Studies on recombinant *Acetobacter xylinum* α-phosphoglucomutase

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The phosphoglucomutase (PGM) from *Acetobacter xylinum*, which had been cloned and expressed in *Escherichia coli*, has been studied. After expression, the enzyme was purified from the *E. coli* in a three-step process consisting of $(NH_4)_2SO_4$ precipitation, gel filtration and anion-exchange chromatography. The purified enzyme gave one band on gel electrophoresis and was judged essentially free of impurities, although it was unstable when diluted without the addition of 15 μM BSA. The isoelectric point for *A. xylinum* PGM was 4.8 and the molar absorbance was 3.9×10^4 $M^{-1} \cdot cm^{-1}$. The enzyme was reasonably heat-stable below 50 °C and was stable throughout the pH 5.5–7.4 range, but was 70% inactivated at pH 10.0 and completely inactivated after standing for 10 min at pH 3.0 or at pH 12.4. When isolated, the recombinant enzyme was fully active without the addition of extra Mg^{2+}. The K_m for glucose 1-phosphate was much higher than that of other PGM species reported, which accords with the production of extracellular cellulose in *A. xylinum*. Glucose 1,6-diphosphate is not considered to be a substrate or coenzyme but an activating cofactor like Mg^{2+}. The following kinetic constants were determined: V_{max} 81.1 units/mg; k_{cat} and the turnover rate 135 s^{-1}; K_m (glucose 1,6-diphosphate) 0.2 μM; K_m (glucose 1-phosphate) 2.6 mM; k_{cat}/K_m (glucose 1-phosphate) 5.2×10^4 $M^{-1} \cdot s^{-1}$. The recombinant enzyme is considered to follow a characteristic substituted enzyme or Ping Pong reaction mechanism.

INTRODUCTION

Phosphoglucomutase (PGM; EC 5.4.2.2) is a key enzyme in the carbohydrate metabolism of a variety of micro-organisms as well as animal cells catalysing in particular the reversible transfer of a phosphate group between C-1 and C-6 of glucose [1]. PGM links the production by anaerobic or aerobic glycolysis of glucose 6-phosphate (G6P) and glucose 1-phosphate (G1P) produced by various glycan phosphorylases. The phosphorylated sugars may enter several different catabolic pathways, to yield energy (ATP) or reducing power (NADPH), or enter anabolic pathways, leading to the synthesis of polysaccharides. Hence PGM plays a key role in directing the metabolic flux towards polymer synthesis or catabolic pathways. Experimental evidence for its key role has been provided by showing that PGM-deficient strains of *Acetobacter xylinum* are unable to synthesize cellulose [2]. The α-PGM gene from *A. xylinum* has been cloned and expressed in *Escherichia coli* [3].

A. xylinum PGM is considered to be specific for α-D-glucose phosphate [3], and without the broad substrate specificity of, for example, rabbit muscle PGM, which interconverts many α-D-hexoses and sugar phosphates [1], in particular, the α anomers of D-G1P and D-G6P (Figure 1). PGM catalysis is usually accomplished by an active phosphoenzyme (Ep) containing a serine phosphate and involving a bound bivalent metal ion, generally Mg^{2+}, and an intermediate α-D-glucose 1,6-phosphate (α-D-G1,6diP) activator complex [1].

The reaction α-D-G1P ↔ α-D-G6P with an equilibrium constant of about 7 at neutral pH favours the forward reaction. However, this is augmented by the anomerization of α-G6P to β-G6P, which the glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) assay measures and gives an overall equilibrium constant of 17.2 [1]. The initial rate of α-G1P turnover is therefore considerably faster than that of α-G6P.

Detailed studies and most published work on PGM have been carried out on the rabbit muscle enzyme [1]. Data also exist on PGM from other sources including *E. coli*, *Bacillus cereus*, *Micrococcus lysodeikticus*, yeast and human placenta [4–7]. Differences in the physical and chemical properties of PGM from rabbit muscle and bacteria have been shown [4,5]. With half the molecular mass, monomeric β-PGM enzymes involved in the conversion of β-G1P have also been described [8,9].

The key role of PGM makes it an ideal target for the protein engineering of micro-organisms to redirect their metabolic flux. Characterization of the enzyme is thus of interest in order to explain the results observed in fermentations of native and engineered organisms. The aim of the present work was to describe the preparation and some important biochemical and kinetic properties of recombinant PGM from *A. xylinum* expressed in *E. coli* and how they compare with the properties of PGM from other sources.

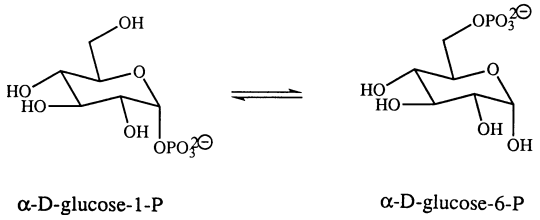

Figure 1 PGM catalysis, by phosphoryl transfer, of the stereospecific interconversion of the α-D-G1P and α-D-G6P anomers

Abbreviations used: PGM, α-phosphoglucomutase; β-PGM, β-phosphoglucomutase; E, the dephospho form of PGM; Ep, the phosphoenzyme form; G1P, α-D-glucose 1-phosphate or α-D-glucosopyranosyl 1-phosphate; G6P, D-glucose 6-phosphate or D-glucosopyranosyl 6-phosphate (equilibrium mixture of α/β-anomers); G1,6diP, α-D-glucose 1,6-bisphosphate or α-D-glucosopyranosyl 1,6-bisphosphate; G6PDH, β-D-glucose-6-phosphate dehydrogenase.

