Identification of the First Fungal NADP-GAPDH from *Kluyveromyces lactis*†

Ritva Verho, Peter Richard,* Per Harald Jonson, Lena Sundqvist, John Londesborough, and Merja Penttilä

VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland

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ABSTRACT: Deletion of the phosphoglucose isomerase gene, *PGI1*, in *Saccharomyces cerevisiae* leads to a phenotype for which glucose is toxic. This is related to overproduction of NADPH through the oxidative part of the pentose phosphate pathway and the incompetence of *S. cerevisiae* to deal with this overproduction. A similar deletion (*rag2*) in *Kluyveromyces lactis* does not lead to such a phenotype. We transformed a genomic library of *K. lactis* in a yeast vector to a *S. cerevisiae* strain with a *pgi1* deletion and screened for growth on glucose. We found a gene (*GDP1*) which encodes a phosphorylating glyceraldehyde-3-phosphate dehydrogenase, NADP-GAPDH (EC 1.2.1.13), that accepts both NADP and NAD. This is the first report of a eukaryotic, nonplant, NADP-linked GAPDH. Presumably, operation of this enzyme in the reverse direction enabled the transformed *S. cerevisiae pgi1* deletion mutant to reoxidize the excess NADPH produced when glucose catabolism was forced through the pentose pathway. On the other hand, transcription of the gene in *K. lactis* was upregulated during growth on D-xylose, which suggests that in *K. lactis* the enzyme is involved in regeneration of NADPH needed for xylose assimilation, but transcription was not detected in a *rag2* mutant grown on glucose. The presence of an asparagine (Asn46 in NADP-GAPDH) instead of the conserved aspartate found in related but NAD-specific enzymes may explain the ability of NADP-GAPDH to work with NADP as well as NAD.

NADH and NADPH are generally used in different reductive biochemical reactions. NADH is primarily used for the generation of ATP through the respiratory chain or, under fermentative conditions, to generate fermentation end products, such as ethanol or lactate. NADPH serves as an electron donor in reductive biosyntheses of fatty acids, sterols, amino acids, and purines. NADPH is also used in fungi and other microorganisms to reduce oxidized nitrogen sources, e.g., NO_3^-, to the NH_3 level.

NADPH is also used in fungi to reduce pentoses such as D-xylose and L-arabinose to sugar alcohols, which are then oxidized by NAD to pentuloses. Thus, NADPH and NAD are consumed in the redox neutral conversion of pentose carbon sources to pentose phosphate pathway metabolites. In nonphotosynthetic microorganisms, it is generally thought that NADPH is generated primarily by the oxidative part of the pentose phosphate pathway, i.e., the oxidation of glucose 6-phosphate to ribulose 5-phosphate using glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Molds growing on pentoses have a higher activity of this part of the pentose phosphate pathway (*1*). However, oxidation of isocitrate by NADP-isocitrate dehydrogenase and of malate by NADP-malic enzyme can also contribute to NADPH generation. All these pathways for NADPH regeneration have in common the fact that they are directly linked to CO_2 production.

Since fungi often use the pentoses D-xylose and L-arabinose as major carbon sources and their catabolism requires NADPH, it might be advantageous for such fungi to have an alternative pathway for the regeneration of NADPH where no carbon is lost through CO_2 production. NADPH can also be formed in a transhydrogenase reaction that is not linked to CO_2 production. However, it is generally believed that fungi do not have such activity (*2*). Also, "transhydrogenase cycles", such as the mannitol cycle, where two dehydrogenases with different cofactor specificities create an effective transhydrogenase in a cyclic reaction, have not been found to be active in NADPH regeneration (*3*).

Saccharomyces cerevisiae with a deletion in the phosphoglucose isomerase (*PGI1*) gene provides a screening system for NADP(H)-linked oxidoreductases (*4*). *S. cerevisiae pgi1* mutants cannot grow on glucose, apparently because rerouting of glucose catabolism through the oxidative part of the pentose phosphate pathway causes an overproduction of NADPH which *S. cerevisiae* cannot tolerate. In the yeast *Kluyveromyces lactis*, a deletion in the phosphoglucose isomerase gene (*RAG2*) does not lead to a similar phenotype; i.e., *K. lactis rag2* mutants can grow on glucose. With this screen, we found a *K. lactis* gene encoding a novel, fungal, NADP-linked glyceraldehyde-3-phosphate dehydrogenase. Apparently, this enzyme enabled the *S. cerevisiae pgi1* deletant to cope with excess NADPH production during growth on glucose. Because this enzyme is on the major glycolytic route from sugar to pyruvate, it may serve in *K. lactis* as a direct method of regenerating NADPH required for xylose assimilation, without CO_2 formation and without diversion of carbon flux through side pathways.

