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The two analogous phosphoglycerate mutases of *Escherichia coli*

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Abstract The glycolytic enzyme phosphoglycerate mutase exists in two evolutionarily unrelated forms. Vertebrates have only the 2,3-bisphosphoglycerate-dependent enzyme (dPGM), whilst higher plants have only the cofactor-independent enzyme (iPGM). Certain eubacteria possess genes encoding both enzymes, and their respective metabolic roles and activities are unclear. We have over-expressed, purified and characterised the two PGMs of *Escherichia coli*. Both are expressed at high levels, but dPGM has a 10-fold higher specific activity than iPGM. Differential inhibition by vanadate was observed. The presence of an integral manganese ion in iPGM was confirmed by EPR spectroscopy.

Key words: Glycolysis; Phosphoglycerate mutase; Vanadate inhibition; Cofactor requirement; *Escherichia coli*

1. Introduction

Phosphoglycerate mutases (EC 5.4.2.1) catalyse the interconversion of 2-phosphoglycerate (2-PGA) and 3-phosphoglycerate (3-PGA) in the Embden-Meyerhoff pathway. Two apparently evolutionarily unrelated enzymes with phosphoglycerate mutase activity have been characterised. One enzyme (dPGM) requires the cofactor 2,3-bisphosphoglycerate (2,3-BPGA) for activity, whilst the other (iPGM) does not. The recent advances in whole-genome sequencing have provided new data on the distribution of these two enzymes over a wide range of organisms [1]. Vertebrates, budding yeast, and various eubacterial species have only dPGM whilst nematodes, archaea, higher plants and various other eubacteria possess only iPGM. In addition, a small number of eubacteria appear to encode both enzymes (Fig. 1). This complex distribution could have arisen if both genes existed early in evolution, followed by selective loss of one or other in different phyla. The absence of iPGM in vertebrates, including humans, makes the enzyme a potential drug target in those organisms containing the gene, which includes many species of pathogenic bacteria, nematodes and other parasites.

dPGM belongs to an enzyme family whose members include bisphosphoglycerate synthase, fructose 2,6-bisphosphatase, pyrophosphatase and acid phosphatase [2]. Sequence motifs suggest that all of these enzymes have a conserved histidine at the active site that becomes reversibly phosphorylated during the catalytic cycle. The crystal structure of dPGM from *Saccharomyces cerevisiae* has been solved at 2.1 Å resolution [3,4], and spectral and kinetic studies, combined with site-directed mutagenesis, have served to define many of the properties of the cofactor-dependent enzyme [3,5–8]. Nevertheless certain fundamental details of the catalytic mechanism, such as the suggested requirement for reorientation of intermediates during catalysis [3], remain unclear.

iPGM is a member of the alkaline phosphatase superfamily [1,9], which is composed of a wide range of enzymes catalysing the transfer of phospho- or sulphate groups. Other members include phosphopentomutase, phosphoglycerol transferase, Ca^{2+}-dependent ATPase and a number of sulphatases [1]. These enzymes share a very limited degree of sequence identity that may reflect conservation of certain active site and metal binding residues. Details of the catalytic mechanism of iPGM are not well understood, though a phospho-enzyme intermediate has been postulated [10] and essential histidine residues have been identified [11].

Given the intriguing distribution of phosphoglycerate mutases, with both enzymes expressed in some bacterial species, and the potential of iPGM as a drug target, we chose to characterise both dPGM and iPGM from *Escherichia coli*. The enzymes were over-expressed and purified to homogeneity. Steady-state kinetic parameters were determined, allowing comparison of the specific activities of the two enzymes. Differential inhibition by vanadate allowed the quantitation of both proteins as a function of growth phase in *E. coli*, and the presence of manganese in *E. coli* iPGM was confirmed by EPR spectroscopy.

