Properties of Phosphoenolpyruvate Carboxykinase from Carp Hepatopancreas and Rainbow Trout Liver

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Phosphoenolpyruvate carboxykinase (EC 4.1.1.32, PEPCK) from rainbow trout liver and carp hepatopancreas were purified and characterized kinetically. There are two forms of PEPCK in the rainbow trout liver, but only one form in the carp hepatopancreas. The molecular weight was 61,000 for rainbow trout liver major isozyme and 59,000 for carp hepatopancreas enzyme when determined by Sephadex G-100 gel filtration. However, when determined by SDS polyacrylamide gel electrophoresis the former was 87,000 and the latter was 83,000. The optimum pH for both enzymes was 7.7 in the direction of oxalacetate (OAA) decarboxylation, and 7.2 in the direction of phosphoenolpyruvate (PEP) carboxylation. Enzyme activities depended on the concentration of substrates (OAA or PEP) and nucleotides in either direction. Both enzymes were also strongly inhibited by several nucleotides. Divalent cation was absolutely necessary for both enzymes in either direction, and Mn2+ was found to be the best activator among several metal ions. Both enzymes were strongly inhibited by Zn2+, especially in the reaction of OAA decarboxylation. Co2+ and Cu2+ also showed obvious inhibition, but Ca2+ showed ambiguous effect on both enzymes. The kinetic properties of PEPCK from both fish liver were similar not only qualitatively but also quantitatively.

Omnivorous fish, such as carp and ayu, utilize carbohydrate very efficiently. Therefore, comparison of the enzymes of these fish with those of carnivorous fish such as rainbow trout will present a valuable and basic knowledge for understanding the mechanism of carbohydrate metabolism in fish.

Pyruvate kinase (EC 2.7.1.40, PK) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32, PEPCK) occupy a key branch-point in glycolytic and gluconeogenic sequences. The characteristic of PK from rainbow trout liver and carp hepatopancreas was reported previously.1) The phosphoenolpyruvate (PEP) is not only the substrate of PK, but also a substrate of PEPCK when performing the backward reaction. So thorough study of the properties of PEPCK from both fish is necessary.

In this paper, the purification and characterization of PEPCK from rainbow trout liver and carp hepatopancreas are described.

Materials and Methods

Samples
Hepatopancreas of the carp Cyprinus carpio and liver of rainbow trout Salmo gairdnerii irideus were obtained from live fish and stored at −20°C.

Chemicals
Hydroxyapatite was purchased from Seikagaku Kogyo. Matrex gel red-A was a product of Amicon and malate dehydrogenase (MDH) was purchased from Oriental Yeast Co. All other reagents were the same as described in previous paper.1)

Enzyme Assay
Two methods of measuring enzyme activity were used. All reactions were started by the addition of nucleotides and the activity was determined spectrophotometrically at 25°C by the decrease or increase of absorbance at 340 nm.

Assay method I: The enzyme activity in PEP carboxylation (reverse reaction) was measured at pH 7.2 in 0.6 ml reaction mixture according to the method of Chang and Lane.2) The reaction mixture contained 100 mM imidazole-HCl buffer, 0.05–0.1 mM reduced form nicotinamide adenine dinucleotide (NADH), 1 mM MnCl2, 2 mM phosphoenolpyruvate (PEP), 4 U/ml MDH, 50 mM NaHCO3, 2 mM inosine 5'-diphosphate (IDP)

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and 6.25 mg/ml bovine serum albumin. One unit of the enzyme activity was defined as the production of one micromole of reaction product per min.

Assay method II: The enzyme activity in oxalacetate (OAA) decarboxylation (forward reaction) was assayed at pH 8.0 in a 0.6 ml reaction mixture according to the method of Roobol and Alleyne. The reaction mixture contained 100 mM imidazole-HCl buffer, 1 mM NAD, 1 mM MnCl₂, 4 U/ml MDH, 2 U/ml lactate dehydrogenase (LDH), 2 mM sodium-l-malate, 2 mM inosine 5'-triphosphate (ITP) and 6.25 mg/ml bovine serum V albumin. Activities were presented as micromoles of oxalacetate utilized per minute.

Protein Determination
Protein concentration was determined by the method of Lowry et al.

SDS-Polyacrylamide Gel Electrophoresis
Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn.

