Purification and Characterization of Phosphoenolpyruvate Carboxylase from *Plasmodium berghei*

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Phosphoenolpyruvate (PEP) carboxylase was purified over 400-fold from *Plasmodium berghei*. The purified enzyme was stable in 0.4 M potassium phosphate buffer (pH 7.4) containing 0.5 M glucose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM MgCl₂. It had a molecular weight of 280,000 determined by sucrose density gradient centrifugation in this buffer, but it aggregated and was unstable in the presence of different salts or a more dilute solution of potassium phosphate. The Kₐ for PEP was 2.6 mM and that for Mg²⁺ was 1.3 mM. The Kₘ for bicarbonate was 2 mM. Citrate, nucleotides, and EDTA inhibited the PEP carboxylase of *P. berghei* by decreasing the concentration of free magnesium ions, but acetyl-coenzyme A, fructose-1,6-diphosphate, and aspartate did not influence its activity. A chloroquine concentration of 1.8 × 10⁻⁴ M inhibited the enzyme 50%.

Over 20 years ago, Trager (30) and Anfinsen (1) showed that the inclusion of 5% carbon dioxide in the atmosphere aided the in vitro cultivation of plasmodia. Although this was initially thought to be due to the buffering effect of bicarbonate, in 1966 Ting and Sherman showed that ¹⁴C-labeled bicarbonate was incorporated into certain amino acids and organic acids (29). At approximately the same time, Siu reported the presence of phosphoenolpyruvate (PEP) carboxylase and PEP carboxykinase in *Plasmodium berghei* (26). We have isolated and characterized PEP carboxylase [orthophosphate:oxalacetate carboxyl-lyase (phosphorylating) EC 4.1.1.31] from *P. berghei* in an effort to learn more about this enzyme and the role it plays in the metabolism of glucose by plasmodia.

MATERIALS AND METHODS

A volume of 150 ml of blood was collected in 350 ml of cold isotonic citrate saline solution (sodium chloride, 161 mM; sodium citrate, 16 mM; and citric acid, 3 mM) from 300 mice (5 to 6 weeks old) that had been inoculated the preceding week with the Walter Reed strain of *P. berghei*. The cells with the plasmodia were isolated by centrifugation, washed, and lysed in distilled water at 4 C for 1 hr. After lysis, the red cell ghost and parasites were washed with distilled water to remove the remaining hemoglobin and were then suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.2. Sonic treatment at 5 amp for 4 min with a Branson Sonifier solubilized the PEP carboxylase activity.

PEP carboxylase and carboxykinase activity was absent from the blood of mice subjected to the same procedure but not inoculated with plasmodia. (There was an equal amount of PEP carboxylase activity in the chloroquine-resistant *P. berghei*.) The sonically treated material was centrifuged at 40,000 × g for 1 hr to remove the cellular debris and was then brought to 40% saturation at 0 C by the addition of solid ammonium sulfate. The ammonium sulfate precipitate was dissolved in 0.4 M potassium phosphate buffer (pH 7.4) containing 0.5 M glucose, 1 mM MgCl₂, and 1 mM ethylenediaminetetraacetic acid (EDTA) and was stored at −12 C until used for further purification. In all subsequent steps, the potassium phosphate buffer had a pH of 7.4, and glucose, MgCl₂, and EDTA were present in the concentrations mentioned above.

Assay of enzymatic activity. PEP carboxylase was assayed by two methods. The first involved the incorporation of ¹⁴C-labeled bicarbonate into oxalacetate. The reaction mixture contained, in a total volume of 0.5 ml: sodium bicarbonate, 16 μmoles (10⁴ counts/min of ¹⁴C); Tris-hydrochloride, 100 μmoles (pH 7.8); MgCl₂, 10 μmoles; PEP, 6 μmoles; and the enzyme preparation. After incubation for 20 min at 37 C, the reaction was stopped by placing the incubation tubes in ice for 1 min and then adding 0.1 ml of 50% trichloroacetic acid. A sample of the

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reaction mixture was agitated for 1.5 min, and the 
radioactivity remaining was counted in a liquid scin-
tillation spectrometer with an ethanol and toluene 
scintillation solution. Gassing with CO₂ and adding 
cold oxalacetate at the end of the incubation period 
were unnecessary if the sample was carefully agi-
tated for 1.5 min and then counted without any 
delay.

