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Phosphopyruvate carboxylase from *Entamoeba histolytica*

Phosphopyruvate carboxylase (Pyrophosphate:oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.38) catalyzes the reversible reaction: phosphoenolpyruvate + CO_2 + P_i ⇌ oxaloacetate + PP_i. The enzyme was found in *Propionibacterium shermanii*[1]. It has been studied by H. G. WOOD and his colleagues who have crystallized the enzyme[2] and investigated its metal requirements[3–5]. This communication deals with the identification and function of this enzyme in *Entamoeba histolytica*. It is the first report of its finding in an organism other than propionibacteria.

Cultures of *E. histolytica*, DKB strain, were grown anaerobically at 37° for 48 h in a complex medium containing penicillin and cells of a penicillin-sensitive bacteria. Growth and harvesting of the amoebae followed procedures previously described[6]. Lyophilized amoebae, 411 million cells representing 2.32 ml of fresh cells, were suspended in 12.6 ml of a buffer containing 0.02 M potassium phosphate, pH 7, and 10^{-4} M cobalt chloride. After centrifugation at 36 000 × *g* for 30 min at 0° the supernatant fluid was concentrated to 6 ml by vacuum dialysis against the buffer. This fluid was applied to a 20 mm × 450 mm column of Biogel A1.5m (Bio-Rad) previously conditioned with the buffer. Enzyme was eluted with the same buffer. The activity in the eluted fractions was assayed at 25° by linking oxaloacetate formation with the oxidation of NADH in the presence of malate dehydrogenase (EC 1.1.1.37) by the procedure of WOOD *et al.*[7]. Fractions containing more than 0.1 unit/ml of enzyme were combined and potassium phosphate, pH 7, was added to bring its total concentration to 0.05 M. This solution was then applied to a 20 mm × 200 mm column of DEAE-cellulose, previously washed with alkali and water, and conditioned with 0.05 M sodium phosphate, pH 7. After collecting the void volume enzyme was eluted with a linear gradient of 0 to 0.1 M NaCl in 0.05 M sodium phosphate, pH 7. Fractions comprising the enzyme peak were combined and concentrated by ultrafiltration on a Diaflo UM-10 membrane (Amicon). There was a 25% loss of enzyme activity during this step. The concentrated enzyme was twice dialyzed for 8 h against 100 volumes of 0.02 M potassium phosphate containing 0.1 mM $CoCl_2$ and stored frozen at −20°. The purified enzyme, 3.2 units, amounted to 58% of the activity present in the original suspension. Its activity was 1.9 units per mg protein, a 50-fold purification. The enzyme was free from phosphopyruvate hydratase, adenylate kinase, and NADH oxidase activities; it contained traces of pyruvate, phosphate dikinase and malate dehydrogenase activity.

The requirements for enzyme activity in the forward reaction were studied using enzyme which had been twice dialyzed for 8 h against 100 volumes of 0.02 M imidazole–HCl, pH 7, containing 10^{-4} M $CoCl_2$. For the complete reaction the cuvettes contained in a final volume of 0.4 ml: 4 μmoles of $MgCl_2$; 0.2 μmoles phosphoenolpyruvate; 4 μmoles potassium phosphate, pH 6.5; 0.1 μmole NADH; 0.04 μmole $CoCl_2$; 0.2 ml of 0.06 M $KHCO_3$ which had been freshly bubbled with CO_2; 0.8 unit of malate dehydrogenase; water; and 0.02 ml of dialyzed enzyme containing 2.4 μg protein. The results, expressed as μmole product/min per mg protein, were the following: complete system, 1.93; *minus* malate dehydrogenase, 0.08; *minus* $MgCl_2$,

0.43; *minus* phosphoenolpyruvate, 0.00; *minus* phosphate, 0.08; *minus* bicarbonate, 0.05; *minus* bicarbonate *plus* one unit lactate dehydrogenase (EC 1.1.1.27), 0.11; *minus* $CoCl_2$, 1.80; *minus* both $MgCl_2$ and $CoCl_2$, 0.08; *minus* phosphate *plus* 0.01 M arsenate, 0.91; and complete, but with enzyme which had been preincubated at 25° for 15 min with 1 mM β-mercaptoethanol, 1.25.

