Metabolic impact of an NADH-producing glucose-6-phosphate dehydrogenase in Escherichia coli

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In Escherichia coli, the oxidative branch of the pentose phosphate pathway (oxPPP) is one of the major sources of NADPH when glucose is the sole carbon nutrient. However, unbalanced NADPH production causes growth impairment as observed in a strain lacking phosphoglucoisomerase (Δpgi). In this work, we studied the metabolic response of this bacterium to the replacement of its glucose-6-phosphate dehydrogenase (G6PDH) by an NADH-producing variant. The homologous enzyme from Leuconostoc mesenteroides was studied by molecular dynamics and site-directed mutagenesis to obtain the NAD-preferring LmG6PDH R46E,Q47E. Through homologous recombination, the zwf loci (encoding G6PDH) in the chromosomes of WT and Δpgi E. coli strains were replaced by DNA encoding LmG6PDH R46E,Q47E. Contrary to some predictions performed with flux balance analysis, the replacements caused a substantial effect on the growth rates, increasing 59% in the Δpgi strain, while falling 44% in the WT. Quantitative PCR (qPCR) analysis of the zwf locus showed that the expression level of the mutant enzyme was similar to the native enzyme and the expression of genes encoding key enzymes of the central pathways also showed moderate changes among the studied strains. The phenotypic and qPCR data were integrated into in silico modelling, showing an operative G6PDH flux contributing to the NADH pool. Our results indicated that, in vivo, the generation of NADH by G6PDH is beneficial or disadvantageous for growth depending on the operation of the upper Embden–Meyerhof pathway. Interestingly, a genomic database search suggested that in bacteria lacking phosphofructokinase, the G6PDHs tend to have similar preferences for NAD and NADP. The importance of the generation of NADPH in a pathway such as the oxPPP is discussed.

INTRODUCTION

Escherichia coli has different metabolic pathways to oxidize available carbon sources, but each of them has a different yield of reduced cofactors NADH and NADPH. The strong segregation in the cofactor specificity among the dehydrogenases of the central pathways suggests that the coupling of the generation of either NADH or NADPH with certain metabolites could be a key property for network function.

For example, generation of pentose phosphate is coupled with NADPH production, whereas pyruvate synthesis is coupled with NADH generation through the Embden–Meyerhof pathway.

In E. coli, the dehydrogenases involved in the oxidative branch of the pentose phosphate pathway (oxPPP) have been regarded as NADP-specific enzymes. Accordingly, this pathway is considered the source of ~30–40% of total NADPH production when glucose is the sole carbon source (Sauer et al., 2004). The generation of NADPH must be balanced with its consumption to maintain optimal growth rates. For example, phosphoglucoisomerase (Δpgi) mutants grown on glucose as the sole carbon source exhibit reduced growth rates (Canonaco et al., 2001). In this case, all the glucose is channelled through oxPPP, leading to an
unbalanced production of NADPH, and growth rates fall ~75% with respect to WT. An additional factor in the redox-balancing capacity of E. coli is the presence of two kinds of transhydrogenases (Fuhrer & Sauer, 2009; Sauer et al., 2004). For example, it was observed that the overexpression of the transhydrogenase UdhA helps to dissipate the NADPH imbalance in the ApgI mutant, increasing the growth rate of this mutant strain (Canonaco et al., 2001). Given the balancing capacity provided by the transhydrogenases, it is not clear why the dehydrogenases in the oxPPP from E. coli have evolved to be specific for NADP. It is also not clear why NADPH production is coupled with carbon flux through the oxPPP. We believe that the high cofactor specificity of the dehydrogenases from oxPPP in E. coli is related to the location of these enzymes after the branching points between the major glycolytic pathways, enabling the regulation of how much carbon flux is coupled to the generation of NADPH. Consequently, the cofactor specificity of these dehydrogenases should affect the magnitude of the carbon flux through these pathways. To test this hypothesis, we depend on the utilization of a dehydrogenase from the oxPPP showing a strong preference for NAD instead of NADP. As we are introducing a single and well-defined stoichiometric change in the metabolic network, we believed flux balance analysis could be a suitable tool to predict the physiological impact of this kind of change.

The preference of the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from E. coli (EcG6PDH) for NADP over NAD has been quantified, showing a specificity constant (kcat/Km) for NADP 410 times higher than for NAD (Olaravaria et al., 2012). The homologous enzyme from Leuconostoc mesenteroides (LmG6PDH) has also been thoroughly studied in terms of structural and functional aspects, with both NAD and NADP (Naylor et al., 2001; Vought et al., 2000). In the present research, we aimed to obtain a G6PDH variant with a higher discrimination power against NADP, use it to replace the native EcG6PDH, and evaluate the metabolic impact of this change. For this reason, we took the well-studied LmG6PDH as a starting point to perform molecular dynamics (MD) simulations of the interactions between this enzyme and the cofactors NAD and NADP, envisioning the design of the point mutations needed to obtain an NAD-preferring G6PDH.

There are previous observations with glyceraldehyde 3-phosphate dehydrogenase, glutamate dehydrogenase, and isocitrate dehydrogenase indicating that cofactor specificity is a key factor in determining the distribution of metabolic fluxes and/or growth in bacteria (Martinez et al., 2008; Marx et al., 1999; Zhu et al., 2005). In spite of its clear contribution to redox balance and growth, in E. coli, the oxPPP has not been studied in this regard.

In this work, we generated an NAD-preferring G6PDH and replaced the native EcG6PDH with the engineered protein. The effects of this change in WT and ApgI genetic backgrounds were compared. We examined the changes in the expression of various genes encoding key enzymes of the central pathways and the growth rates during aerobic growth on glucose. We compared predictions performed by flux balance analysis with the experimental results and found that in spite of the prediction, the flux through the oxPPP was active after the replacement. A search in genome databases suggested that the cofactor specificity in G6PDH is related to the bifurcation of the carbon flux between the Embden–Meyerhof pathway and the oxPPP.

