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The *PGM3* gene encodes the major phosphoribomutase in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

The phosphoglucomutases (PGM) Pgm1, Pgm2, and Pgm3 of the yeast *Saccharomyces cerevisiae* were tested for their ability to interconvert ribose-1-phosphate and ribose-5-phosphate. The purified proteins were studied in vitro with regard to their kinetic properties on glucose-1-phosphate and ribose-1-phosphate. All tested enzymes were active on both substrates with Pgm1 exhibiting only residual activity on ribose-1-phosphate. The Pgm2 and Pgm3 proteins had almost equal kinetic properties on ribose-1-phosphate, but Pgm2 had a 2000 times higher preference for glucose-1-phosphate when compared to Pgm3. The in vivo function of the PGMs was characterized by monitoring ribose-1-phosphate kinetics following a perturbation of the purine nucleotide balance. Only mutants with a deletion of *PGM3* hyper-accumulated ribose-1-phosphate. We conclude that Pgm3 functions as the major phosphoribomutase in vivo.

1. Introduction

Phosphoribomutase (PRM) catalyzes the interconversion of ribose-1-phosphate (ribose-1P) and ribose-5-phosphate (ribose-5P). The molecular identity of this protein in *Saccharomyces cerevisiae* is not yet established unequivocally. A recent phylogenetic study assigned a putative PRM function to ORF YMR278w [1]. However, Tiwari and Bhat [2] proposed that this gene rather encodes the third phosphoglucomutase, and consequently termed it *PGM3*. Their conclusion derived from the observations that overexpression of *PGM3* complemented the growth defect of a *pgm1 pgm2* double mutant during growth on galactose, and that purified Pgm3, albeit being active on both glucose-1-phosphate (glucose-1P) and ribose-1P, exhibited a very weak affinity for the latter substrate in vitro [2].

Our interest in the identification of PRMs in *S. cerevisiae* derived from our work on the regulation of energy homeostasis in yeast. In a recent study we found that adenosine nucleotides were converted into the purine salvage pathway metabolite, inosine, upon perturbation of energetic equilibrium under fermentative conditions [3]. Inosine was recycled into the purine nucleotide pool via the consecutive action of purine nucleoside phosphorylase, Pnp1, and the phosphoribosyltransferases, Hpt1 and Xpt1 (Fig. 1). Upon the recycling of inosine, a transient accumulation of ribose-1P was observed that was strictly dependent on the function of Pnp1 [3,4]. One question that remained unanswered in this study was to know which enzyme catalyzed the conversion of ribose-1P into ribose-5P, thus, enabling the recycling of the nucleoside's ribose moiety through the pentose phosphate pathway.

In the present study we set out to identify the PRM(s) in *S. cerevisiae*. The Pgm1, Pgm2, and Pgm3 proteins were overexpressed in *Escherichia coli*, purified, and their kinetic properties on glucose-1P and ribose-1P were studied. All enzymes were active on both substrates but Pgm1 exhibited only residual activity on ribose-1P. The Pgm2 and Pgm3 proteins had almost equal kinetic properties on ribose-1-phosphate, but only in mutants defective in *PGM3* we observed a strongly increased concentration of ribose-1P and completely defective recycling of ribose-1P upon glucose-induced purine nucleoside recycling via the purine salvage pathway. From these results we conclude that Pgm3 functions as the major phosphoribomutase in vivo.

2. Materials and methods

2.1. Strains media and cultivation conditions

Glucose-limited growth was mimicked by cultivating the yeast strains in a medium containing 10 g/l trehalose (Sigma), 5 g/l $(NH_4)_2SO_4$, 1.76 g/l YNB (Yeast Nitrogen Base w/o amino acids and ammonium sulfate) (Difco), supplemented with uracil at a final concentration of 100 mg/l. The medium was buffered at pH 5.5 by 50 mM potassium phthalate. Cells were cultivated in Erlenmeyer flasks at 30 °C, and shaken on a rotary shaker (Infors) at 200 rpm. When yeast cultures reached a biomass concentration of 1 gDW/l (DW = cellular dry weight), glucose was added at a final

