Excess Mannose Limits the Growth of Phosphomannose Isomerase
PMI40 Deletion Strain of Saccharomyces cerevisiae* ❯

Received for publication, September 15, 2004, and in revised form, October 18, 2004
Published, JBC Papers in Press, November 1, 2004, DOI 10.1074/jbc.M410619200

Juha-Pekka Pitkänen‡, Anssi Törma§§, Susanne Alff‡, Laura Huopaniemi‡§, Pirko Mattila‡, and Risto Renkonen‡‡
From ‡MediCel Ltd., Haartmaninkatu 8, FIN-00290 Helsinki, Finland and the §Rational Drug Design Program,
Department of Bacteriology and Immunology, Haartman Institute and Biomedicum, University of Helsinki, P.O. Box 63,
FIN-00014 Helsinki, Finland

Phosphomannose isomerase (PMI40) catalyzes the conversion between fructose 6-phosphate and man-
noise 6-phosphate and thus connects glycolysis, i.e. energy production and GDP-mannose biosynthesis or cell wall synthesis in Saccharomyces cerevisiae. After PMI40 deletion (pmi−) the cells were viable only if fed with extracellular mannose and glucose. In an attempt to force the GDP-mannose synthesis in the pmi− strain by increasing the extracellular mannose concentrations, the cells showed significantly reduced growth rates without any alterations in the intracellular GDP- mannose levels. To reveal the mechanisms resulting in reduced growth rates, we measured genome-wide gene expression levels, several metabolite concentrations, and selected in vitro enzyme activities in central metabo-
lic pathways. The increasing of the initial mannose concentration led to an increase in the mannose 6-phosphate concentration, which inhibited the activity of the second enzyme in glycolysis, i.e. phosphoglu-
cose isomerase converting glucose 6-phosphate to fruc-
tose 6-phosphate. As a result of this limitation, the flux through glycolysis was decreased as was the median expression of the genes involved in glycolysis. The expression levels of RAPI, a transcription factor in-
volved in the regulation of the mRNA levels of several enzymes in glycolysis, as well as those of cell cycle regulators CDC28 and CLN3, decreased concomitantly with the growth rates and expression of many genes encoding for enzymes in glycolysis.

PMI40 gene (1). In a PMI40 deletion strain (pmi−), the synthe-
sis of Man-6-P from Fru-6-P is not possible, disabling the growth of such a strain on medium without mannose. The inability to grow, caused by defective glycosylation of a temper-
ature-sensitive pmi40 mutant of S. cerevisiae, and repairing the defects by addition of mannose to the growth medium have been described previously (2). In humans PMI4 deficiency is the cause of carbohydrate-deficient glycoprotein syndrome type Ib, but the condition can be successfully treated by mannose ad-
ministration (3). Man-6-P produced either from Fru-6-P or mannose serves as a precursor for the de novo biosynthesis of GDP-mannose. Man-6-P is converted to mannose 1-phosphate (Man-1-P) by phosphomannomutase encoded by SEC53. Sub-
sequently, Man-1-P is ligated with the guanosine 5-triphos-
phate molecule (GTP) to form GDP-mannose by Man-1-P gua-
nylyltransferase encoded by PSA1 (4). The de novo formation of the purine ring of GTP, required for the biosynthesis of GDP-
mannose, starts from ribose 5-phosphate in the pentose phos-
phate pathway and requires also 3-phosphoglycerate in the glycolysis as a precursor. Taken together, the biosynthesis of GTP is more complex than the mannose pathway (4). GDP-
mannose is needed in S. cerevisiae for mannosylation of various structures such as lipopolysaccharides and glycoproteins (4). Additionally, GDP-mannose is necessary as a precursor for other sugar nucleotides including GDP-fucose and GDP-
ram-
nose (5–7). In S. cerevisiae GDP-mannose biosynthesis, as nu-
cleotide sugar biosynthesis generally, is carried out in the cytosol (8). Hypothetically, a strain lacking the gene for PMI4 would direct all its mannose into the biosynthesis of GDP-
mannose.

We applied novel measurement techniques to study the syn-
thesis of GDP-mannose and main metabolic pathways in a S. cerevisiae pmi− strain. Samples taken from carefully controlled bioreactor cultivations at different mannose concentrations were analyzed using genome-wide transcription profiling, anal-
ysis of selected enzyme activities, and analysis of selected in-
tracellular metabolite concentrations. In this study we demon-
strated that in batch cultivation increasing amounts of initial mannose inhibit the growth of the pmi− strain but not that of the control strain. We postulate the mechanisms leading to the growth inhibition and demonstrate the effects it causes.