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EXPERIMENTAL

Materials

Sodium phosphate (0.02 M) was used at pH 6.0 and 7.4 and Tris/0.1 M HCl at pH 7.4. In the study of buffer and anion effects on the PGM assay, the Tris buffers used contained 0.1 M acid anion titrated with Tris to pH 7.4. BSA, $NADP^+$, G6P, G1,6diP, G6PDH, glycerol and Tris were from Sigma. α-D- and β-D-G1P were from Boehringer. All chemicals were the purest commercially available.

Strain and growth conditions

The *E. coli* strain used in this work contained a PGM plasmid from *A. xylinum* [2] which was provided by UNIGEN (Trondheim, Norway) as a part of our collaboration. It was kept at -80 °C, and was revived by growth on L-agar-broth [10]. Inoculum was prepared by transferring one colony from the agar plate to a 500 ml baffled shake flask containing 100 ml of L-agar-broth. The flask was incubated at 30 °C overnight on a rotary shaker (240 rev./min) to give a cell density of approx. 10^8/ml. The composition of the L-agar-broth was in g/l: bactotryptone, 10; yeast extract, 5; NaCl, 10; ampicillin, 0.10; kanamycin, 0.05. Ampicillin and kanamycin were filtered (0.22 μm; Millipore) and added to the medium after being autoclaved.

The fermentor used had an 8-litre working volume. The inoculum level was 1% (v/v), and the cells were grown in L-broth at 30 °C to an A_{600} of 1.2. T_7 RNA transcription was induced by heat [11]. The temperature was increased to 42 °C for 30 min, whereupon the culture was maintained at 33 °C for 2 h. Biomass concentration was followed by measuring A_{600} and dry weight. Throughout the entire fermentation run, pH was maintained at 7.0.

PGM purification

The harvested cells were concentrated and washed with phosphate buffer, pH 7.4, in a cross-flow filtration system (Millipore; pore size 0.22 μm). At 4 °C, the cells were then disrupted by pulsed sonication (2 s on, 8 s off) at 245 W and 20 kHz using a Virsonic 475 ultrasonic cell disrupter (Virtis, New York, NY, U.S.A.), and cell debris was removed by centrifugation (12000 g; 1 h).

The proteins in the supernatant were precipitated by the addition of solid $(NH_4)_2SO_4$ to 45% saturation at 0 °C, the pH being maintained at 7.1 with NaOH. After being stirred for 30 min, the suspension was centrifuged (17000 g; 1 h). The precipitate was discarded and the PGM activity in the supernatant was then recovered by the addition of solid $(NH_4)_2SO_4$ to 75% (w/v) saturation. After being stirred for 30 min, the suspension was centrifuged, and the concentrated PGM in the pellet was dissolved in phosphate buffer, pH 6.0, to give a final volume of 35 ml.

The dissolved pellet was then subjected to further purification on an automated FPLC system (Pharmacia, Uppsala, Sweden). The sample was applied to a 430 ml Sephacryl S-300 (Pharmacia) column (5.0 cm × 25 cm) and fractionated under the following conditions: sample concentration, 5–10 mg of protein/ml; sample volume, 6 ml; eluent, phosphate buffer, pH 6.0; flow rate, 5.0 ml/min; detection, UV absorption at 280 nm. The PGM fraction was eluted 42–63 min after injection. PGM fractions from five runs were collected and applied to an 8 ml Resource S anion-exchange column (Pharmacia) previously equilibrated with phosphate buffer, pH 6.0, and run under the following conditions: sample concentration, 0.3–0.6 mg/ml; sample volume, 100–200 ml; eluent, (A) phosphate buffer, pH 6.0, (B) phosphate buffer, pH 6.0, containing 0.2 M NaCl; linear gradient, 0–60% B for 50 min; flow rate, 2.5 ml/min; detection, UV absorption at 280 nm. The PGM fraction was eluted with approx. 25% (v/v) B.

PAGE

Samples of PGM were analysed for purity, molecular mass and pI using the Phast system (Pharmacia). Samples containing 0.2–2 mg/ml were applied using six- or eight-well sample applicators. SDS/PAGE was performed using 20% homogeneous gels with SDS buffer strips, native PAGE using 20% homogeneous gels with native buffer strips and isoelectric focusing with pH 3–9 gels. All electrophoresis runs were performed under the conditions recommended by Pharmacia.

Protein determination

Protein was determined according to the Bio-Rad protein assay Bulletin 1069 (Bio-Rad Laboratories GmbH, Munich, Germany) using BSA as standard.