Purification Procedure
All operations were conducted at 0-4°C. After crude enzyme was extracted from carp hepatopancreas homogenate, it was fractionated by ammonium sulfate; chromatographed by DEAE-Sephadex A-50 column (45.0×3.4 cm), first hydroxyapatite column (25.5×2.0 cm), second hydroxyapatite column (6.3×2.0 cm), and phosphocellulose column (10.7×1.0 cm); then filtered with Sephadex G-100 column (35.6×2.5 cm).

Rainbow trout liver PEPCK was purified with a little different procedure. After extraction, ammonium sulfate fractionation, DEAE-Sephadex column chromatography, hydroxyapatite column (24.5×2.0 cm) chromatography, phosphocellulose column chromatography, Matrex gel red-A affinity chromatography and then Sephadex G-100 gel filtration were manipulated accordingly.

Results and Discussion
Purification
During purification, carp hepatopancreas PEPCK was consistently chromatographed as a single peak (Fig. 1. A), while two isozymes of rainbow trout liver PEPCK were separated on a DEAE-Sephadex column (Fig. 1. F). Further purification and characterization for the minor peak was omitted, because its total activity was only 6.7% of the major one. The final enzyme preparations for both fishes appeared to be homogeneous on SDS-polyacrylamide gel electrophoresis.

Molecular Weight
The molecular weight of the major PEPCK from rainbow trout liver was estimated to be 61,000 by Sephadex G-100 gel filtration, but it was calculated to be 87,000 by SDS-polyacrylamide gel electrophoresis. For carp hepatopancreas PEPCK, the molecular weight was estimated to be 59,000 by Sephadex G-100 gel filtration and 83,000 by SDS-polyacrylamide gel electrophoresis, respectively (Fig. 2). Such discrepancy has also been observed by Kochi et al. for chicken liver PEPCK. The reason of the discrepancy remains unclear, but the adsorption of PEPCK to the Sephadex gel specifically seems to account for the discrepancy.

Effect of pH
In the direction of OAA decarboxylation, the optimum pH was 7.7 for both enzymes, but in the direction of PEP carboxylation, the optimum pH was 7.2 for both enzymes (Fig. 3). These values were slightly alkaline than that reported for bullfrog liver PEPCK by Goto et al.

Effect of Nucleotides
As shown in Table 1, we found ITP, GTP and UTP had active effect but ATP, CTP had no effect for both enzymes when performing OAA decarboxylation, however UTP only showed little effect on carp enzyme. On the other hand, when performing PEP carboxylation, GDP and IDP had active effect of which effect of GDP was stronger. We also found UDP had little effect and ADP, CDP had no effect for both enzymes.

Nucleotides also inhibit the activity of PEPCK. In the direction of OAA decarboxylation, GDP and IDP showed strong inhibition for both PEPCK. And in the direction of PEP carboxylation, ITP, ATP, CTP, GTP and UTP all showed strong inhibition for both PEPCK (Table 2).

Substrate Affinities
When determined in the direction of OAA decarboxylation, the Km values of rainbow trout liver PEPCK and carp hepatopancreas PEPCK were 4.0 µM and 6.7 µM for OAA, 12.5 µM and 10.0 µM for ITP, respectively (Fig. 4: A, D, B, E). On the other hand, when determined in the direction of PEP carboxylation, the Km values of
rainbow trout enzyme and carp enzyme were 200 \( \mu M \) and 170 \( \mu M \) for PEP, 45 \( \mu M \) and 54 \( \mu M \) for IDP, respectively (Fig. 4: G, J, H, K). The \( K_m \) values of both enzymes are summarized in Table 3. For OAA, both enzymes’ lower \( K_m \) values corresponded with liver PEPCK from rat, sheep, chicken\(^9\) and bullfrog.\(^9\) The \( K_m \) values of both enzymes for ITP, IDP and PEP were
Table 1. Nucleotide specificity of PEPCK

<table>
<thead>
<tr>
<th>Nucleotide (2 mM)</th>
<th>OAA decarboxylation</th>
<th>PEP carboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITP</td>
<td>ATP</td>
</tr>
<tr>
<td>Trout PEPCK activity (%)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Carp PEPCK activity (%)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Nucleotide inhibition of PEPCK

<table>
<thead>
<tr>
<th>Nucleotide (2 mM)</th>
<th>OAA decarboxylation</th>
<th>PEP carboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>IDP</td>
</tr>
<tr>
<td>Trout PEPCK inhibition (%)</td>
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<td>47</td>
</tr>
<tr>
<td>Carp PEPCK inhibition (%)</td>
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<td>41</td>
</tr>
</tbody>
</table>

Fig. 2. Molecular weights determined by Sephadex G-100 column chromatography (A) and SDS-polyacrylamide gel electrophoresis (B).

