participate in isoindole formation.

**DISCUSSION**

Chicken mitochondrial P-enolpyruvate carboxykinase is
inactivated irreversibly by o-phthalaldehyde in a pseudo first-
order process. Replots of the kinetic data yield a second-order
rate constant of 29 M−1 s−1 for the inactivation process at 25 °C and pH 7.5. The kinetic analysis provides no evidence for the reversible formation of a binary enzyme-(o-phthalal-
dehyde) complex prior to inactivation. The spectroscopic
properties of the inactivated enzyme are consistent with the
formation of isoindole derivatives via the cross-linking of
proximal cysteine and lysine residues. There is a direct cor-
relation between isoindole formation and the loss of enzyme-
activity in either the physiologic reaction (Equation 1) or in
the decarboxylation of oxaloacetate (Equation 2). In the
early stages of inactivation there is a linear relationship
between the percent of residual activity and isoindole forma-
tion, which extrapolates to a stoichiometry of 1 mol of isoind-
ole/mol of enzyme. Analysis of tryptic digests from partially
inactivated enzyme has implicated cysteine 31 and lysine 39
as the rapidly reacting residues. Complete inactivation of the
enzyme by o-phthalaldehyde is accompanied by the incorpo-
ration of 3 mol of isoindole/mol of enzyme.

There is considerable evidence to suggest that at least one of
the cysteine-lysine pairs modified by o-phthalaldehyde is
near the P-enolpyruvate/oxaloacetate binding site. Thus,
complete protection from inactivation and isoindole forma-
tion was afforded by β-sulfopyruvate, an isoelectronic ana-
logue of oxaloacetate (15), and greater than 90% protection
was observed in the presence of 10 mM oxaloacetate. Signifi-
cantly less protection was provided by P-enolpyruvate or
oxalate, a reaction intermediate analogue of the enolate of
pyruvate (15). These results and the lack of protection by
HCO3− would suggest that occupation of the oxaloacetate
binding site results in a protein conformation change that
shields all three cysteine-lysine pairs from reaction with o-
phthalaldehyde.

The pH dependence of the inactivation process indicates that
a group with a pK of 7.7 must be deprotonated to permit
reaction with o-phthalaldehyde. This pK is most likely due to
the rapidly reacting cysteine 31-lysine 39 pair identified in
Fig. 3. Although the group responsible for this ionization
cannot be unambiguously assigned, it is likely the lysine
residue of this pair, which must exist in a deprotonated state
according to the proposed mechanisms of isoindole formation
(28, 29).

o-Phthalaldehyde has been employed in chemical modifi-
cation studies aldolase (26), CAMP- and cGMP-dependent
protein kinases (30, 31), hexokinase (32), 6-phosphofructo-2-
kinase/fructose-2,6-bisphosphatase (33), and 5-enolpyruvyl-
shikimate-3-phosphate synthase (34). Although the isoindole
derivatives of proteins are relatively stable (35), the deriva-
tives of the resulting peptides are subject to reactions that
result in the loss of absorbance at 337 nm and fluorescence (reviewed in 28). Thus, isolated tryptic peptides from the o-phthalaldehyde modification of 5-enolpyruvoylshikimate-3-phosphate synthase showed no absorbance at 337 nm (34), and it was necessary to detect modified peptides by absorbance at 280 nm. Sequence analysis of these peptides identified 2 modified lysine residues, but the cysteine residues involved in cross-link formation could not be identified because of the decomposition of the isoindole derivatives (34).

The isoindole derivatives of P-enolpyruvate carboxykinase are stable to prolonged dialysis. The fluorescent properties of the isolated tryptic peptides, however, are lost rapidly in aqueous solution. Isolation and sequencing of fluorescent peptides were possible only when the time between protein denaturation and peptide isolation was minimized and when samples were stored dry at –70 °C. Even with these precautions, peptide C was not obtained in sufficient quantity for sequence analysis. Peptides A and B retained the fluorescent isoindole derivative and contained only 1 cysteine-lysine pair. Thus, assignment of the modified residues was straightforward. Peptides in which the cross-linked cysteine-lysine pairs reside on separate tryptic peptides would have given characteristic sequence patterns which were not observed.

Peptides A and B are adjacent peptides in the primary sequence of chicken mitochondrial P-enolpyruvate carboxykinase. Of the 17 residues in peptide A, 8 are expected to carry a net positive or negative charge at physiologic pH whereas 6 of the 21 residues of peptide B should carry a charge at this pH. The relative hydrophilic nature of these peptides is reflected by the molar transition energy of 187 kJ/mol for the o-phthalaldehyde-derivatized enzyme. This value is higher than those reported for most isoindole-modified proteins (116–136 kJ/mol (33)) and comparable to that for the isoindole derivative of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, indicating that the microenvironment of the site modified by o-phthalaldehyde in P-enolpyruvate carboxykinase is less hydrophobic than in most other enzymes.