2. Materials and methods

2.1. Cloning the phosphoglycerate mutase genes

The gene for cofactor-dependent phosphoglycerate mutase (*gpmA*, accession number P31217) was a kind gift from Dr W.N. Hunter, and was supplied as an insert in the vector pET3a, allowing over-expression of the native protein in *E. coli* strain BL21 (DE3). The gene for cofactor-independent phosphoglycerate mutase, *pgmI* (accession number P37689), was amplified by PCR using Vent exo+ proof-reading polymerase (NEB), from *E. coli* strain DH5α, using oligonucleotide primers:

5′ primer: 5′- CGTCGGATCCCATATGGCCATGGCGGTTTCTAAAAAA-CCTATGGTACTGGTGATTC
3′ primer: 5′- GGCAGGATCCGTCGACTTATTCCACGATGAACAGCGG-CTTACCAG

The oligonucleotides introduced several restriction sites at either end of the amplified gene to facilitate subcloning. Amplified *pgmI* was subcloned into the *Bam*HI site of vector pUC119 (Clontech), creating the plasmid pUC119-*pgmI*. The *pgmI* gene was sequenced completely to ensure that no errors had been introduced in the amplification process; the sequence was identical to that published. The *pgmI* gene was subcloned from pUC119-*pgmI* into the *Bam*HI and *Sal*I sites of the expression vector pET15b (Novagen), allowing expression of iPGM with a six-histidine tag at the N-terminus, which resulted in the 22 amino acid extension MGSSHHHHHHSSGLVPRGSHMA at the N-terminus compared to the published sequence. Additionally, at position 2 in the amino acid sequence, a leucine residue has been replaced by an alanine. iPGM expressed with a native N-terminal sequence had very similar kinetic constants (data not shown).

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2.2. Over-expression and purification of phosphoglycerate mutase enzymes

For both proteins, cells were grown in LB medium containing 100 μg/ml carbenicillin in shaker flasks at 28°C to an A_{600} of 0.8. IPTG was added to a final concentration of 0.1 mM, and the cells were incubated for an additional 2.5 h under the same growth conditions. Cells were harvested by centrifugation, subjected to one freeze-thaw cycle to aid lysis, and resuspended in 5 ml per g (wet weight of cells) of lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.2 mM DTT). Cells were lysed by sonication (three 45 s bursts on ice), and the lysate was cleared by centrifugation (4°C; 20 min; 40 000 × *g*). The cleared lysate was subjected to ammonium sulphate fractionation.

2.2.1. dPGM. The protein precipitating between 50% and 85% ammonium sulphate saturation was resuspended in buffer F (20 mM Tris-HCl, pH 7.5, 300 mM KCl), and applied to a Superdex 200 26/70 size exclusion column (Amersham Pharmacia) equilibrated with buffer F, with a flow rate of 2.5 ml/min. dPGM eluted with an apparent molecular mass of 49 kDa, suggesting that the protein is a dimer of 28 kDa subunits. Fractions containing dPGM were pooled and dialysed against buffer A (20 mM Tris-HCl pH 8.5) at 4°C overnight. The dialysed protein was next applied to a POROS HQ 20/100 cation exchange column (Perseptive Biosystems) pre-equilibrated with buffer A, and eluted with a gradient of 0–0.2 M KCl in buffer A. Fractions collected from the column were analysed by SDS-PAGE, and those containing essentially pure dPGM were pooled and concentrated.

2.2.2. iPGM. The protein precipitating between 25% and 50% ammonium sulphate saturation was resuspended in buffer MC (20 mM $NaPO_4$, pH 7.5, 0.5 M KCl), and applied to a 5 ml HiTrap metal chelating column (Amersham-Pharmacia) pre-loaded with nickel chloride. The column was washed with three column volumes of buffer MC+50 mM imidazole to release weakly bound proteins, followed by three volumes of buffer MC+250 mM imidazole to elute iPGM. The protein was further purified by size exclusion chromatography as described for the dPGM enzyme. iPGM eluted with an apparent molecular mass of 61 kDa, consistent with the calculated molecular mass of the monomeric protein of 56 kDa.