PGM assay

PGM activity was measured at pH 7.4 and 25 °C by using α-G1P and determining the initial rate of α-G6P formation after anomerization of β-G6P utilizing a coupled assay involving G6PDH (specific for β-G6P) and $NADP^+$ [1]. No preincubation of the enzyme was necessary. The reaction mixture contained 0.1 M Tris/HCl (adjusted to pH 7.4 with NaOH), 8 μM G1,6diP, 2 mM G1P, 0.5 mM $NADP^+$ and 1.25 units/ml G6PDH (1 unit will oxidize, in the presence of $NADP^+$, 1.0 μmol of β-G6P to 6-phosphogluconate/min at pH 7.4 and 25 °C) and a suitable amount of enzyme protein in a total volume of 0.2 ml. The PGM was diluted in 0.02 M phosphate buffer, pH 6.0, containing 15 μM BSA. Enzyme activity was determined by measuring the rate of increase in A_{340} due to NADPH formation from $NADP^+$ using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Taking 0.2 ml assay volumes with a light path length of 0.6 cm, the change in absorbance as a function of time was always linear in the time interval measured. The effect of buffer anions on the assay was examined by replacing at pH 7.4 the Tris/0.1 M HCl with other acids such as H_3PO_4 at 0.1 M.

Steady-state kinetics

The kinetic constants V_{max} and K_m were determined by monitoring the PGM activity at 25 °C and pH 7.4, in assay solutions containing a constant concentration of G1P and various concentrations of G1,6diP or vice versa. For each constant concentration of G1P (0.25–60 mM), the concentration of G1,6diP was varied from 0 to 5 mM, and for each constant concentration of G1,6diP (1 μM–4 mM), the concentration of G1P was varied from 0–5 mM. Double-reciprocal plots of PGM activity against G1P or G1,6diP concentration were constructed.

Demetalization

After anion-exchange chromatography, all the equipment was washed in 10% HNO_3 to remove loosely bound metal ions. Demetalization was performed by dialysis (Spectra/Por® membranes; molecular-mass cut off at 12–14 kDa) overnight at 4 °C in 20 mM phosphate buffer, pH 5.5/5 mM EDTA, thereafter in 20 mM phosphate buffer at pH 6.0. Demetalization was also performed at 40 °C for 1 h in 20 mM phosphate buffer, pH 6.0.

PGM stability

Aliquots of the PGM solution were placed in Eppendorf tubes, and using the PGM assay, enzyme stability was tested at room temperature as a function of time with and without the addition of 5 mM $MgCl_2$ or 5 mM $ZnCl_2$.

Enzyme stability was also tested at different dilutions (1:50, 1:100, 1:200) as a function of time. The enzyme was diluted in 0.02 M phosphate buffer, pH 6.0, with and without the addition of 5% (v/v) glycerol or BSA (0.015, 0.15 and 1 mM).

pH stability was tested with enzyme diluted 1:3 by activity measurements after being left at room temperature for 10 min in 0.1 M Tris/HCl buffer (pH 3.0, 7.4, 10.0 and 12.0 containing 0.015 mM BSA). The enzyme solution was neutralized before the PGM assay was carried out.

Heat stability as a function of time was likewise tested in 0.1 M Tris/HCl buffer, pH 7.4, using enzyme (diluted 1:3). The PGM assay solution was added after incubation at 25, 37, 50 and 65 °C. Enzyme activity was tested 0, 5, 20 and 30 min after incubation.

RESULTS

Production and purification

The PGM yield increased rapidly with sonication time reaching a maximum level after 2 min of sonication, whereas the number of cells declined with sonication time, being essentially zero after 2 min of sonication. Hence 2 min is an appropriate sonication time under the sonication conditions chosen for cell concentrations below 50 mg/ml. At cell concentrations exceeding 50 mg/ml there was a decrease in the PGM yield under otherwise identical sonication conditions.

The procedure developed for the purification of recombinant PGM from *E. coli* consists of ultrasonication, $(NH_4)_2SO_4$ fractionation, gel filtration and anion-exchange chromatography. After anion-exchange chromatography, the PGM fraction had a specific activity of 98 units/mg of protein.

As judged by SDS/PAGE (Figure 2) and native PAGE (not shown), which resulted in a single band, the purified PGM was essentially free of impurities. Isoelectric focusing experiments with pH 3–9 gels also gave a single band, positioned at a pI of 4.8 ± 0.2.

The A_{280} of PGM (1 mg/ml) was 0.65.

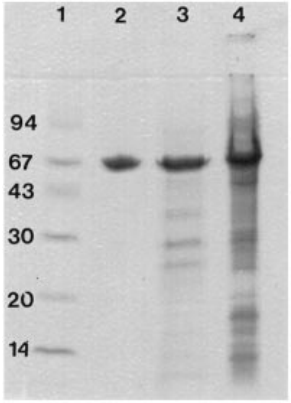


Figure 2 SDS/PAGE of samples taken during and after PGM purification

The samples were run using 20% homogeneous gel. Lane 1, Low-molecular mass markers (Pharmacia) (molecular mass is given in kDa); lane 2, PGM after anion-exchange chromatography; lane 3, PGM fraction after gel filtration; lane 4, $(NH_4)_2SO_4$ precipitate (45–75% saturation).

Stability

Freezing and thawing resulted in almost total loss of activity, whereas approx. 80% of activity was retained in freeze-dried enzyme (results not shown).