MATERIALS AND METHODS

Yeast Strains—A genetically modified S. cerevisiae yeast strain with PMI40 knock-out (MATα URA3 TRP1 LEU2 his3-D1 MAL2–Δ6 suc2 PMI40::HIS3) was constructed as follows. Most of the reading frame (nucleotides 147–1233) of the PMI40 gene was PCR-amplified from S. cerevisiae chromosomal DNA and subsequently cloned into Xhol/XbaI sites of vector pRS403 (Stratagene). HIS3 gene with its regulatory regions was amplified by PCR from pRS403 with primers (5’ ggaattc-
CGTTTCGCGTATGACGTTGAAAT GC 3’ and 5’ ggaattcTTCCTGTATCGGG-

This paper is available on line at http://www.jbc.org

‡‡This work was supported by the Academy of Finland (Graduate School in Chemical Engineering (to J.-P. P. )) and the National Techno-
lology Agency (TEKES Neobio project). The costs of publication of this
article were defrayed in part by the payment of page charges. This
advertisement □□ This work was supported by the Academy of Finland (Graduate
School in Chemical Engineering (to J.-P. P. )) and the National Techno-
lology Agency (TEKES Neobio project). The costs of publication of this
article were defrayed in part by the payment of page charges. This

□□ This work was supported by the Academy of Finland (Graduate
School in Chemical Engineering (to J.-P. P. )) and the National Techno-
logy Agency (TEKES Neobio project). The costs of publication of this
article were defrayed in part by the payment of page charges. This

*S The abbreviations used are: PMIe, phosphomannose isomerase en-
zyme; Fru-6-P, fructose 6-phosphate; Man-6-P, mannose 6-phosphate;
CDW, cell dry weight; PGle, phosphoglucone isomerase; PKF, phos-
phofruktokinase; PPy, pyruvate kinase; Fru-1,6-P, fructose 1,6-
biphosphate; CIT/ICIT, citrate and isocitrate; Glu-6-P, glucose 6-phos-
phate; GO, gene ontology.

†† To whom correspondence should be addressed: Dept. of Bacteriol-
ogy and Immunology, Haartman Institute, P.O. Box 63, FIN-00014
University of Helsinki, Finland. E-mail: Risto.Renkonen@helsinki.fi.

‡‡ This work was supported by the Academy of Finland (Graduate
School in Chemical Engineering (to J.-P. P. )) and the National Techno-
lology Agency (TEKES Neobio project). The costs of publication of this
article were defrayed in part by the payment of page charges. This
article must therefore be hereby marked “advertisement” in accordance
with 18 U.S.C. Section 1734 solely to indicate this fact.

* The abbreviations used are: PMIe, phosphomannose isomerase en-
zyme; Fru-6-P, fructose 6-phosphate; Man-6-P, mannose 6-phosphate;
CDW, cell dry weight; PGle, phosphoglucone isomerase; PKF, phos-
phofruktokinase; PPy, pyruvate kinase; Fru-1,6-P, fructose 1,6-
biphosphate; CIT/ICIT, citrate and isocitrate; Glu-6-P, glucose 6-phos-
phate; GO, gene ontology.

†† To whom correspondence should be addressed: Dept. of Bacteriol-
ogy and Immunology, Haartman Institute, P.O. Box 63, FIN-00014
University of Helsinki, Finland. E-mail: Risto.Renkonen@helsinki.fi.

The Journal of Biological Chemistry © 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 279, No. 53, Issue of December 31, pp. 55737–55743, 2004
Printed in U.S.A.
Phosphomannose Isomerase Deletion in Yeast

TATTICTCTCT(3) and cloned into the unique restriction site of EcoRI in PMI40 (nucleotide 681 in CDS). Digestion of the resulting construct with EcoRI and BamHI (nucleotides 207 and 384, respectively) revealed a 2400-bp fragment with PMI40-specific tails. This DNA construct was transformed into the S. cerevisiae strain CEN.PK13-7A (MATa URA3 TRPI LEU2 his3-D1 MAL2-8° SUC2) (9), a derivative of strain CEN.PK2-1D (MATa ura3-52 trpl-289 leu2-3,112 his3-D1 MAL2-8° SUC2) (10). The cells were plated on selective medium (HIS) with 5 g/liter mannose and 20 g/liter glucose. The control strain CEN.PK13-7D (MATa URA3 TRPI LEU2 HIS3 MAL2-8° SUC2) was obtained from Eppendorf (Frankfurt, Germany) (9).