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* To whom correspondence should be addressed: VTT Biotechnology, Tietotie 2, Espoo, P.O. Box 1500, FIN-02044 VTT, Finland. Telephone: +358 9 456 7190. Fax: +358 9 455 2103. E-mail: Peter.Richard@vtt.fi.

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EXPERIMENTAL PROCEDURES

Constructing the Host Strain for the Library Screening; Deleting the PGI1 Gene in S. cerevisiae. The *PGI1* gene of the *S. cerevisiae* haploid strain CEN.PK2 was deleted. A *S. cerevisiae PGI1* fragment was obtained by PCR using the following primers: 5′-CGACCGGTCGACTACCAGCCTAAAAATGTC-3′ (*Sal*I site underlined) and 5′-GGCACGCTGCAGAGAGCGATTTGTTCACAT-3′ (*Pst*I site underlined). The *PGI1* fragment was digested with *Sal*I and *Pst*I and ligated into the pBluescript SK− vector (Stratagene). The resulting plasmid (B1186) was digested with *Eco*RI and *Bst*BI to remove a 715 bp fragment from the middle of the *PGI1* gene, i.e., a fragment between base pairs 109 and 824.

The *HIS3* gene was obtained by *Drd*I digestion from the yeast expression vector pRS423 (*5*). The *HIS3* fragment was blunted with T4 DNA polymerase and ligated into the pBluescript SK− *Eco*RV site. This plasmid (B1185) was digested with *Eco*RI and *Cla*I, and the 1.5 kb fragment carrying the *HIS3* gene was ligated into the *Eco*RI- and *Bst*BI-digested B1186 plasmid. The resulting plasmid was named B1187.

The *PGI1*+*HIS3* fragment was released from the B1187 plasmid with *Sal*I and *Mun*I digestion, and *S. cerevisiae* strain CEN.PK2 was transformed with the fragment. The lithium acetate method (*6*, *7*) was used for the yeast transformation. The yeast transformants were confirmed by Southern blot analysis using a fragment from the *S. cerevisiae PGI1* gene as the probe. The resulting strain, CEN.PK2 Δ*pgi1*, was then used for the screening.

Construction and Screening of the K. lactis Genomic Library. The *K. lactis* genomic library was constructed from strain CBS 2359 as described previously (*8*). The library was transformed into the *S. cerevisiae* CEN.PK2 Δ*pgi1* strain; the transformants were plated on SC-leu, 2% D-fructose, and 0.05% D-glucose, and 1.3×10^6 transformants were pooled in 0.9% NaCl. The transformants were then again plated first on SC-leu, 2% D-fructose, and 0.1% D-glucose and after 3 days replica plated on SC-leu and 0.1% glucose. Approximately 100 colonies, which appeared after 9 days, were streaked and restreaked on SC-leu and 0.1% glucose. Clones that contained the *K. lactis RAG2* gene, which encodes phosphoglucose isomerase, were identified by PCR with primers specific for the *K. lactis RAG2* gene (5′-CACTGAAGGACGTGCTGTGT-3′ and 5′-AGCTGGGAATCTGTGCAAGT-3′).

Identifying the Product of the Redox Screening. The plasmid, which was obtained in the screening procedure, had an insert of an estimated 10 kb. A transposon was randomly inserted into the plasmid with the Template Generation System (Finnzymes). Different transposon insertions (as judged by PCR with primers from the transposon and the yeast vector) were retransformed to the CEN.PK2 Δ*pgi1* strain and tested for growth on 0.1% D-glucose. From strains, which were maintained on 2% D-fructose and 0.05% D-glucose, but showed no growth on 0.1% D-glucose, the plasmids were recovered and sequenced with primers of the transposon sequence.

Enzyme Assay for Measuring NADP-GAPDH[1] *Activity.* The standard assay for measuring NADP-GAPDH activity in the reverse direction was a coupled enzyme assay with phosphoglycerate kinase. A sample of the yeast extract or the histidine-tagged purified protein was added to a reaction buffer containing 500 mM triethanolamine (pH 7.8), 1 mM ATP, 2 mM $MgCl_2$, 200 μM NADPH, and 10 μg/mL phosphoglycerate kinase (Boehringer). The reaction was started by the addition of 3-phosphoglycerate to a final concentration of 5 mM. The NAD-GAPDH activity was measured in the same way except that NADH was used instead of NADPH.