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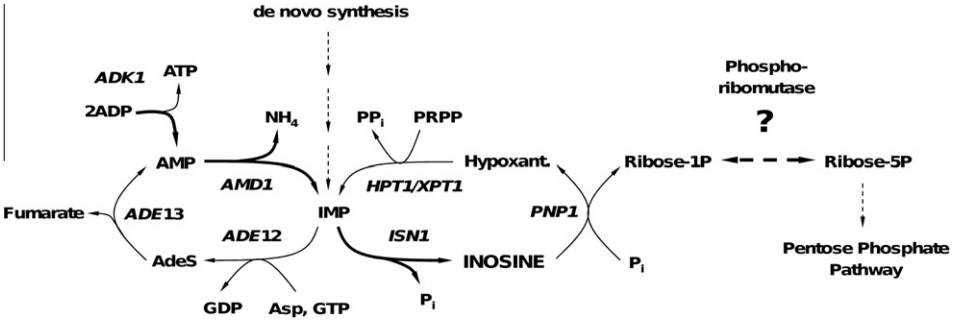

Fig. 1. Pathway responsible for purine nucleotide conversions during energetic adaptation to fermentative conditions.

Table 1
List of yeast strains used in this study.

Strain	Relevant genotype	Source
Wild-type	CEN.PK133-5D Mat **a**,*ura3-52*	[5]
pgm1	Mat **a**, *pgm1Δ*::*kanMX4ura3-52*	This study
pgm2	Mat **a**,*pgm2Δ*::*kanMX4*, *ura3-52*	This study
pgm3	Mat **a**,*pgm3Δ*::*natMX4*,*ura3-52*	This study
pgm1,2	Mat **a**, *pgm1Δ*::*kanMX4*, *pgm2Δ*::*kanMX4*, *ura3-52*	This study
pgm1,2,3	Mat **a**, *pgm1Δ*::*kanMX4*, *pgm2Δ*::*kanMX4*, *pgm3Δ*::*natMX4*, *ura3-52*	This study

Table 2
Primers used to amplify *PGM* genes from genomic DNA. Restriction sites are underlined.

Primer	Sequence (5′–3′)	Linker
PGM1_clon_for	TATAATGCTAGCATGTCACTTCTAATAGATTC	*Nhe*I
PGM1_clon_rev	TATAATCTCGAGCTATGTGCGGACTGTTGGTT	*Xho*I
PGM2_clon_for	TATAATGCTAGCATGTCATTTCAAATTGAAAC	*Nhe*I
PGM2_clon_rev	TATAATCTCGAGTTAAGTACGAACCGTTGGTT	*Xho*I
PGM3_clon_for	TATAAGCTAGCATGTTGCAAGGAATTTTAGA	*Nhe*I
PGM3_clon_rev	TATAATCTCGAGTCAAAATTTTGTAACTATAT	*Xho*I

concentration of 10 g/l using a concentrated stock solution of 400 g/l.

All mutants analysed in this study were derived from the *S. cerevisiae* CEN.PK133-5D ura3-52 strain [5] (referred to as wild-type, Table 1). Disruption cassettes containing the *kanMX4* cassette flanked by ~200 base pairs homologous to the upstream and downstream region of the targeted ORF were amplified from single deletion mutants of the EUROSCARF collection [6] and transformed into the corresponding strains using standard genetic protocols. The *pgm1 pgm2* double mutant was obtained by crossing and sporulation of the corresponding single mutants. The *natMX4* cassette for the deletion of *PGM3* was amplified from the vector pFvL99 [7]. Disruptions were verified by PCR.