Bioreactor Cultivations—The inocula for bioreactor cultivations were grown in 250-ml Erlenmeyer shake flasks on YPD-medium containing 10 g/liter yeast extract, 20 g/liter peptone (International Diagnostics Group, Lancashire, UK), 20 g/liter v-glucose (BDH Laboratory Supplies, Poole, UK), and 1 g/liter n-mannose (Fluka, Buchs, Switzerland). The inocula were started from 20 mg cell dry weight (CDW) of cells in glycerol stocks. The inocula were incubated at 30 °C, 1000 rpm (model C2, New Brunswick Scientific, Edison, NJ). Roughly, 0.5 g CDW of cells in 0.9% NaCl were used to inoculate the bioreactor cultivations. Bioreactor cultivations were performed in 3.2-liter Braun Biostat CT2-DCU 3 instruments (B. Braun Biotech International, Melsungen, Germany). The cultivation temperature was 30 °C, agitation speed 200 rpm (model C2, New Brunswick Scientific, Edison, NJ).

RESULTS

The phosphomannose isomerase (PMI40) deletion strain, here called the pmi− strain, and a wild type control strain were cultivated in carefully controlled batch cultivations using a bioreactor. The initial glucose concentration was 15 g/liter in all cultivations, whereas the initial mannose concentration varied. The pmi− strain was cultivated on 0.75, 1, 1.5, 3, and 5 g/liter mannose and the control strain on 0.75 and 5 g/liter mannose. As shown in Table I, the specific growth rate (h−1) of the pmi− strain decreased as the initial mannose concentration increased. The negative effect of mannose in growth could not be seen with the control strain as the growth rates of the control strain on both 0.75 or 5 g/liter mannose were higher than that of the pmi− strain on 0.75 g/liter mannose. Because the growth of the control strain was seemingly unaffected by the initial mannose concentration, this study focuses mainly on presenting the effects of mannose in the pmi− strain. In addition to the specific growth rates, Table I gives the specific residual concentration of hexoses (sum of mannose and glucose) (g/g CDW), the residual glucose concentration (g/liter), the specific mannose consumption (μmol/g CDW), the specific GDP-mannose accumulation (μmol/g CDW), and the yield of GDP-mannose from mannose (%). Both residual glucose concentration and specific residual concentration of hexoses increased in the elevated initial mannose concentrations. Simultaneously, the specific mannose consumption decreased. There was no particular improvement observed in the accumulation of GDP-mannose in the pmi− strain when compared with the...
control strain. The yield of GDP-mannose from mannose was improved only at the highest initial mannose concentration. The concentrations used define the values 6 h after inoculation of the control strain and 4 h after inoculation of the control strain. The samples of the control strain were taken earlier from mannose (%) in batch cultivations of a S. cerevisiae PMI40 deletion strain (pmi−) after 6 h and of a control (ctr) strain after 4 h in media containing varying initial mannose concentrations and a constant initial glucose concentration of 15 g/liter. S.D.

The concentrations used define the values 6 h after inoculation of the control strain. The yield of GDP-mannose from mannose was still growing on hexoses and not shifting to growth on ethanol. The pmi− strain was allowed a longer time for growth to provide an adequate cell mass for later analysis. At these time points samples were taken also for gene expression, enzyme activity, and metabolite concentration analysis, as we wanted to evaluate what was the mechanism that limited the growth and reduced the accumulation of GDP-mannose at the higher mannose concentrations in the pmi− strain.

Transport and Phosphorylation of Hexoses—The expression of genes encoding for hexose transporters is shown in Fig. 1 as a function of the specific concentration of residual hexoses (sum of glucose and mannose concentrations). The expression of low affinity transporter HXT1 and high affinity transporter HXT6 (17) responded to the elevation of the hexose concentration as expected: HXT1 with ascending levels and HXT6 with descending levels. The expression of intermediate hexose affinity transporters, HXT3 and HXT4 (17) stayed practically constant between 4 and 24 g/g CDW. The transporter induced by starvation, HXT5 (18, 19), was induced at the lowest residual hexose concentration and again at the two topmost hexose concentrations. The expression of the genes encoding for hexokinases HXK1 and GLK1 followed that of the hexose transporter HXT6. Similarly, the expression of the hexokinase HXK2 followed that of the hexose transporter HXT4.