**METHODS**

**MD analysis.** MD simulations were performed using a model of the 3D structure of LmG6PDH in complex with NADP (Protein Data Bank ID: 1H9A) (Naylor et al., 2001). The in silico mutations R46E and Q47E were generated using the Mutagenesis plugin implemented in PyMOL (http://www.pymol.org/) over the 1H9A structure and were submitted to 150 000 steps of conjugate gradient energy minimization prior to the MD simulations. Starting from these structures we generated the NAD complexes by removing the 2’-phosphate group of NADP in each case, leaving a hydroxyl group. The complexes were simulated under explicit solvent (i.e. simulating the presence of water molecules in the systems) using NAMD 2.9 software (Phillips et al., 2005) and the Amber99SB-ILDN force field (Lindorff-Larsen et al., 2010). The systems were neutralized using Na+ counter-ions. The parameters and topology for the NAD and NADP were taken from simulations performed over alcohol dehydrogenase (Ryde, 1995) and lactate dehydrogenase (Holmberg et al., 1999), respectively. The simulations were performed for 10 ns using a 1 fs timestep measuring the non-bonded interactions in a radius of 12 Å every 2 fs. The simulated temperature was 300 K without additional user-defined restraints. The calculations were performed on a 64-core AMD Opteron server.

**VMD 1.9.1 software** (Humphrey et al., 1996) was used to analyse the trajectories. The interactions described in Fig. 1 were quantified from the distances between the following pairs of atoms (protein and ligand): (I) the main-chain nitrogen hydrogen of position 46 and the N3A nitrogen in the adenine moiety; (II) any nitrogen hydrogen in the side-chain of R46 and the 2’-oxygen of NAD or any oxygen of the 2’-phosphate group of NADP (WT systems); any oxygen in the side-chain of E46 and the 2’-oxygen of NAD or any oxygen of the 2’-phosphate group of NADP (mutant systems); (III) the main-chain nitrogen hydrogen of T14 and 3’-oxygen in the ribose moiety; (IV) the γ-oxygen of T14 and 3’-hydrogen of the ligand (NAD systems); the γ-hydrogen of T14 and either 3’-oxygen or any oxygen in the 2’-phosphate (NADP systems); and (V) the ε-oxygen of residue 47 and the γ-hydrogen of T14. These distances were evaluated every 50 ps; one interaction was counted when the distance between the involved atoms was <2.5 Å.

**Enzyme mutagenesis and purification.** The double-mutant R46E,Q47E of LmG6PDH was obtained by site-directed mutagenesis using the plasmid pET25-LmG6PDH (lactO, lacI, ColE1, AmpR) as the template. Dr Michael Cosgrove (Syracuse University, New York, NY, USA) kindly donated this plasmid and it contained the amino acid codifying sequence for the WT LmG6PDH (GenBank accession number M64446.1). The primers employed were: 5’-CATTGTGTTGGA-ACGGCCCAGGAAGCCCTCAATGATG-3’ and 5’-GGCCGTTCCA-ACAATGGCAAAATGTTTGGCAAG-3’. The bold bases in the first primer denote the intended mutations. The mutant sequence was then cloned in plasmid pTEV (Rocco et al., 2008) to facilitate the overexpression and His-tag-based purification of the mutant enzyme. E. coli BL21 DE3 cells transformed with the derived plasmid were aerobically grown in Luria–Bertani (LB) medium at 37 °C to OD600
0.5. After 4 h of induction with 1 mM IPTG (US Biologicals), the cells were harvested by centrifugation (10 min, 2000 g, 4 °C), resuspended in 50 mM cold Tris (Merck), 10 mM MgCl₂ (Merck) and 1 mM PMSF (Calbiochem), pH 8.2, and sonicated. The cellular extract was centrifuged for 30 min at 80 000 g, 4 °C and the supernatant was inoculated in a HiTrap Q HP (GE Healthcare) ion-exchange column previously equilibrated with a buffer of 50 mM Tris, 10 mM MgCl₂ and 40 mM NaCl (Merck), pH 8.2. The bound proteins were fractionally eluted by increasing the concentrations of NaCl inside the column from 40 mM to 1 M. The fractions with the highest G6PDH activities were supplemented with 40 mM imidazol (Merck) and inoculated in a HisTrap (GE Healthcare) column previously charged with NiSO₄ (Merck) according to the manufacturer's instructions. The bound proteins were eluted using a buffer 50 mM Tris and 10 mM MgCl₂, pH 8.2, supplemented with increasing concentrations of imidazol from 40 to 600 mM. Before setting the proteolytic cut to remove the His₆ tail from the molecules of LmG6PDH₄₆E,Q₄₇E, the fractions with the highest activities were pooled and dialysed against the buffer 25 mM Tris/HCl, pH 8.0, 50 mM NaCl and 1 mM EDTA to chelate the soluble nickel that interfered with the activity of the protease TEV. A home-made preparation of the protease TEV, expressed from plasmid pRK793 (Addgene), which had its own poly-His tail, was utilized. The proteolysis was performed for 1 h at 25 °C with 100:1 (w/w) substrate/protease and tris(2-carboxyethyl)phosphine (Sigma-Aldrich) was added up to a final concentration of 1 mM (β-mercaptoethanol provoked the precipitation of the protease TEV). After the digestion, the protein mixture was inoculated again in a HisTrap column, and the unbound protein was collected whilst the His-tagged TEV and the His₆ tails remained bound to the column. The purity of the protein obtained was checked by running a sample with 10–20 μg total protein in SDS-PAGE and staining with Coomasie blue (Merck). The effectiveness of the proteolytic cut was checked by Western blotting using an Ni-NTA HRP Conjugate kit (Qiagen). The purified LmG6PDH₄₆E,Q₄₇E was concentrated to a final concentration of 1 mg ml⁻¹ after the addition of 50 % (v/v) pure glycerol (Merck) and stored at −20 °C until the kinetic assays were performed.

