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PURIFICATION AND PROPERTIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM THE THERMOPHILIC FUNGUS *PENICILLIUM DUPONTII*

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SUMMARY

1. D-Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was purified 565-fold from the thermophilic fungus *Penicillium dupontii*.

2. The enzyme exhibited sigmoidal saturation curves both with glucose 6-phosphate and $NADP^+$. Double reciprocal plots of these data produced parabolic curves. From Hill plots n values of 1.61 for glucose 6-phosphate and 1.08 for $NADP^+$ were calculated. Co-operative interaction between substrate and enzyme was therefore demonstrated.

3. Mg^{2+} activated the enzyme at concentrations of less than 10 mM and inhibited the enzyme at concentrations greater than 10 mM.

4. NADPH inhibited the enzyme competitively.

5. The energy of activation of the enzyme was calculated to be 5.5 kcal/mole.

6. No unusual thermostability properties appear to be associated with this enzyme.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADPH oxidoreductase, EC 1.1.1.49) has been isolated from a wide range of sources including mammalian tissues[1–7], fish[8], bacteria[9–12], yeast[13,14], molds[15–18] and plants[19].

This investigation of glucose-6-phosphate dehydrogenase from the thermophilic fungus *Penicillium dupontii* forms part of a general comparison of the properties of enzymes of thermophilic fungi with enzymes isolated from mesophiles.

Glucose-6-phosphate dehydrogenase is ideally situated for regulation of the pentose phosphate pathway since it is the first enzyme on this pathway. The factors affecting the regulation of this enzyme would be of considerable importance in maintaining an adequate pool of the important biosynthetic cofactor NADPH (ref. 17).

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This communication demonstrates that glucose-6-phosphate dehydrogenase from *P. dupontii* exhibits sigmoidal saturation curves with both glucose 6-phosphate and the coenzyme $NADP^+$. Thus co-operative interaction is occurring between substrate and enzyme indicating that glucose-6-phosphate dehydrogenase from *P. dupontii* behaves as an allosteric enzyme.

MATERIALS AND METHODS

Materials

$NADP^+$, NAD^+, NADPH, ATP and Fru-1,6-P_2 were obtained from Sigma Chemical Co., St. Louis, Mo. Yeast glucose-6-phosphate dehydrogenase was obtained from Boehringer and Soehne, Mannheim, Germany.

Cells

The thermophilic fungi examined for glucose-6-phosphate dehydrogenase were *Penicillium dupontii*, *Malbranchae pulchella* var. *sulfurea*, *Chaetomium thermophile* var. *coprophile* and *Humicola lanuginosa*. These cultures were obtained from Dr. G. M. Gaucher, Chemistry Department, University of Calgary, Alberta, Canada, and are derived from cultures isolated by Dr. R. Emerson, Botany Department, University of California, Berkeley, San Francisco, Calif. *P. dupontii* was selected as the source of glucose-6-phosphate dehydrogenase since it produced more activity than any of the other organisms tested.

Culture conditions and preparation of crude cell-free extract

Cultures of *P. dupontii* were prepared by a modification of the method of GAUCHER AND SHEPHERD[20]. Czapek–Dox agar slants of the organism were maintained at 45° for approx. 10 days. Spores from a single agar slant were suspended in 5 ml of a 500 parts per million Tween 80 (Atlas Powder Co., Delaware) solution. This spore suspension was used to inoculate a 2-l erlenmeyer flask containing 600 ml of modified Czapek–Dox liquid medium.

The 2-l erlenmeyer flask was shaken at 45° on a New Brunswick gyrotory shaker (250 rev./min). After 48 h this culture was used to inoculate a 14-l New Brunswick Microferm fermentor jar containing 8 l of modified Czapek–Dox liquid medium. This culture was then grown at 45° with an aeration rate of 5 l/min and a stirring rate of 600 rev./min for 40 h.

