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STUDIES ON A PYROPHOSPHATASE AND GLUCOSE-6-PHOSPHATASE FROM *ASPERGILLUS ORYZAE*

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SUMMARY

An inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) isolated from *Aspergillus oryzae* is associated with a glucose-6-phosphatase (glucose 6-phosphate phosphohydrolase, EC 3.1.3.9). It is also associated with a pyrophosphate–glucose phosphotransferase. These enzyme activities are heat stable, have acidic pH optima, and require no metallic ions for activity. Studies, including the determination of kinetic parameters, indicate that these catalytic activities are due to a single enzyme. This enzyme has a mol. wt of about 60 800 as determined by gel filtration. The enzyme also has low levels of acid phosphatase activity. *A. oryzae* possesses a second inorganic pyrophosphatase which has a low pH optimum and could be separated from the other catalytic activities.

INTRODUCTION

The inorganic pyrophosphatase of rat liver has been shown to be identical with glucose-6-phosphatase and pyrophosphate–glucose phosphotransferase[1,2]. This enzyme has been found in microsomal fractions of kidney, pancreas, and intestinal mucosa as well as the liver of rat and many other mammals[3–5]. Purification of the enzyme has been hindered by its apparent integral association with the membrane of the endoplasmic reticulum. A review by Nordlie[5] describes many of the studies performed on detergent-solubilized preparations of the enzyme, including the responses of catalytic activity to hormonal and dietary factors *in vivo*.

The three enzymatic activities are present in crude extracts of the mold *Aspergillus oryzae*[6]. This report describes the purification of the enzyme, the investigation of some of its properties, and the demonstration that the three catalytic activities are due to a single enzyme.

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METHODS

Purification of enzyme

The crude enzyme preparation was obtained as a powder resulting from an acetone precipitation of *A. oryzae*. This is sold commercially as pyrophosphatase (Calbiochem, catalog number 5503). 50 g of the powder (5.5 g protein) was dissolved in 400 ml of 0.2 M acetate buffer (pH 4.5) which contained 0.1 M pyrophosphate. This mixture was heated at 60 °C for 30 min, then cooled. Precipitated material was centrifuged at 10 000 × *g* at 0 °C. The supernatant was dialyzed against three 2-l portions of 0.02 M acetate buffer, pH 4.5. An equal volume of precooled acetone was added at −10 °C. After standing for 10 min the solution was centrifuged at 10 000 × *g* at 0 °C. The residue was redissolved in 0.02 M acetate buffer, pH 4.5. The protein concentration was 20–25 mg/ml.

The enzyme was eluted from a column of CM-cellulose (Bio Rad Cellex CM) with a linear 0–1 M NaCl gradient in 0.02 M acetate buffer, pH 4.5. Column dimensions were 1.5 cm × 60 cm. 5-ml samples were collected at 15 °C at flow rates of between 75–100 ml/h. Tubes exhibiting the enzyme activity were pooled. This fraction was then washed with distilled water and concentrated at 0 °C in a 50-ml ultrafiltration cell (Amicon) fitted with a UM 10 membrane. Sephadex G-100 chromatography was then performed in columns of 1.5 cm × 40 cm. Protein was eluted with 0.05 M acetate buffer, pH 4.5. The chromatography was run as described for the cellulose column except that the flow rates were 15–25 ml/h. A second CM-cellulose chromatography was run in a column of 1.5 cm × 20 cm. The procedure was the same as followed for the first CM-cellulose column. Total yield of enzyme was 4 mg. See Table I for summary of purification.

Disc gel electrophoresis

Gel electrophoresis was carried out at pH 9.5 at 0 °C. Gels were stained for protein with aniline black dye. The gels were stained for enzyme activity by locating the inorganic phosphate released after incubating the gel in 10 mM substrate for

TABLE I

SPECIFIC ACTIVITIES OF ENZYME AT VARIOUS STAGES OF PURIFICATION

Enzymatic activity was tested at each step of purification. Specific activities are in units/mg protein. One unit of enzymatic activity is defined in Methods.