**Enzyme kinetics.** Just before the assays, a sample of pure LmG6PDH₄₆E,Q₄₇E was dialysed against a buffer of 100 mM Tris, 10 mM MgCl₂ and β-mercaptoethanol, pH 8.0, at 25 °C. The assay
buffer had similar characteristics: it was supplemented with the substrates and did not contain β-mercaptoethanol. The substrates NAD, NADP and glucose-6-phosphate were prepared for the kinetic assays as described previously (Olavarria et al., 2012). The minimal enzymatic concentration that could be used safely during the assays without appreciable inactivation was determined using the test described previously (Selwyn, 1965). The reactions were triggered by the addition of glucose-6-phosphate. The velocities were assessed by monitoring the formation of NAD(P)H following the temporal changes in $A_{340}$ at 25 °C in a Synergy 2 (Biotek) UV/visible spectrophotometer. The protein concentration was determined using a Bio-Rad protein assay taking the dialysis buffer as the blank. The kinetic parameters of the mutant LmG6PDH_R60Q,Y74T were determined by an initial velocities approach. We set up the conditions to minimize the accumulation of products during the mixing before beginning the measurements. All the kinetic assays were performed using low-protein-absorption 96-well plates (Nunc).

**Generation of mutant strains.** The FRT-Kan′-FRT cassette [containing the FLP recognition target (FRT)] from the plasmid pKD13 (Datsenko & Wanner, 2000) was amplified using ad hoc designed primers that included restriction sites for the endonucleases Xhol and SalI. Both the amplified FRT-Kan′-FRT cassette and the plasmid pET-LmG6PDH_R60E,Q74T were cut with the endonucleases Xhol and SalI. After gel purification, the cassette and the cleaved vector were joined using a Quick Ligation kit (New England Biolabs). The resultant plasmid had the FRT-Kan′-FRT cassette just downstream of the amino-acid-encoding sequence for LmG6PDH_R60E,Q74T. The ligation product was used to transform *E. coli* TOP10 cells (Invitrogen) to propagate the resultant plasmid. The cells transformed with the resultant plasmid gained resistance to kanamycin conferred by the Kan′ gene. The correctness of the ligation was checked by DNA sequencing. Following a routinely used recombination protocol (Datsenko & Wanner, 2000), the second step was to generate, by PCR, a linear DNA molecule with the sequence LmG6PDH_R60E,Q74T-FRT-Kan′-FRT flanked with nucleotide sequences identical to the chromosomal 36 bp upstream and the 36 bp downstream of the 1476 bp native amino-acid-encoding sequence for the enzyme EGD6PDH.

The parental strains for the recombination procedure were: WT (MG1655 K12) and Δpgi (MG1655 K12 Δpgi; Charusanti et al., 2010). The linear DNA molecule obtained was purified and introduced into electrocompetent cells from the parental strains transformed previously with plasmid pKD46 (Datsenko & Wanner, 2000). To accomplish the recombination, the electrocompetent cells were grown to OD₅₆₀ 0.4–0.5 at 30 °C and the expression of the Red recombinase system was induced with the addition of 10 mM arabinose to the culture for 1 h before the treatment to transform the bacteria into electrocompetent cells. It is highly recommended to introduce the linear DNA into freshly prepared electrocompetent cells. The recombinant bacteria were detected by their ability to grow in LB-agar supplemented with the corresponding antibiotics. Colony PCR, chromosomal DNA sequencing, quantitative PCR (qPCR) and measurement of G6PDH specific activity in the cellular extracts were useful to confirm the success of the recombination procedure. A detailed description of the method for homologous recombination can be found elsewhere (Datsenko & Wanner, 2000). In this way, the following recombinant strains were obtained: NAD-G6PDH (MG1655 K12 Δzwf::zwf<sup>ΔmgaB-ΔmgaE-ΔmgaF</sup>), inact-G6PDH (MG1655 K12 Δzwf::zwf<sup>ΔmgaB-ΔmgaE-ΔmgaF</sup>) and Δpgi-NAD (MG1655 K12 Δpgi Δzwf::zwf<sup>ΔmgaB-ΔmgaE-ΔmgaF</sup>), where inact-G6PDH refers to a strain bearing an inactive form of G6PDH.

All the resultant strains were whole-genome sequenced and no other unintended mutations were detected.

**Specific activities in cell extracts.** The cells obtained by centrifugation (2500 g, 10 min) during the exponential growth phase in LB were washed twice with ice-cold 100 mM Tris, 10 mM MgCl₂ and 5 mM NaCl, pH 8.0, and centrifuged again (2500 g, 10 min). The resultant pellets were dissolved in the same buffer, but this time supplemented with a protease inhibitor cocktail (Roche). The cells were disrupted by sonication and the micelles were centrifuged at 20000 g for 20 min at 4 °C. The supernatants were rescued and placed on ice, and the pellets were discarded. The specific activities were checked by adding aliquots of the supernatants to defined volumes of a buffer of 50 mM Tris, 10 mM MgCl₂, and 1 mM glucose-6-phosphate, pH 8.0, supplemented without NAD or NAD at 0.4, 1 or 3 mM, and monitoring the initial rate of formation of NAD(P)H following the changes in the absorbance at 340 nm at 25 °C. A coupled assay was used to determine the specific activity of phosphoglucomutase (Salas et al., 1965). The concentration of protein in the cellular extracts was estimated using a commercial protein assay (Bio-Rad). The enzymic activities were normalized by the concentration of total proteins in the respective cellular supernatants.

**Growth conditions and sampling.** During the genetic manipulations and the assessment of the enzymic specific activities in the cellular extracts, the strains were grown up in LB rich medium with or without agar, supplemented with ad hoc antibiotics. All physiological experiments were done by growing the cells in full aerobic conditions, with vigorous agitation (300 r.p.m.), at 37 °C in M9 minimal medium supplemented with 0.2 μM-filtered glucose as the sole carbon source (4.5 g l⁻¹) (M9-glucose). M9 minimal medium was prepared as described previously (Fuhrer & Sauer, 2009). All experiments were performed in biological triplicates and every measurement in a biological replicates had technical triplicates.

In all the experiments, the starting biological material was bacteria sampled from isolated colonies previously grown up overnight in 50 ml M9-glucose before being inoculated into the flasks for the experiments. To measure the growth rate, the glucose uptake rate and the acetate excretion rate, samples were taken every 30 min to measure the OD₅₆₀ and broth samples were filtered using a 0.2 μm filter. These samples were immediately chilled at −80 °C until analysis. When the cultures reached OD₅₆₀ 1.0, samples of biomass were treated and saved for measurement of mRNA. Samples of extracellular media and measurements of optical density were taken until the cultures approached the stationary phase.

