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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 (*p*H 7.4) containing 0.5 M glucose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM $MgCl_2$. 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 K_m for PEP was 2.6 mM and that for Mg^{2+} was 1.3 mM. The K_m 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 ^{14}C-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 carboxy-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, *p*H 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 (*p*H 7.4) containing 0.5 M glucose, 1 mM $MgCl_2$, 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 *p*H of 7.4, and glucose, $MgCl_2$, 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 ^{14}C-labeled bicarbonate into oxalacetate. The reaction mixture contained, in a total volume of 0.5 ml: sodium bicarbonate, 16 μmoles (10^6 counts/min of ^{14}C); Tris-hydrochloride, 100 μmoles (*p*H 7.8); $MgCl_2$, 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 scintillation spectrometer with an ethanol and toluene scintillation solution. Gassing with CO_2 and adding cold oxalacetate at the end of the incubation period were unnecessary if the sample was carefully agitated 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 (*p*H 7.8), 100 μmoles; $MgCl_2$, 8 μmoles; NADH, 100 nmoles; sodium bicarbonate, 25 μmoles; malate dehydrogenase, 1 μM unit (6 μg); 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 (*p*H 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 phosphate, but at a concentration of 50 mM and above there was inhibition of the reaction through the precipitation of magnesium phosphate salts. The specific activity of the purified enzyme with the coupled spectrophotometric assay was 60% of that with the radioactive assay.

Sucrose density gradient centrifugation. Sucrose density gradient centrifugation was done according 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. Polyacrylamide-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 plasmodial 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 incubated at room temperature for 5 min with 0.025 mg of PEP carboxylase. After this incubation, the carboxylase was assayed spectrophotometrically.

Materials. Diethylaminoethyl (DEAE) cellulose (reagent grade) was obtained from the Brown Co. It was washed with 0.5 M HCl and NaOH before equilibration with 0.15 M potassium phosphate buffer. Cellulose phosphate, obtained from Schleicher & Schuell Co., was also washed with 0.5 M HCl and NaOH before being equilibrated with 0.15 M phosphate buffer. The following enzymes and chemicals were purchased from commercial sources: 2-phosphoenolpyruvic acid and malate dehydrogenase from Sigma Chemical Co.; hexokinase and glucose-6-phosphate dehydrogenase from P-L Biochemicals; ^{14}C-labeled sodium bicarbonate (20 mCi/mmole) from New England Nuclear Corp.; ammonium sulfate and glucose from Fisher Scientific Co.

RESULTS

Purification. The 0 to 40% ammonium sulfate precipitate contained both PEP carboxylase and carboxykinase activity, but the carboxykinase activity was completely lost on freezing. After thawing, the ammonium sulfate precipitate was dialyzed against 0.15 M potassium phosphate buffer for 2 hr to remove the excess salt. It was then passed through a DEAE cellulose column (20 cm^2 by 45 cm) which had been equilibrated with 0.15 M potassium phosphate buffer. Under these conditions, 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. The fractions from this column with PEP carboxylase activity were applied to a cellulose phosphate column (6 cm^2 by 30 cm) which had been equilibrated with 0.15 M potassium phosphate. The cellulose phosphate column was washed with 0.3 M potassium phosphate until all of the protein being eluted at this concentration 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 Diaflow 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 centrifugation, 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 $MgCl_2$. When 0.4 M ammonium sulfate was substituted for the 0.4 M potassium phosphate, with glucose, EDTA, and $MgCl_2$ 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 $MgCl_2$ overnight re-

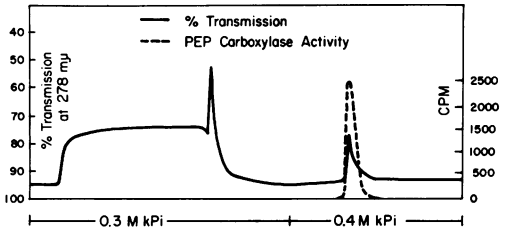

FIG. 1. *Elution of phosphoenolpyruvate (PEP) carboxylate with potassium phosphate buffer. The 0.3 and 0.4 M solutions of potassium phosphate had a pH of 7.4 and contained 0.5 M glucose and 1 mM $MgCl_2$ and EDTA. PEP carboxylase activity was measured by the incorporation of ^{14}C-bicarbonate into oxalacetate as described in the text.*

TABLE 1. *Purification of phosphoenolpyruvate (PEP) carboxylase*[a]

Fraction	Specific activity	Total activity	Purification	Recovery (%)
Crude	0.0031	2.68	1	100
Ammonium sulfate	0.0121	3.67	4	137
DEAE cellulose	0.0248	1.32	8	49
Cellulose phosphate	1.512	0.37	480	14

[a] PEP carboxylase activity was assayed by the incorporation of ^{14}C-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 $MgCl_2$ 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 K_m for PEP was 2.6 mM (Fig. 4) and that for Mg^{2+} was 1.3 mM (Fig. 5). The K_m for bicarbonate was 2 mM. The *p*H 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 $MgCl_2$. Increasing the concentration of PEP or bicarbonate did not alter the degree of inhibition. There was no alteration of the K_m for PEP or bicarbonate or of the V_{max} of the reaction by acetyl-coenzyme A (CoA), fructose-1,6-diphosphate, nucleotides, or aspartate. A chloroquine concentration of 1.8×10^{-4} 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