Fraction	*Pyrophosphatase pH 6.1*		*glucose-6-phosphatase*		*Pyrophosphate-glucose phosphotransferase*	
	Activity	*Yield (%)*	*Activity*	*Yield (%)*	*Activity*	*Yield (%)*
Original (dialyzed)	0.17	—	0.14	—	0.004	—
Heated 60 °C 30 min	0.62	95	0.51	89	0.010	92
Acetone fractionation	2.75	77	1.75	60	0.074	66
CM-cellulose chromatography	7.8	39	6.12	35	0.262	30
Sephadex chromatography	12.4	25	7.88	23	0.348	22
Second CM-cellulose chromatography	13.1	15	9.54	13	0.381	12

30 min at 37 °C. The inorganic phosphate was observed by treating the incubated gel with a solution of triethylamine molybdate[7].

Assays of enzymatic activity

Inorganic pyrophosphatase was assayed in a mixture containing 2.0 ml of 0.2 M glycine–HCl buffer, pH 3.0, or 2.0 ml of 0.2 M Tris–HCl buffer, pH 6.1, 1.0 ml of 0.01 M $Na_4P_2O_7 \cdot 10\ H_2O$, enzyme, and distilled water to a volume of 4.4 ml. The reaction was initiated by the addition of the enzyme. After incubation at 37 °C the reaction was terminated by adding 0.4 ml of acid molybdate reagent consisting of 2.5 M H_2SO_4 and 3.0% molybdate. This addition, which brought the concentration of molybdate to 0.202 M, was found to stop the reaction as effectively as using trichloroacetic acid. The phosphate released was determined by a modification[8] of the method of Fiske and SubbaRow. Reducing agent was the only addition required for color development because of the previous addition of acid molybdate reagent. Glucose-6-phosphatase was assayed by the same method except that the buffer used was 0.2 M Tris–HCl buffer, pH 5.7, and the substrate was 1.0 ml of 0.01 M glucose 6-phosphate. A unit of pyrophosphatase or glucose-6-phosphatase activity was defined as that amount of enzyme catalyzing the hydrolysis of 1 μmole of substrate per min.

Phosphotransferase assays were carried out as described by Nordlie and Arion[1] except that the buffer used was 0.6 ml of 0.2 M Tris–maleate–NaOH, pH 6.9. The assay mixture contained 20 mM pyrophosphate and 525 mM glucose. Glucose 6-phosphate formed was thus measured by observing the formation of NADPH using glucose 6-phosphate dehydrogenase. A unit of phosphotransferase activity was defined as that amount of enzyme which produced 1 μmole of glucose 6-phosphate per min. All enzyme assays were run in duplicate and a blank, in which enzyme was added after the reaction was stopped, was run with each determination.

Determination of protein

Protein was measured as described by Lowry *et al.*[9] using crystalline bovine serum albumin (Pentex) as standard. Eluates from columns were scanned for protein by measuring the absorption at 280 nm.

RESULTS

pH optimum

The effect of pH on enzyme activity is illustrated in Fig. 1. Pyrophosphatase has two pH optima, one at pH 3.0, the other at pH 6.1–6.2. All enzyme preparations that had pyrophosphatase activity at pH 6.1 also had some activity at pH 3.0. The pH optimum for glucose-6-phosphatase is 5.7 and for phosphotransferase is 6.9. All the enzyme activities had acidic pH optima. *A. oryzae* grows best in acidic media; therefore, it is not unexpected to find enzymes with acidic pH optima.

Stability of enzyme

Stability studies as a function of pH at 20 °C and 60 °C indicated that the enzyme was particularly stable at pH 4.5. The effect of heating at 60 °C and 65 °C on pyrophosphatase, glucose-6-phosphatase and phosphotransferase is presented in