**qPCR.** An aliquot of 1 ml broth from each culture was added to 2 vol. RNAprotect Bacterial Reagent (Qiagen), and the total RNA was isolated by using RNEasy columns (Qiagen) and treated with DNase I (Promega). Total RNA yields were measured by quantifying $A_{260}$ and quality was checked by visualization on agarose gels and by measuring the $A_{260}/A_{280}$-ratios. The DNAs were obtained by using SuperScript II reverse transcriptase (Invitrogen). The transcript levels of the genes *aek*, *gapA*, *gnd*, *icd*, *mdh*, *nadE*, *pntA*, *salA* and *zwf* were determined by qPCR (SYBR GreenER qPCR SuperMix Universal; Invitrogen).

The reaction mixtures were monitored on an MX3000P (Stragane) qPCR analyser with the following protocol: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, annealing at 56 °C for 60 s and extension at 72 °C for 30 s. The specificity of the products was verified by melting curves analysis. The levels of the target mRNAs were normalized to the level of mRNA encoding the housekeeping protein DnaA. The sequences of the primers used can be found in the Supplementary Material.

**Computational systems analysis of physiological and qPCR data.** The latest comprehensive genome-scale model of *E. col*
ijO1366, was used as the WT default Systems Biology Markup Language model (Orth et al., 2011).

Modelling was performed by solving flux balance analysis problems with targeted constraints. Generally, the flux balance analysis problem is a linear optimization problem formulated as:

$$\min \ c^Tv$$

s.t.

$$Sv=0$$

$$v_{lb} \leq v \leq v_{ub}$$

where $c$ is the objective vector containing the reaction to be maximized or minimized (for negative and positive elements, respectively), $v$ is the vector of fluxes, $S$ is the stoichiometric matrix, and $v_{lb}$ and $v_{ub}$ are the lower and upper flux bounds, respectively. Additional constraints to model the effects of gene deletions can be added by constraining flux bounds to zero for reactions catalysed by deleted genes. The formalism was previously described in detail (Orth et al., 2010). All the computational simulations were performed with COBRA Toolbox 2.0.3 (Schellenberger et al., 2011) in MATLAB.

For qPCR and robustness analysis, a mutant G6PDH model with the cofactor specificity of G6PDH changed from NADP to NAD was created. Robustness analyses were performed using the methodology developed previously (Varma et al., 1993) as implemented in the COBRA Toolbox (Schellenberger et al., 2011). Additional constraints were added to specific reactions (transhydrogenase and phosphoglucoisomerase) as indicated in the Results and Discussion during the analysis. A G6PDH knockout was simulated by constraining the G6PDH reaction to have zero flux. For the *in silico* robustness analysis, a glucose uptake rate of 8 mmol [g dry weight (DW)]$^{-1}$ h$^{-1}$ and oxygen uptake rate of 10 mmol gDW$^{-1}$ h$^{-1}$ was assumed. qPCR measurements were then incorporated as follows.

The WT flux state was first calculated by using flux balance analysis with the WT model, by maximizing ATP production, and subsequently minimizing the length of the flux vector necessary to achieve the measured growth rate given measured glucose uptake and acetate excretion. To enforce known flux splits, previously measured fractions of NADPH production from the membrane-bound transhydrogenase and flux through oxPPP and the NADP-specific malic enzyme were constrained in the WT model (Perrenoud & Sauer, 2005; Sauer et al., 2004). Then, to estimate the mutant flux state in each case, for each one of the genes with its level of expression measured using qPCR, the mutant flux was constrained to be equal to the WT flux multiplied by the ratio of mutant/WT glucose uptake and the ratio of mutant/WT gene expression. This procedure was based on the assumption that expression changes correlate directly with flux changes, which has obvious exceptions but is simply intended to provide a systems analysis of the possible integrated effects of gene expression changes (Fig. S1). The reaction catalysed by 6-phosphogluconate dehydrogenase was excluded from analysis in the G6PDH ‘knockout’ model as the oxPPP was assumed to not carry flux once G6PDH was inactive. Glyceraldehyde 3-phosphate dehydrogenase was left unconstrained in all models despite the availability of qPCR measurements because constraining flux based on gene expression was found to lead to an unfeasible flux solution. We found this to be due to an inflexibility of glycolysis due to the lack of alternate pathways that could sustain energy production and metabolite secretion production given a downregulation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase in the mutant strain. Flux solutions for mutant strains were then calculated as with the WT model by maximizing ATP production and subsequently minimizing the length of the flux vector necessary to satisfy all constraints. This choice of objective is most consistent with both physiological data and reports that bacterial flux states tend to fall near a Pareto surface determined by the competing objectives of growth, energy production and minimal use of enzymes (Schuetz et al., 2012).

A number of existing expression integration methods exist, as recently reviewed (Machado & Hertrgård, 2014). These methods have been shown to provide little increased accuracy in flux prediction from incorporation of gene expression data. For this reason, we did not attempt to quantitatively predict flux levels, a deviation from the goal of previous methods, but instead only sought to determine the possible systems-level impact of observed gene expression changes, taking into account the limitations of expression as a proxy for flux levels.

**Phenotypic measurements.** Glucose and acetate concentrations in the extracellular filtrates were analysed by HPLC using an Aminex HPX-87H (Bio-Rad) ion-exchange column at 65 °C and an IR HP 1047A (Hewlett Packard) detector. No other organic compound was detected consistently. The mobile phase was degassed with 5 mM H$_2$SO$_4$ at a flow rate of 0.5 ml min$^{-1}$. The concentration was assessed by comparison with standards of known concentrations run previously under the same conditions. For the conversion of the optical density (OD) to gDW, we previously studied the relationship between these two variables, observing a linear relationship for values of OD<sub>600</sub><0.6. The broth samples with OD<sub>600</sub><0.5 were diluted and measured again, considering the dilution factor (Neidhardt et al., 1990). From this study we obtained the conversion factor 0.45 gDW l$^{-1}$ OD$^{-1}$.