The cells were harvested by filtration and washed twice with distilled water. The mycelial mat was resuspended in 5 vols. of ice-cold 0.10 M Tris–HCl (pH 7.4)–0.01 M $MgCl_2$ buffer. This suspension was sonicated for 20 min in an MSE 100-W ultrasonic disintegrator and centrifuged at 27 000 $\times g$ for 20 min in a Sorvall RC2-B centrifuge. The 27 000 $\times g$ supernatant constituted the crude cell-free extract.

Enzyme assays

Enzymatic assays were performed at 25° by a modification of the method of COHEN AND ROSEMEYER[3]. The reduction of $NADP^+$ was measured spectrophotometrically at 340 mμ in quartz cuvettes of 1 cm light path using a Gilford Model 2000 continuous recording spectrophotometer with a Beckman DUR monochromator. The recorder was adjusted to give a full scale deflection equivalent to 0.2 absorbance

units at 340 mμ. The standard assay mixture (final volume 3.0 ml) contained 0.67 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 94 mM Tris–HCl (pH 7.4)–9.4 mM $MgCl_2$ and a suitable dilution of enzyme. The reaction was initiated by the addition of $NADP^+$. Controls without glucose 6-phosphate and controls without $NADP^+$ were routinely performed. They consistently showed no activity.

Preparation of the enzyme

Glucose-6-phosphate dehydrogenase was purified from the crude cell-free extract by modification of the method of BRODY AND TATUM[17].

A solution of 1 M $MnCl_2$ adjusted to pH 6.7 with NaOH, was added to the crude cell-free extract to a final concentration of 16.7 mM $MnCl_2$. The mixture was stirred for 10 min before centrifuging at 10 000 × *g* for 10 min. The supernatant was adjusted to 45% satn. $(NH_4)_2SO_4$ and centrifuged. The pellet was discarded. The $(NH_4)_2SO_4$ content of the supernatant was increased to 60% satn. and the pellet formed after centrifuging was dissolved in 10 ml of 0.01 M Tris–HCl (pH 7.4)–1 mM $MgCl_2$. This solution was desalted by centrifuging through 7 cm × 2 cm columns of coarse Sephadex G-25 (Pharmacia, Uppsala, Sweden), using specially adapted centrifuge tubes.

The desalted preparation was stirred for 10 min with calcium phosphate gel prepared by the method of KEILIN AND HARTREE[21]. At a gel to protein ratio of 3:1 by weight, the enzyme was completely adsorbed. More than 90% of the enzyme was eluted from the gel with 50 ml of 0.01 M Tris–HCl (pH 7.4)–1 mM $MgCl_2$ containing 8% $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ content of the eluate was raised to 50% satn. and the mixture was stirred for 10 min before centrifuging. The resulting supernatant was adjusted to 60% satn. by the addition of solid $(NH_4)_2SO_4$. The precipitate collected by centrifugation was dissolved in 5 ml of 0.01 M Tris–HCl (pH 7.4)–1 mM $MgCl_2$ and was desalted as previously described. This desalted preparation was applied to a 22 cm × 1 cm column of DEAE-cellulose, Cellex-D with an exchange capacity of 0.72 mequiv/g, (Bio-Rad Laboratories, Richmond, Calif.). The DEAE-cellulose was pre-treated with 0.1 M HCl and then with 0.1 M NaOH followed by exhaustive washing and equilibration with 0.01 M Tris–HCl (pH 7.4)–1 mM $MgCl_2$. The column was eluted with a linear gradient of 0–0.25 M NaCl in 0.01 M Tris–HCl (pH 7.4)–1 mM $MgCl_2$ (200 ml per bottle). The flow rate was 30 ml/h. All operations were performed at 4°.

Polyacrylamide gel electrophoresis

This was performed by a modification of the method of ORNSTEIN AND DAVIS[22]. The samples were applied in 2% sucrose without a sample gel; 7% running gels were used throughout. Electrophoresis was performed at pH 9.5. The protein was stained with Amido Schwarz.

Protein estimation

Protein was measured by a modification of the method of LOWRY *et al.*[23] where sodium citrate was used instead of sodium tartrate[24].