Metal ion concentration

Rabbit muscle PGM is activated by Mg^{2+} ions and deactivated by Zn^{2+} ions. These metal ions were therefore tested on PGM from *A. xylinum*, and the activity was followed as a function of time with and without the addition of either 5 mM $MgCl_2$ or $ZnCl_2$. Experiments were performed with PGM from two different batches isolated by exactly the same procedure. The results showed a rapid deactivation of *A. xylinum* PGM from batch no. 1 when metal ions were added. The activity was reduced to almost 65% (Mg^{2+}) and 60% (Zn^{2+}) of the initial activity in only a few minutes, but continued to fall with time, reaching 30% (Zn^{2+}) and 40% (Mg^{2+}) after 1 week. When no metal ions were added, PGM activity remained stable over the same time interval. When Mg^{2+} was added to native PGM from batch no. 2, no significant change was observed. The enzyme was also stable over time. The variations in the stability experiments was generally less than $\pm 5\%$.

Demetalization

The activity of *A. xylinum* PGM was the same before and after demetalization at 4 °C. In addition, a sample of the demetalized PGM that was re-treated with 1 mM EDTA for 1 h and the activity compared with the sample without EDTA showed no difference in PGM activity (results not shown). When demetalization was performed at 40 °C for 1 h, PGM activity was reduced to 5% of initial activity. The addition of Mg^{2+} or Zn^{2+} to samples of the demetalized enzyme resulted in an increase in enzyme activity. The activity was increased to 17 and 37% by adding 1 and 2 mM Mg^{2+} respectively. A 100% active enzyme was only obtained by the addition of 5 mM Mg^{2+}, and this activity was more or less the same as the native enzyme without the addition of Mg^{2+}. The addition of 1 and 2 mM Zn^{2+} to samples of the demetalized enzyme increased the enzyme activity to 11 and 13% respectively. On the addition of 5 mM Zn^{2+}, 65% of the activity was obtained. The dialysed enzyme was not stable when stored without the addition of metal ions, but continued to lose activity as a function of time (result not shown).

Buffer and anion effects

When Tris/0.1 M HCl was replaced with Tris/0.1 M H_3PO_4 in the pH 7.4 assay buffer, PGM activity was 5–6-fold less.

Glycerol and BSA

Without the addition of BSA or glycerol, the activity of PGM diluted in phosphate buffer was reduced to half in less than 1 min. The presence of 5% glycerol reduced the loss of PGM activity to some extent for the 1:50 dilution but not significantly

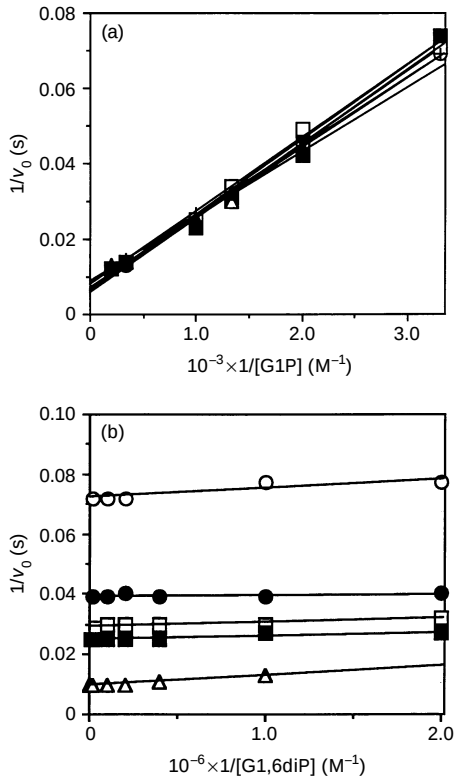


Figure 3 Double-reciprocal plots of PGM activity against concentration to determine the apparent V'_{max} and K'_m

(**a**) $1/v_0$ versus 1/[G1P] at seven constant concentrations of G1,6diP: ○, 4 μM; ●, 8 μM; □, 16 μM; ■, 40 μM; △, 80 μM; ▲, 0.4 mM; +, 0.8 mM. (**b**) $1/v_0$ versus 1/[G1,6diP] for five constant concentrations of G1P: ○, 0.25 mM; ●, 0.5 mM; □, 0.75 mM; ■, 1 mM; △, 3 mM.

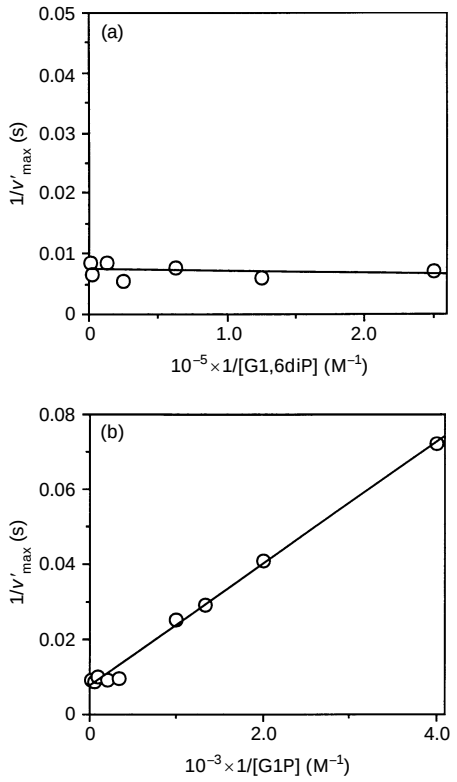


Figure 4 Determination of V_{max}

Secondary plots of 1/[G1,6diP] (**a**) and 1/[G1P] (**b**) based on the maximal velocities obtained from Figures 3(a) and 3(b).

for higher dilutions. However, addition of BSA to the phosphate buffer completely stabilized the enzyme. Enzyme stability was the same whether 0.015, 0.15 or 1 mM BSA was used (results not shown).

pH

A. xylinum PGM was stable throughout the pH 5.5–7.4 range. Titrated back to neutral pH, the enzyme was inactive after standing for 10 min at pH 3.0 or pH 12.4. At pH 10.0 the activity was reduced by 70 % (not shown).