The PEP carboxylase activity was also assayed by 
coupling the reaction with malate dehydrogenase and 
measuring the decrease in absorbance at 340 nm 
as reduced nicotinamide adenine dinucleotide 
(NADH) was oxidized. The assay mixture contained, 
in a total volume of 1 ml (at 25 C): Tris-hydrochloride 
(pH 7.8), 100 μmoles; MgCl₂, 8 μmoles; NADH, 100 
moles; sodium bicarbonate, 25 μmoles; malate de-
hydrogenase, 1 μM unit (6 μM); PEP, 12 μmoles; and 
an appropriate amount of the enzyme preparation.

The change in absorption at 340 nm was measured 
with a Gilford recording spectrophotometer. From 
4 to 10 μmoles of potassium phosphate (pH 7.4) 
was also present in the assay mixture as the result of 
adding the enzyme solution containing phosphate. 
The reaction was not altered by this amount of phos-
phate, but at a concentration of 50 mM and above 
there was inhibition of the reaction through the pre-
cipitation of magnesium phosphate salts.

Sucrose density gradient centrifugation. Su-
crose density gradient centrifugation was done ac-
cording to the procedure of Martin and Ames (13). 
Catalase was used as a standard, and its activity was 
measured by the procedure of Chance and Maehly 
(2).

Gel disc electrophoresis. Polycrylamide-gel disc 
electrophoresis was performed by the method of 
Ornstein (17). The gels were stained with Amido 
Schwarz dye and destained with 7% acetic acid. A 
100-μg amount of the purified plasmoidal enzyme 
was applied to the gel.

Protein measurements. Protein was measured by 
the ultraviolet method of Warburg and Christian (11, 
33) and by the method of Waddell (32).

Assay of ATP. Adenosine triphosphate (ATP) 
was measured by use of hexokinase and glucose-6-
phosphate dehydrogenase (10).

Avidin incubation. One unit of avidin was in-
cluded at room temperature for 5 min with 0.025 mg 
of PEP carboxylase. After this incubation, the car-
boxylase was assayed spectrophotometrically.

Materials. Diethylaminomethyl (DEAE) cellulose 
(reagent grade) was obtained from the Brown Co. It 
was washed with 0.5 M HCl and NaOH before equili-
bration with 0.15 M potassium phosphate buffer. Cel-
lulose phosphate, obtained from Schleicher & 
Schuell Co., was also washed with 0.5 M HCl and 
NaOH before being equilibrated with 0.15 M phos-
phate buffer. The following enzymes and chemicals 
were purchased from commercial sources: 2-phos-
phoenolpyruvic acid and malate dehydrogenase from 
Sigma Chemical Co.; hexokinase and glucose-6-
phosphate dehydrogenase from P-L Biochemicals;

RESULTS

Purification. The 0 to 40% ammonium sul-
fate precipitate contained both PEP carbox-
ylase and carboxykinase activity, but the car-
boxykinase activity was completely lost on 
freezing. After thawing, the ammonium sulfate 
precipitate was dialyzed against 0.15 M potas-
sium phosphate buffer for 2 hr to remove the 
excess salt. It was then passed through a 
DEAE cellulose column (20 cm² by 45 cm) 
which had been equilibrated with 0.15 M potas-
sium phosphate buffer. Under these condi-
tions, the enzyme was not absorbed on the 
DEAE cellulose column and was recovered by 
washing the column with approximately 350 
ml of 0.15 M potassium phosphate buffer. 
Phosphate from this column with PEP carbox-
ylase activity were applied to a cellulose phos-
phate column (6 cm² by 30 cm) which had 
been equilibrated with 0.15 M potassium phos-
phate. The cellulose phosphate column was 
washed with 0.3 M potassium phosphate until 
all of the protein being eluted at this concen-
tration of potassium phosphate was removed. 
Then the PEP carboxylase was eluted with 0.4 
M potassium phosphate buffer (Fig. 1). This 
fraction from the cellulose phosphate column 
was concentrated in a Dialflow ultrafiltration 
cell from Amicon with an XM-50 membrane.