Requirements for enzyme activity in the reverse direction were studied using enzyme which had been dialyzed for 4 h against the imidazole buffer. Conditions for the back reaction were similar to those previously employed by DAVIS *et al.*[3]. Observed spectrophotometrically was the reduction of $NADP^+$ due to the production of phosphoenolpyruvate in the presence of glucose, Mg^{2+}, ADP, and the linking enzymes, pyruvate kinase (EC 2.7.1.40), hexokinase (EC 2.7.1.1), and glucose-6-P dehydrogenase (EC 1.1.1.49). For the complete reaction the cuvettes contained in a final volume of 0.4 ml: 20 mM Tris–HCl buffer, pH 7.4; 4 mM $MgCl_2$; 1 mM ADP; 1 mM glucose; 20 mM KCl; 0.3 mM NADP; 0.6 mM oxalocetate; 0.5 mM sodium pyrophosphate (PP_i); 0.1 mM $CoCl_2$; 0.5 unit of glucose-6-P dehydrogenase; 0.4 unit of hexokinase; 0.32 units of pyruvate kinase; and 0.01 ml of enzyme containing 2.4 μg protein. The results expressed as μmole product/min per mg protein were the following: Complete system, 0.57; *minus* oxaloacetate, 0.00; *minus* PP_i, 0.00; *minus* $CoCl_2$, 0.54 decreasing sharply with time; and *minus* pyruvate kinase, 0.08.

The results of a study on the stoichiometric balance in the phosphopyruvate carboxylase reaction are shown in Table I. Except for the method of assay of PP_i the procedures for this experiment were similar to those described by SIU AND WOOD[8].

TABLE I

STOICHIOMETRY OF THE PHOSPHOPYRUVATE CARBOXYLASE REACTION

In addition to the substances and concentrations shown in the Table the reaction mixture contained the following (in μmoles): $MgCl_2$, 48; $KHCO_3$, freshly bubbled with CO_2, 120 (22,280 counts/min per μmole); NADH, 1.2; also, 12.8 units of malate dehydrogenase and 0.2 ml of dialyzed amoebal enzyme containing 48 μg protein. The final volume was 4 ml. Incubation was for 40 min at 25°. Reaction was terminated by withdrawing samples into an equal volume of cold 10 percent trichloroacetic acid. Oxalacetate was determined by the change in optical density at 340 nm during the incubation period; bound $^{14}CO_2$, by acid stable counts fixed; pyruvate, by lactate dehydrogenase; phosphoenolpyruvate, following pyruvate by pyruvate kinase in the presence of KCl and ADP; P_i, by the method of FISKE AND SUBBAROW[13]; PP_i, by the method of REEVES AND MALIN[14]. All assays were made in duplicate. No pyruvate was found.

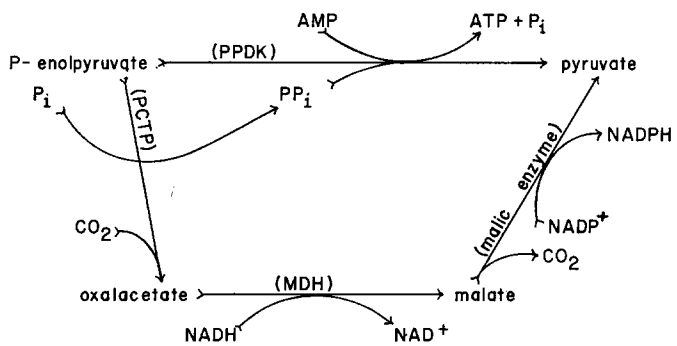
	Components measured (μmoles)				
	phospho enol-pyruvate	*P_i*	*$^{14}CO_2$ fixed*	*Oxaloacetate*	*PP_i*
Initial time	1.55	2.48	0.04	(0)	0.13
After 40 min	0.99	1.87	0.65	0.56	0.72
Net change	−0.56	−0.61	+0.61	+0.56	+0.59