2.3. Estimation of protein concentrations

Protein concentrations were estimated both by computer predictions of extinction coefficients at 280 nm ($\varepsilon^{0.1\%}_{280nm} = 1.9$ for dPGM and 0.65 for iPGM), and by dye binding assay using bovine serum albumin as a standard [12]. These techniques were in agreement to within 10%.

2.4. Enzyme assays

Phosphoglycerate mutase activities in the 3-PGA to 2-PGA (forward) and 2-PGA to 3-PGA (reverse) directions were monitored at 30°C using assays coupled to the oxidation of NADH [6]. For the forward direction, the assay mixture comprised 30 mM Tris-HCl pH 7.0, 20 mM KCl, 5 mM $MgSO_4$, 1 mM ATP, 0.15 mM NADH, 3-PGA (concentration varied), 0.1 mM 2,3-BPGA (for assay of dPGM activity), 0.08 U enolase (Roche), 0.5 U pyruvate kinase (Sigma), 0.5 U lactate dehydrogenase (Sigma).

For the reverse direction, the assay mixture comprised 30 mM Tris-HCl pH 7.0, 20 mM KCl, 5 mM $MgSO_4$, 0.2 mM ADP, 0.15 mM NADH, 2-PGA (concentration varied), 0.1 mM 2,3-BPGA (for assay of dPGM activity), 0.5 U phosphoglycerate kinase (Sigma), 0.5 U glyceraldehyde 3-phosphate dehydrogenase (Roche).

2.5. Vanadate inhibition

Vanadate inhibition experiments were carried out using the NADH-linked assay in the forward direction. Sodium metavanadate was added to the reaction mixture containing the phosphoglycerate mutase enzyme, and after 10 min the assay was initiated by addition of a saturating concentration of substrate 3-PGA (5 mM). Enzyme activity was assayed as described above, with triplicate measurements for each concentration of vanadate used.

2.6. Investigation of mutase expression and activity levels in E. coli

In order to measure the levels of phosphoglycerate mutase activity in *E. coli* extracts, an overnight culture of *E. coli* was used to inoculate 100 ml SOB medium, to an initial OD_{600} of approximately 0.03. Cells were grown at 37°C with vigorous shaking. Cell growth was monitored by measuring the optical density of the culture at 600 nm, with appropriate dilutions to ensure linearity. At intervals, an aliquot of cells was removed from the culture, cells were pelleted by centrifugation and resuspended in 60 μl chilled buffer (30 mM Tris-HCl pH 7.0, 20 mM KCl, 5 mM $MgSO_4$). Cells were lysed by sonication, and cell debris was pelleted by centrifugation at top speed in a microcentrifuge for 2 min. The supernatant, containing the soluble protein fraction, was used to measure dPGM and iPGM activity, and total protein concentration. Activity and protein assays were carried out in triplicate, and standard errors did not exceed 10%.

2.7. Electron paramagnetic resonance

EPR spectra were recorded on a Bruker 200D spectrometer (Bruker UK Ltd., Coventry, UK) fitted with an Oxford Instruments ER900 liquid helium cooled flow cryostat (Oxford Instrument Company Ltd., Osney Mead, Oxford, UK). Instrument settings are given in the figure legend.

3. Results

3.1. Expression and purification

Both of the phosphoglycerate mutase enzymes were expressed to a very high level in *E. coli* under the control of the T7 RNA polymerase promoter. dPGM was purified by size exclusion and anion exchange chromatography, whilst iPGM was purified by immobilised metal affinity chromatography, followed by size exclusion chromatography (Fig. 2). By calibrating the size exclusion column with a range of proteins of known molecular mass, the apparent molecular mass of dPGM was estimated at 49 kDa, corresponding to a dimer of 27 kDa subunits. iPGM eluted with an apparent molecular mass of 61 kDa, suggesting the 56 kDa protein is a monomer in solution.