In the forward direction, i.e., dehydrogenase reaction, the activity was measured by adding the enzyme to a buffer containing 350 mM triethanolamine (pH 9.2), 30 mM sodium phosphate (pH 9.2), and NAD or NADP. D-Glyceraldehyde 3-phosphate (GAP) was then added to start the reaction. When the affinity for NAD and NADP was measured, GAP was added to a final concentration of 14 mM. For the affinity measurement of GAP, the NAD or NADP concentration was 1 mM. The protein concentration was measured with the Bio-Rad protein assay using BSA as a standard. All enzyme assays were made with a Mira Automated Analyser (Roche) at 30 °C.

Cloning the GAPDH Homologue to Expression Vectors. The gene encoding the GAPDH homologue was amplified by PCR using the following primers for the sense and antisense directions: 5′-ATAAAGCTTAAGATGCCCGATATGACAAAACGAATCTTC-3′ and 5′-AAGGATCCAAGCGTCTCCTTAAACACCAGC-3′, respectively. The restriction sites *Hin*dIII and *Bam*HI were introduced by PCR and are underlined in the primer sequence. The PCR product was then digested with *Hin*dIII and *Bam*HI and ligated into the corresponding sites of the pYES2 vector (Invitrogen). The pYES2 vector is a yeast expression vector with a galactose inducible promoter and a *URA3* gene for selection.

In another construct, the GAPDH gene was amplified by PCR as described above except that the primer in the sense direction contained a *Bam*HI restriction site. A third construct with the GAPDH homologue with a C-terminal histidine tag was generated by PCR by using the primer in the sense direction from above (with a *Bam*HI site) and the 5′-AAGGATCCTTAATGATGATGATGATGATGAACACCAGCTTCGAAGTCCTTTTGAGCC-3′ primer for the antisense direction. The PCR products were digested with *Bam*HI. The *PGK1* promoter and terminator (*9*) were ligated into the multicopy vector YEplac195 (*10*), and the GAPDH homologue was ligated into the *Bgl*II site located between the *PGK1* promoter and terminator. The resulting plasmids were then transformed to the CEN.PK2 yeast strain with the *pgi1* deletion.

Purification of the Histidine-Tagged Protein. The CEN-.PK2 strain containing the plasmid with the histidine-tagged GAPDH homologue was grown on selective medium with D-glucose as a carbon source. The yeast cells were extracted by vortexing with glass beads in a buffer containing 10 mM sodium phosphate (pH 7.0) and 0.17 mg/mL PMSF. The histidine-tagged protein was then purified with a Ni−NTA agarose column (Quiagen) according to the manufacturer's descriptions, and desalted with a Hightrap (Pharmacia) gel filtration column. The purified protein was obtained in 10 mM sodium phosphate (pH 7.0) and 10 mM DTT.

Northern Blot Analysis of the Expression of the GAPDH Homologue Gene. K. lactis strain CBS 2359 was cultivated on SC medium with 2% D-glucose, glycerol, or D-xylose as

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAP, glyceraldehyde 3-phosphate.

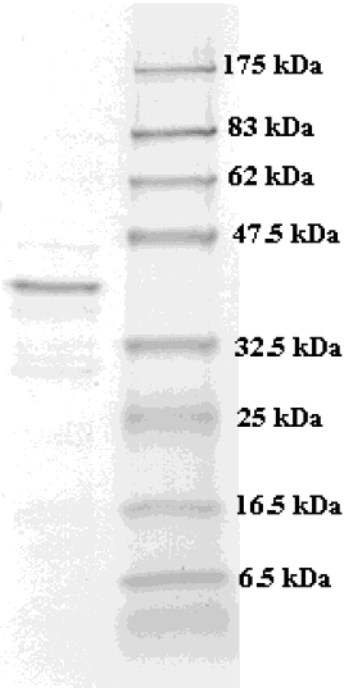

FIGURE 1: SDS−PAGE of the purified protein with a C-terminal histidine tag.

the carbon source. The *K. lactis rag2* mutant was grown on SC and 2% D-glucose. The *S. cerevisiae* strain having the GAPDH homologue gene in a multicopy plasmid under the *PGK1* promoter and a *S. cerevisiae* control strain carrying the empty plasmid were cultivated on selective medium with 2% D-glucose as the carbon source. The RNA was extracted from the yeast cells with the Trizol reagent kit (Life Technologies Inc.); 1−2 μg of the total RNA per sample was used in the analysis. The Northern hybridization was carried out using standard methods.