2.2. Construction of plasmids for expression of his-tagged enzymes

The coding sequences of the *PGM1*, *PGM2*, and *PGM3* genes were amplified using high fidelity polymerase Phusion™ (Finnzymes) and the primers listed in Table 2 that introduced a NheI and a XhoI restriction site upstream of the start codon and downstream of the stop codon, respectively. Genomic DNA of *S. cerevisiae* BY4741 was used as the template. The PCR products were digested with *Nhe*I and *XhoI*, ligated into the corresponding sites of the pET28a+(Novagen) expression vector using T4 DNA ligase (Biolabs), and transformed into *E. coli* DH5α cells. The resulting pET28-pgm1, pET28-pgm2, and pET28-pgm3 plasmids were isolated and shown by DNA sequencing to contain the full-length *PGM* genes having the correct sequence:

2.3. Expression and purification of enzymes

E. coli BL21 (DE3) cells were transformed with the appropriate plasmids using standard genetic protocols [8]. Recombinant bacteria were inoculated in 250 ml LB cultures containing 50 μg/l kanamycin from an overnight culture at OD_{600} of 0.1. Protein expression was induced at OD_{600} of 0.6 by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium. After 3 h of protein expression, cells were harvested by centrifugation at 13 000×*g* for 10 min. The supernatant was discarded and cell pellets were stored at −20 °C until further analysis. Growth and protein expression were carried out at 37 °C.

Frozen cell pellets of expression cultures were resuspended in 0.5 ml of breakage buffer (50 mM Hepes, 300 mM NaCl, pH 7.5) and broken open by four successive rounds of sonication (sonication interval: 30 s, power output: 30%, sonicator: Bioblock Scientific, VibraCell™ 72437). Cell debris was removed by centrifuging the crude extracts for 15 min at 4 °C at 13 000×*g* and retaining the clear supernatant. RNA and DNA were removed from the extracts by adding 15 mg/ml streptomycin (Sigma), centrifuging the samples at 13 000×*g* for 7 min at 4 °C and retaining the supernatant. Clear protein extract was incubated and gently mixed for 20 min at room temperature with 0.75 ml (bed volume) of Talon™ Cobalt affinity resin (Clontech). The suspension was centrifuged at 700×*g* in a table top centrifuge and supernatant was removed. The resin was washed with 10 bed volumes of wash buffer (50 mM Hepes, 300 mM NaCl, 15 mM Imidazole, pH 7.5) before proteins were eluted with 0.5 ml of elution buffer (50 mM Hepes, 300 mM NaCl, 250 mM Imidazole, pH 7.5). Protein concentrations were estimated with the method of Bradford.

2.4. Phosphoglucomutase assay

Phosphoglucomutase activity was assayed in a reaction mixture that contained a suitable amount of purified enzyme, 60 mM Hepes (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$, 0.5 mM NADP, 3 U/ml glucose-6-phosphate dehydrogenase, and 5 μM glucose-1,6-bisphosphate. The reactions were started by adding appropriate amounts of glucose-1-phosphate (all products from Sigma). Assays were carried out at 30 °C in 96-well flat bottomed microtiter plates in a final volume of 250 μl. The reactions were followed by the characteristic absorption of NADPH at 340 nm (εNADPH = 6.22 mM^{-1} cm^{-1}) in a microplate reader (BioRad 680XR).

2.5. Phosphoribomutase assay

Phosphoribomutase activity was assayed based on the method proposed by Tiwari and Bhat [2] in a reaction mixture that

Table 3
Kinetic parameters of purified His-tagged Pgm proteins on glucose-1-phosphate and ribose-1-phosphate.

Enzyme	Glucose-1-phosphate			Ribose-1-phosphate		
	V_{max} [μmol/(min mg_{prot})]	k_m^* [μM]	k_{cat}/k_m [10^3mol/(L s)]	V_{max} [μmol/(min mg_{prot})]	k_m^* [μM]	k_{cat}/k_m [10^3mol/(L s)]
Pgm1	0.24 ± 0.09	60 ± 2.1	4.2	0.06 ± 0.01	nd	nd
Pgm2	33.7 ± 8.1	26 ± 0.4	1388	0.32 ± 0.01	530 ± 3	0.63
Pgm3	0.11 ± 0.05	112 ± 1.9	1.16	0.29 ± 0.06	750 ± 35	0.46

nd – not determined, (*) k_m values were estimated using the method of Eadie and Hofstee.

contained a suitable amount of purified protein, 60 mM Hepes (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$, and 5 μM glucose-1,6-bisphosphate. The reactions were started by adding appropriate amounts of ribose-1-phosphate (all products from Sigma). Assays were carried out at 30 °C in 96-well flat bottomed microtiter plates in a final volume of 250 μl. After 0, 10, 20, 30, and 40 min of incubation, 30 μl aliquots of the reactions were mixed with 30 μl of 0.5 M H_2SO_4, and incubated for at least 10 min. Acid labile phosphate released from the remaining ribose-1-phosphate was quantified by a colorimetric method [9].