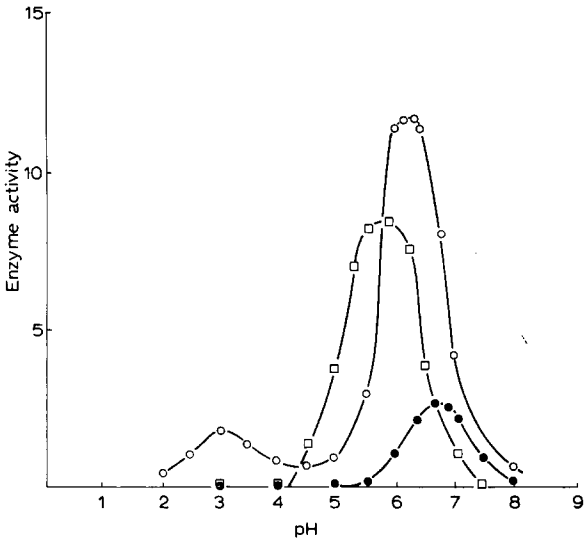

Fig. 1. pH–activity profile of enzymatic activity. The buffers employed were glycine–HCl, pH 2.2–3.6; acetate, pH 3.8–5.5; Tris–maleate–NaOH, pH 5.5–8.5; glycine–NaOH, pH 8.6–10.5. The concentration of each of these buffers in the incubation mixture was 90 mM. The pyrophosphatase (○—○) and glucose-6-phosphatase (□—□) assays involved 5-min incubations using 0.015 mg purified enzyme. The pyrophosphate-glucose phosphotransferase (●—●) assays involved 5-min incubations using 0.150 mg of the purified enzyme.

Fig. 2. The three enzyme activities are denatured at a relatively similar rate. Approximately 50–55% of each of the enzymatic activities remains after heating at 60 °C for 30 min. No substrate was added during heating in these experiments. The inclusion of substrate (0.1 M pyrophosphate) could prevent the denaturation of 90–95% of the three catalytic activities when crude extracts of the mold were heated at 60 °C

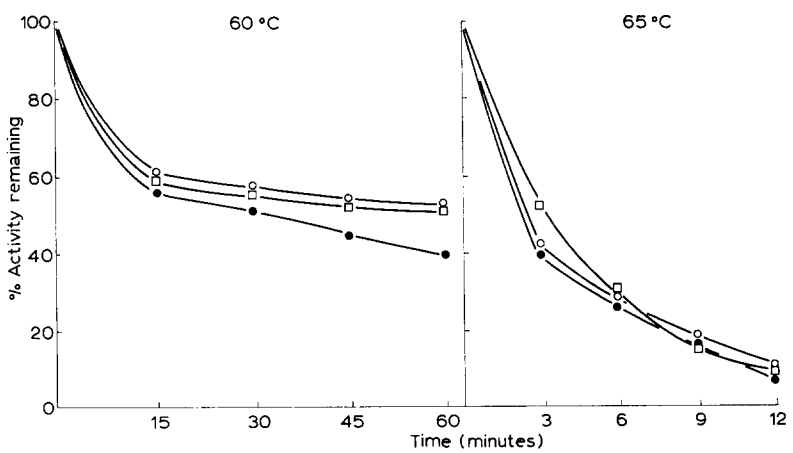

Fig. 2. Effect of heating on enzymatic activity. 2 ml of crude dialyzed enzyme (12 mg protein) in 0.02 M acetate buffer, pH 4.5, were heated in a hot water bath at 60 °C or 65 °C for the time indicated. Activity was measured on aliquots of the enzyme for pyrophosphatase (○—○), glucose-6-phosphatase (●—●), and phosphotransferase (□—□).

for 30 min. This stability is in contrast to the effect of heat on the mammalian enzyme, which exhibits marked heat lability[1].

Kinetic parameters

The kinetic constants for the enzyme are listed in Table II. The apparent K_m for pyrophosphate in the hydrolase reaction at pH 6.1 is 0.20 mM and in the transferase reaction at pH 6.9 is 1.78 mM. These two constants should be approximately equal if the two reactions share a common mechanistic pathway. The discrepancy

TABLE II

KINETIC CONSTANTS OF PYROPHOSPHATASE, GLUCOSE-6-PHOSPHATASE AND PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE OF *Aspergillus oryzae*

Kinetic constants were calculated from data of Fig. 3 and other double reciprocal plots of initial velocity *versus* the concentration of substrate.