In the exponential growth phase, the temporal evolution of the dry weight DW(t) is described by the equation:

$$\text{lnDW}(t)=kt+\text{lnDW}_0$$

where DW<sub>0</sub> is the initial dry weight, $k$ is the specific growth rate of the strain and $t$ is time (the independent variable). Given this linear form, it was possible to statistically compare the specific growth rates (slopes) of the different strains by an analysis of covariance, with the null hypothesis of no difference among the specific growth rates.

For the concentration of any metabolite detected in the extracellular media, the instantaneous concentration X(t) is described by:

$$X(t)=r \times (\text{DW}(t)/k)+X_0$$

where $r$ is the specific uptake/excretion rate and X<sub>0</sub> is the initial concentration of the metabolite X. The above equation also represents a line where the slope is the relevant specific uptake/excretion rate, so it was also possible to statistically compare the glucose uptake rates and the acetate excretion rates of the different strains.

**RESULTS AND DISCUSSION**

In this work, we used a multidisciplinary approach to understand the importance of the reduced cofactor produced in the reaction catalysed by EcG6PDH. To the best of our knowledge, the present work is the first study in *E. coli* where a dehydrogenase from the oxPPP was replaced by a homologue with a different specificity. Furthermore, the inserted gene was not an intact, heterologous gene, but rather an engineered gene, which we name a heteromutant gene.

**Design and kinetic study of an NAD-preferring G6PDH**

According to previous work (Vought et al., 2000), the presence of a carboxylate group at the side-chain of
position 46 or 47 antagonizes the negative charge of the 2’-phosphate of NADP. We wanted to evaluate to what extent two negative charges would reinforce the discrimination against NADP without disturbing the interactions with NAD. With this aim, we simulated the effect of the double-mutant R46E,Q47E over NAD(P) discrimination by MD. Trajectories of 10 ns were run for the different enzyme-cofactor complexes in explicit solvent, with either NAD or NADP for both \textit{Lm}G6PDH and \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} (Fig. 1). For \textit{Lm}G6PDH, the side-chain atoms of the residues T14 and R46 establish the network of interactions with the adenosine moiety of NADP. Conversely, when the 2’-phosphate is absent from the ligand (NAD molecule), the interactions are mostly provided by the main-chain atoms of those residues, together with a stabilizing hydrogen bond formed between the side-chains of Q47 and T14. In the case of the mutant, the hydrogen-bond network around NADP is weak because of the absence of the strong ionic interaction provided by the guanidinium group of R46 to the 2’-phosphate. Indeed, the simulation shows that the negative charge of the carboxylate in E46 lies very close to the phosphate group (interaction II), increasing the instability of the system. The scheme is not so complicated in the double-mutant active site when it comes to the interactions network around the adenosine moiety of NAD. In fact, it seems that the carboxylate of E46 could establish relatively weak interactions with the 2’-hydroxyl of ribose. These observations suggest that the double-mutant R46E,Q47E will be able to use NAD as the substrate more effectively than NADP, providing a discrimination against the 2’-phosphate stronger than the single-mutant R46E (Vought et al., 2000), due to an increased coulombic repulsion. Thus, we evaluated \textit{in vitro} the effect of the proposed double mutation.

Using site-directed mutagenesis, we obtained the double-mutant enzyme \textit{Lm}G6PDH\textsuperscript{R46E,Q47E}, which was studied kinetically (Table 1, Fig. S2) using either NAD or NADP as cofactor in the presence of glucose-6-phosphate at 800 μM (a typical concentration for this metabolite in the cell; Lowry et al., 1971) or 10 mM. For the coenzyme NADP, a change was seen from a $K_m$ of 8 μM with \textit{Ec}G6PDH to almost 4.8 mM with \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} (Table 1). The change in $K_m$ for NAD was not as drastic as that for NADP; however, it was more than three times lower than the $K_m$ for NAD of \textit{Ec}G6PDH. The MD simulations predicted that in \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} the interactions with NAD around the adenosine moiety would not be severely compromised after the amino acid replacements. Therefore, the increase in the $K_m$ for NAD in comparison with the unmodified \textit{Lm}G6PDH could be related to the loss of interactions in other regions of the molecule or the effect of the presence of the glucose-6-phosphate at the active site, which was not considered in the simulations. The most noteworthy effect of the double mutation is that the $k_{cat}$ for NADP was almost 13 times lower than the $k_{cat}$ for NAD (Table 1). With a specificity constant ($k_{cat}/K_m$) for NAD 37 times higher than for NADP, we almost doubled the discrimination power against NADP observed in the mutant with the highest NAD preference obtained previously (Vought et al., 2000). When we increased the glucose-6-phosphate concentration to 10 mM to obtain more saturating conditions for this substrate, we observed an increase in the specificity constant for NAD up to a value 50 times higher than the specificity constant for NADP (Fig. S2). Moreover, considering the $K_m$ for NADP and the physiological concentration of this nucleotide, the event of oxidation of a glucose-6-phosphate by an NADP catalysed by \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} must be very infrequent under physiological conditions. Therefore, the kinetic properties of the double-mutant G6PDH allow its use \textit{in vivo} as an almost exclusive NADH-generating enzyme.