2.6. Metabolite sampling and analysis

Sampling for intracellular metabolites was carried out by filtering 5 ml of culture medium on a polyamide membrane (pore size 0.45 μm, Sartorius), rinsing the cells on filter using 10 ml water containing only the corresponding carbon source, and quenching the cells metabolism in 80 °C hot ethanol (75%). Processing of the samples was done as described earlier [10,11]. Metabolites were quantified using LC–MS: Liquid anion exchange chromatography was performed on an ICS-3000 system from Dionex (Sunnyvale, USA) equipped with an automatic eluent (KOH) generator system (RFIC, Dionex), and an autosampler (AS50, Dionex) holding the samples at 4 °C. Analytes were separated on an IonPac AS11 (250 × 2 mm, Dionex) column protected by an AG11 (50 × 2 mm, Dionex) pre-column. Column temperature was held at 25 °C, flow rate was fixed at 0.25 ml/min, and analytes were eluted applying the KOH gradient described earlier [12]. Injected sample volume was 15 μl. For background reduction, an ASRS ultra II (2 mm, external water mode, 75 mA) anion suppressor was used. Analytes were quantified using a photo diode array detector (Ultimate 3000, Dionex), a conductivity detector (part of ICS-3000, Dionex) and a mass-sensitive detector (MSQ Plus, Thermo) running in ESI mode (split was 1/3, nitrogen pressure was 90 psi, capillary voltage was 3.5 kV, probe temperature was 450 °C).

3. Results and discussion

3.1. The Pgm1, Pgm2 and Pgm3 proteins have phosphoribomutase activity in vitro

The coding sequences of the *PGM1*, *PGM2*, [13] and *PGM3* [2] genes were amplified from genomic DNA, cloned into the pET28a+ expression vector and overexpressed in *E. coli* BL21 (DE3). The proteins expressed from these constructs carried a hexa-His tag at their N-terminus enabling their purification by cobalt affinity chromatography. SDS–page analyses of the protein preparations showed only one band migrating at the expected sizes of the purified proteins (data not shown). The purified proteins were then characterized with regard to their PGM and PRM activity using glucose-1P or ribose-1P, respectively, as the substrate. All three tested enzymes were active on glucose-1P. The k_m value of His-tagged Pgm2 was estimated with 26 μM (Table 3). This result agreed with the k_m value observed for the untagged purified enzyme [14] suggesting that the His-tag does not interfere with the substrate affinity of the Pgm proteins. Pgm2 had the highest catalytic efficiency (in terms of k_{cat} over k_m) on glucose-1P exhibiting both the highest maximum specific activity and the highest substrate affinity of the tested enzymes (Table 3). In agreement with their minor role in the interconversion of glucose-1P and glucose-6P, Pgm1 and Pgm3 had catalytic efficiencies on glucose-1P that were three orders of magnitude lower than for Pgm2.

Furthermore, the three tested PGM enzymes had catalytic activity on ribose-1P. However, maximum specific activity (V_{max}) of Pgm1 was only one fifth of Pgm2 and Pgm3 (Table 3). The k_m value of this enzyme could not be determined. Pgm2 and Pgm3 had nearly identical kinetic parameters (k_m and V_{max}) on ribose-1P. Contrary to [2] who found that Pgm3 could not be saturated at ribose-1P concentrations of up to 3 mM, we estimated a k_m value of 750 μM for this enzyme. While Pgm3 accepted glucose-1P and ribose-1P as a substrate with very similar catalytic efficiency, Pgm2 acted $\sim 2 \times 10^3$ better on glucose-1P than on ribose-1P (Table 3).