The results of the purification procedure are 
shown in Table 1. There was a greater than 
400-fold purification with the recovery of about 
10% of the initial activity. The specific activity 
was 1.5 μmoles of bicarbonate incorporated 
into acid-stable material per min per mg of 
protein.

Purity. On sucrose density gradient centrifri-
gation, there were two protein peaks. The 
PEP carboxylase activity peak overlapped the 
major protein peak (Fig. 2). On acrylamide-gel 
disc electrophoresis, there were two major 
bands and some material at the origin of the 
separating gel (Fig. 3).

Stability. The purified enzyme had no loss of 
activity over a 3-month period of time at 
-12 C in 0.4 M potassium phosphate buffer 
containing 0.5 M glucose, 1 mM EDTA, and 1 
mm MgCl₂. When 0.4 M ammonium sulfate 
was substituted for the 0.4 M potassium phosphate, 
with glucose, EDTA, and MgCl₂ being present 
in the same concentrations as before, the 
activity decreased 25% after 1 month at -12 C. 
Dialysis of the purified enzyme against 0.4 M 
potassium phosphate containing 0.5 M glucose 
and 1 mM EDTA and MgCl₂ overnight re-

from New England Nuclear Corp.; ammonium sul-
fate and glucose from Fisher Scientific Co.
VOL. 109, total cellulose pressed was 2.6

Table 1. Purification of phosphoenolpyruvate (PEP) carboxylase with 0.05 M KCl and EDTA. PEP carboxylase activity was measured by the incorporation of 14C-bicarbonate into oxalacetate as described in the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>0.0031</td>
<td>2.68</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.0121</td>
<td>3.67</td>
<td>4</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>0.0248</td>
<td>1.32</td>
<td>8</td>
</tr>
<tr>
<td>Cellulose phosphate</td>
<td>1.512</td>
<td>0.37</td>
<td>480</td>
</tr>
</tbody>
</table>

*PEP carboxylase activity was assayed by the incorporation of 14C-bicarbonate into oxalacetate as described in Materials and Methods. The crude fraction is the 40,000 × g supernatant fraction of sonically treated plasmodia. Specific activity is expressed as micromoles per minute per milligram; total activity, as micromoles per minute.

sulted in the loss of 5% of the initial activity. Dialysis overnight against 0.05 M potassium phosphate or 0.8 M sodium chloride, with glucose, EDTA, and MgCl₂ being present as before, resulted in a loss of 35% of the initial activity with 0.05 M potassium phosphate and a loss of 90% with 0.8 M sodium chloride.

Molecular-weight determination. The PEP carboxylase of P. berghei had a molecular weight of 280,000 by sucrose density gradient centrifugation in the presence of 0.4 M potassium phosphate buffer. In the presence of a lower ionic strength or different ions, the enzyme tended to aggregate, as shown in Table 2.

Kinetic properties. The apparent Kₘ for PEP was 2.6 mM (Fig. 4) and that for Mg²⁺ was 1.3 mM (Fig. 5). The Kₘ for bicarbonate was 2 mM. The pH optimum for the reaction was 7.4 in Tris-hydrochloride and sodium cacodylate buffers. The reaction did not require phosphate or monovalent ions, but was inhibited by a phosphate concentration above 50 mM through the precipitation of magnesium phosphate salts. Biotin was not required and there was no inhibition by avidin. The activity with manganese was about 60% of that with magnesium.

