Correction. In the article "Comparative inhibition of mitochondrial and cytosolic phosphoenolpyruvate carboxykinases by stereospecific substrate analogues" by Thomas H. Duffy, Paul J. Markovitz, David T. Chuang, Merton F. Utter, and Thomas Nowak, which appeared in the November 1981 issue of Proc. Natl Acad. Sci. USA (78, 6680–6683), the authors request the following correction. The synthesis and purification of the E and the Z isomers of phosphoenolpyruvate used in our studies were performed previously according to the methods detailed by Adlersberg et al. [Adlersberg, M., Dayan, J., Bondinell, W. E. & Sprinson, D. B. (1977) Biochemistry 16, 4382–4387]. We gratefully acknowledge the advice and the sample of Z-P-eBut supplied by David Sprinson at the early stages of this work and regret the omission of this reference from the original manuscript.
Comparative inhibition of mitochondrial and cytosolic phosphoenolpyruvate carboxykinases by stereospecific substrate analogues

(thphosphoenolpyruvate analogues/enzyme inhibition/active sites/isozymes)

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ABSTRACT Phosphoenolpyruvate carboxykinase [GTP:oxaloacetate carboxyl-lyase (transphosphorylating), EC 4.1.1.32] was purified from the liver of several species; the mitochondrial enzyme and the cytosolic enzyme were separated from each other. The species included guinea pig, monkey, rat, and chicken. Each enzyme was assayed for inhibition by the substrate analogues E-2-phosphoenolbutyrate and Z-2-phosphoenolbutyrate. Each enzyme tested displayed the same stereospecificity: the E diasteroisomer was the more potent inhibitor than the Z. These results suggest an active site homology for all carboxykinases. The absolute values for $K_i$ measured show that in almost every case the mitochondrial enzyme is more susceptible to inhibition by these analogues than is the cytosolic enzyme. The $K_i$ values are one-fifth those for the mitochondrial enzymes. These results imply subtle differences in ligand interactions at the active sites of these enzymes.

The conversion of pyruvate into glucose—i.e., gluconeogenesis—occurs primarily in the cytosolic fraction of the cell. The initial step, the carboxylation of pyruvate to give oxalacetate, occurs exclusively in the mitochondria (1). The following step, the committed step in gluconeogenesis, is catalyzed by the enzyme phosphoenolpyruvate carboxykinase [PEPCKase; GTP:oxaloacetate carboxyl-lyase (transphosphorylating), EC 4.1.1.32]. This enzyme catalyzes the conversion of oxaloacetate to phosphoenolpyruvate at the expense of GTP or ITP. This reaction is readily reversible but under gluconeogenic conditions the reaction proceeds readily in the direction of phosphoenolpyruvate formation.

The compartmentalization of this reaction and its regulation have been of great interest. The localization of PEPCKase appears to be species dependent; thus, the mechanism of regulation also would be expected to be species dependent. The enzyme in rat liver appears to be located almost entirely in the cytosol (2) whereas the enzyme in chicken liver is entirely in the mitochondria (3). Other species of animals, including man, have a sizeable fraction of both cytosolic and mitochondrial enzymes (4). It would appear that both enzymes are of integral importance in the process of gluconeogenesis. The apparent regulation of the compartmentally different enzymes appears to vary, however. The cytosolic enzyme responds to various stimuli such as fasting, glucagon administration, and diabetes which increase its rate of synthesis (5, 6). The cytosolic enzyme reportedly also is activated by a cytosolic iron-carrying protein, ferroactivator, which stimulates activity of PEPCKase (7). Both the cytosolic enzyme from rat (8, 9) and the mitochondrial enzyme from chicken (unpublished data) are activated by divalent transition metal ions in the presence of Mg$^2+$ (as Mg-GTP). The regulation of cation flux may play an important role in regulation of both the enzymes.

The structural and functional similarities and differences between the two varieties of PEPCKase have not been adequately elucidated. Most of the animal species of PEPCKase yield similar kinetic values and have similar molecular weights (10). Antibody prepared against the rat cytosolic enzyme shows no crossreactivity with the mitochondrial enzyme (11), and the cytosolic and mitochondrial forms of human (12) and monkey (13) liver enzymes have been separated by ion exchange and polyacrylamide gel electrophoresis, respectively. These results also indicate both similarities and differences among the isozymes.