An inactive form of the double-mutant enzyme was obtained by the additional mutation D16G that nullifies the activity of the enzyme with both NAD and NADP. The D16 side-chain was observed to contact the nicotinamide amide in the 3D structures of \textit{Lm}G6PDH with both NAD and NADP (Naylor et al., 2001), as well as in the course of our simulations (Fig. S3). In the absence of this side-chain, the nicotinamide moiety of the cofactor must be unable to acquire the correct orientation for hydride transfer.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
 & \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} & \textit{Lm}G6PDH & \textit{Ec}G6PDH \\
\hline
\textbf{NAD} & & & \\
$K_m$ (μM) & 1626 ± 243 & 162 ± 18 & 5909 ± 400 \\
\$k_{cat}$ (s$^{-1}$) & 736 ± 32 & 727 & 288 ± 5 \\
\hline
\textbf{NADP} & & & \\
$K_m$ (μM) & 4757 ± 724 & 8.0 ± 0.7 & 7.5 ± 0.8 \\
\$k_{cat}$ (s$^{-1}$) & 58 ± 3 & 337 & 174 ± 2 \\
$(k_{cat}/K_m)^{\text{NADP}} : (k_{cat}/K_m)^{\text{NAD}}$ & 0.027 & 9.4 & 410 \\
\hline
\end{tabular}
\caption{Apparent kinetic parameters for the double-mutant \textit{Lm}G6PDH\textsuperscript{R46E,Q47E} at 800 μM glucose-6-phosphate, using either NAD or NADP as the cofactor}
\end{table}

For comparison, the corresponding parameters of \textit{Lm}G6PDH and \textit{Ec}G6PDH are shown as reported by Lee & Levy (1992) and Olavarria et al. (2012), respectively.
**Active LmG6PDH**<sup>R46E,Q47E</sup> **is expressed from the chromosomal locus of zwf**

Taking advantage of the homologous recombination mediated by the Red recombinase from phage lambda (Datsenko & Wanner, 2000), the amino-acid-encoding sequence from the chromosomal copy of zwf was replaced by the amino-acid-encoding sequence for LmG6PDH<sup>R46E,Q47E</sup>, preserving the endogenous cis-regulatory elements (Fig. S4). The replacement was done both in the WT strain and in an isogenic Δpgi strain. In this way, two recombinant strains (NAD-G6PDH and Δpgi-NAD-G6PDH) were obtained. A third recombinant strain (inact-G6PDH) was generated where the mutant gene (encoding LmG6PDH<sup>D16G,R46E,Q47E</sup>) should be transcribed and translated, but the protein was inactive. Thus, it was expected that the strain harbouring the gene encoding LmG6PDH<sup>D16G,R46E,Q47E</sup> would resemble the metabolic phenotype of a Δzwf strain (Fig. 2c). Cellular extracts from batch cultures of all the strains studied were used to test the specific G6PDH activities using either NADP or NAD as the cofactor. The results indicated that LmG6PDH<sup>R46E,Q47E</sup> was translated in an active form and suggested that it could sustain the flux through the oxPPP in the recombinant strains while producing NADH instead of NADPH (Fig. 2f). The specific activities observed with both cofactors were consistent with the kinetic parameters obtained for the EcG6PDH (Olavarria et al., 2012) and the engineered LmG6PDH<sup>R46E,Q47E</sup> (Table 1, Fig. S2). No phosphoglucoisomerase activity was detected in the cellular extracts from Δpgi and Δpgi-NAD-G6PDH strains (data not shown), confirming that the upper part of the Embden–Meyerhof pathway is blocked in these strains.

**Modelling optimum growth predicts a low impact for the shift of cofactor specificity of G6PDH over different genetic backgrounds**

Constraint-based modelling, as used here, is particularly useful for examining the limits of functionality for a particular organism (Price et al., 2004). Fig. 3 shows a robustness analysis (Schellenberger et al., 2011) where the maximum possible growth rate is predicted given various amounts of flux passing through the G6PDH reaction (either through the WT G6PDH or through the NAD-specific G6PDH). Simulations were run in the WT, Δpgi and ΔpntAB (the membrane-bound transhydrogenase) backgrounds. Overall, the impact that the NAD-specific G6PDH had on the maximal possible growth rate was rather modest: in the most extreme case (ΔpntAB background), the growth of the strain bearing the NAD-specific G6PDH was ~2% lower than the value for the strain bearing the NAD-specific G6PDH form. For the WT background, nearly half of the glucose-6-phosphate must be oxidized through the oxPPP to accomplish the maximum growth rate and this maximal growth rate is only ~1% higher than the growth rate attainable when there is no flux through G6PDH. The simulations also predicted that after the replacement of the NADP-specific EcG6PDH by the NAD-specific LmG6PDH<sup>R46E,Q47E</sup>, any flux through the oxPPP is detrimental to the theoretical maximum growth rate, albeit a small change. In the case where PntAB supplied 40% of the NADPH production, no appreciable changes were expected. In fact, the membrane-bound transhydrogenase has been reported to be responsible for between 35 and 45% of NADPH production during aerobic batch growth of *E. coli* (Fuhrer & Sauer, 2009; Sauer et al., 2004). In other words, the use of an NAD-specific G6PDH would not be optimal for the formation of biomass at any flux through oxPPP. Therefore, we should expect that if the internal fluxes are distributed such that the formation of biomass is maximal, the flux through oxPPP will be zero and the metabolic phenotype of the NAD-G6PDH strain must resemble the metabolic phenotype of the inact-G6PDH strain.

**A shift in the cofactor preference of G6PDH significantly affects the growth rate and depends on the genetic background**

In order to test the modelling predictions, the growth rates and the glucose uptake rates were observed first on the Δpgi and Δpgi-NAD-G6PDH strains, where all the catabolic carbon flows through the oxPPP when glucose is the sole carbon source. This was also an *in vivo* test of functionality for LmG6PDH<sup>R46E,Q47E</sup>, as a Δpgi strain will not grow under these conditions in the absence of flux through the oxPPP (Sauer et al., 2004). When these strains were grown aerobically with glucose as the sole carbon source, the mutant Δpgi-NAD-G6PDH showed a growth rate 59% faster than that observed for the parental Δpgi (Table 2). Canonaco et al. (2001) demonstrated that the impaired growth rate of the Δpgi strain is caused by the relative excess of NADPH produced when almost the entire assimilated glucose flows through the oxPPP. The results shown here are consistent with the results of Canonaco et al. (2001) because the substitution of an NADPH-producing enzyme by an NADH-producing enzyme would partially relieve the relative excess of NADPH observed in Δpgi, improving its growth rate. However, the glucose uptake rate and the growth rate of the Δpgi-NAD-G6PDH strain remained far from those observed in the WT strain, giving additional support for the hypothesis suggested by Canonaco et al. (2001) about the inability of the pentose phosphate pathway alone to carry a glycolytic flux of the same magnitude observed in the WT strain. Taken together, the specific G6PDH activities measured in the cellular extracts and the growth rate observed in the Δpgi-NAD-G6PDH strain show that the expressed molecules of LmG6PDH<sup>R46E,Q47E</sup> were catalytically capable of sustaining flux through the oxPPP.