Regulatory properties. The effect of various compounds on the plasmodial PEP carboxylase was investigated by use of the spectrophotometric assay. Citrate, nucleotides, oxalacetate, and EDTA inhibited the carboxylase reaction (Table 3). The relative degree of inhibition by these compounds corresponds to their ability to chelate magnesium (4), and the original activity was restored by the further addition of MgCl₂. Increasing the concentration of PEP or bicarbonate did not alter the degree of inhibition. There was no alteration of the Kₘ for PEP or bicarbonate or of the Vₘₐₓ of the reaction by acetyl-coenzyme A (CoA), fructose-1,6-diphosphate, nucleotides, or aspartate. A chloroquine concentration of 1.8 × 10⁻⁶ M inhibited the reaction 50%.

DISCUSSION

The metabolism of glucose by plasmodia is incompletely understood because of the difficulties involved in cultivating them in the absence of red blood cells. Except for P. lophurae and P. falciparium, the erythrocytic forms of plasmodia do not possess mitochondria with cristae (20), and it is generally accepted that this stage is primarily glycolytic, with the citric acid cycle being present but not functioning as the major source of energy (2). Lactate is the major product of glucose metabolism, and the oxidation of glucose to carbon dioxide accounts for only a small percentage of the glucose metabolized (15, 16, 23). The exo-erythrocytic

Fig. 1. Elution of phosphoenolpyruvate (PEP) carboxylase with potassium phosphate buffer. The 0.3 and 0.4 M solutions of potassium phosphate had a pH of 7.4 and contained 0.5 M glucose and 1 mM MgCl₂ and EDTA. PEP carboxylase activity was measured by the incorporation of 14C-bicarbonate into oxalacetate as described in the text.

Fig. 2. Sucrose density gradient centrifugation. The sucrose gradient (5 to 20%) contained 0.4 M potassium phosphate (pH 7.4) and 1 mM MgCl₂. Thirty 1-ml samples were collected from the gradient tube, and carboxylase activity was measured as described. The protein was determined by the difference in absorbance at 215 and 225 nm. The specific activity of the peak tube is 600,000 counts per min per mg of protein. The bottom of the gradient is on the left and the top is on the right.
Table 2. Effect of salt concentration on the apparent molecular weight of phosphoenolpyruvate carboxylase

<table>
<thead>
<tr>
<th>Salt</th>
<th>Calculated mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate (pH 7.4), 0.4 M</td>
<td>280,000</td>
</tr>
<tr>
<td>Potassium phosphate (pH 7.4), 0.05 M, with ammonium sulfate, 0.2 M</td>
<td>375,000</td>
</tr>
<tr>
<td>Potassium phosphate (pH 7.4), 0.05 M</td>
<td>500,000</td>
</tr>
</tbody>
</table>

*The sucrose gradients (5 to 20%) were made up containing the concentrations of potassium phosphate and ammonium sulfate shown. They also contained 1 mM MgCl₂. The sedimentation of catalase, the reference protein, is not altered by this variation in ionic strength (21).*

FIG. 4. Effect of increasing the concentration of phosphoenolpyruvate on the formation of oxalacetate. This was assayed by use of malate dehydrogenase (see Materials and Methods). DPNH = NADH (reduced nicotinamide adenine dinucleotide).

FIG. 5. Effect of increasing the concentration of Mg²⁺ on oxalacetate formation. The same curve for the phosphoenolpyruvate carboxykinase of P. berghei is S-shaped. DPNH = NADH (reduced nicotinamide adenine dinucleotide).
stages of plasmodia possess more typical mitochondria with cristae (8), and the citric acid cycle appears to have a more active role in this form.