The results presented here provide physical evidence for differences between the two isozymes and kinetic evidence that, although homology exists among PEPCKases, there are subtle differences at the catalytic sites of these two isozymes.

MATERIALS AND METHODS

Malate dehydrogenase was purchased from Boehringer Mannheim. Phosphoenolpyruvate, IDP, and NADH were purchased commercially available.

Synthesis, purification, and characterization of the E and the Z isomers of phosphoenolbutyrate (P-eBut) will be reported in detail elsewhere. Briefly, 2-ketobutyric acid was treated with bromine to yield 3-bromo-2-ketobutyric acid. This product was then treated with diethyl phosphate to yield diethyl[1-hydroxy-1-carboxy-2-bromopropyl] phosphate which, upon treatment with NaOH, yielded Z-P-eBut. Exposure of a solution of Z-P-eBut to a UV source yielded a 45:55 mixture of the E and Z isomers. Treatment of the mixture with pyruvate kinase and hexokinase in the presence of ADP, glucose, and MgSO$_4$ preferentially utilized the Z isomer, leaving only E-P-eBut. The reaction was quenched with HCl, and $\alpha$-ketobutyrate was decomposed with H$_2$O$_2$. Each isomer was purified by elution on a Dowex 1-X8 column. Unwanted material was eluted at 0.06 M HCl, and the $P$-eBut isomers were eluted at 0.1 M HCl. The NMR spectra ($^2$H$_2$O) showed resonances at $\delta = 2.44$ and 6.75 for $Z$-P-eBut and at $\delta = 2.55$ and 6.35 for $E$-P-eBut.

The PEPCKase from rat liver cytosol was a gift from Richard W. Hanson. Purification of the PEPCKase from chicken liver mitochondria will be described elsewhere. In summary, the list of the paper presented here.

The abbreviations: PEPCKase, phosphoenolpyruvate carboxykinase; F-P-eBut, E-2-phosphoenolbutyrate; Z-P-eBut, Z-2-phosphoenolbutyrate.

† Deceased, Nov. 28, 1980. Dr. Utter died after this paper had reached its final form.

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ers were homogenized in a sucrose buffer and the mitochondrial fraction was isolated, washed, and lyophilized. Upon lysis, the mitochondrial solution was fractionated by ammonium sulfate precipitation followed by sequential chromatography on calcium phosphate gels and Sepharose-blue dextran. This procedure yielded protein of >98% purity as determined by disc polyacrylamide gel electrophoresis and NaDodSO4/polyacrylamide gel electrophoresis.

To purify the mitochondrial and the cytosolic enzymes from guinea pig liver and from rhesus monkey liver, the livers were homogenized in an isotonic sucrose buffer, with care taken to cause as little mitochondrial damage as possible. The cytosolic and mitochondrial fractions were separated by centrifugation (14), and both fractions were lyophilized. The two isozymes were purified to homogeneity by ammonium sulfate fractionations and sequential chromatography on DEAE-Bio-Gel (Bio-Rad), Sephadex G-100, and cellulose phosphate. Each isozyme from guinea pig and from monkey was purified from other proteins and from the other PEPCKase isozyme. Purity was determined by using disc polyacrylamide gel electrophoresis, NaDodSO4/polyacrylamide gel electrophoresis, and isoelectric focusing. The details will be reported elsewhere.

Initial velocity studies of PEPCKase were performed by measuring the rate of formation of oxalacetate from phosphoenolpyruvate with the continuous assay coupled to malate dehydrogenase. The assay mixture contained in 1.0 ml: Tris-HCl, pH 7.4, 65 μmol; KCl, 100 μmol; MnCl2, 4 μmol; phosphoenolpyruvate, 2 μmol; IDP, 2 μmol; 2-mercaptoethanol, 143 μmol; KHCO3, 200 μmol; NADH, 0.14 μmol; and 22 enzyme units of malate dehydrogenase. The reaction was initiated by the addition of 15–20 milliliters of PEPCKase (1 milliliter is the amount of PEPCKase needed to produce 1 nmol of oxalacetate per min at 25°C). The decrease in absorption of NADH at 340 nm was followed as a function of time in a Gilford 240 or 230 recording spectrophotometer.