Phosphoglucose Isomerase Nodule—In the upper glycolysis, PGle encoded by PGI1 catalyzes the isomerization between Glu-6-P and Fru-6-P. Fig. 2 displays the decreasing PGI1 expression as a function of the initial mannose concentration. Simultaneously, the in vitro activity of the PGle increased. Intracellular, specific concentration of the substrate of the PGle, Glu-6-P, was constant in all other mannose concentrations except on 1 g/liter mannose where it was elevated over 2-fold. Interestingly, it has been reported that Man-6-P can inhibit the activity of PGI1 (20). We set up an in vitro assay in which the concentration of Man-6-P was increased to verify the inhibition of PGle by Man-6-P. Indeed, the decreasing activity of PGle as a function of Man-6-P in an in vitro assay demonstrates the inhibition by Man-6-P (Fig. 2). In this assay with Man-6-P, the PGle activity was measured using a lysate of the cells grown on 3 g/liter mannose. The actual measured in vivo concentration of Man-6-P increased in the upper mannose concentration levels (3 and 5 g/liter).

Phosphofructokinase Nodule—The next step in glycolysis is further phosphorylation of Fru-6-P to Fru-1,6-P by PFKe. Fig. 3 demonstrates how the expression of the gene encoding the major subunit of the first PFKe, PFK1, stayed rather constant when compared with that of PFK26, a gene encoding for the second PFKe, which catalyzes the reaction to Fru-2,6-P (fructose 2,6-bisphosphate). Fru-2,6-P activates the first PFKe (4). The concentration of the product of the first PFKe, Fru-1,6-P, was elevated at the mannose concentration of 1 g/liter. PFKe is also inhibited by other metabolites, e.g. by citrate (21), the measured value of which is a sum of the concentrations of CIT/ICIT. The in vivo concentration of CIT/ICIT increased substantially. Unlike the PGle activity, the in vitro PFKe activity was seen to descend at the upper mannose concentrations.

Lower Glycolysis, Citric Acid Cycle, and Pentose Phosphate Pathway—Further steps of the main metabolism, lower glycolysis, citric acid cycle, and pentose phosphate pathway, demonstrated milder effects to the elevated initial mannose concentration. In the lower glycolysis (supplemental Fig. 1), pyruvate and phosphoenolpyruvate concentrations were lowest at the initial mannose concentration of 1.5 g/liter, but otherwise were rather stable. The expression levels of pyruvate kinase encoding genes decreased slightly whereas the PYKe activity increased simultaneously with the PGle activity. Taken together, the median expression of genes involved in glycolysis decreased in the elevated initial mannose concentrations. In the citric acid cycle (supplemental Fig. 2), many of the carboxylic acids accumulated. Median expression of the genes involved in the citric acid cycle increased in the elevated initial mannose concentrations. Also the measured isocitrate dehydrogenase activity increased steadily. In the pentose phosphate pathway (supplemental Fig. 3), concentrations, activities, and expression levels were rather constant at the initial mannose concentrations 1.5–5 g/liter after decreasing from the slightly higher res.

### Table 1

| Strain               | Control | Control | pmi− | pmi− | pmi− | pmi− | pmi− |
|----------------------|---------|---------|------|------|------|------|------|
| Initial mannose (g/liter) | 0.75    | 0.75    | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| Growth rate (1/h)     | 0.35    | 0.35    | 0.34 | 0.32 | 0.29 | 0.29 | 0.29 |
| Residual hexoses (g/g CDW) | 3.3     | 3.3     | 5.3  | 5.3  | 5.3  | 5.3  | 5.3  |
| Residual glucose (g/liter) | 5.9     | 5.9     | 6.4  | 6.4  | 6.4  | 6.4  | 6.4  |
| Mannose consumption (μmol/g CDW) | 591     | 3707    | 703  | 652  | 440  | 547  | 435  |
| GDP-Man accumulation (μmol/g CDW) | 0.75    | 1.6     | 0.87 | 1.2  | 0.63 | 0.68 | 1.4  |
| GDP-Man yield from mannose (%) | 0.13    | 0.04    | 0.20 | 0.19 | 0.14 | 0.12 | 0.31 |