The 150 bp GAPDH homologue probe was released by *Eco*RI and *Xba*I digestion from a plasmid containing the GAPDH homologue gene. The probe was labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using the randomly primed DNA labeling kit (Roche Molecular Biochemicals).

Modeling of the NADP Binding Pocket. A crude model of the structure of the *K. lactis* GDP1 and the *S. cerevisiae* GAPDH1 was made by homology modeling using Swiss-Pdb Viewer version 3.7b2 (*11*). The structure that was used is the NAD-dependent GAPDH from *Palinurus versicolor* (South China Sea lobster) called 1DSS (*12*) in the Protein Data Bank (*13*). The structure contains the same sequence element as the *S. cerevisiae* GAPDH1 and the *K. lactis* GDP1 around the conserved aspartate residue as indicated in Figure 2.

RESULTS

Screening the K. lactis Genomic Library for Redox Enzymes. A *S. cerevisiae* strain with a deletion in the phosphoglucose isomerase gene was transformed with a genomic library from *K. lactis*. After the transformation, the cells were maintained on 2% D-fructose and 0.05% D-glucose. Under these conditions, the glucose is not toxic for the *pgi1* mutant. Cells were then transferred to 0.1% D-glucose, which is toxic for the *pgi1* mutant. In cells, which expressed the *K. lactis* phosphoglucose isomerase gene, growth on glucose was restored. To identify these transformants, we used PCR with specific primers for the *K. lactis* phosphoglucose isomerase. From ~100 transformants which could restore growth on glucose, two were identified that did not contain the phosphoglucose isomerase gene. These two clones turned out to be identical, containing a DNA fragment of ~10 kb. A transposon was randomly inserted into the plasmid, and the resulting plasmids were transformed back to the *pgi1* mutant. A transposon-containing plasmid that did not restore growth on glucose was then sequenced, starting from the transposon sequence. This plasmid had the transposon in an open reading frame encoding a protein with 356 amino acids and a molecular mass of 39 030 Da. The open reading frame was very similar to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), indicating that this GAPDH homologue was responsible for restoring growth on glucose. The nucleotide sequence has been submitted to EMBL, GenBank, and DDBJ Nucleotide Sequence Database and will appear under accession number AJ430565.

Expression of the GAPDH Homologue under a Galactose-Inducible Promoter. The GAPDH homologue was expressed under a galactose-inducible promoter in the pYES2 yeast expression vector. This vector and a control vector without the GAPDH homologue were then transformed to the *S. cerevisiae* strain with the *pgi1* deletion. The strains were kept on plates with selective medium with 2% D-fructose and 0.05% D-glucose as a carbon source and transferred to plates with 2% D-galactose and 0.3 or 2% D-glucose. The strain with the GAPDH homologue grew in the presence of 0.3 and 2% D-glucose. The control strain with an empty plasmid showed no growth on either medium.

To test if the GAPDH homologue had NADP-GAPDH activity, the GAPDH homologue was expressed under the galactose-inducible promoter in the yeast strain CEN.PK2. The strain and a control strain carrying the empty vector were grown on selective medium with 2% D-glucose and 2% D-galactose as a carbon source and extracts prepared from the cells. The extracts were analyzed for NADP-GAPDH enzyme activity with the standard assay. In the crude extract of the strain with the GAPDH homologue, we found an activity of 0.05 nkat/mg of extracted protein. In the control strain, the activity was below the detection limit, which was ~0.005 nkat/mg. For comparison, the GAPDH activity with NAD was in both strains 15 ± 3 nkat/mg. We named the gene for the GAPDH homologue *GDP1* for glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent.

Expression of the GAPDH Homologue under a Constitutive PGK1 Promoter. The *GDP1* gene was also expressed under a constitutive promoter in the *pgi1* mutant. The *pgi1* mutant carrying the *GDP1* under the *PGK1* promoter in a multicopy vector can grow on 2% D-glucose as the sole carbon source. We estimated a doubling time of 22 h for this strain in shake flask cultivation under this aerobic condition. The NADP-GAPDH activity was ~0.3 nkat/mg in the crude extract.