Enzymatic activity	*Apparent Michaelis constant (mM)*	*Apparent inhibitor constant (mM)*
Pyrophosphate hydrolysis (pH 6.1)	0.20	—
Pyrophosphate–glucose phosphotransferase (pyrophosphate-variable substrate)	1.78	—
Pyrophosphate–glucose phosphotransferase (glucose-variable substrate)	456	—
Glucose 6-phosphate hydrolysis	0.51	—
Glucose 6-phosphate as inhibitor of pyrophosphatase (pH 6.1)	—	0.62
Glucose as inhibitor of pyrophosphatase (pH 6.1)	—	690

may be accounted for by the fact that each K_m measurement was determined at a different pH. An experiment was carried out to determine the K_m for pyrophosphate in the hydrolase reaction at pH 6.9. The K_m obtained was 1.32 mM. Another parameter determined was the K_m for pyrophosphate in the hydrolase reaction at pH 3.0, which was found to be 0.55 mM.

The effect of the presence of glucose 6-phosphate or glucose on pyrophosphatase is shown in Fig. 3. Glucose 6-phosphate is a competitive inhibitor and glucose is a non-competitive inhibitor of pyrophosphatase. These findings are in accordance with the proposal that the three catalytic activities are due to a common enzyme. The K_i for glucose 6-phosphate (0.62 mM) is near the observed K_m of glucose 6-phosphate (0.51 mM). The K_i for glucose as an inhibitor of pyrophosphatase at pH 6.1 was found to be 0.690 M. The apparent K_m for glucose in the phosphotransferase reaction, which was assayed at pH 6.9 was 0.456 M.

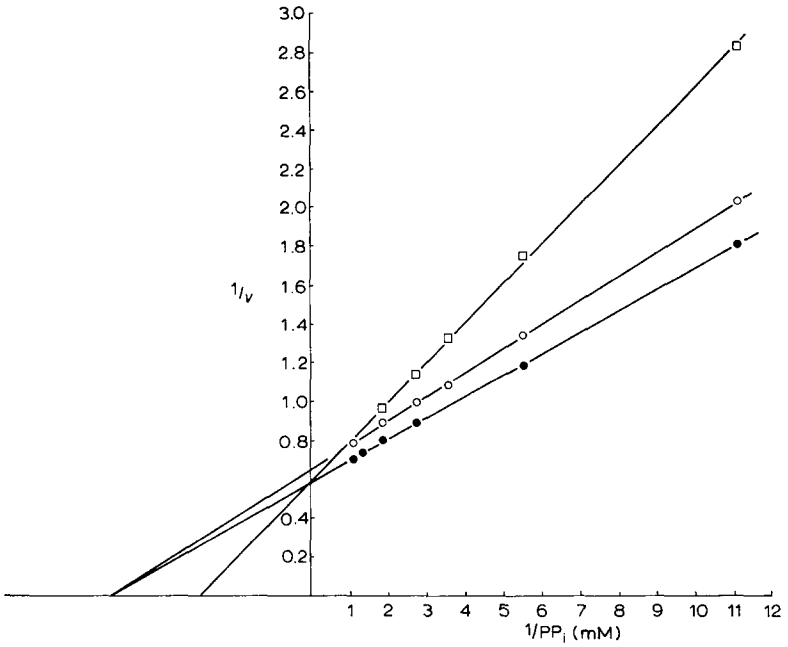

Fig. 3. Effect of pyrophosphate concentration on pyrophosphate hydrolysis. In normal assay mixture (●—●) and in the presence of 92 mM glucose (○—○), $v = 1/2$ (μmoles phosphate consumed per min). In the presence of 2 μmoles glucose 6-phosphate (□—□) $v = 1/2$ (μmoles phosphate produced − μmoles glucose 6-phosphate hydrolyzed). Glucose 6-phosphate consumed was determined by assaying the incubation mixture at the end of the experiment with glucose 6-phosphate dehydrogenase.