Heat

Regarding heat stability as a function of time, the enzyme was stable at 25 and 37 °C, but 35 and 90 % of the activity was lost over 30 min at 50 and 65 °C respectively (not shown).

Kinetics

Steady-state kinetics

Linear double-reciprocal primary and secondary plots were obtained when G1P and G1,6diP were varied. Figure 3(a) shows double-reciprocal plots of $1/v_0$ versus 1/[G1P] for seven constant concentrations of G1,6diP. Figure 3(b) shows the same data plotted as $1/v_0$ versus 1/[G1,6diP] at five constant concentrations of G1P. Both primary plots are parallel and characteristic of Ping Pong kinetics and a substituted phosphoenzyme isomerization mechanism. In Figure 3(a) the parallel plots show that there is no substrate inhibition in the concentration area shown. At 4 mM G1,6diP and higher, substrate inhibition occurred (not shown). In Figure 3(b) substrate inhibition started at 3 mM G1P, and at higher concentrations the slopes increased with increasing substrate concentration (not shown), while the intercepts were constant and the same as with 3 mM G1P. At these concentrations, inhibition of G1P competed with G1,6diP. Extrapolation of the plots in Figure 3 to $1/[G1P] = 0$ or $1/[G1,6diP] = 0$ gives the reciprocals of the maximal velocities obtained with each of the concentrations of G1P and G1,6diP respectively. Figures 4(a) and 4(b) show secondary plots of the intercepts from Figures 3(a) and 3(b), which result in a common V_{max} of 22.7 units/ml. Given the protein concentration (0.28 mg/ml), V_{max} becomes 81.1 units/mg and V_{max} or k_{cat}, the number of molecules of substrate turned over/s per molecule of PGM, is 135. In Figure 4(b), $1/V_{max}$ remained almost constant with increasing concentration of G1,6diP. In Figures 3(a) and 3(b) the slopes give K'_m/V_{max} for G1P and G1,6diP respectively. In Figure 5(a) the intercept gives $K_m(G1P)/V_{max}$, and the true $K_m(G1P)$ is calculated as 2.6 mM. In Figure 5(b) the intercept gives $K_m(G1,6diP)/V_{max}$, and $K_m(G1,6diP)$ is 0.2 μM. V_{max} (k_{cat}) and the K_m values give k_{cat}/K_m, the 'on rate' of the reaction of G1P and G1,6diP with PGM. This is 5.2×10^4 $M^{-1} \cdot s^{-1}$ for G1P and 6.8×10^8 $M^{-1} \cdot s^{-1}$ for G1,6diP. The latter agrees with G1,6diP being a cofactor/activator [1,12]. In the absence of added G1,6diP, enzymic activity was observed with G1P which was stated to be 98 % pure.

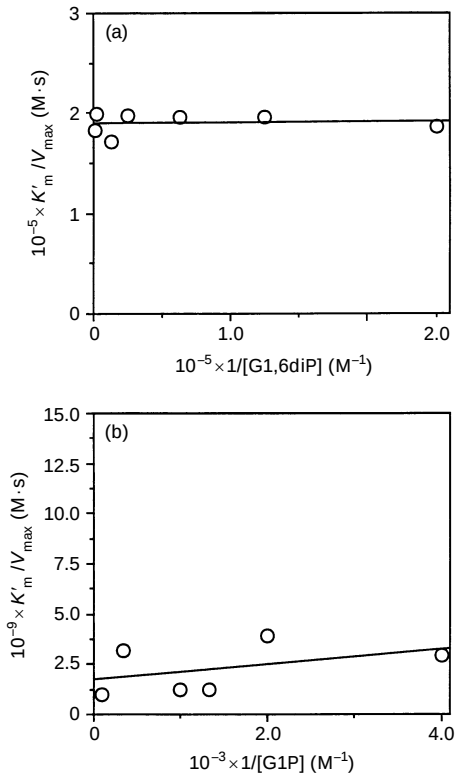


Figure 5 Determination of K_m

Secondary plots of 1/[G1,6diP] (**a**) and 1/[G1P] (**b**) based on the slopes in Figures 3(a) and 3(b).

DISCUSSION

Production and purification

Initially, purification of the rabbit muscle enzyme consisted of pH, heat and $(NH_4)_2SO_4$ precipitation followed by column chromatography [1,13]. The presence of bound glucose phosphates had been noted on the isolated rabbit muscle enzyme even after $(NH_4)_2SO_4$ precipitation, gel filtration and/or extensive dialysis [1]. Freshly isolated rabbit muscle enzyme is predominantly in the phosphorylated-serine form, whereas commercial enzyme is largely in the dephospho form [1]. Substantial differences in the methods of purification used here (gel-filtration and anion-exchange chromatography) may have resulted in different properties of the PGM isolated. Thus *A. xylinum* PGM appeared to be in the phosphorylated form since enzyme activity was obtained without the addition of G1,6diP. The traces of activator G1,6diP necessary could be tightly bound to the enzyme or might arise from G1P substrate (see the Experimental section).