3.2. Mutants carrying deletions in PGM3 hyper-accumulate ribose-1-phosphate in vivo

In order to test whether Pgm1, Pgm2, and Pgm3 act as PRM in vivo, we investigated ribose-1P dynamics in mutants carrying single or multiple deletions in *PGM* genes. In a recent study, we found that *S. cerevisiae* cells responded to the perturbation of energetic equilibrium during the respiro-fermentative transition by conversion of adenosine nucleotides ([AXP] = [ATP] + [ADP] + [AMP]) into inosine. Inosine recycling into the AXP pool coincided with a transient Pnp1-dependent accumulation of ribose-1P [3]. Thus, addition of glucose to respiring yeast cells represents a convenient means to trigger ribose-1P accumulation. Monitoring of ribose-1P dynamics in *pgm* mutants under these conditions was used to identify the Pgm enzyme(s) that act(s) as PRM in vivo.

Wild-type and mutant strains with single or multiple deletions of *PGM* genes were cultivated under respiratory conditions in shake flask cultures using trehalose as the only carbon source: When biomass concentration reached 1 gDW/l, glucose was added at a final concentration of 10 g/l. At the indicated time intervals, cells (5–10 mg DW) were rapidly collected by filtration and their metabolism was instantaneously quenched upon transfer to 75% hot ethanol. Metabolite concentrations were estimated using IC-MS. Ribose-5P, xylose-5P and xylulose-5P could not be separated by the applied method. Concentrations of these metabolites were measured as a single pool denoted pent-5P (Fig. 2).

In agreement with earlier data, respiring wild-type cells responded to the addition of glucose by a transient decrease of ATP and AXP concentrations (Fig. 2A). The apparent loss of AXP nucleotides resulted in the accumulation of IMP and inosine (Fig. 2B). Concomitantly, a transient but very weak increase of ribose-1P from a basal concentration of ~5 to ~150 nmol/gDW could be observed (Fig. 2C) indicating active Pnp1. The same behavior was found for the *pgm1*, *pgm2*, and *pgm1 pgm2* strains (Fig. 2F, I, P) indi-