RESULTS

Purification of the enzyme

Table I shows that glucose-6-phosphate dehydrogenase was purified 565-fold

TABLE I

PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Fraction	*Vol. (ml)*	*Units/ml**	*Total units*	*Protein/ml (ml)*	*Total protein (ml)*	*Spec. act.*	*Purification*	*Yield (%)*
Crude sonicate	230	4.42	1020	3.95	910.0	1.12	—	100
$MnCl_2$ supernatant	230	3.15	725	2.90	668.0	1.09	—	71
45% $(NH_4)_2SO_4$ supernatant	230	3.15	725	2.00	460.0	1.58	1.375 ×	71
60% $(NH_4)_2SO_4$ pellet	4.5	96.5	435	10.8	48.5	8.95	7.8 ×	42.6
8% $(NH_4)_2SO_4$ eluate from $Ca_3(PO_4)_2$ gel	19.5	11.0	233	4.66	21.0	11.1	9.9 ×	22.8
Desalted 60% $(NH_4)_2SO_4$ pellet of 8% eluate	4.5	51.4	233	4.22	19.0	12.2	10.9 ×	22.8
Combined DEAE fractions	50		196		0.31	632.0	565.0 ×	19.2

* 1 unit = 1 μmole NADP hydrolysed/min per mg protein at 25°.

with a 19.2% recovery. Purified enzyme was compared with commercially purified yeast glucose-6-phosphate dehydrogenase by polyacrylamide gel electrophoresis. Three bands were present in both preparations. The slowest-moving band of the yeast enzyme migrated faster than the corresponding band in our preparation.

Stability of the enzyme

The purified enzyme was found to be unstable both at 4° and −85° and lost its total activity in 3 days. The following reagents did not impart any stability to the enzyme: $NADP^+$ (10 μM), NADPH (10 μM) bovine serum albumin (1%), sucrose (2%), glycerol (1%), tripalmitin (0.1%), 2-mercaptoethanol (5 mM), dithiothreitol (1 mM), $(NH_4)_2SO_4$ (2% and 50%), NaCl (1%), $MgSO_4$ (1%). In the presence of 1 mM EDTA however, approx. 10% of the original activity was retained after 3 days at 4°. The rate of decay was increased at temperatures above 4° and after heating at 55° for 5 min there was a loss of 55% of the activity. When the enzyme was heated at 70° for 5 min no activity remained.

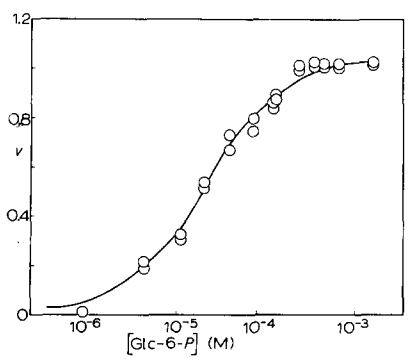


Fig. 1. Saturation curve for glucose-6-phosphate dehydrogenase with glucose 6-phosphate. Initial velocity v = μmoles NADPH formed per min per mg protein.

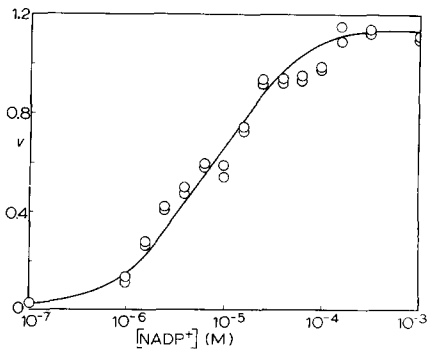
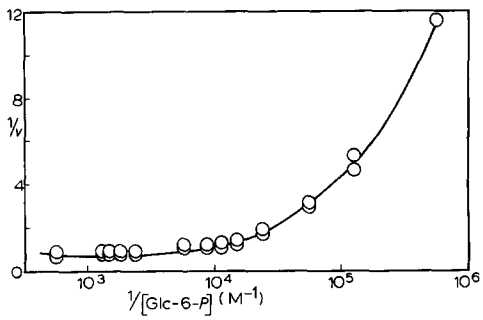


Fig. 2. Saturation curve for glucose-6-phosphate dehydrogenase with $NADP^+$. Initial velocity v = μmoles NADPH formed per min per mg protein.