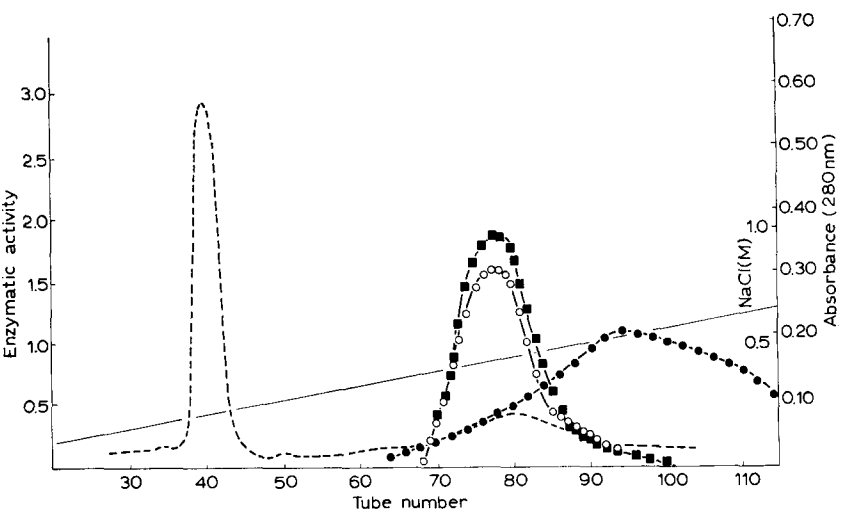

Fig. 4. CM-cellulose chromatography of acetone-precipitated material. Protein (— — —) was followed by measuring the absorbance of column eluates at 280 nm. Pyrophosphatase at pH 6.1 (■—■), pyrophosphatase at pH 3.0 (●—●) and glucose-6-phosphatase (○—○) activities in μmoles substrate consumed per min were measured on 0.2-ml aliquots of each tube. The column (1.5 cm × 60 cm) was equilibrated with 0.02 M acetate buffer, pH 4.5, and eluted with a linear NaCl gradient. Tubes 70–82 were pooled and rechromatographed as described in Fig. 5.

Chromatography

Elution of the enzyme from CM-cellulose is illustrated in Fig. 4. Phosphotransferase and pyrophosphatase at pH 6.1 eluted in the same fractions as glucose-6-phosphatase. This catalytically active peak was rechromatographed on Sephadex G-100 (Fig. 5). Again the three enzymatic activities remained together. Pyrophos-

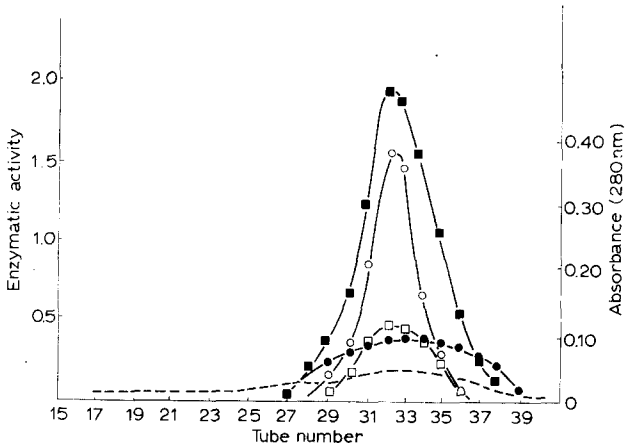

Fig. 5. Sephadex G-100 chromatography of pooled fraction from CM-cellulose. Phosphotransferase activity (□—□) in μmoles product produced per min was determined on 0.5-ml aliquots of each tube. Pyrophosphatase at pH 3.0 (●—●), pyrophosphatase at pH 6.1. (■—■), and glucose-6-phosphatase (○—○) were measured on 0.05-ml aliquots of each tube as described in the legend of Fig. 4. Protein (— — —) was eluted from the column (1.5 cm × 40 cm) with 0.05 M acetate buffer, pH 4.5.

phatase at pH 3.0 was present in all fractions which exhibited the other enzyme activities. Chromatography of crude enzyme preparations on DEAE-cellulose (Whatman DE 11) in 0.02 M acetate buffer, pH 5.6, also failed to separate the three enzyme activities. A molecular weight determined from retention on a Sephadex G-100 column resulted in a value of 60 800 (Fig. 6).