RESULTS

The purification of PEPCKase from rat liver leads to pure cytosolic enzyme (11). The pure protein obtained from chicken liver contained only mitochondrial enzyme (3). The enzymes from monkey and from guinea pig were pure isozymes based on three criteria. The two enzymes of each species were separable on either DEAE-Bio-Gel or on cellulose phosphate; both steps are used during enzyme purification. Second, small differences in migration were observed on NaDodSO4 gels. Native gels of the monkey enzymes show clear separation and >98% purity of the enzymes (13). Third, immunotitration using antibodies against purified isozymes from the monkey demonstrate no crossreactivity (13). These same experiments were performed with antibodies against purified enzymes from the guinea pig. The antibody toward the mitochondrial enzyme showed no reactivity with the cytosolic enzyme and the antibody to the cytosolic enzyme showed no crossreactivity with the mitochondrial enzyme (unpublished data).

The Z isomer of P-eBut was >95% free of the E isomer and the E isomer was >94% free of the Z isomer as determined by 1H NMR at 100 MHz. These values are limited by the signal-to-noise ratio of the spectra. No contaminating isomer was observed.

The cytosolic and the mitochondrial PEPCKases from guinea pig both showed stereoselectivity inhibited by the two diastereoisomers of P-eBut (Figs. 1 and 2). Both isomers showed linear competitive inhibition with respect to the substrate phosphoenolpyruvate. For the cytosolic enzyme (Fig. 1), the E isomer was 20 times more potent than the Z isomer as an inhibitor: K1 for E-P-eBut, 1.9 ± 0.2 μM; K1 for Z-P-eBut, 39 ± 3 μM. For the mitochondrial enzyme (Fig. 2), K1 = 0.43 ± 0.07 μM with E-P-eBut and K1 = 46.4 ± 1.3 μM with Z-P-eBut. The K1 values for phosphoenolpyruvate for both enzymes were equal. The E isomer was a more potent inhibitor of the mitochondrial enzyme than the cytosolic enzyme. The K1 values for Z-P-eBut were approximately equal. The stereoselectivity of inhibition was more dramatic for the mitochondrial enzyme. These results are summarized in Table 1.

The isozymes from monkey were also tested for inhibition by these substrate analogues. Both showed linear competitive inhibition of the cytosolic and mitochondrial enzymes with phosphoenolpyruvate: cytosolic enzyme, K1 = 7.7 ± 0.5 μM with E-P-eBut and K1 = 161 ± 3 μM with Z-P-eBut; mitochondrial enzyme, K1 = 0.77 ± 0.06 μM with E-P-eBut and K1 = 24 ± 4 μM with Z-P-eBut. These results demonstrate the same stereoselectivity of inhibition, with the E isomer being the more potent inhibitor by a factor of 20. These results are summarized in Table 1.

These analogs also demonstrated stereoselective inhibition
of the enzyme from rat liver cytosol. The $K_i$ values (for $E$, 7.4 ± 0.3 μM; for $Z$, 157 ± 9 μM) were nearly identical to the values obtained for the cytosolic enzyme from monkey. The inhibitor constants for these diastereoisomers found with chicken liver mitochondrional enzyme agreed more closely with the values obtained for mitochondrial enzymes. The kinetic results are shown in Table 1 for comparison.

**DISCUSSION**

Ion exchange chromatography of PEPCKase from guinea pig and from monkey on DEAE-Bio-Gel and on cellulose phosphate separates the mitochondrial and the cytosolic isoforms. These two proteins can also be distinguished by discontinuous polyacrylamide gel electrophoresis and by NaDodSO₄ gel electrophoresis (13). These results indicate that, although similar in size and charge, the two isoforms are not identical proteins. The lack of crossreactivity among the isoforms from rat (11), monkey (13), and guinea pig with isozyme-specific antibody formed against pure enzyme shows that the compartmentalized enzymes are also immunologically distinct and have different antigenic determinant domains. Because the antigen–antibody complexes also appear to retain their catalytic activities for the chicken (3), rat (11), monkey (13), and guinea pig (data not shown) enzymes, the antigenic determinants are apparently not at the catalytic sites.