The impact on the growth rate of the replacement of the NADP-specific EcG6PDH by the NAD-specific LmG6PDH<sup>R46E,Q47E</sup> was then studied in the WT background. This mutation provoked a fall of 44% in the growth rate in comparison with the WT strain. Surprisingly, this effect was even more negative than that observed in the inact-G6PDH strain, where the growth rate

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*K. Olavarria and others*
fell just 17% (Table 2). As no other change beyond the intended gene replacement was observed, the differences in the growth rates could be explained by the generation in the oxPPP of some NADH instead of only NADPH.

Interestingly, despite the differences in the growth rates, no changes in the yield of biomass per consumed substrate were observed between WT and NAD-G6PDH strains (Table 2). Therefore, flux balance analysis correctly predicted no changes in the growth yields, but failed to predict the existence of some flux through the modified oxPPP.

These results suggest that the effect on the growth rate of the generation of NADH by G6PDH depends on the presence of a branching point for the carbon flux: this effect was positive when the upper Embden–Meyerhof pathway was blocked and was negative when both the upper Embden–Meyerhof pathway and the oxPPP were active. To obtain further evidence for this statement, we searched for the presence of phosphofructokinases (which catalyse the irreversible committed step of the upper Embden–Meyerhof pathway) in the genomes of the

Fig. 2. Metabolic pathways around the node of glucose-6-phosphate in the five strains involved in this study. Blue circles represent NADP-specific dehydrogenases; red circles represent NAD-specific dehydrogenases. (a) WT, (b) NAD-G6PDH, (c) inact-G6PDH, (d) Δpgi and (e) Δpgi-NAD-G6PDH. G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; RL5P, ribulose 5-phosphate; F6P, fructose 6-phosphate; E4P, erythrose 4-phosphate; X5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphosphoglyceric acid. (f) Mean specific G6PDH activities, detected in the cellular extracts from the represented strains, using NAD or NADP as the cofactor at different concentrations. The concentration of glucose-6-phosphate used in these assays was 1 mM. No activities were detected with NAD or NADP in the inact-G6PDH strain (data not shown).
prokaryotic organisms whose G6PDHs have been characterized using NAD and NADP as cofactors. We considered the four kinds of phosphofructokinases (EC 2.7.1.11, EC 2.7.1.56, EC 2.7.1.105 and EC 2.7.1.146) described so far (Table 3). It is noteworthy that, for the 16 cases analysed, the presence of a dual G6PDH was associated with the absence, non-confirmed functionality or very weak phosphofructokinase activity. Phosphofructokinase activity has been reported in the three organisms where the G6PDHs were 100 times more specific for NADP than for NAD.

Integrating qPCR and phenotypic data with in silico modelling

One possible explanation for the differences in the growth rates could come from changes in the expression levels of different enzymes involved in the NAD(P)(H) balance. We addressed this issue by performing qPCRs. The results with the primers designed for hybridization with the cDNAs derived from the mRNA transcribed from the zwf loci in WT, inact-G6PDH and NAD-G6PDH strains were consistent with the genetic manipulations performed, and their expression levels were similar under the conditions studied here: the normalized values were 0.041 ± 0.070 in WT, 0.035 ± 0.080 in inact-G6PDH and 0.034 ± 0.090 in NAD-G6PDH. For the other loci explored, the levels of expression did not change more than 1.7-fold (Table 4). Therefore, despite the elimination of at least one source of NADPH, the transcriptional regulatory network did not provoke important changes in the loci observed. These results indicate that the mechanisms that regulate the transcription of the enzymes associated with NAD(P)(H)

Table 2. Physiological parameters during the exponential growth phase for the different strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth rate (h⁻¹)</th>
<th>Glucose uptake rate (mmol gDW⁻¹ h⁻¹)</th>
<th>Acetate excretion rate (mmol gDW⁻¹ h⁻¹)</th>
<th>Yield (gDW mmol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δpgi</td>
<td>0.145 ± 0.003</td>
<td>1.63 ± 0.04</td>
<td>ND</td>
<td>0.089</td>
</tr>
<tr>
<td>Δpgi-NAD-G6PDH</td>
<td>0.231 ± 0.003</td>
<td>3.04 ± 0.04</td>
<td>ND</td>
<td>0.076</td>
</tr>
<tr>
<td>WT</td>
<td>0.64 ± 0.02</td>
<td>8.08 ± 0.20</td>
<td>1.91 ± 0.17</td>
<td>0.079</td>
</tr>
<tr>
<td>inact-G6PDH</td>
<td>0.53 ± 0.02</td>
<td>7.19 ± 0.18</td>
<td>2.93 ± 0.15</td>
<td>0.074</td>
</tr>
<tr>
<td>NAD-G6PDH</td>
<td>0.36 ± 0.02</td>
<td>4.53 ± 0.17</td>
<td>ND</td>
<td>0.079</td>
</tr>
</tbody>
</table>

ND, Not detected.
metabolism did not change their patterns in the short term after a shift in the cofactor specificity in G6PDH.

We then sought to estimate computationally the possible impact of transcriptional changes that did occur on the NADH and NADPH production pathways. The growth rates, glucose uptake rates, acetate excretion rates and qPCR data were considered as constraints to calculate the possible internal flux distributions in the WT, inact-G6PDH and NAD-G6PDH strains. The production and consumption patterns of NADH and NADPH were compared considering the most relevant sources and sinks in each strain. For the NAD-G6PDH strain, the simulation shows an operative zwf flux contributing to the NADH pool and the possible metabolic response to achieve a new balance in the presence of NADH production by oxPPP (Fig. 4). However, using this steady-state analysis we could obtain no inferences regarding changes in the size of the NADH or NADPH pools.