Previous chemical modification studies of rat cytosolic (16) and chicken mitochondrial (19) P-enolpyruvate carboxykinases have shown that modification of a single sulfhydryl group results in the total loss of enzymatic activity in the physiologic reaction (Equation 1). For both enzymes, significant protection against inactivation was provided by nucleotide substrates whereas P-enolpyruvate and oxaloacetate provided little protection. Cysteine 288 of the rat cytosolic enzyme is modified by maleimide derivatives (16), and a comparison of sequences suggests that cysteine 273 of the chicken liver enzyme is modified by iodoacetate and 5,5'-dithiobis(2-nitrobenzoic acid)2 (8). Although the modification of a single sulfhydryl group by 5,5'-dithiobis(2-nitrobenzoic acid) results in complete inactivation of chicken P-enolpyruvate carboxykinase in the physiologic reaction, the oxaloacetate decarboxylase activity of the enzyme is inhibited by only 35% (19). In contrast, o-phthalaldehyde modifies cysteine-lysine pairs that appear to be at or near the oxaloacetate binding site. Peptides A and B contain cysteine 31 and cysteine 60, respectively, and prior modification of the enzyme with iodoacetate does not alter the stoichiometry of isoindole formation. Furthermore, modification of the enzyme with o-phthalaldehyde results in a parallel loss of activity in the physiologic reaction and in the decarboxylation of oxaloacetate.

Peptide A (residues 27–43), which contains the rapidly reacting cysteine-lysine pair, is partially contained within the putative P-enolpyruvate binding domain of P-enolpyruvate carboxykinase (7). The assignment of this binding domain is based on studies of deletion mutants of 5-enolpyruvoylshikimate-3-phosphate synthase from Escherichia coli and the comparison with sequences for 3-deoxynarabinono-7-heptulosonate 7-phosphate synthase and the cytosolic P-enolpyruvate carboxykinases from rat and chicken liver. These binding domains are summarized in Table IV and compared with the sequence for the corresponding region of chicken mitochondrial P-enolpyruvate carboxykinase (8). There are 5 conserved amino acid residues in this domain, each separated by several nonconserved amino acids. The conserved residues in chicken mitochondrial P-enolpyruvate carboxykinase correspond to Pro-9, Ala-19, Pro-25, Cys-31, and Glu-36, with Cys-31 being the rapidly reacting cysteine residue modified by o-phthalaldehyde. The P-enolpyruvate binding domain of cat muscle pyruvate kinase has been identified by x-ray diffraction (22). This domain is not homologous to the binding domain proposed by Cook et al. (7) for P-enolpyruvate carboxykinase but is highly conserved among pyruvate kinases from cat muscle, chicken muscle, rat liver, and yeast. A glutamate residue of pyruvate kinase interacts with the enzyme-bound metal ion, and a lysine residue serves as an acid-base catalyst in the interconversion of pyruvate and the enolate of pyruvate. Since chicken mitochondrial P-enolpyruvate carboxykinase does not catalyze the enolization of pyruvate (15), the lack of homology in the P-enolpyruvate binding domains for these two enzymes may reflect the different mechanistic requirements of the catalytic sites.

A comparison of amino acid residues 27–64 for chicken mitochondrial P-enolpyruvate carboxykinase with the homologous regions of the enzymes from chicken cytosol, rat cytosol, and Drosophila indicates that the cysteine-lysine pairs modified by o-phthalaldehyde (Fig. 6) are highly conserved. Only for the rat cytosolic enzyme does a substitution of glycine for lysine 54 occur. This finding would suggest that the stoichiometry of o-phthalaldehyde labeling with this enzyme should differ from that observed with the chicken mitochondrial enzyme. Comparison of the o-phthalaldehyde inactivation for these two enzymes should provide some insight into the role of these amino acids in catalysis.

In summary, o-phthalaldehyde inactivates chicken mitochondrial P-enolpyruvate carboxykinase by cross-linking proximal cysteine and lysine residues to form fluorescent isoindole derivatives. Initial loss of enzymatic activity correlates with isoindole formation between cysteine 31 and lysine 39; however, 3 mol of isoindole/mol of enzyme is found in the fully inactivated enzyme. Protection experiments as well as sequence analysis have localized at least one of the reactive cysteine-lysine pairs, cysteine 31-lysine 39, within putative the oxaloacetate/P-enolpyruvate binding site of the enzyme. None of the reactive cysteine-lysine pairs corresponds to the reactive cysteine residues identified previously for P-enolpyruvate carboxykinase.

REFERENCES


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2 This assignment has been confirmed recently by K. C. Cheng and T. Nowak, personal communication.