Expression with His Tag, and Activity of the His-Tagged Protein. The *GDP1* was expressed with a C-terminal histidine tag in strain CEN.PK2 and in the strain with the *pgi1* deletion. The strain with the *pgi1* deletion was tested for growth on D-glucose to test if the enzyme was still functional with the histidine tag. The *pgi1* mutant with the histidine-tagged *GDP1* was able to grow on D-glucose at

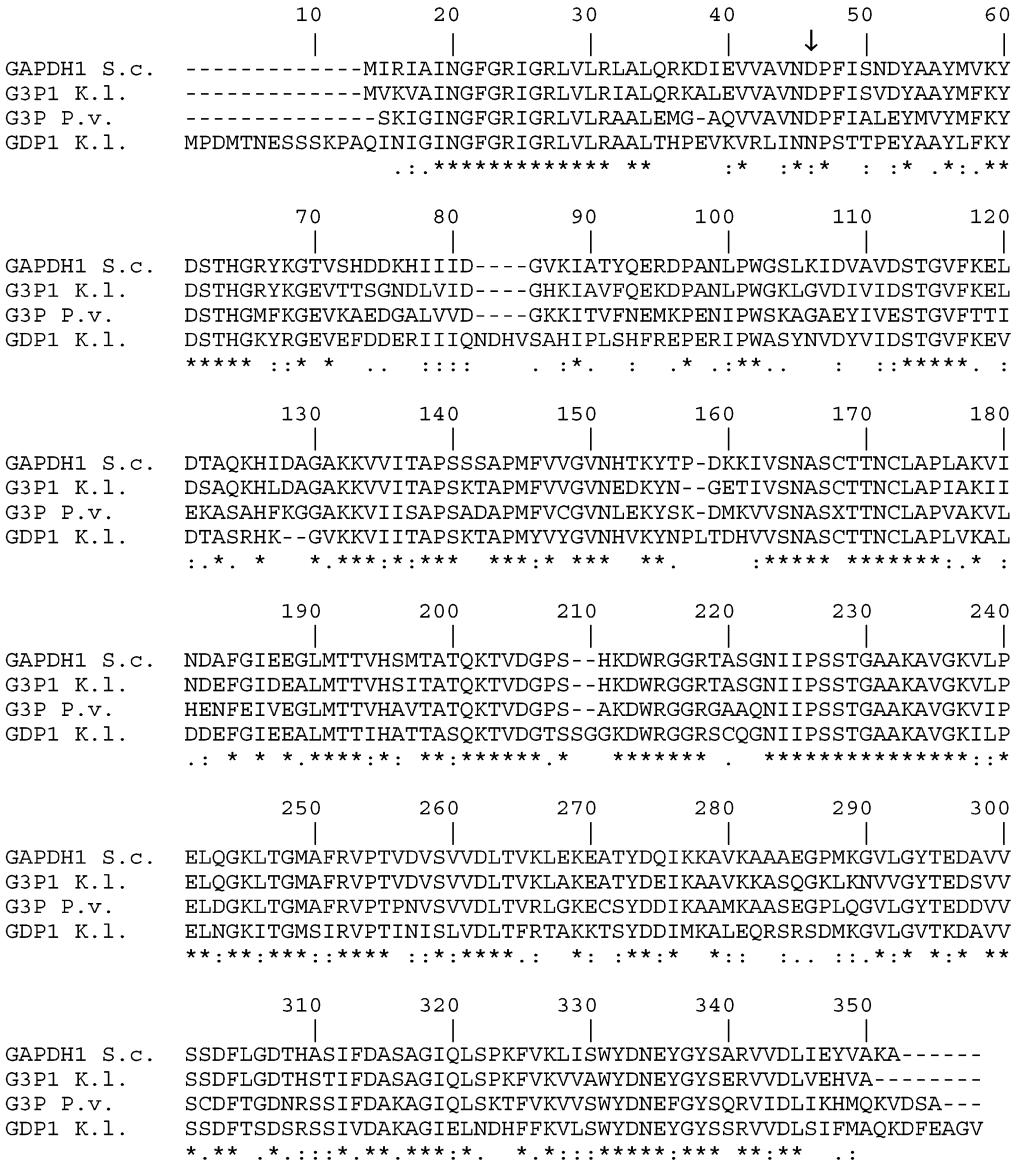

FIGURE 2: Amino acid sequence alignment of the glyceraldehyde-3-phosphate dehydrogenase from *S. cerevisiae* (GAPDH1 S.c.), the NAD-dependent GAPDH from *K. lactis* (G3P1 K.l.), the NAD-dependent GAPDH from *P. versicolor* (G3P P.v.), and the GDP1. The locations of the conserved aspartate in NAD-dependent enzymes are marked with an arrow (position 46 in *K. lactis* GDP1). Asterisks denote identity. Colons indicate a high degree of conservation. Periods indicate a lower degree of conservation.

the same rate as without the histidine tag, indicating that the addition of the histidine tag did not greatly influence the enzyme activity. The histidine-tagged protein was then purified. From SDS−PAGE, we estimated that the protein was ~90% pure and had a molecular mass of ~40 kDa (see Figure 1). This is in good agreement with a calculated molecular mass of the histidine-tagged protein of 39.852 kDa. With the purified enzyme, we found an activity with NADPH of 70 nkat/mg of protein, using our standard assay. When NADPH was replaced with NADH, the activity was similar.