The enzyme was dialyzed against a buffer containing 0.01 M potassium phosphate and 10^{-4} M $CoCl_2$. The reaction occurred in a stoppered quartz cuvette which was continuously monitored at 340 nm in a spectrophotometer. ^{14}C-Labelled $NaHCO_3$ was introduced at an arbitrary initial time and samples were withdrawn to acid for the initial assays. The reaction continued for 40 min after which samples were again withdrawn for assay. Oxaloacetate formation was calculated from the oxidation of

NADH which occurred in the presence of malate dehydrogenase during the observation period.

The amoebal phosphopyruvate carboxylase is stable in the presence of cobalt ions and may be readily purified. The reversible reaction catalyzed by the amoebal enzyme is identical to that catalyzed by the enzyme from propionibacteria, although minor differences in enzyme properties are indicated.

Anaerobic resting cells of *E. histolytica* at 37° consume glucose at the rate of approximately 1 μmole/min per ml of fresh cells. They produce acetate, ethanol, CO_2 and H_2 as end products[9]. Since the cells lack pyruvate kinase[10] some unusual step between phospho enolpyruvate and pyruvate is indicated to sustain the active glycolysis. The finding of phosphopyruvate carboxylase provides an interesting explanation for this. The enzyme pyruvate, phosphate dikinase is present in amoebae in amounts sufficient to convert P-enolpyruvate to pyruvate at the rate of 2 μmoles/min per ml fresh cells, at 25°. This reaction proceeds according to the following equation: phospho enolpyruvate + AMP + PP_i ⇌ pyruvate + ATP + P_i. The pyruvate, phosphate dikinase reaction requires inorganic pyrophosphate (PP_i) as substrate in the forward direction. On the other hand, the reaction catalyzed by phosphopyruvate carboxylase produces PP_i (and oxaloacetate) from phospho enolpyruvate, CO_2, and P_i. Its activity in the fresh cells could provide PP_i in excess of 2 μmoles/min per ml of cells, at 25°. These two enzymes functioning together produce pyruvate and oxaloacetate from glucose and CO_2. Oxaloacetate is converted to malate by the NAD-linked malate dehydrogenase, and malate to pyruvate and CO_2 by the NADP-linked malate dehydrogenase (decarboxylating) EC 1.1.1.40 (malic enzyme). The last two enzymes have been identified in amoebae[11] and are present at activities greater than those of pyruvate, phosphate dikinase and phosphopyruvate carboxylase (REEVES, unpublished observations). Together these four enzymes provide a system capable of converting phospho enolpyruvate to pyruvate by a branched pathway as is shown in the scheme below.



Scheme depicting the 4-enzyme step of amobal glycolysis. PPDK is pyruvate, phosphate dikinase; PCPT, phosphopyruvate carboxylase; and MDH, malate dehydrogenase.

The alcohol dehydrogenase in amoebae is an NADP-linked enzyme[12]. Ethanol production by this enzyme requires NADPH. The enzymes malate dehydrogenase and malic enzyme, when catalyzing the conversion of oxaloacetate to pyruvate also catalyze

the reaction: NADH + $NADP^+ \rightarrow NAD^+$ + NADPH. Pyruvate formed via the lower branch in the scheme results in the oxidation of NADH and the reduction of $NADP^+$.

In summary, evidence which compels serious consideration of a step involving four linked enzymes in amoebal glycolysis is the following: (1) The amoeba appears to lack pyruvate kinase. (2) The four enzymes are present in excess of the activity required. (3) The 4-enzyme scheme provides for maintenance of a P_i, PP_i balance in the organism. (4) The scheme provides a means for oxidizing NADH and makes NADPH available in quantity for the subsequent alcohol dehydrogenase step.

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