3.2. Investigation of iPGM by EPR

As there has been some controversy over the presence of metal ions in cofactor-independent mutases, we used EPR spectroscopy to look for the presence of manganese in the *E. coli* iPGM protein. As can be seen in Fig. 3, the characteristic hyperfine peaks of manganese were observed, and the broadening of the peaks relative to the spectrum for free manganese suggested that the manganese was integrally

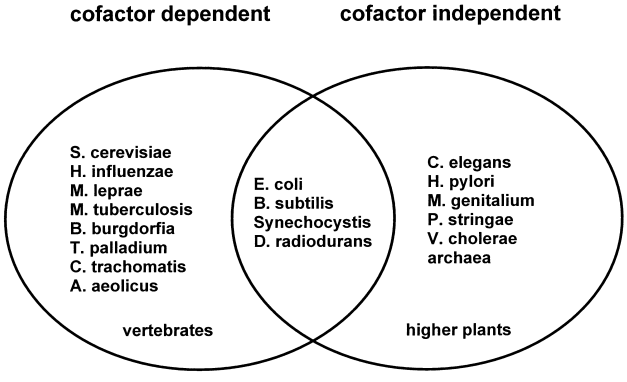

Fig. 1. Distribution of cofactor-dependent and -independent phosphoglycerate mutases. The *E. coli* dPGM and iPGM protein sequences were used to search the SwissProt database. Organisms with completed genome sequences were placed in one of three categories, according to the identification of sequence matches of high significance ($P < 10^{-6}$): only dPGM present; only iPGM present; both dPGM and iPGM present. Although no complete genome sequences are available for vertebrates or higher plants, there is good biochemical evidence that the former possess only dPGM and the latter iPGM, and they have been classified accordingly. The archaeal homologue of iPGM has been identified based on weak sequence similarity, and has not yet been confirmed biochemically [15].

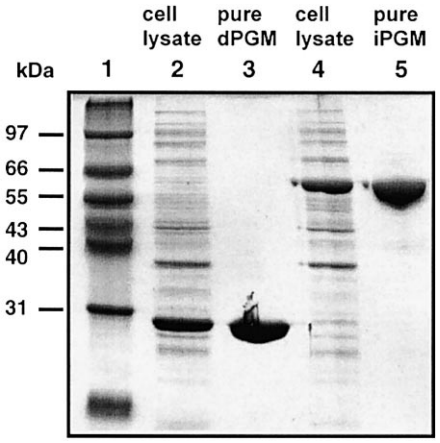

Fig. 2. SDS-PAGE analysis of protein expression and purification. Lane 1, size markers; lane 2, total soluble protein for cells expressing dPGM; lane 3, purified dPGM; lane 4, total soluble protein for cells expressing iPGM; lane 5, purified iPGM.

bound by the protein. These results therefore confirm the presence of manganese in *E. coli* iPGM.

3.3. Determination of kinetic constants

Steady-state kinetic analyses of the two purified enzymes were carried out for the forward (glycolytic) and reverse (gluconeogenic) reactions catalysed by phosphoglycerate mutases, as described in Section 2. Both enzymes displayed Michaelis-Menten kinetics, and the kinetic constants obtained are summarised in Table 1. The values obtained previously for dPGM from *S. cerevisiae* under the same assay conditions are included to allow comparison. For both *E. coli* enzymes, K_m values for the substrates 2-PGA and 3-PGA were in the range 100–200 μM. This differed from the *S. cerevisiae* enzyme, which had a 10-fold higher K_m for 3-PGA compared to 2-PGA. The catalytic constants for dPGM are similar for the forward and reverse reactions, and were approximately 10-fold higher than those for iPGM. dPGM therefore appears to be the more active enzyme in both the glycolytic and gluconeogenic reactions, but of course total mutase activity in vivo is dependent on the relative expression levels of the two enzymes.