Physical and chemical properties

A. xylinum PGM appears, like rabbit muscle PGM, to consist of one polypeptide chain, one active site and one metal-binding site, but has only one cysteine residue (Cys^{283}) [3], in contrast with the rabbit muscle enzyme which has five [14].

No overall sequence similarity exists between *A. xylinum* and other PGMs. However, two highly conserved regions, the catalytic site and the metal-binding site, are also seen in *A. xylinum*. In the sequence of the metal-binding site in *A. xylinum* PGM, one amino acid, Thr^{307}, is unique when compared with PGM from rabbit and many other species, where the corresponding amino acid is glycine. In addition, Ala^{309} in *A. xylinum* contrasts with glycine and phenylalanine in PGM from other sources [3].

The molecular mass of PGM from different sources is in the range 58–69 kDa: rabbit muscle, 61 609 Da; *E. coli* 62–65 kDa; *B. cereus* 63 kDa; *M. lysodeikticus* 58 kDa; yeast 65.5 kDa; shark and flounder 63 kDa; human placenta 58.5–69 kDa [1,6,7,14]. *A. xylinum* PGM with a 558-amino acids sequence and a molecular mass of 59.6 kDa [3] is similar to rabbit muscle PGM with 561 amino acids and a molecular mass of 61 609 Da [13]. The molecular mass of *A. xylinum* PGM, like its α-G1P substrate specificity, also distinguishes it from the bacterial β-PGM enzymes, which are also monomeric, but half the size with molecular masses around 28 kDa [8,9].

The isoelectric point of PGM exhibits substantial variation between different species. The present data show a pI of 4.8 for *A. xylinum* PGM, which is similar to that of 4.4 for *Lactococcus lactis* [9] but lower than that of PGM from many other sources. For the rabbit muscle enzyme the pI is 7.0 [15], whereas that from blood cells and human placenta has a pI in the range 5.3–6.0 [7].

The 0.65 mg/ml absorbance of *A. xylinum* PGM at 280 nm gives a molar absorbance of $3.9 \times 10^4\ M^{-1} \cdot cm^{-1}$, similar to $4.3 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 278 nm for the rabbit muscle enzyme [14], which, although having a similar polypeptide chain size, has no overall sequence similarity [3].

Stability

A. xylinum PGM was stable at neutral pH and room temperature but lost activity at high and low pH and above 50 °C.

A problem with PGM is the retention of full activity when stored. The experiments showed the more dilute the enzyme, the greater the activity loss with time. A certain protein concentration was necessary to stabilize the enzyme. However, when BSA was added, PGM diluted up to 1:200 with phosphate buffer, pH 6.0, remained stable with time. There were no differences in PGM activity with concentration of BSA greater than 15 μM. Glycerol, which is often used to stabilize proteins in solutions, had little or no effect on diluted PGM solutions.

PGM can be stored as an $(NH_4)_2SO_4$ precipitate, although some authors have suggested that this results in the formation of a less active enzyme [4]. In the present work $(NH_4)_2SO_4$-precipitated PGM was redissolved in phosphate buffer within 1–2 h.

Activation and inhibition

According to the literature, salts are, in general, inhibitory because of an anionic effect. Anions have been shown to inhibit PGM competitively with both mono-(G1P and G6P) and bis-(G1,6diP) glucose phosphates [12]. Anions can inhibit by both binding to the enzyme and reducing the concentration of free Mg^{2+}. Univalent anions inhibit PGM activity to a smaller extent than polyvalent anions [1]. Buffer anion effects are seen at pH 7.4 where Tris/HCl results in a 5–6-fold higher enzyme activity than when Tris/phosphate is used as assay buffer.

Many metal ions can activate PGM but to different extents, with the best being Mg^{2+}. Metal ions such as Zn^{2+} and Cu^{2+} are inhibitors of the rabbit muscle enzyme which do not bind to the active site [16], but Zn^{2+} can activate the yeast enzyme (84 % of the Mg^{2+} activity) [6]. The present results suggest that *A. xylinum* PGM was saturated with Mg^{2+} when isolated, since the activity was the same as demetalized (at 40 °C) enzyme after the addition of 5 mM Mg^{2+}. That Zn^{2+} can activate *A. xylinum* PGM (65 % as much as Mg^{2+}) is similar to the situation in yeast PGM. Both *A.*

xylinum PGM batches, the purification procedure of which was the same, were active without the addition of Mg^{2+}, but had different responses to Mg^{2+} addition. The differences are perhaps due to a different degree of saturation of PGM with Mg^{2+} when isolated.