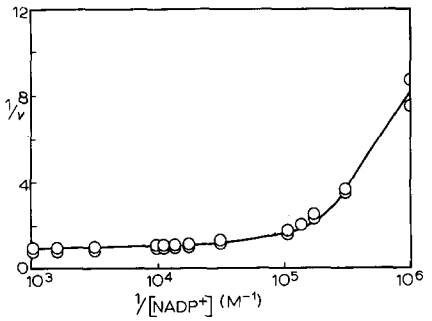


Fig. 3. Double reciprocal saturation curve for glucose-6-phosphate dehydrogenase with glucose 6-phosphate. Initial velocity v = μmoles NADPH formed per min per mg protein.

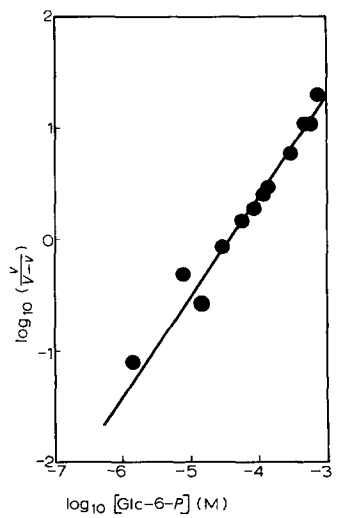
Fig. 4. Double reciprocal saturation curve for glucose-6-phosphate dehydrogenase with NADP+. Initial velocity v = μmoles NADPH formed per min per mg protein.

Kinetic studies

All kinetic data were obtained using the purified enzyme. Figs. 1 and 2 show the initial velocity of reaction plotted against substrate (glucose 6-phosphate) and coenzyme (NADP+) concentrations, respectively. Sigmoidal saturation curves were produced in both cases.

Double reciprocal plots of the same data are shown in Figs. 3 and 4 for substrate and coenzyme, respectively. Parabolic curves were produced in both instances.

From Hill plots of the above data, the values n = 1.61 and $[S_{0.5}]$ = 37 μM were calculated for glucose 6-phosphate (Fig. 5) and n = 1.08 and $[S_{0.5}]$ = 6 μM for NADP+ (Fig. 6).



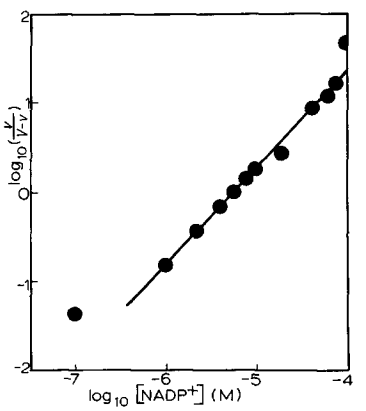


Fig. 5. Hill plot of the saturation curve of glucose-6-phosphate dehydrogenase with glucose 6-phosphate. V = 1.186; $[S_{0.5}]$ = 37 μM; n = 1.61. Initial velocity v = μmoles NADPH formed per min per mg protein.

Fig. 6. Hill plot of the saturation curve of glucose-6-phosphate dehydrogenase with NADP+. V = 1.0; $[S_{0.5}]$ = 6 μM; n = 1.08. Initial velocity v = μmoles NADPH formed per min per mg protein.

These results indicate that co-operative interaction between the binding sites for both substrate and coenzyme occurs and suggests that glucose 6-phosphate dehydrogenase may be an allosteric enzyme.