3.4. Vanadate inhibition

Vanadate is known to be a potent inhibitor of a number of cofactor-dependent phosphoglycerate mutases in the micromolar range, but does not inhibit cofactor-independent enzymes [13]. We tested the effect of vanadate on the activity of purified *E. coli* dPGM and iPGM. Under saturating substrate concentrations (5 mM 3-PGA), iPGM was found to be insensitive to vanadate at concentrations up to 100 μM, whereas dPGM was strongly inhibited. By fitting the data for dPGM inhibition with an equation for reversible, competitive inhibition, an apparent K_I of 15 nM was calculated (Fig. 4). Inhibition by vanadate therefore appears to be a diagnostic test for discrimination between the cofactor-dependent and -independent phosphoglycerate mutases of *E. coli*.

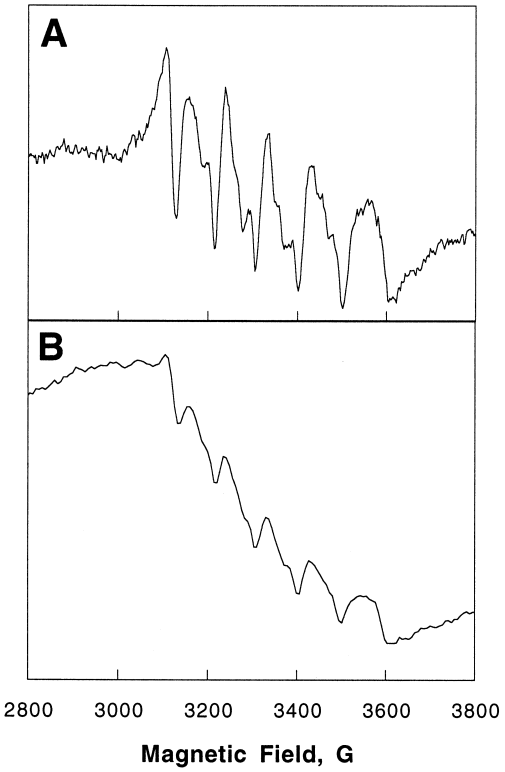

Fig. 3. X-band EPR spectra of 100 μM free manganese in solution (A) and iPGM (B). Experimental conditions: protein concentration in B, 2 mg/ml; temperature, 30 K; modulation amplitude, 5.2 Gauss; modulation frequency, 100 kHz; microwave frequency 9.42 GHz; microwave power 2.0 mW; receiver gain 2.5e^5.

3.5. Phosphoglycerate mutase activity during the E. coli growth cycle

The differential inhibition by vanadate of the two phosphoglycerate mutase enzymes allowed us to quantify the activity of each enzyme in *E. coli* cell lysates, and thus to compare relative expression and activity levels as a function of cell growth. Total phosphoglycerate mutase activity was first

Table 1
Kinetic constants for *E. coli* dPGM and iPGM and *S. cerevisiae* PGM

	dPGM	iPGM	*S. cerevisiae* PGM[a]
K_m 3-PGA (μM)	200 ± 27	210 ± 39	510 ± 60
K_m 2-PGA (μM)	190 ± 35	97 ± 14	52 ± 7
k_{cat} forward (s^{-1})	330 ± 11	22 ± 1	380 ± 10
k_{cat} reverse (s^{-1})	220 ± 13	10 ± 0.5	520 ± 25
Forward k_{cat}/K_m (M^{-1} s^{-1})	1.6×10^6	1.0×10^5	7.4×10^5
Reverse k_{cat}/K_m (M^{-1} s^{-1})	1.1×10^6	1.0×10^5	1.0×10^7

Kinetic constants were ascertained as described in Section 2. All points were measured in triplicate, and values are the mean ± S.D. The kinetic parameters of the cofactor-dependent mutases were measured at saturating concentrations of the 2,3-BPGA cofactor, and are therefore *apparent* k_{cat} and K_m values.
[a]Data from [16].

