PHOSPHOGLYCERATE KINASE (GTP). AN ENZYME FROM ENTAMOEBA HISTOLYTICA SELECTIVE FOR GUANINE NUCLEOTIDES.

Richard E. Reeves and Dorothy J. South
Department of Biochemistry
Louisiana State University Medical Center
New Orleans, La. 70112

Received April 29,1974

SUMMARY

The phosphoglycerate kinase of Entamoeba histolytica shows a profound preference for guanine nucleotides over adenine nucleotides. The enzyme has been purified 60-fold and freed from interferring enzyme activities. In the forward (glycolytic) direction the selectivity for GDP over ADP is 150-fold, and selectivity for GTP over ATP is about 50-fold in the reverse reaction. The inosine nucleotides are intermediate between the guanosine and adenine nucleotides in both directions. In order to distinguish the amebal enzyme from the usual enzyme (EC 2.7.2.3) it is given the trivial name phosphoglycerate kinase (GTP).

The functioning of a phosphoglycerate kinase in one strain of Entamoeba histolytica was reported by Bragg and Reeves (1). Subsequent work in this laboratory demonstrated the enzyme in extracts from several other strains of the organism, but the amounts of activity found was always low. The assayed values in the forward reaction with ADP as phosphate acceptor were less than would be required to account for the known glycolytic flux of the organism. Further investigations revealed that guanine nucleotides elicited much more activity from the amebal enzyme than did the adenosine nucleotides. This nucleotide selectivity is unusual since the adenine nucleotides are the preferred substrates for this enzyme from all other investigated sources (2, 3). This report describes the partial purification of amebal phosphoglycerate kinase and the characterization of its nucleotide requirements.

MATERIALS AND METHODS

E. histolytica was grown with penicillin-inhibited cells of Bacteroides symbiosus as described by Reeves and Ward (4). Cells were harvested, washed by centrifugation and the supernatant fluid was with-

drawn. The pellet was frozen, lypophilized, sealed in glass, and stored at -20° until used.

Assay enzymes and substrates - Boehringer enzymes in ammonium sulfate suspension were centrifuged and the pellets were dissolved in 1 mM EDTA, pH 7. Aldolase and the mixture of glycerol-3-phosphate dehydrogenase-triosephosphate isomerase were further treated by dialysis overnight against the EDTA.

Stock solutions of nucleoside diphosphates were assayed by the extent of oxidation of DPNH in the presence of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. Solutions of nucleoside triphosphates, except ATP, were assayed by the extent of oxidation of DPNH in the presence of fructose 6-phosphate, 6-phosphofructokinase, aldolase, and a mixture of glycerol-3-phosphate dehydrogenase-triosephosphate isomerase. ATP was assayed by the reduction of TPN in the presence of glucose, hexokinase, and glucose-6-phosphate dehydrogenase.

Stock solutions of Sigma D, L-glyceraldehyde 3-phosphate were assayed for the D-isomer by the extent of oxidation of DPNH in the presence of the mixture of glycerol-3-phosphate dehydrogenase-triosephosphate isomerase. Solutions of Boehringer 3-phosphoglycerate were assayed for the D-isomer under the conditions of the standard reverse enzyme assay (see below), but with Boehringer phosphoglycerate kinase replacing the amebal enzyme and ATP substituted for the GTP.

Standard enzyme assays - All assays were made at 30° employing a millimolar extinction coefficient (340 nm) of 6.22 for DPNH and TPNH. Forward assays were conducted in 50 mM sodium α-glycerol phosphate, pH 7.0, since this buffer strongly inhibited the action of the triosephosphate isomerase present in crude enzyme preparations (5). Standard assays in this direction contained in addition 50 mM potassium phosphate, pH 7; 2.5 mM $MgCl_2$; 1 mM D-glyceraldehyde 3-phosphate; 0.25 mM DPN; 50 µg glyceraldehyde phosphate dehydrogenase; 1.25 mM DTT; 1.25 mM EDTA; 0.38 mM GDP; enzyme; and water to a final volume of 0.40 ml. After 5 minutes of thermal equilibration reaction was initiated by addition of the enzyme. The observed rate of DPN reduction was corrected for that occurring in a control curette which lacked enzyme.

In the reverse direction the cuvettes contained 50 mM imidazole-HCl, pH 7; 2 mM 3-phospho-D-glycerate; 0.125 mM DPNH; 0.5 mM GTP; 13 µg of the dialyzed mixture of glycerol-3-phosphate dehydrogenase-triosephosphate isomerase; $MgCl_2$, DTT, glyceraldehyde-phosphate dehydrogenase, and EDTA as in the forward reaction; enzyme; and water to a final volume of 0.40 ml. After thermal equilibration reaction was initiated by addition of the enzyme and the results were corrected for any reaction noted in the absence of added enzyme.

