Occurrence of Phosphoenolpyruvate Carboxylase in the Extremely Thermophilic Bacterium Thermus aquaticus

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In the extreme thermophile Thermus aquaticus, phosphoenolpyruvate carboxylase catalyzes carbon dioxide fixation on the C3 metabolite phosphoenolpyruvate, producing oxaloacetate. In a moderately thermophilic Bacillus species this function is fulfilled by pyruvate carboxylase. Like several of its mesophilic counterparts, the Thermus enzyme exhibits a requirement for acetyl coenzyme A.

Two well-documented enzymes serving to produce oxaloacetate by carbon dioxide fixation onto C3 metabolites are phosphoenolpyruvate (PEP) carboxylase (orthophosphate:oxaloacetate carboxylase [phosphorylating]; EC 4.1.1.31) and pyruvate carboxylase (pyruvate: carbon dioxide ligase [adenosine 5'-diphosphate forming] EC 6.4.1.1). PEP carboxylase is distributed in the plant kingdom widely and in several bacterial genera (9), and pyruvate carboxylase is distributed in animal tissues, yeasts, fungi, and several bacteria (6). In a moderately thermophilic Bacillus species carbon dioxide fixation is mediated by pyruvate carboxylase rather than PEP carboxylase (7, 8). We now find, interestingly, that the extremely thermophilic bacterium Thermus aquaticus apparently uses PEP carboxylase for this purpose. This communication presents the evidence pointing to this conclusion and some properties of the Thermus PEP carboxylase.

T. aquaticus YT-1 was grown aerobically at 70 C in a salts-tryptone-yeast extract medium (4) at the Microbiological Research Establishment, Porton Down, England, and supplied to us as a frozen cell paste. To prepare a cell-free extract the thawed cells, suspended in 50 mM Tris-hydrochloride buffer (pH 7.6) containing 0.15 M potassium chloride and 1 mM ethylenediaminetetraacetate, were disrupted in a French press. The supernatant portion (cell-free extract) was collected after centrifugation of the cell homogenate at 145,000 g and 4 C for 90 min. PEP carboxylase was assayed by spectrophotometric and radiochemical methods. The reaction mixture in the spectrophotometric assay contained (in micromoles, unless otherwise stated) in 1 ml: Tris-hydrochloride (pH 8), 100; MgCl2, 5; KHCO3, 50; reduced nicotinamide adenine dinucleotide, 0.65; acetyl coenzyme A, 0.25; malate dehydrogenase, 1 IU; enzyme, 0.02 to 0.04 U (Table 1); and PEP, 10. The rate of oxidation of reduced nicotinamide adenine dinucleotide consequent on the addition of PEP was measured at 45 C by decrease in absorbance at 340 nm in a recording spectrophotometer. The reaction mixture for the radiochemical assay contained, in 0.3 ml, the same ingredients at similar concentrations and, in addition, 1.6 C14 of Na2 14CO3. The reaction was initiated by the addition of PEP, and after incubation at 45 C for 10 min, 0.9 ml of ethanol was added and the mixture was centrifuged. Acid-stable radioactive material in the supernatant liquid was determined as described elsewhere (8). Pyruvate carboxylase was assayed by similar spectrophotometric and radiochemical methods (1, 8). Protein contents were measured by the method of Lowry et al. (2).

A cell-free extract of T. aquaticus cells, when assayed by both radiochemical and spectrophotometric methods, was devoid of pyruvate carboxylase but possessed what was apparently PEP carboxylase activity. In order to confirm the presence of PEP carboxylase, the extract was fractionated as follows, primarily to remove the low-molecular-weight nucleoside phosphates. The extract (16 ml) was treated with 200 C14 of deoxyribonuclease at 30 C for 30 min and osmotically concentrated to 2 ml in a dialysis sac surrounded by solid sucrose. The concentrate was applied to the top of a Sephadex G-200 column (3 by 60 cm) equilibrated in 50 mM Tris-hydrochloride (pH 7.6) containing 0.15 M potassium chloride and 1 mM ethylenediaminetetraacetate. The column was eluted with the same buffer at a flow rate of 10 ml/h. The most active enzyme fractions, which were substantially free of the intense yellow pigment produced by T. aquaticus, were pooled and concen-
trated osmotically, and the concentrate was dialyzed against 50 mM Tris-hydrochloride (pH 7.6) buffer containing 1 mM ethylenediaminetetraacetate for 18 h at 4°C. This final preparation catalyzed the oxidation of 0.7 \( \mu \text{mol} \) of reduced nicotinamide adenine dinucleotide per min per mg of protein at 45°C in the spectrophotometric assay and was estimated to be purified more than fivefold over the cell-free extract. In the absence of added PEP there was very little reduced nicotinamide adenine dinucleotide oxidation in this assay system and almost no \(^{14}\text{CO}_2\) fixation in the radiochemical assay; pyruvate and adenosine 5'-triphosphate could not substitute for PEP (Table 1).