**FIG. 1.** Normalized expression levels of hexose transporter encoding genes HXT1 (●), HXT3 (○), HXT4 (△), HXT5 (□), and HXT6 (●) presented as a function of specific residual hexoses (sum of glucose and mannose concentrations) (g/g CDW) in batch cultivations of a S. cerevisiae PMI40 deletion strain. S.D. < 20%, n = 4.
The phosphoglucone isomerase (PGIe) nodule in the upper glycolysis. Normalized expression level of PGI1 (●), normalized in vitro activity of PGI1 (○), and normalized specific intracellular concentrations of G6P (●), and Man-6-P (□) presented as a function of initial mannose concentration (g/liter) in batch cultivations of a S. cerevisiae PMI40 deletion strain. Further, normalized in vitro activity of PGI1 (○) is given as a function of added mannose 6-phosphate in the activity assay of the cells grown in the presence of 3 g/liter initial mannose. S.D. for expression levels was < 20%, n = 3, for enzyme activities < 30%, n = 2, and for metabolite concentrations < 30%, n = 4.

FIG. 3. The phosphofructokinase (PFKe) nodule in the upper glycolysis. Normalized expression levels of PFK1 (●) and PFK26 (○), normalized in vitro activity of PFKe (□), and normalized specific intracellular concentrations of Fru-6-P (G6P, ○), Fru-1,6-P (F16P, ○), and the sum of CIT/ICT (□) are presented as a function of initial mannose concentration (g/liter) in batch cultivations of a S. cerevisiae PMI40 deletion strain. S.D. for expression levels was < 20%, n = 3, for enzyme activities < 30%, n = 2, and for metabolite concentrations < 30%, n = 4.

values in the lowest initial mannose concentrations.

Regulatory Connections—Networks of the main metabolic pathways were constructed at three regulatory levels, regulation of the expression of genes, protein-protein interactions, and post-translational covalent and allosteric modifications. The networks were constructed based on the transcriptional regulatory network by Lee et al. (22) and three Internet databases, Brenda, Bind, and YPD. The genes encoding for hexokinases and hexose transporters are regulated in a tight manner (supplemental Fig. 4), as are also the genes in the lower glycolysis. The genes in the upper glycolysis, e.g. the PGIe- and PFKe-encoding genes, have no apparent regulatory connections. However, according to the transcription factor binding network (22), the RAP1 transcription factor binds directly to HXT5, PGI1, PFKe, and PFKe and has eventually regulatory connections to all steps of glycolysis either directly or via other transcription factors such as YAP6 and CIN5 (supplemental Fig. 5). In our study, the expression of RAP1 decreased in the elevated initial mannose concentrations. The protein-protein binding network (supplemental Fig. 6), however, connects proteins PfKlp1p and PfK2p tightly to several steps of glycolysis (23). Interestingly, Pmi40p has been reported to be involved in a complex with PfK2p (23). Pf1p seems to have only two memberships in protein complexes, and those complexes do not involve Pmi40p or the PFKe proteins (24, 25). Supplemental Figs. 4 and 5 compare the gene expression levels controlling the mRNA levels of the enzymes in glycolysis. Fig. 4 displays the comparison of gene expression levels, enzyme activities, and metabolite concentrations in the context of metabolic reactions and allosteric and covalent regulation in the glycolysis. The comparisons are shown as log<sub>2</sub> ratios of the 5 and 1 g/liter initial mannose concentration. The expression levels of the majority of the genes involved in glycolysis were down-regulated, and many of the metabolite concentrations were decreased in the high mannose concentration.

Genome-wide Analysis—A quality-clustering analysis was performed to identify groups of strongly correlating transcripts, enzyme activities, and metabolites. The initial mannose concentration of 0.75 g/liter was discarded in statistical analysis because of a diauxic shift at the time of sampling. The number of clusters obtained with quality clustering was 23, with sizes ranging from 51 to 279 genes. The median profiles of the seven selected clusters are shown in Fig. 5, and the GO biological processes enriched in these clusters are in Table II. Cluster 1, which was enriched with genes involved in ribosome biogenesis and assembly, stayed fairly constant. Biological processes related to energy generation, protein biosynthesis, and translation were represented in repressed clusters 3 and 7. Processes such as response to stress and various stimuli and also coenzyme and amino acid biosynthesis were enriched in the induced clusters. Correlations were studied between enzyme activities, metabolite concentrations, and gene expression. Each metabolite and enzyme activity curve was compared against the expression profiles and each other using Pearson’s correlation coefficient. The median of gene expression levels correlating positively with PFKe activity is given in Fig. 5. The GO biological processes enriched in this PFKe cluster are presented in Table II, indicating that the protein phosphorylation and protein complex assembly processes correlated with the decreasing PFKe activity. These genes most significantly affected by the initial mannose concentration (1–5 g/liter) were identified using an analysis of variance, and the resulting subset was clustered hierarchically using Spearman’s correlation as the distance metric. A total of 71 genes was identified as being monotonically induced, and 23 were monotonically repressed in elevated initial mannose concentrations. Cell wall organization and biogenesis were enriched in the repressed set, and vitamin metabolism was enriched in the induced set (Table II).