In bacteria with an active citric acid cycle, PEP carboxylase supplies oxalacetate for the citric acid cycle and is regulated to insure an adequate supply of oxalacetate for various levels of cycle activity. Despite this decrease in Krebs cycle activity, the fixation of carbon dioxide at the level of PEP appears to be an important reaction in the erythrocytic forms of plasmodia, in that the addition of PEP and 5% carbon dioxide to an in vitro culture of plasmodia aids their development (31). Ting and Sherman (29) showed that the initial products of carbon dioxide fixation in P. lophurae were oxalacetate and \( \alpha \)-ketoglutarate. Aspartate and glutamate, which are readily formed from these two organic acids, are not required in the media of P. knowlesi for growth in vitro and are not incorporated from the media into the plasmodial proteins in the presence of glucose (18, 19). If plasmodia are capable of generating ATP in the conversion of fumarate to succinate, as has been shown to occur in the earthworm and beef heart mitochondria (22), this would further explain the seeming importance of the carboxylation of PEP in plasmodia. In keeping with this point, succinate, in addition to lactate, is a major product of glucose catabolism in P. lophurae (24). The PEP carboxylase of P. berghei is inhibited by citrate and ATP through the chelation of magnesium ions. Unlike the PEP carboxylase of Enterobacteriaceae, there is no activation of the enzyme by acetyl-CoA, fructose-1,6-diphosphate, or nucleotides (5, 12). This basic difference in metabolite regulation may be related to the overall difference in the relative role of the citric acid cycle in the metabolism of Enterobacteriaceae and plasmodia.

While attempting to purify the enzyme by gel filtration in 0.05 m Tris-hydrochloride (pH 7.4) containing 0.01 m CoCl\(_2\), we obtained, in addition to the large-molecular-weight fraction, a peak of activity which came off the column after hemoglobin, indicating that it had a molecular weight of less than 68,000. This fraction was extremely unstable and was not further characterized. This finding, along with the fact that the three molecular weights obtained by sucrose density gradient centrifugation differed by approximately 100,000, suggests that the 280,000 species is made up of two or more subunits. In spite of this, there is no indication that any type of subunit inter-

<table>
<thead>
<tr>
<th>Table 3. Alteration of phosphoenolpyruvate carboxylase activity*</th>
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<tbody>
<tr>
<td>Compound added</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Succinate</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphate</td>
</tr>
<tr>
<td>Aspartate</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
</tr>
<tr>
<td>Citrate</td>
</tr>
<tr>
<td>Oxalacetate</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetate</td>
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</tbody>
</table>

*The coupled spectrophotometric assay with malate dehydrogenase described in Materials and Methods was used except in the case of oxalacetate where the radioactive assay was used. The concentration of each compound was 10 \( \mu \)moles/ml. The addition of 10 \( \mu \)moles of MgCl\(_2\) in the case of adenosine triphosphate and 20 \( \mu \)moles in the case of ethylenediaminetetraacetate and citrate overcame inhibition by these compounds and restored the activity to its original level.

action occurs under metabolite control which alters the kinetic characteristics of the enzyme.

Biotin has been reported to aid the growth of P. knowlesi in vitro (25). However, it certainly does not appear to be required by the PEP carboxylase of P. berghei. This is in keeping with the known enzymatic reactions by which PEP is carboxylated to oxalacetate (14, 28, 34). Biotin may be required for the carboxylation reaction which is suggested by the work of Ting and Sherman to exist in plasmodia at the level of \( \alpha \)-ketoglutarate. The concentration of chloroquine required for inhibition of the plasmodial PEP carboxylase (10\(^{-4}\) M) is of the same magnitude as that required for inhibition of the PEP carboxykinase of P. berghei (26) and the deoxyribonucleic acid polymerase from bacteria (3). The coenzyme Q-requiring NADH oxidase and succinoxidase systems are inhibited by a chloroquine concentration of 10\(^{-2}\) M (27), which is more in keeping with the concentration that Fitch has shown to exist in plasmodia (7). Therefore, inhibition of PEP carboxylase is probably not important in the mechanism of action of chloroquine (6, 9).

LITERATURE CITED