The product of the reaction was identified as follows. The supernatant liquid from the radiochemical assay was acidified with a few drops of glacial acetic acid and evaporated to dryness over solid sodium hydroxide in a vacuum desiccator. The acidification and drying was repeated, and the residue was dissolved in a small volume of water. This concentrate was co-chromatographed on Whatman filter paper with standards of malate, citrate, and fumarate, using the solvent system \( n\)-butanol-formic acid-water (10:2:15, vol/vol/vol). The dried chromatogram was exposed to X-ray film for 10 days. The autoradiogram thus prepared showed the products in the enzyme assay system to be similar chromatographically to malate, citrate, and fumarate (Fig. 1). These compounds were obviously produced in the assay.

### Table 1. Characteristics of \( T. \) aquaticus PEP carboxylase

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Enzyme activity (% of complete)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Mg(^{2+}) omitted</td>
<td>0.3</td>
</tr>
<tr>
<td>Acetyl CoA omitted</td>
<td>0.5</td>
</tr>
<tr>
<td>PEP omitted</td>
<td>0.5</td>
</tr>
<tr>
<td>PEP omitted; pyruvate (10 mM) and ATP (2 mM) added</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Details of the complete reaction mixture are given in the text. The partially purified enzyme was used, 0.02 to 0.04 U in the spectrophotometric assay and 0.05 to 0.1 U in the radiochemical assay. One unit of enzyme catalyzes the oxidation of 1 \( \mu \text{mol} \) of reduced nicotinamide adenine dinucleotide per min in the spectrophotometric assay. Abbreviations: Acetyl CoA, Acetyl coenzyme A; ATP, adenosine 5'-triphosphate.

**FIG. 1.** Autoradiogram developed from the \( T. \) aquaticus PEP carboxylase reaction mixture. The partially purified enzyme was used. Other details are in the text. Radioactive spot \( C \) corresponds to citrate, spot \( M \) corresponds to malate, and spot \( F \) corresponds to fumarate.
mixture from the oxaloacetate generated by CO₂ fixation on to PEP. When PEP was omitted from the reaction mixture or pyruvate and adenosine 5'-triphosphate were substituted for it, no radioactive spot could be detected on the chromatogram.

Thermus PEP carboxylase requires Mg²⁺ and is virtually inactive in the absence of acetyl coenzyme A (Table 1). In this respect it resembles many mesophilic bacterial PEP carboxylases (9). Fructose 1,6-diphosphate, which also activates several of these mesophile enzymes, has, however, no similar effect on the Thermus carboxylase.

The distribution of pyruvate carboxylase and PEP carboxylase, which in effect fulfills the same metabolic role, in biological systems follows no obvious pattern. While animal tissues, yeasts, and fungi contain pyruvate carboxylase and a number of plants form PEP carboxylase, the two enzymes are present in gram-positive and gram-negative representatives of bacteria (3, 6, 9). Only Azotobacter vinelandii and Pseudomonas citronellolis, both gram-negative bacteria, have so far been observed to contain both carboxylases. Curiously in these two organisms pyruvate carboxylase is inducible and not constitutive (5). T. aquaticus is the first thermophile in which PEP carboxylase has been shown to be present. Whether this extreme thermophile, like A. vinelandii and P. citronellolis, would produce pyruvate carboxylase if grown in an appropriate medium is not clear at present. Several of the mesophile PEP carboxylases are subject to regulation by positive and negative effector metabolites. A study of the molecular and regulatory properties of the Thermus enzyme, which is obliged to function at the very high growth temperature of this organism, should be of great interest.

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LITERATURE CITED