DISCUSSION

Our study combined a great variety of measurement and modeling techniques to study the effect of initial mannose concentration in the phosphomannose isomerase deletion strain of yeast S. cerevisiae. The samples from five different initial mannose concentrations were analyzed for gene expression levels, selected enzyme activities, and selected intracellular metabolite concentrations. The increased initial mannose concentrations led to an imminent decrease in the growth rate of the pmi<sup>−</sup> strain. The growth rate in the highest mannose
concentration was more than three times lower in the pmi/H11002 than in the control strain. Because of the negative effects caused by the increasing initial mannose concentrations, we were not able to push mannose to GDP-mannose by increasing the initial mannose concentration. The deletion of the PMI40 had unexpected effects, some of which were observed also in the low mannose concentration.

According to the metabolite concentrations, there was a bottleneck in the upper glycolysis as hexose 6-phosphates accumulated in elevated initial mannose concentrations. The bottleneck lies either in the reaction catalyzed by phosphoglucose isomerase or phosphofructokinase. This hypothesis is further strengthened by the measured concentrations of Fru-1,6-P and dihydroxyacetone phosphate, which decreased in the higher mannose concentrations. The expression of the genes encoding for PGIe and PFKe decreased slightly in the elevated mannose concentrations. Very little is known about the control of the PGI1 and PFK genes. However, we know that especially PFK proteins participate in several protein complexes (23). Also Pmi40p should be involved in some of those complexes (23), but as it is absent in the pmi− strain, its absence might decrease the functionality of the PFK complex. One should note, however, that although most of these observations come from the comparisons of the pmi− strain on various mannose/glucose mixtures, also the lowest mannose concentration of the pmi− strain batch showed the accumulation of hexose phosphates compared with the control strain. Thus, it is reasonable to claim that in this respect the accumulation could be because of less functional protein complexes at the upper glycolysis.
The connections of allosteric control (Fig. 4) demonstrate the traditional three control sites of glycolysis, HXKe, PFKe, and PYKe. PFKe can be inhibited by citrate in the tricarboxylic acid cycle (21), the concentration of which was high in the high mannose concentrations of the pmi− strain. Further, PG1e, which is not regarded as a control site of glycolysis, can be inhibited by Man-6-P (20). The concentration of Man-6-P was directly correlated with the increasing initial mannose concentration. Thus, although the in vitro PG1e activity increased, the real in vivo PG1e activity decreased because of an inhibition caused by higher in vivo Man-6-P concentrations. This regulatory connection links the otherwise disconnected GDP-mannose-synthesizing pathway back to glycolysis and is hypothetically the main reason why the growth of the pmi− strain decreased in the elevated initial mannose concentrations. Interestingly, the PFKe activity measured in vitro and the genes involved in protein phosphorylation (e.g., PKC1, MAP1, HOG1, RIM15, ORC1, CLN1) had a positive correlation. PFKe is modified after translation by phosphorylation (26), and the Fru-2,6-P concentrations that activate PFKe increase after phosphorylation of the second PFKe encoded by PFKe2 and PFKe7 by phosphokinase A (Fig. 4) (27, 28). Therefore, decreased activity of PFKe can be explained by the inactivation of a second PFKe that results in decreased concentrations of Fru-2,6-P, which in turn decreases the activity of PFKe. Also PG11 is phosphorylated after translation (29), and cAMP has a role in regulation of PG1e (30). These observations suggest that PG1e activity can be modulated. Referring to our results, as the in vitro measured concentration of active PFKe increased, and as the expression of genes encoding for protein phosphorylation decreased, the activity of PG1e could be reduced by protein phosphorylation. Nevertheless, the most severe limitation is caused by the inhibition of PG1e activity by Man-6-P. This limitation could be avoided by modifying the structure of the Pgi1p so that it would not bind Man-6-P. Alternatively, applying a mannose feed strategy where only an adequately low concentration of mannose is provided for the cells so that they are able to consume it steadily without accumulating Man-6-P could increase the production of GDP-mannose.