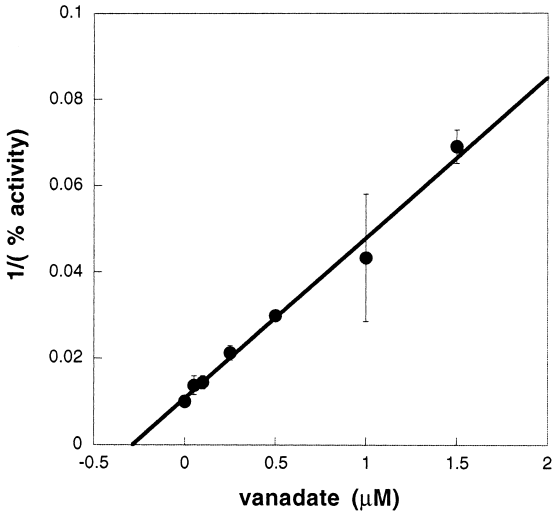

Fig. 4. Inhibition of dPGM by vanadate. Vanadate inhibition of dPGM was measured using the forward NADH-linked assay under saturating substrate concentrations (5 mM 3-PGA). Increasing concentrations of vanadate were added to dPGM in the reaction mixture and incubated for 10 min prior to initiation of the assay by addition of the substrate 3-PGA. All data points were measured in triplicate, and means ± S.D. are shown. Assuming competitive inhibition by vanadate, the K_I for the inhibitor can be calculated as 0.015 μM. If non-competitive inhibition is assumed, the K_I for vanadate is 0.29 μM. Clearly, vanadate is a potent inhibitor of dPGM at sub-micromolar concentrations.

measured using the NADH-linked forward assay, and the assay was then repeated in the presence of 10 μM vanadate. Under the latter conditions only the cofactor-independent enzyme was active, allowing calculation of the individual activities of the two enzymes in the lysate. Total protein concentrations in lysates were estimated using the Bradford dye binding assay, and by using the values obtained for the specific activities of dPGM and iPGM an estimate of the concentrations of the two proteins could be made. Phosphoglycerate mutase activities were measured at all stages of the growth cycle for *E. coli* strain DH5α grown in rich medium containing glucose (Fig. 5). Data obtained using *E. coli* strain JM101 were qualitatively similar (data not shown). iPGM was expressed most strongly in early exponential growth, with levels falling off as cells reached late log phase and stationary phase. dPGM was expressed slightly later, with peak expression observed in mid to late log phase. The levels of expression of the two enzymes each peaked at 20 μg/mg total protein, showing that both are abundant enzymes in the cell. As the cofactor-dependent enzyme has a much higher specific activity, the differential temporal expression of the two enzymes resulted in a wide variation in the ratio of dPGM to iPGM activity, ranging from 5-fold to 35-fold higher during cell growth.

4. Discussion

It has long been appreciated that cofactor-dependent and -independent phosphoglycerate mutases have a complex distribution in nature [14]. Whilst it was established that vertebrates possessed the former and higher plants the latter, the distribution in bacteria and fungi was known to be complicated to the point of appearing haphazard. The advent of whole-genome sequencing projects has provided us with a wealth of unequivocal information on the occurrence of the two enzymes (Fig. 1), and has highlighted the unexpected finding that some bacteria encode both proteins. Although the cofactor-independent mutase is a potential drug target in many organisms of biomedical relevance, it remains perhaps the least characterised of all the glycolytic enzymes, with fundamental questions regarding its structure and mechanism unresolved. One example of this is the controversy over the presence and importance of metal ions in iPGMs. Purification of milligram quantities of *E. coli* iPGM allowed us to demonstrate the presence of bound manganese directly using EPR. Given this finding, and the conservation of metal binding