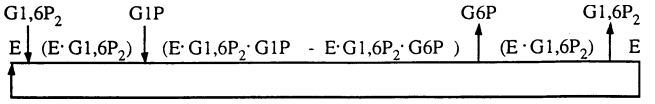
Several chelating agents and other biological compounds can activate PGM, as the result of their metal-binding properties or by accelerating the enzyme–metal dissociation rate [16]. EDTA binds strongly to metals, but does not increase metal–enzyme dissociation as effectively as histidine and imidazole. Thus EDTA is often used in combination with either histidine or imidazole to remove metals from PGM. Lower pH may also increase enzyme–metal dissociation [12]. The PGM activity of *A. xylinum* was not changed before or after demetalization when an EDTA/phosphate buffer at pH 5.5 was used. Demetalization succeeded only when performed for 1 h at 40 °C. The metal is so tightly bound that it is not removed under conditions that would normally result in demetalization (4 °C).

Cysteine and mercaptoethanol can activate rabbit muscle PGM, since an excess of these compounds can maintain the protein thiol groups in a reduced state or remove inhibiting metal ions by chelation [12]. An activating effect of cysteine and mercaptoethanol was also observed on *A. xylinum* PGM, but storage experiments revealed a destabilizing effect on the enzyme when compared with phosphate buffer alone (results not shown). Perhaps cysteine rapidly removes inhibiting metal ions but with storage slowly removes tightly bound Mg^{2+} activating ions. It has been reported that PGM from *E. coli*, *M. lysodeikticus* and *B. cereus* failed to respond to treatment with chelating agents before assay [1], but that the first two enzymes required cysteine for maximal activity [4]. The requirement may be the result of the sensitivity of the thiol groups toward chemical modification by metals. However, the *B. cereus* enzyme showed no stimulation by and no requirement for cysteine, but a cysteine concentration above 0.01 M resulted in 10–15% inhibition of activity [5], perhaps as the result of the removal of metal activation.

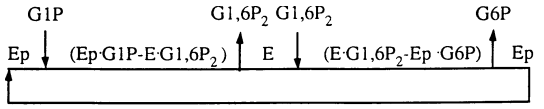
Kinetics and the reaction mechanism

For PGM isolated from *A. xylinum* the product time plots with G1P as substrate are linear because of the favourable equilibrium constant [1], although the back reaction has so far not been studied. For PGM, V_{max} and K_m vary considerably from organism to organism [1]. Thus for *A. xylinum* PGM, the k_{cat} or turnover rate of 135 s^{-1} is less than the 730 s^{-1} for the rabbit muscle enzyme [17], but higher than 54 s^{-1} for *E. coli* [6]. Likewise the K_m (G1P) of 2.6 mM for *A. xylinum* is higher than the 8 μM value for rabbit muscle PGm [12,17] and is higher than that of other species, varying from 20 to 130 μM [1]. This accords with the production of extracellular cellose in *A. xylinum*, where a high K_m(G1P) favours the metabolic flux towards polymer synthesis rather than catabolic pathways. For the rate of G1P binding to *A. xylinum* PGM, the 'on velocity' k_{cat}/K_m(G1P) of 5.2×10^4 $M^{-1} \cdot s^{-1}$ is much lower than the 90×10^6 $M^{-1} \cdot s^{-1}$ for rabbit muscle [17] because of a slower turnover number and a much higher K_m.

A. xylinum PGM is seen only to use α-G1P and not β-G1P, and is thus, like the rabbit muscle enzyme and most PGMs, an α-PGM. This suggests that it only uses α-G1,6diP as β-G1,6diP is not an activator for the rabbit muscle enzyme [1,12]. This agrees with the single polypeptide chain molecular mass of 59.6 kDa of *A. xylinum* PGM [3], as α-PGM enzymes are monomeric and of similar size, unlike β-PGM enzymes, which, while also monomeric, have half the molecular mass at around 28 kDa [8,9].



Scheme 1 Bi Bi sequential compulsory-order mechanism for the catalysis of G1P to G6P



Scheme 2 Ping Pong mechanism for the catalysis of G1P to G6P by *A. xylinum* PGM

Different reaction mechanisms have been suggested for PGM enzymes. Those from *B. cereus* and *M. lysodeikticus* have been considered to follow a single-displacement reaction mechanism or a Bi Bi sequential compulsory-order mechanism (Scheme 1). For these enzymes, G1,6diP is the first substrate and second product of the central complexes, the interconversion of which is rate-limiting [5]. These enzymes are stated not to be phosphorylated (do not serve as glucose bisphosphate phosphorylases) and are inactive without added G1,6diP [5]. Scheme 2 is a Ping Pong mechanism followed by *A. xylinum* PGM similar to that of rabbit muscle PGM, where the product of the first reaction acts as a substrate for the second reaction [13], and E is not formed during each catalytic cycle [1,12].

For *A. xylinum* PGM, activity was observed in the absence of added G1,6diP and increased with increasing [G1P]. This suggests that the enzyme is in the phospho form (Ep) when isolated or that it obtains the small amounts of G1,6diP (activating cofactor) necessary from the G1P substrate. Our findings suggest that high concentrations of both G1,6diP and G1P inhibit PGM activity, with the inhibition constants depending on each other. Thus the higher the G1P concentration, the greater the inhibition. For enzymes that exhibit Ping Pong kinetics, competitive inhibition by higher substrate concentrations is common [1].