Affinity for NADP, NAD, and D*-Glyceraldehyde 3-Phosphate.* The purified histidine-tagged protein was used to measure the substrate affinity for NAD, NADP, and D-glyceraldehyde 3-phosphate at 30 °C and pH 9.2. For NADP and NAD, the K_m values were 400 ± 100 and 500 ± 60 μM, respectively. The V_{max} for NADP was 250 nkat/mg of protein, and the V_{max} for NAD was 500 nkat/mg. For D-glyceraldehyde 3-phosphate, the K_m was 750 μM with NAD or NADP as a cofactor.

Northern Blot Analysis of the GDP1. K. lactis was grown on different carbon sources to study *GDP1* expression. The *K. lactis* wild-type strain was cultivated on D-glucose, glycerol, and D-xylose as carbon sources. The *GDP1* gene expression was also studied in the *K. lactis rag2* mutant when it was grown on glucose. The *S. cerevisiae* strain carrying the *GDP1* in the multicopy plasmid under the *PGK1* promoter and a control strain with the empty vector were used as controls in the Northern blot analysis. The *GDP1* probe was chosen from the region of the NADP-GAPDH sequence that is least similar with the *K. lactis GAP1* gene encoding the NAD-dependent GADPH. A Southern blot analysis was performed to confirm that the probe does not bind any genes other than the *GDP1* (not shown). The Northern blot result is shown in Figure 3. In *K. lactis*, the transcription can only be seen when the cells were grown on D-xylose. The *GDP1* mRNA of the *S. cerevisiae* strain where the *GDP1* was overexpressed is ~250 bp smaller than the *GDP1* mRNA of the *K. lactis* wild-type strain. This is

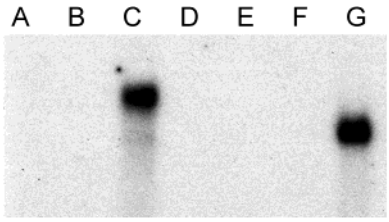

FIGURE 3: mRNA levels of *GDP1* in *K. lactis* (wild type) grown on D-glucose (lane A), glycerol (lane B), and D-xylose (lane C). *K. lactis rag2* mutant grown on D-glucose (lanes D and E), *S. cerevisiae* overexpressing the *GDP1* (lane G), and the control where the *S. cerevisiae* strain is not expressing *GDP1* (lane F). The labeled mRNA in lane C is ~250 bases larger than that in lane G (see the text).

due to differences in the transcription between the *K. lactis* strain and the construct in *S. cerevisiae*. The polyadenylation signal in the *K. lactis GDP1* terminator is ~250 bp downstream from the stop codon. In the construct where the *GDP1* is expressed under the *S. cerevisiae PGK1* promoter, the polyadenylation signal is immediately after the stop codon.

DISCUSSION

Redox Screening. A strain of *S. cerevisiae* with a deletion in the gene (*PGI1*) encoding phosphoglucose isomerase cannot grow on glucose (*14*, *15*). In such a strain, the glucose has to be metabolized through the pentose phosphate pathway. For *S. cerevisiae*, this is believed to be lethal because it cannot cope with the NADPH produced in the oxidative part of the pentose phosphate pathway (*4*).

This is different in other microorganisms. Mutants of *Escherichia coli* and the yeast *K. lactis* lacking phosphoglucose isomerase activity can grow on glucose (*16*, *17*). Since *K. lactis* also produces NADPH in the oxidative part of the pentose phosphate pathway, it must have a means of utilizing the excess NADPH. To identify possible genes that are involved in the NADPH utilization in *K. lactis*, we screened a *K. lactis* genomic library in a *S. cerevisiae pgi1* mutant for growth on glucose. In this screen, we found a gene encoding an NADP-GAPDH (EC 1.2.1.13) which we called *GDP1*.