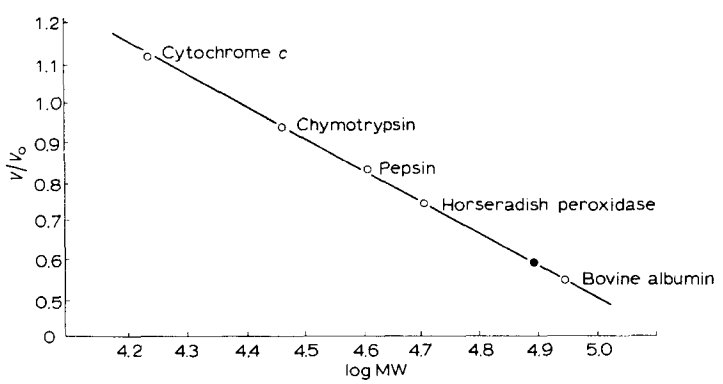

Fig. 6. Determination of molecular weight of enzyme by gel filtration on Sephadex G-100. Plot of log molecular weight *versus* elution volume/column void volume. Void volume was determined with Dextran Blue. The column (1.5 cm × 40 cm) was equilibrated with 0.05 M acetate buffer, pH 4.5. ● represents pyrophosphatase and related activities.

Disc gel electrophoresis

Pyrophosphatase and glucose-6-phosphatase were located in each gel as described in Methods. This activity was found to be substantially decreased by electrophoresis. A prolonged incubation with substrate (30 min) was required in order to find the inorganic phosphate released in the gel. Even then the resulting light yellow band was not intense. The phosphate released in the gel after incubation with glucose 6-phosphate at pH 5.7 or with pyrophosphate at either pH 3.0 or 6.1 produced bands which all appeared at the same place in the gel. A single band was obtained when the gels were stained for protein. This band appeared at the same place in the gel as the enzyme activity bands.

Effect of ions

The effect of metal ions on enzyme activity is illustrated in Table III. Low concentrations of molybdate inhibit pyrophosphatase, glucose-6-phosphatase and phosphotransferase activities in *A. oryzae* as they do in rat liver[1]. The presence of molybdate inhibited phosphotransferase to a lesser extent than it did the other two

TABLE III

PER CENT INHIBITION OF ENZYME ACTIVITY BY METAL IONS

Enzymatic activity was measured as described in Methods. The reaction mixtures were preincubated for 2 min at 37 °C in the presence of the indicated reagent. The reaction was started by the addition of 0.1 mg partially pure enzyme (6.1 units glucose-6-phosphatase/mg).

Reagent	*Concentration (mM)*	*Enzymatic activity lost (%)*		
		Pyrophosphatase pH 6.1	*Glucose 6-phosphatase*	*Phosphotransferase*
MoO_4^{2-}	0.06	36	41	80
MoO_4^{2-}	0.12	11	16	71
$MgSO_4$	1.1	3	1	2
$MnCl_2$	1.1	0	2	2
$ZnSO_4$	1.1	10	6	4
EDTA	1.1	2	0	2

activities. A reason for this may be that the assay medium for the phosphotransferase contained higher levels of substrate, which partially overcame the inhibition. The pyrophosphatase activity at pH 3.0 is only slightly inhibited by the presence of molybdate.

Experiments in which the enzyme was assayed in the presence of 20 mM concentrations of divalent cations (Mg^{2+}, Mn^{2+}, Zn^{2+}) indicated that these cations caused significantly more inhibition of pyrophosphatase activity than glucose 6-phosphate activity. Pyrophosphate forms metal complexes with these cations which are unsuitable substrates. Glucose 6-phosphate probably does not interact with the cations. The results of investigations using low concentrations of cations show that there is little inhibition of any of the catalytic activities in the presence of 1.1 mM Mg^{2+} or Mn^{2+}, but all the catalytic activities are inhibited by Zn^{2+} at a cation concentration of 1.1 mM. The inhibition caused by Zn^{2+} is most likely due to an interaction with the enzyme rather than an interaction with the substrate, since the hydrolysis of both pyrophosphate and glucose 6-phosphate is inhibited. The addition of 1.1 mM EDTA

to the incubation mixture had little effect on any of the enzymatic activities. This indicates that there is probably no metal ion requirement for any of these enzyme activities.