The genome-wide response to increasing the initial mannose concentration demonstrated especially repressed energy pathways (including glycolysis), repressed protein biosynthesis, and repressed cell wall biogenesis. Similarly, stress responses, vitamin, coenzyme, and amino acid biosynthesis were induced in the pmi− strain as a function of the increasing initial mannose concentration. The expression of ribosome biogenesis and assembly were relatively constant under the mannose concentrations studied. The observed gene patterns were similar to those characterized in starved or stressed cells (31, 32). Further, it seems that the bottleneck in the upper glycolysis in PG1 and PFK results in a smaller flux of carbon downstream, which resembles a deletion of either of the genes PG11, PFK1, or PFK2 (33, 34). Newcomb et al. (35) have studied the relation of flux through glycolysis to the expression of genes encoding for cell cycle regulatory proteins CLN3, BCK2, and CDC28, whose expression causes a passage from phase G1 (growth 1) into S phase (start). They found that deletion of either gene encoding for PFKe blocks glucose induction of CLN3, BCK2, and CDC28, resulting in a prolonged G1 and thus slower growth (35). In our setup, the expression of cell cycle-related genes CLN3 and CDC28 was also decreased in the lower growth rates in elevated initial mannose concentrations. Newcomb et al. (35) postulate that an uncharacterized pathway carries a signal of low flux through glycolysis, which regulates the expression levels for a set of cell cycle genes. In principle this pathway could operate via RAP1, known to be involved in the regulation of several steps in glycolysis (36), but also has a major role in telomere structuring (37). Rap1p binds also to CLN3 and via other transcription factors (YAP6 and HMS1) to CDC28 (22). However, as Rap1p binds to several genes, the real role of
Raplp as a link between glycolysis and cell cycle progress needs further clarification. Another possible mechanism connecting glycolysis to the rate of proliferation could be the availability of the building block precursors, located in glycolysis, the pentose phosphate pathway, and the citric acid cycle (4). Induced amino acid and vitamin biosynthesis pathways observed in our study may indicate a low availability of precursors or intermediates in those biosynthesis pathways. The rate of amino acid biosynthesis and protein biosynthesis would eventually control the rate that new cells can be generated.

Overall, our study demonstrates how crucial the measurements of metabolites and even in vitro enzyme activities are in the quest of finding the metabolic rate-limiting steps. This still holds true, although the whole transcriptome was analyzed simultaneously. Explaining the results based on gene expression data alone would not be enough to truly unravel the limitations we discovered here.

Acknowledgments—We thank Dr. Heikki Ojamo for initiating the test of the pon1 strain and for his comments on the manuscript. We thank Satu Bruun, Sirkka-Liisa Holm, Aki Aittola, and Kati Venäläinen for their indispensable help in the laboratory.