The K. lactis rag2 Mutant Is Not Able To Grow on Glucose when Mitochondrial Respiration Has Been Blocked. Gonzalez Siso et al. (*18*) suggested that *K. lactis* has an external mitochondrial dehydrogenase that could pass electrons to the mitochondrial respiratory chain, similar to *NDE1* and *NDE2* in *S. cerevisiae* (*19*), but which could use NADPH instead of NADH. This dehydrogenase would oxidize the NADPH generated in the oxidative part of the pentose phosphate pathway. This would be consistent with the observation that the *rag2* mutant cannot grow on glucose when mitochondrial respiration is blocked.

An alternative explanation for this observation would be an NADP-GAPDH. An NADP-GAPDH activity in the presence of NAD-GAPDH activity would form a transhydrogenase catalyzing the reaction between NADPH and NAD and between NADP and NADH. The excess NADPH could be converted into an excess of NADH, which is subsequently oxidized by a mitochondrial NADH reductase. The inability to grow on glucose when mitochondrial respiration has been blocked might be due to the inability to oxidize the excess NADH.

Is the NADP-GAPDH Responsible for the Phenotype that Allows the K. lactis rag2 Mutant To Grow on Glucose? The NADPH-GAPDH can restore the ability to grow on glucose in a *S. cerevisiae pgi1* mutant, so it could be the same principle that enables the *K. lactis rag2* mutant to grow on glucose.

The activity of NADP-GAPDH when expressed in *S. cerevisiae* under the *PGK1* promoter was low (~0.3 nkat/mg), when compared to the NAD-GAPDH activity (15 nkat/mg) when measured in our standard assay, i.e., in the direction of NAD(P)H oxidation. The *S. cerevisiae pgi1* mutant with the *GDP1* overexpressed has a correspondingly slow rate of growth on glucose, i.e., a doubling time on glucose of ~20 h. The *K. lactis rag2* mutant also grows slowly on glucose (*18*). In the Northern blot analysis, however, no transcription of the *GDP1* gene was detected in the *K. lactis rag2* mutant grown on glucose (Figure 3). This suggests that other factors are responsible for the ability of the *K. lactis rag2* mutant to grow on glucose. However, we have not examined the effect of deleting *GDP1* from the wild type or *rag2* mutants of *K. lactis*.

NADP-GAPDH (EC 1.2.1.13). The phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) is an enzyme in the Embden Meyerhoff pathway. It catalyzes the reversible reaction from glyceraldehyde 3-phosphate, inorganic phosphate, and NAD to 1,3-bisphosphoglycerate and NADH. It is essential in both glycolysis and gluconeogenesis. In fungi and all other eukaryotic cells except plants, known examples of this enzyme are strictly specific for NAD.

In plants and photosynthetic bacteria, an NADP-dependent GAPDH (EC 1.2.1.13) has been found. However, it is not part of the Embden Meyerhoff pathway, but involved in the carbon fixation of photosynthesis. In nonphotosynthetic bacteria, an NADP-GAPDH is known to exist besides NAD-GAPDH. However, the two different enzymes are suggested to have different physiological roles, the NAD enzyme being involved in glycolysis and the NADP enzyme in gluconeogenesis (*20*).

In this paper, we report a glyceraldehyde-3-phosphate dehydrogenase from the yeast *K. lactis*, which uses NADP and NAD equally well as cofactors and is to our knowledge the first eukaryotic, nonphotosynthetic, GAPDH that is able to use NADP.

Role of an NADP-GAPDH in Pentose Fermentation. NADP-GAPDH activity can be beneficial in pentose fermentation, which is redox neutral but uses different redox cofactors. In the catabolism of 1 mol of D-xylose to xylulose phosphate, 1 mol of NADP and NADH is generated (*21*), and in the catabolism of 1 mole of L-arabinose, 2 mol NADP and NADH is generated (*22*, *23*). NADP is usually regenerated through the oxidative part of the pentose phosphate pathway, which is coupled to CO_2 production. In contrast, the NADP regeneration through an NADP-GAPDH is not directly linked to CO_2 production (see Figure 4). Furthermore, unlike recycling through the pentose phosphate pathway, use of NADP-GAPDH provides for stoichiometric regeneration of both the NADPH and the NAD required for assimilation of xylose. Conversion of 1 mol of xylose to $^5/_3$ mol of glyceraldehyde 3-phosphate (GAP) provides 1 mol each of NADPH and NAD. If 1 mol of GAP is oxidized by the NADP-GAPDH, thereby regenerating the NADPH, then $^2/_3$ mole is oxidized by the NAD-linked GAPDH, generating