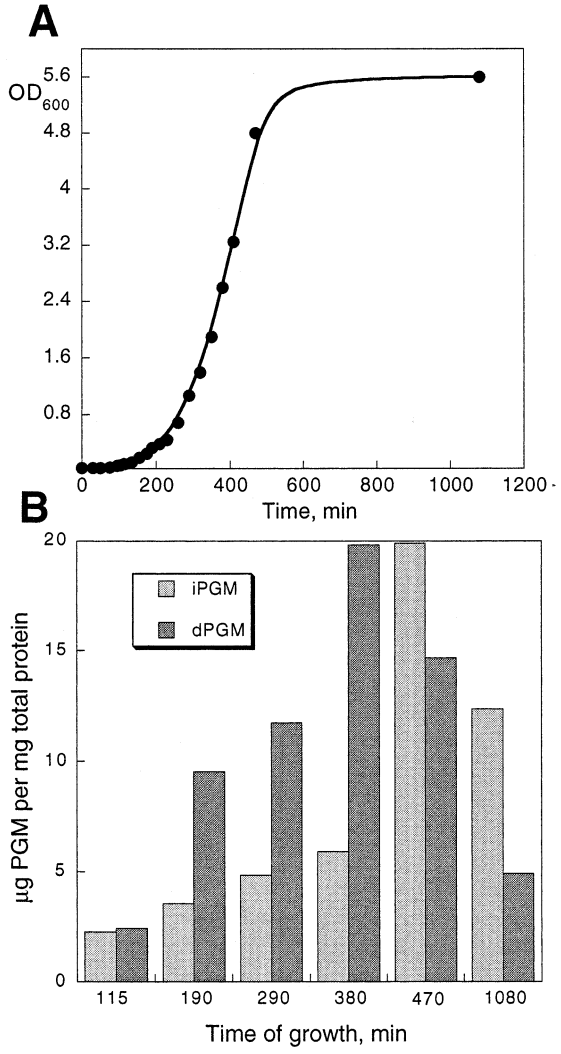

Fig. 5. dPGM and iPGM expression as a function of *E. coli* growth phase. The details of the assay are described in Section 2. A: The growth of a culture of *E. coli* strain DH5α was followed by measuring the optical density of suitably diluted aliquots at 600 nm. B: Utilising the differential inhibition of dPGM and iPGM by vanadate to distinguish between the two enzymes, activities were measured during cell growth. PGM activity was measured in triplicate at saturating substrate concentrations using the forward (glycolytic) assay. Standard deviations were found to be below 10% for each point. The expression levels of iPGM and dPGM were calculated by dividing the observed activity of the two enzymes by their respective specific activities, yielding the amount of each enzyme present in the cell lysate. Total protein concentration was determined by a dye binding assay [12] in triplicate (standard errors $< 10\%$), and μg mutase per mg total protein was plotted for the two enzymes at the six growth stages.

residues between iPGM and alkaline phosphatase [1], we can now predict with some confidence that all cofactor-independent mutases are, like the *Bacillus* iPGMs, metalloenzymes with bound manganese perhaps fulfilling a catalytic role.

Comparison of the kinetic properties and expression levels of the two phosphoglycerate mutases of *E. coli* has allowed the metabolic role of each to be assessed. Both catalyse the interconversion of 2- and 3- phosphoglycerate in the glycolytic and gluconeogenic directions, but dPGM has at least a 10-fold higher specific activity for both reactions. The observation that vanadate is a potent inhibitor specific for dPGM has allowed us to determine the relative expression and specific activity levels of the two enzymes in *E. coli*. Variation in the expression levels with growth phase was observed, with iPGM expressed slightly earlier than dPGM. Although both enzymes are highly expressed in *E. coli*, the 10-fold higher specific activity of dPGM ensures that dPGM is the dominant mutase activity in *E. coli*.

In addition to identifying the predominance of dPGM, our results also question the role of iPGM in *E. coli*. One possibility is that iPGM catalyses an alternative reaction that is important for *E. coli* metabolism. Wheat germ iPGM has previously been shown to act as a phosphatase for a variety of small compounds including phosphoenolpyruvate, 2-phospholactate and 3-phosphohydroxypropionate [10], and these or related activities might have biological relevance under certain growth conditions. Creation of iPGM and dPGM null mutants in *E. coli* will allow the role and importance of both enzymes to be tested in more detail.

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