DISCUSSION

An enzyme has been found in *A. oryzae* that can catalyze the hydrolysis of inorganic pyrophosphate and glucose 6-phosphate, as well as catalyze the transfer of a phosphate group from pyrophosphate to the 6-hydroxyl group of glucose. The three enzyme activities are relatively heat stable and have acidic pH optima. In all the column chromatographies performed, the three enzymatic activities elute together. Electrophoresis indicates that they all have similar electrophoretic mobilities. They apparently require no metal ion and are inhibited by Zn^{2+} and low levels of molybdate. The inorganic pyrophosphatase is inhibited by glucose and glucose 6-phosphate. These results lead to the conclusion that these three catalytic activities are due to a single enzyme.

The mold also possesses an inorganic pyrophosphatase with a pH 3.0 optimum. This enzyme is also relatively heat stable. Purification procedures have produced fractions which possess only the pH 3.0 pyrophosphatase. However, it was not possible to obtain the pH-6.1 pyrophosphatase and related activities, which did not have pyrophosphatase activity at pH 3.0. The possibility that the pH-6.1 pyrophosphatase also has some activity at pH 3.0 cannot be ruled out.

The significance of two acidic inorganic pyrophosphatases in *A. oryzae* is unclear, but this phenomenon is not unique. Jungnickel[10] has shown the existence of two inorganic pyrophosphatases in *Candida utilis*, an organism which is very similar to *A. oryzae*. In *C. utilis*, one pyrophosphatase has an activity optimum at pH 2.5, the other at pH 6.0. Both of these enzymes are heat stable, but no other enzymatic activities are reported to be associated with either pyrophosphatase. Silva and Zancan[11], reporting on the purification of an alkaline, Mg^{2+}-dependent inorganic pyrophosphatase from *Polyporus circinatus*, found that crude extracts of the mold had detectable levels of pyrophosphate-glucose phosphotransferase activity. However, the phosphotransferase activity was not associated with the pyrophosphatase.

The phosphotransferase activity in *A. oryzae* is relatively low compared to the other two catalytic activities. In the purified enzyme fraction the specific activity of the phosphotransferase is only 4% of that exhibited by glucose-6-phosphatase. In the mammalian system the phosphotransferase is approximately 33% as active as the glucose-6-phosphatase[1]. The disparity may reflect a difference in importance to the mold and to higher organisms of this catalytic activity *in vivo*.

The high apparent K_m for glucose in the phosphotransferase reaction makes the physiological significance of this reaction in *A. oryzae* questionable. Similar doubts have been raised concerning the phosphotransferase reaction catalyzed by the mammalian enzyme, which also has a high apparent K_m value for glucose[1,12]. However, typical growth media contain 3% sucrose or dextrose, but sometimes contain as much as 20, 40, or even 60% (ref. 13). Nehira[14] reports that if glucose is the carbon source of the growth media it should be 6%, a glucose concentration of about 0.33 M. In addition, intracellular compartmentalization may cause large concentrations of glucose to exist in the cell.

The mechanism outlined for the mammalian enzyme by Arion and Nordlie[12], which would seem to be applicable to the enzyme from *A. oryzae*, makes it likely that there are other metabolic substrates for the enzyme. While substrate specificity of the enzyme studied here remains to be established, it was found that the enzyme could hydrolyze β-glycerol phosphate at about 35% the rate of hydrolysis of glucose 6-phosphate, indicating that the enzyme possesses some acid phosphatase activity. Glucose, with its high apparent K_m, may not be the most important phosphate acceptor for phosphotransferase. The phosphate donor for the phosphotransferase may include compounds other than inorganic pyrophosphate. Preliminary work indicates that carbamyl phosphate can be utilized as substrate, and the phosphorylation of glucose proceeds at approximately the same rate as with inorganic pyrophosphate. It has been shown that the mammalian enzyme can utilize carbamyl phosphate as well as phosphoenolpyruvate and most nucleoside di- and triphosphates in place of inorganic pyrophosphate[5,15].

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