REFERENCES

1. Smith, D. J., Proudfoot, A., Friedli, L., Klig, L. S., Paravicini, G., and Payton, M. A. (1992) Mol. Cell. Biol. 12, 2924–2930
2. Payton, M. A., Rheinheimer, M., Klig, L. S., DeTiani, M., and Bowden, E. (1991) J. Bacteriol. 173, 2006–2010
3. Nielson, K., Hasilik, M., Alton, G., Kern, C., Schiehe-Sukumar, M., Koch, H. G., Zimmer, K. P., Wu, R., Harms, E., Reiter, K., von Figura, K., Freeze, H. H., Harms, H. K., and Marquardt, T. (1998) J. Clin. Invest. 101, 1414–1420
4. Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002) Biochemistry, W. H. Freeman & Co., New York
5. Jarvien, N., Maki, M., Rabina, J., Roos, C., Mattila, P., and Renkonen, R. (2001) Eur. J. Biochem. 268, 6458–6464
6. Maki, M., Jarvien, N., Rabina, J., Roos, C., Maaheimo, H., Mattila, P., and Renkonen, R. (2002) Eur. J. Biochem. 269, 583–601
7. Wu, B., Zhang, Y., Zheng, R., Guo, C., and Wang, P. G. (2002) FEBS Lett. 519, 87–92
8. Gao, X. D., Nishikawa, A., and Dean, N. (2001) J. Biol. Chem. 276, 4424–4432
9. Entian, K. D., and Kötter, P. (1998) in Methods in Microbiology (Brown, J. P. and Tuite, M. F., eds) Vol. 26, pp. 431–449, Academic Press Ltd., San Diego
10. Boles, E., Gohlmann, H. W., and Zimmermann, F. K. (1996) Mol. Microbiol. 20, 65–76
11. Verduyn, C., Postma, E., Scheffers, W. A., and Van Dijken, J. P. (1992) Yeast 8, 501–517
12. Bergmeyer, H. U. (ed) (1983) Methods of Enzymatic Analysis, John Wiley & Sons, New York
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. de Koning, W., and van Dam, K. (1992) Anal. Biochem. 204, 118–123
15. Gonzalez, B., Francois, J., and Renaud, M. (1997) Yeast 13, 1347–1355
16. van Dam, J. C., Eman, M. R., Frank, J., Lange, H. C., van Dedem, G. W. K., and Heijnen, S. J. (2002) Anal. Chem. Acta 469, 209–218
17. Ozcan, S., and Johnston, M. (1999) Microbiol. Mol. Biol. Rev. 63, 554–569
18. Buziol, S., Becker, J., Baumeister, A., Jung, S., Mauch, K., Reuss, M., and Boles, E. (2002) FEMS Yeast Res. 2, 283–291
19. Diderich, J. A., Schuurmans, J. M., Van Gaalen, M. C., Krukeberg, A. L., and Van Dam, K. (2001) Yeast 18, 1515–1524
20. Noltmann, E. A. (1972) in The Enzymes (Boyer, P. D., ed) Vol. 8, pp. 219–278, Academic Press, New York
21. Bloxham, D. P., and Lardy, H. A. (1973) in The Enzymes (Boyer, P. D., ed) Vol. 5, pp. 204, 209–218, Academic Press, New York
22. Lee, T. I., Rinaldi, N. J., Roberts, A. M., Schaar, B. M., Rouy, D., Hannon, G. J., Hannon, N. M., Harbison, C. R., Thompson, C. M., I., S., Z., Jennings, E. G., Murray, H. L., Gordon, D. B., Ren, B., Wyrick, J. J., Tagne, J. T., L., V., Fraenkel, E. D. K., and Young, R. A. (2002) Science 298, 799–804
23. Ho, Y., Gruhler, A., Heilbut, A., Bader, G., Moore, L., Adams, S., Millar, A., Taylor, F., Bennett, K., Boultier, K., Yang, W., Wolting, C., Donaldson, I., Schandorf, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A., Sassi, H., Nielsen, P., Rasmussen, K., Andersen, J., Johansen, L., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Pawson, T., Moran, M. F., Durocher, D., Mann, H., Hogue, C. W., Figeys, D., and Tyers, M. (2002) Nature 415, 183–189
24. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, M., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hopfert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Basch, A., Bastuck, S., Huhse, B., Leutwein, C., Heruttier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rhydin, V., Drewes, G., Rada, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Nature 414, 141–147
25. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001) Methods 24, 218–229
26. Piccarro, S. B., McCleland, M. L., Stakenberg, P. T., Burke, D. J., Ross, M. M., Shabanozvit, J., Hunt, D. F., and White, F. M. (2002) Nat. Biotechnol. 20, 391–395
27. Weichert, A., and Holzer, H. (1988) Yeast 4, 227–232
28. Rider, M. H., Bertrand, L., Vertommen, D., Michels, P. A., Rousseau, G. G., and Hue, L. (2004) Biochem. J. 381, 561–579
29. Zhou, H., Watts, J., and Aebersold, R. (2001) Nat. Biotechnol. 19, 375–378
30. Boy-Marcotte, E., Tudi, D., Perrot, M., Boucherie, H., and Jacquet, M. (1996) Microbiology 142, 459–467
31. Gasch, A. P., Spellman, P. T., Kao, C. M., Carnel-Haret, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Mol. Biol. Cell 11, 4241–4257
32. Hohmann, S. (2002) Microbiol. Mol. Biol. Rev. 66, 300–372
33. Corominas, J., Clotet, J., Fernandez-Banares, L., Boles, E., Zimmermann, F. K., Guinovart, J. M., and Arias, J. (1992) FEBS Lett. 210, 182–186
34. Klinger, A., Kirchberger, J., Edelmann, A., and Koppeschaeger, G. (1998) Yeast 14, 325–334
35. Newcomb, L. L., Slattery, M. G., and Heideman, W. (2003) Methods in Microbiology 33, 276–334
36. Giese, R., Rother, M., Giraldo, R., Rhodes, D., and Gasser, S. M. (1993) J. Mol. Biol. 231, 293–310