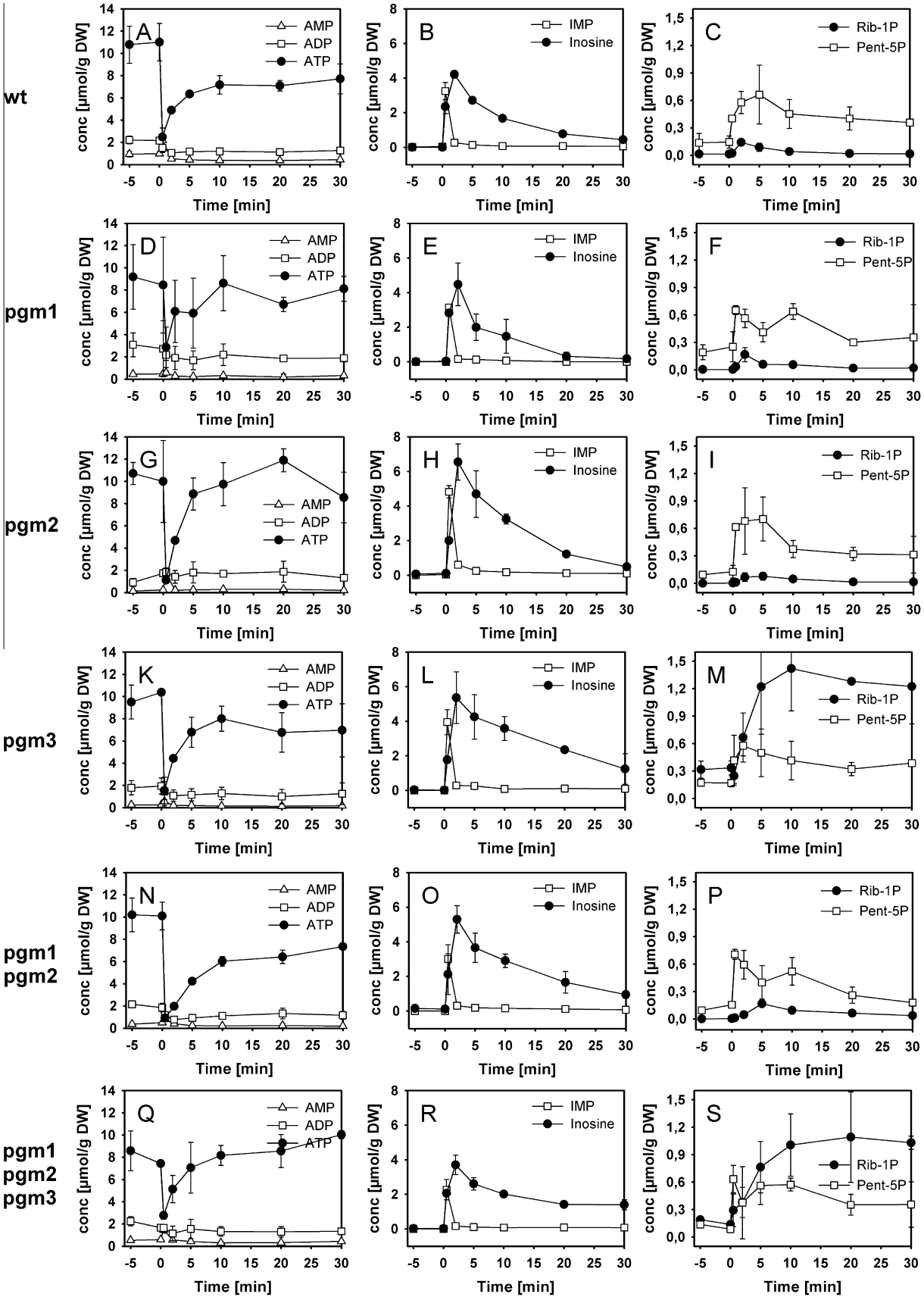

Fig. 2. Metabolite dynamics in cells exposed to a sudden increase of glucose concentration. Left panel: adenosine nucleotide concentrations. Middle panel: IMP and inosine concentrations. Right panel: ribose-1P and pentose-5P concentrations. Data shown represent the average of at least two independent experiments.

cating that the corresponding proteins were not implicated in ribose-1P to ribose-5P conversion. Mutants carrying deletions in *pgm3* (*pgm3* and *pgm*1 *pgm*2 *pgm*3) exhibited a different behavior: during unperturbed growth on trehalose, the ribose-1P concentration in these strains was ~170 nmol/gDW and thus significantly higher than in wild type, *pgm1* or *pgm2* mutant cells. This concentration readily increased to values larger than 1 μmol/gDW upon glucose addition (Fig. 2M, S) which triggered ribose-1P release through inosine formation and recycling (Figs. 1 and 2L, R). Once ribose-1P was accumulated in the *pgm*3 or *pgm*1 *pgm*2 *pgm*3 strains, its concentration remained nearly constant during the observed time span of 60 min after glucose addition (60 min time point not shown).

Combining the in vitro kinetic characterizations of the PGM proteins on ribose-1P and in vivo ribose-1P dynamics of *pgm* mutants we conclude that Pgm3 functions as the major phosphoribomutase in vivo. Our results are therefore at variance to those of Tiwari and Bhat [2] who found a very weak affinity of Pgm3 for Glucose1-P (that is k_m 2 mM, compared to 0.112 mM in our study) and who were unable to determine a k_m for ribose-1P. Based on these data, they concluded that Pgm3 could not function as PRM in vivo. The reasons for the discrepancies of these in vitro data are not clear since we have used similar experimental protocols and methods for enzymatic assays. More importantly, our results indicate that inferring in vivo metabolic function from in vitro enzymatic assays alone may be misleading: in our study, Pgm2 and Pgm3 were found to have nearly identical catalytic efficiency on ribose-1P (Table 3). Nevertheless, Pgm2 cannot compensate for the lack of Pgm3 to convert ribose 1-P to ribose 5-P in vivo. This fact may be explained by the preference of Pgm2 for glucose-1P which is three orders of magnitude higher than for ribose-1P.

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