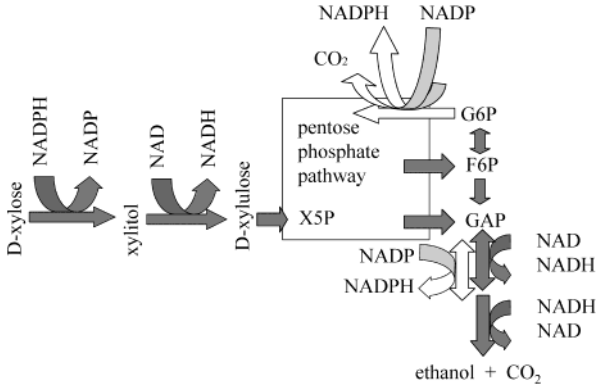

FIGURE 4: D-Xylose catabolic pathway and the possible role of the NADP-GAPDH in the regeneration of both NADPH and NAD. D-Xylose is converted to the pentose phosphate pathway intermediate, D-xylulose 5-phosphate (X5P), via xylose reductase, xylitol dehydrogenase, and xylulokinase. X5P is then converted, partially via D-fructose 6-phosphate (F6P), to D-glyceraldehyde 3-phosphate (GAP, $^5/_3$ mol/mol of xylose). NADPH, which is utilized in the D-xylose pathway, can be regenerated either by using the oxidative part of the pentose phosphate pathway, thereby generating CO_2, or by using the NADP-GAPDH. The latter process can also regenerate the NAD stoichiometrically (see the text).

a total of $^5/_3$ mol of NADH. Just this amount is reoxidized by alcohol dehydrogenase, also producing $^5/_3$ mol of ethanol. At least in theory, this permits the anaerobic fermentation of xylose to ethanol, which is of some biotechnological relevance. The result from the Northern blot (Figure 3) suggests that the *GDP1* has a role in D-xylose utilization in *K. lactis*. The transcription of *GDP1* was observed in cells grown on D-xylose, but not in cells grown on D-glucose or glycerol.

Simultaneous Expression of NAD- and NADP-GAPDH Allows the Transhydrogenase Reaction To Take Place. It remains an open question why there are not more examples of an eukaryotic nonphotosynthetic NADP-GAPDHs. Overexpression of the NADP-GAPDH in the presence of an NAD-GAPDH had apparently no negative effect in a strain of *S. cerevisiae* (CEN.PK2) without a *pgi1* deletion. However, the NADP-GAPDH activity was much lower (0.3 nkat/mg) than the NAD-GAPDH activity (15 nkat/mg) in the crude extract. Having an NAD and NADP-GAPDH expressed simultaneously would tend to equilibrate the NAD/NADH and NADP/NADPH couples. It remains to be seen whether an efficient transhydrogenase system has a negative effect on metabolism.

The Glyceraldehyde-3-phosphate Dehydrogenase Can Use Both Cofactors NAD and NADP. The *GDP1*-encoded (His-tagged) enzyme accepts both NAD and NADP as cofactors, with approximately the same affinity. This suggests that the phosphate group of the nucleotide is not directly involved in protein binding.

NAD-dependent GAPDH enzymes contain a highly conserved aspartate in position 46 of the alignment in Figure 2. This negatively charged residue will, as illustrated in Figure 5, prevent binding of NADP due to electrostatic repulsion between the negative charges of the phosphate group and the negative charge in the aspartate side chain (*20*). The corresponding site of the *K. lactis* GAPDH homologue is an asparagine, which is not negatively charged and therefore will allow binding of NADP. The negative charge in the NAD enzymes thus prevents the NADP from binding,

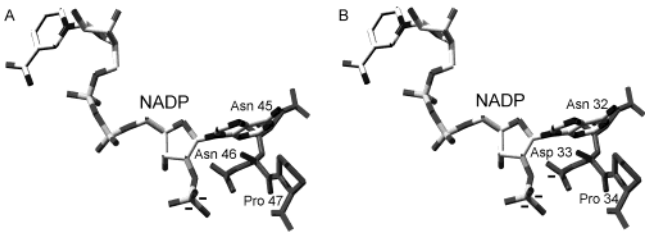

FIGURE 5: Model of the potential binding of NADP to GAPDH: (A) model of the *K. lactis* enzyme and (B) NAD-specific *S. cerevisiae* enzyme. The model indicates why NADP can bind to GDP1 (A) but not to other GAPDHs (B).

whereas the absence of this negative charge allows NADP to bind, but does not interfere with the binding of NAD. This might explain why the *K. lactis* enzyme can use both cofactors.

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