Effect of Mg^{2+}

The effect of Mg^{2+} on the activity of glucose-6-phosphate dehydrogenase was examined at two different concentrations of glucose 6-phosphate and $NADP^+$. The enzyme was activated at concentrations of Mg^{2+} less than approx. 10 mM and inhibited at concentrations greater than approx. 10 mM. The fact that the inhibition of activity is more marked with non-saturating concentrations of glucose 6-phosphate, suggests that magnesium ions inhibit binding of the substrate to the enzyme.

Inhibition of activity by NADPH

Inhibition of glucose-6-phosphate dehydrogenase by NADPH (45.6 mM final concn.) was competitive with $NADP^+$. No inhibition of the enzyme by NADPH was demonstrated in the presence of saturating concentrations of $NADP^+$ and variable concentrations of glucose 6-phosphate.

Energy of activation of the enzyme

The energy of activation, calculated from an Arrhenius plot, was 5.5 kcal/mole over the temperature range 25–60°. In calculating this value, it was assumed that the concentrations of substrate and coenzyme which were saturating at 25° were also saturating at 60°.

Coenzyme specificity of the enzyme

In the presence of saturating concentrations of substrate, the enzyme is highly specific for $NADP^+$. With 0.67 mM glucose 6-phosphate, NAD^+ at final concentrations of 0.3 mM–3 μM, produced less than 1% of the activity obtained from $NADP^+$ at a concentration of 0.5 mM.

Effect of ATP and Fru-1,6-P_2

In Hydrogenomonas[12] and groundling fish embryos[8] ATP inhibits glucose 6-phosphate dehydrogenase whereas Fru-1,6-P_2 activates the enzyme[8]. We performed preliminary studies with both compounds at saturating (0.67 mM) and non-saturating (26.6 μM) concentrations of glucose 6-phosphate in conditions otherwise similar to those in the standard assay. Neither ATP nor Fru-1,6-P_2 at final concentrations ranging from 1 to 10 μM, produced any alteration of the reaction velocity. Further work with both compounds at low concentrations of glucose 6-phosphate will be necessary to elucidate their effects on the sigmoidicity of the saturation curve of enzyme with substrate.

DISCUSSION

This appears to be the first reported isolation of glucose-6-phosphate dehydrogenase from a thermophilic fungus.

The saturation curves of glucose-6-phosphate dehydrogenase are sigmoidal and the resulting Lineweaver–Burk plots are non-linear. These data, which suggest more

than one binding site for both $NADP^+$ and glucose 6-phosphate, are characteristic of the kinetics of numerous regulatory enzymes. Sigmoidal saturation curves have been reported for glucose-6-phosphate dehydrogenase isolated from human erythrocytes[25], lactating rat mammary gland[7] and Hydrogenomonas[12].

The effects of Mg^{2+} on this enzyme are in agreement with those reported in other systems. Increasing concentrations of Mg^{2+} produced activation and then inhibition of the yeast enzyme[13] and of highly purified human erythrocyte enzyme[3]. Only slight activation was observed with the enzymes from *Aspergillus niger*[15] and *Leuconostoc mesenteroides*[9].

Inhibition of glucose-6-phosphate dehydrogenase with NADPH has now been shown to be competitive in *P. dupontii*, yeast[13], Hydrogenomonas[12] and lactating rat mammary gland[7]. Both competitive and non-competitive inhibition by NADPH was reported for the human erythrocyte[2] enzyme.

LEVY *et al.*[7] have reported that glucose-6-phosphate dehydrogenase of rat mammary gland, when activated with NAD^+, shows a sigmoidal saturation curve in the presence of NADPH.

Yeast glucose-6-phosphate dehydrogenase[13] was found to have an apparent activation energy of 7.1 kcal/mole whereas in *P. dupontii* the activation energy was only 5.5 kcal/mole. This finding may be significant but it remains to be seen what the activation energy is in other closely related mesophilic fungi. It is interesting to note that *P. dupontii* glucose-6-phosphate dehydrogenase does not appear to have any extra thermostability when compared with the enzyme from *Neurospora crassa*[17]. This is in contrast to the situation observed for thermophilic bacteria[26].

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