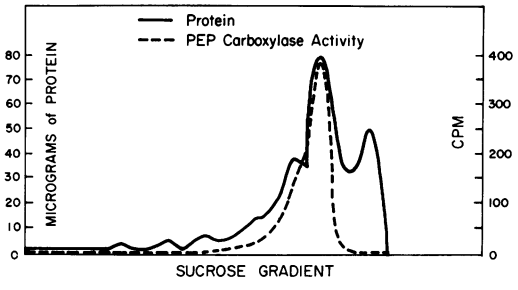

FIG. 2. *Sucrose density gradient centrifugation. The sucrose gradient (5 to 20%) contained 0.4 M potassium phosphate (pH 7.4) and 1 mM $MgCl_2$. Thirty 1-ml samples were collected from the gradient tube, and carboxylase activity was measured as described. The protein was determined by the difference 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.*

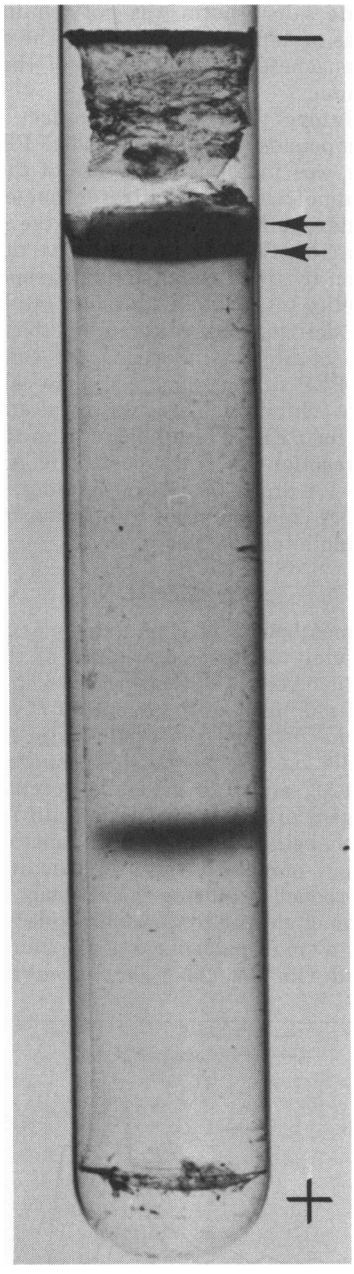

FIG. 3. *Polyacrylamide-gel disc electrophoresis. The 7% gel was run at pH 8.5 and 4 C. A 100-μg amount of the purified enzyme (after cellulose phosphate) was applied to the gel. The upper staining material is composed of two distinct bands (arrows). One of these bands just entered the separating gel, and the other is at the interface of the stacking gel and the separating gel.*

TABLE 2. *Effect of salt concentration on the apparent molecular weight of phosphoenolpyruvate carboxylase*[a]

Salt	Calculated mol wt
Potassium phosphate (*p*H 7.4), 0.4 M . .	280,000
Potassium phosphate (*p*H 7.4), 0.05 M, with ammonium sulfate, 0.2 M	375,000
Potassium phosphate (*p*H 7.4), 0.05 M .	500,000

[a] The sucrose gradients (5 to 20%) were made up containing the concentrations of potassium phosphate and ammonium sulfate shown. They also contained 1 mM $MgCl_2$. The sedimentation of catalase, the reference protein, is not altered by this variation in ionic strength (21).

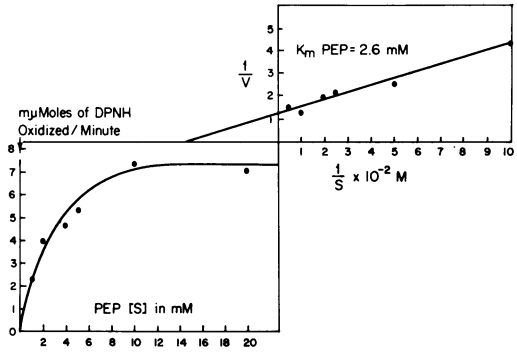

FIG. 4. *Effect of increasing the concentration of phosphoenolpyruvate on the formation of oxalacetate. This was assayed by use of malate dehydrogenase (see Materials and Methods). DPNH = NADH (reduced nicotinamide adenine dinucleotide).*

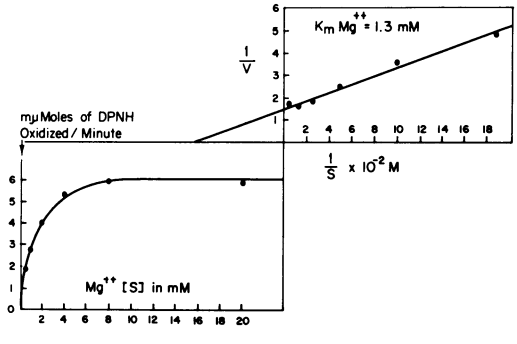

FIG. 5. *Effect of increasing the concentration of Mg^{2+} on oxalacetate formation. The same curve for the phosphoenolpyruvate carboxykinase of P. berghei is S-shaped. DPNH = NADH (reduced nicotinamide adenine dinucleotide).*

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 α-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 (*p*H 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 interaction 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 α-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^{-7} 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).

TABLE 3. *Alteration of phosphoenolpyruvate carboxylase activity*[a]

Compound added	Change from the initial rate (%)
Glucose	0
Succinate	0
Acetyl-CoA	0
Fructose-1,6-diphosphate	0
Aspartate	0
α-Ketoglutarate	−5
Citrate	−65
Oxalacetate	−30
Adenosine diphosphate	−25
Guanosine diphosphate	−22
Adenosine triphosphate	−50
Guanosine triphosphate	−40
Ethylenediaminetetraacetate	−100

[a] 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 μmoles/ml. The addition of 10 μmoles of $MgCl_2$ in the case of adenosine triphosphate and 20 μmoles in the case of ethylenediaminetetraacetate and citrate overcame inhibition by these compounds and restored the activity to its original level.

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