The interactions of several PEPCKases with a diastereoisomeric pair of substrate analogues was investigated by steady-state kinetics to determine if homology exists at the active sites of these enzymes. Each of the enzymes studied showed homology of stereoselectivity, with the $E$ isomer being the more potent inhibitor. The enzymes demonstrated a selectivity factor of 20 for the $K_i$ ratio of the $Z$ to $E$ isomers with the exception of the guinea pig mitochondrial enzyme. This enzyme showed even greater selectivity, with a $K_i$ ratio of 116. The stereoselectivity of PEPCKase for these analogues is also observed in the PEPCKase found in the parasitic flatworm Ascaris. This enzyme plays a different role in these parasites: it catalyzes the conversion of phosphoenolpyruvate to oxalacetate as the terminal step in glycolysis. There is no significant gluconeogenesis.

**Table 1. Summary of inhibitor constants of the P-eBut analogues for PEPCKase from various sources**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Analogue</th>
<th>$K_i$, μM</th>
<th>$K_m/K_i$</th>
<th>$K_d/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol, guinea pig</td>
<td>Z-P-eBut</td>
<td>39 ± 3</td>
<td>7.1</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>1.9 ± 0.2</td>
<td>145.8</td>
<td></td>
</tr>
<tr>
<td>Mitochondria, guinea pig</td>
<td>Z-P-eBut</td>
<td>46.4 ± 1.3</td>
<td>5.67</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>0.63 ± 0.07</td>
<td>687.5</td>
<td></td>
</tr>
<tr>
<td>Cytosol, monkey</td>
<td>Z-P-eBut</td>
<td>161 ± 3</td>
<td>1.8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>7.7 ± 0.5</td>
<td>41.4</td>
<td></td>
</tr>
<tr>
<td>Mitochondria, monkey</td>
<td>Z-P-eBut</td>
<td>24 ± 4</td>
<td>5.9</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>0.77 ± 0.06</td>
<td>186.8</td>
<td></td>
</tr>
<tr>
<td>Cytosol, rat</td>
<td>Z-P-eBut</td>
<td>157 ± 9</td>
<td>1.06</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>7.4 ± 0.3</td>
<td>22.57</td>
<td></td>
</tr>
<tr>
<td>Mitochondria, chicken*</td>
<td>Z-P-eBut</td>
<td>32 ± 1</td>
<td>5.2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E-eBut</td>
<td>1.5 ± 0.1</td>
<td>110.7</td>
<td></td>
</tr>
</tbody>
</table>

* Data from other work.
in Ascaris (15). These results suggest an evolutionary homology among all PEPCKases. The mechanistically homologous enzyme pyruvate kinase shows the opposite stereoselectivity for this analogue pair.

The difference in sensitivity of the mitochondrial enzymes and cytosolic enzymes to inhibition suggests differences in the active sites between these enzymes. Because the values for \( K_i \) are dependent upon the measured \( K_m \) for phosphoenolpyruvate (an apparent \( K_m \) because we did not determine if substrates and the Mn\(^{2+} \) are all at saturating conditions for each enzyme), the \( K_m/K_i \) ratio also was calculated for each experiment (Table 1). The \( K_i \) values are usually the same as thermodynamic constants for these complexes. Therefore, the forces that affect the interactions between these ligands and PEPCKase must differ between the mitochondrial and the cytosolic enzymes. These differences reflect subtle variations in the structures of the active sites of these distinct enzymes.

The authors wish to express their appreciation to Dr. Richard W. Hanson for his advice and assistance in this work. We acknowledge the support of the National Institutes of Health (Grants AM17049 to T.N. and AM21859 to M.F.U.) for this work. T.N. was the recipient of Research Career Development Award AM00486.