Fig. 3. Effect of pH on PEPCK in the direction of OAA decarboxylation (●) and PEP carboxylation (○). Enzyme activity; µmol/min per ml.

found lower than bullfrog liver PEPCK, which were 4,000 µM, 2,500 µM and 800 µM for ITP, IDP and PEP, respectively. However, these Km values for PEP were considerably higher than the Km values of 49–54 µM for chicken liver PEPCK.
Table 3. Kinetic constants of PEPCK

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Compound</th>
<th>Trout Km (μM)</th>
<th>Carp Km (μM)</th>
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<tbody>
<tr>
<td>OAA decarboxylation</td>
<td>OAA</td>
<td>4.0</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>ITP</td>
<td>12.5</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Mn²⁺</td>
<td>385.0</td>
<td>294.0</td>
</tr>
<tr>
<td>PEP carboxylation</td>
<td>PEP</td>
<td>200.0</td>
<td>170.0</td>
</tr>
<tr>
<td></td>
<td>IDP</td>
<td>45.0</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>Mn²⁺</td>
<td>190.0</td>
<td>250.0</td>
</tr>
</tbody>
</table>

Table 4. Divalent cation specificity of PEPCK

<table>
<thead>
<tr>
<th>Divalent cation (1 mM)</th>
<th>OAA decarboxylation activity (%)</th>
<th>PEP carboxylation activity (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Trout PEPCK</td>
<td>Carp PEPCK</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺</td>
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<td>83</td>
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<tr>
<td>Co²⁺</td>
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</tr>
<tr>
<td>Ca²⁺</td>
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<td>0</td>
</tr>
<tr>
<td>Cu²⁺</td>
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</tr>
<tr>
<td>Zn²⁺</td>
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<tr>
<td>None</td>
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</table>

Effect of Metal Ions

Both enzymes required divalent cation for OAA decarboxylation and PEP carboxylation. Mn²⁺ was found to be the best activator for both enzymes in both reactions (Table 4). Mg²⁺ was found to be partially capable in substitution of Mn²⁺ for both enzymes in the PEP carboxylation, whereas can up to 83% of Mn²⁺ can be replaced by Mg²⁺ for both enzymes in the OAA decarboxylation. Ca²⁺, Cu²⁺ and Zn²⁺ showed no activity for both enzymes. The need of divalent...
cation to activate their reactions has also been reported by Kapke et al.\textsuperscript{11} for PEPCK of the oral bacterium \textit{Capnocytophaga ochracea}.

The \(K_m\) values of rainbow trout liver PEPCK for \(\text{Mn}^{2+}\) in the reactions of OAA decarboxylation and PEP carboxylation were 385 \(\mu\text{M}\) and 170 \(\mu\text{M}\), respectively. On the other hand, the \(K_m\) values of carp enzyme for \(\text{Mn}^{2+}\) in the reactions of OAA decarboxylation and PEP carboxylation were 294 \(\mu\text{M}\) and 250 \(\mu\text{M}\), respectively (Fig. 4: C, F, I, L).

As shown in Table 5, both enzymes were strongly inhibited by \(\text{Zn}^{2+}\), especially in the reaction of OAA decarboxylation. \(\text{Co}^{2+}\) and \(\text{Cu}^{2+}\) also showed obvious inhibition on both enzymes, but \(\text{Ca}^{2+}\) showed ambiguous effect.

The kinetic properties of PEPCK from both fishes showed similarity not only qualitatively but also quantitatively. The activities of both PEPCKs depended on the concentrations of substrates (OAA or PEP) and nucleotides in the direction of OAA decarboxylation and PEP carboxylation. Nucleotides also inhibited the activities of both PEPCKs in the reverse direction.

It is suggested that the concentration of nucleotides may be physiologically important in switching on and off the gluconeogenesis and glycolysis. Therefore, the detailed analysis of metabolite levels in the liver under relevant condition is also necessary to understand the mechanism of the regulation of carbohydrate metabolism in fish.

\textbf{Acknowledgement}

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\textbf{References}