In the substrate-velocity profiles, where no substrate inhibition occurs, increasing concentration of G1P gave parallel lines for G1,6diP kinetics, and vice versa, indicating a Ping Pong mechanism. The facts that the parallel lines overlap with increasing concentrations of G1P for G1,6diP kinetics and that the slopes of the lines in plots of G1P kinetics with increasing concentration of G1,6diP are close to zero support the suggestion that G1,6diP is not a real substrate in the reaction, but rather an activating cofactor only needed in catalytic quantities to phosphorylate the enzyme (see Scheme 2). The K_m(G1,6diP) was experimentally estimated to be 2×10^{-7} M. This accords with a slow dissociation of E·G1,6diP compared with interconversion to Ep·G6P, and with E having high affinity for G1,6diP. A comparatively slow dissociation of E·G1,6diP has also been indicated for rabbit muscle PGM [1,12,18]. For G1,6diP, the kinetics suggest that it is not a substrate or coenzyme but rather, like Mg^{2+}, a tightly bound activating cofactor, functioning to maintain the active-site Ser^{148} of *A. xylinum* PGM [3] in the active phosphorylated form, as in the case of Ser^{116} of rabbit muscle PGM [14], where G1,6diP is free only once in many turnovers [12,19]. The active-site Ser^{148}, which is conserved in *A. xylinum* PGM, fits in with Scheme 2 and the characteristic Ping Pong enzyme kinetics observed. Scheme 2 represents such a Ping Pong substituted enzyme or enzyme isomerization mechanism, where enzyme

isomerization via phosphorylation–dephosphorylation occurs with central complexes and a G1,6diP bisphosphate intermediate which is a first product and second substrate [1,13,18]. In Scheme 2 in either direction, the second half reaction maintains the enzyme in the active phosphoenzyme form.

G1P and G6P have phosphate on opposite sides of the α-D-glucopyranosyl ring (Figure 1), with the result that a conformation change of the enzyme to reposition the active-site serine to give and then receive phosphate should occur. Alternatively, the G1,6diP intermediate must rotate or flip and there must be two distinct glucose-ring-binding sites, as has been reported for rabbit muscle PGM [20,21].

The present work has described biochemical and kinetic properties of a recombinant PGM from *A. xylinum* with some properties differing from those of other PGM enzymes. The K_m(G1P) of 2.6 mM for *A. xylinum* is higher than that of other species and accords with the production of extracellular cellulose in *A. xylinum*, where a high K_m(G1P) favours the metabolic flux towards polymer synthesis rather than catabolic pathways. *A. xylinum* PGM is considered to follow a characteristic substituted enzyme or Ping Pong type of reaction mechanism in accordance with previous studies of rabbit muscle PGM [1,13]. The next step in this work is the characterization of recombinant *A. xylinum* PGM mutants.

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REFERENCES

1 Ray, W. J. and Peck, E. J. (1972) in Enzymes 3rd Ed. **6**, 407–457
2 Fjærvik, E., Frydenlund, K., Valla, S., Huggirat, Y. and Benziman, M. (1991) FEMS Microbiol. Lett. **77**, 325–330
3 Brautaset, T., Standal, R., Fjærvik, E. and Valla, S. (1994) Microbiology **140**, 1183–1188
4 Joshi, J. G. (1982) Methods Enzymol. **89**, 599–605
5 Hanabusa, K., Dougherty, H. W., Del Rio, C., Hashimoto, T. and Handler, P. (1966) J. Biol. Chem. **241**, 3930–3939
6 Joshi, J. G. and Handler, P. (1964) J. Biol. Chem. **239**, 2741–2751
7 Fazi, A., Piacentini, M. P., Piatti, E. and Accorsi, A. (1990) Prep. Biochem. **20**, 219–240
8 Ben-Zivi, R. and Schramm, M. (1961) J. Biol. Chem. **236**, 2186–2189
9 Qian, N., Stanley, G. R., Hahn-Hagerdal, B. and Rådstrøm, P. (1994) J. Bacteriol. **176**, 5304–5311
10 Valla, S., Coucheron, D. H., Fjærvik, E., Kjosbakken, J., Weinhouse, H., Ross, P., Amikam, D. and Benziman, M. (1989) Mol. Gen. Genet. **217**, 26–30
11 Tabor, S. and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1074–1078
12 Ray, W. J. and Roscelli, G. A. (1964) J. Biol. Chem. **239**, 1228–1236
13 Najjar, V. A. (1962) Enzymes 2nd ed. **6**, 161–178
14 Ray, W. J., Hermodson, M. A., Puvathingal, J. M. and Mahoney, W. C. (1983) J. Biol. Chem. **258**, 9166–9174
15 Filmer, D. L. and Koshland, D. E. (1963) Biochim. Biophys. Acta **77**, 334–336
16 Ray, W. J. (1967) J. Biol. Chem. **242**, 3737–3744
17 Ray, Jr., W. J., Post, C. B. and Puvathingal, J. M. (1993) Biochemistry **32**, 38–47
18 Britton, H. G. and Clarke, J. B. (1968) Biochem. J. **110**, 161–179
19 Ray, Jr., W. J., Post, C. B. and Puvathingal, J. M. (1989) Biochemistry **28**, 559–569
20 Ma, C. and Ray, W. J. (1980) Biochemistry **19**, 751–759
21 Percival, M. D. and Withers, S. G. (1992) Biochemistry **31**, 498–505