A unit of enzyme activity is defined as that amount which produces one micromol/min of 3-phosphoglycerate under the conditions of the standard forward assay. Specific activity is units of enzyme per mg protein. Protein was determined by the method of Lowry et al (6).

RESULTS AND DISCUSSION

Preparation of amebal phosphoglycerate kinase - Lyophilized powder representing 4 g of amebal cells was suspended in 16 ml of cold 20 mM α-glycerol phosphate, pH 7.0, containing 2 mM DTT. This suspension was

centrifuged for 30 min at 36,000 g in the cold. The supernatant solution was placed on a column containing 580 ml of Biogel P-300 resin previously equilibrated with 20 mM imidazole-HCl, pH 7, and eluted with the same buffer. Fractions containing the enzyme were combined and applied to an 18 x 240 mm column of DEAE cellulose previously equilibrated with the same buffer. Elution was by means of a linear gradient of 0-0.2 M NaCl in the imidazole buffer and 17 ml fractions were collected. Enzyme elution began as soon as the gradient entered the column. The first two enzyme fractions were free from triosephosphate isomerase. They were combined and reserved for the subsequent kinetic studies. Assays at various stages of the above preparation are given in Table 1.

Table I

Purification of phosphoglycerate kinase from 4 g lyophilized amebae.

Treatment	PGK, units	Protein mg	Sp. Act.
Supernatant	167	182	0.86
Biogel column, combined fractions	139	29	4.7
DEAE column, combined fractions	40	0.77	52
DEAE column, other fractions	54	-	-

The combined fractions were free from detectable amounts of triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate mutase (<0.5%). Upon concentration by vacuum dialysis against 20 mM imidazole buffer, pH 7, a loss of about 40% of enzyme activity occurred. The concentrated enzyme solution was stored at 4° and lost about 10% of its activity per week during storage.

Nucleotide substrates - Employing the conditions for the standard forward or reverse enzyme assays, except for nucleotide concentrations, the apparent K_m and V_{max} values were determined for the purine nucleo-

Table II

Apparent K_m and V_{max} values for amebal phosphoglycerate kinase. Enzyme was purified through the DEAE cellulose column and concentrated by vacuum dialysis.

Substrate	K_m, mM	V_{max}, $\frac{\mu mol}{min \times mg\ enzyme}$	$\frac{V_{max}}{K_m}$
GDP	0.3	45	150
IDP	0.7	41	58
ADP[a]	10	10	1
GTP	0.25	14	56
ITP	0.8	13	16
ATP	1.2	1.5	1
3-Phosphoglycerate	0.4[b]	-	-

a.) Ten mM $MgCl_2$ was employed in this determination.

b.) The fixed substrate was 0.38 mM GTP.

tides. These values are listed in Table II. The last column of the table lists values for the ratio, V_{max}/K_m. The uracil and cytosine nucleotides were less than 0.1% as effective as substrates as the corresponding guanine nucleotides and results with these pyrimidine nucleotides are not included in the table.

As indicated by the V_{max}/K_m ratio GDP is 150-fold more effective than ADP as substrate for the amebal enzyme in the forward reaction. In the reverse reaction the preference for GTP over ATP was also pronounced. In either direction the inosine nucleotides were intermediate between the guanine and adenine nucleotides. The preference for guanine nucleotides suggests that the amebal phosphoglycerate kinase functions, *in vivo*, to produce GTP rather than ATP. In support of this idea evidence will be

presented elsewhere indicating that ATP comprises no more than 50% of the total nucleotide triphosphate pool in E. histolytica.

Because of its unusual nucleotide specificity we hesitate to classify the amebal phosphoglycerate kinase as EC.2.7.2.3. To distinguish it from the usual enzyme we suggest the trivial name phosphoglycerate kinase (GTP).

ACKNOWLEDMENTS

This work was supported, in part, by grants AI-02951, AI-10788, and GM-14023 from the National Institutes of Health.

REFERENCES

1. Bragg, P.D. and Reeves, R.E. (1962) Exptl. Parasitol. 12, 393-400.
2. Krietsch, W.K.G. and Bücher, T. (1970) Eur. J. Biochem. 17, 568-580.
3. Scopes, R.L. in The Enzymes, 3rd Ed. (P. Boyer, ed.) Vol. 8, p 335-351. Academic Press (1973).
4. Reeves, R.E. and Ward, A.B. (1965) J. Parasitol. 51, 321-324.
5. Johnson, L.N. and Wolfenden, R. (1970) J. Molec. Biol. 47, 93-100.
6. Lowry, O.H., Rosebrough, N.J., Farr, L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.