Taken together, the physiological results suggest that the drop in the growth rate observed in the NAD-G6PDH strain could be the consequence of a limited systemic capacity to overcome, in the short term, the effects of the production of NADH by G6PDH. The qPCR data suggest that the regulatory network did not respond in the short term to compensate for the shortage in NADPH production through the oxPPP. It would be possible, however, that the growth rate of the NAD-G6PDH strain could be augmented after selecting for the cells with the fastest growth in the context of adaptive laboratory evolution. Furthermore, given the finding that the response was not near the optimal

<table>
<thead>
<tr>
<th>Locus</th>
<th>ID in iJO1366</th>
<th>inact-G6PDH</th>
<th>NAD-G6PDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>zwf</td>
<td>b1852</td>
<td>0.82</td>
<td>0.81</td>
</tr>
<tr>
<td>gnd</td>
<td>b2029</td>
<td>1.47</td>
<td>1.35</td>
</tr>
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<td>icd</td>
<td>b1136</td>
<td>1.31</td>
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</tr>
<tr>
<td>nadvk</td>
<td>b2615</td>
<td>1.22</td>
<td>1.16</td>
</tr>
<tr>
<td>pntA</td>
<td>b1603</td>
<td>1.63</td>
<td>1.31</td>
</tr>
<tr>
<td>maeA</td>
<td>b1479</td>
<td>0.76</td>
<td>0.70</td>
</tr>
<tr>
<td>maeB</td>
<td>b2463</td>
<td>1.21</td>
<td>1.05</td>
</tr>
<tr>
<td>ackA</td>
<td>b2296</td>
<td>1.62</td>
<td>1.32</td>
</tr>
<tr>
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<td>1.54</td>
<td>1.29</td>
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<tr>
<td>udhA</td>
<td>b3962</td>
<td>0.69</td>
<td>0.85</td>
</tr>
<tr>
<td>mdh</td>
<td>b3236</td>
<td>1.18</td>
<td>1.23</td>
</tr>
<tr>
<td>gapA</td>
<td>b1779</td>
<td>1.33</td>
<td>1.61</td>
</tr>
</tbody>
</table>

*Data refer to G6PDH committed to the Entner–Doudoroff pathway; there is also an NADP-preferring G6PDH in the same organism.

Table 3. Cofactor specificity of G6PDHs and presence/absence of phosphofructokinase in 16 bacterial species

Table 4. Fold change in the expression levels from different loci for the inact-G6PDH and NAD-G6PDH strains relative to the values in WT
predicted phenotype, additional algorithms, such as MOMA (Segrè et al., 2002) or ROOM (Shlomi et al., 2005), could be pertinent for examining the flux state of the cells immediately post-perturbation.

It seems that metabolic balances are insufficient to explain why EcG6PDH enzyme is highly specific for NADP. It is important to note that among the NADPH generation pathways of E. coli, the oxPPP is dedicated exclusively to NADPH production and the flux through this pathway does not affect NADH metabolism directly. The importance of having NADP-specific dehydrogenases is explained as follows.

Despite the structural similarities, NAD(H) and NADP(H) have very different metabolic roles. The reoxidation of NADH is coupled to energy-conserving processes fuelled by the difference between the redox potential of the electron sources and the redox potential of the final acceptors. However, the reducing power of NADPH is used to drive antioxidant and biosynthetic reactions. The majority of the reducing equivalents harnessed in the NADPH pool remain in the cell, unlike the reducing equivalents in the NADH pool, which leave the cell. Given these differences in their physiological roles, the redox status of these pools must be regulated by different means. The redox status of the internal NAD:NADH pair depends on the redox potential of the external electron acceptors (de Graef et al., 1999), but the NADP:NADPH redox status must be regulated by controlling the flux through the NADPH-producing branching points. One way to control the generation of NADPH is by partitioning the carbon flux at branching points under some kind of regulatory control where only one of the emerging pathways bears NADPH-producing enzymes. The amount of carbon going through each branch varies according to the particular system. It depends on the kinetic parameters and relative abundances of the enzymes.

Fig. 4. In silico modelling of the production and consumption of NADH and NADPH pools in the different strains generated in this work by integrating phenotypic and qPCR data. The top panel shows the production and consumption of NADPH, whereas the bottom panel shows the same for NADH. The amount of flux (expressed as mmol gDW$^{-1}$ h$^{-1}$) is scaled to the amount of glucose coming into the cell for each case. As expected, dehydrogenases from oxPPP, isocitrate dehydrogenase and PntAB are the major contributors of NADPH. In the presence of an NAD-preferring G6PDH, oxPPP is used at an increased relative rate, but its contribution to the NADPH pool falls by 33%. However, the simulation indicates that the contribution of the engineered G6PDH to the NADH pool must be balanced mainly by a higher consumption, particularly by PntAB transhydrogenase.
competing for the same metabolite, the rate of generation of the shared metabolite and other regulatory mechanisms (LaPorte et al., 1984). The best-studied case so far is the branching point between isocitrate dehydrogenase and isocitrate lyase where the behaviour of the emerging fluxes upon different changes in the concentration of isocitrate and the maximum velocity of the isocitrate dehydrogenase were modelled (LaPorte et al., 1984). It is also well known that isocitrate dehydrogenase can be transiently inactivated by phosphorylation, enabling an increase in the flux through the glyoxylate cycle (Garnak & Reeves, 1979). In the case of the partitioning of the flux around glucose-6-phosphate, it was observed that it does change after an increase in the demand (Rui et al., 2010) or the offer of NADPH (Martínez et al., 2008). To the best of our knowledge, there are no studies on how much control EcG6PDH could exert upon the flux through the oxPPP, although increasing its concentration above the typical level did not increase significantly the flux through this pathway (Nicolas et al., 2007; Orthner & Pizer, 1974).

The branching points described could function as valves because NADPH is usually generated in reactions that are irreversible under physiological conditions. Interestingly, all the sources of NADPH in the central pathways of E. coli are irreversible reactions (under physiological conditions) and are placed at branching points (with the exception of the membrane-bound transhydrogenase). In the case of the oxPPP, there are two steps with the ability to generate NADPH: the irreversible conversion of glucose-6-phosphate into 6-phospho-glucuronolactone (at the branching point between the Embden–Meyerhof pathway and the oxPPP) and the decarboxylative oxidation of 6-phosphogluconate to form ribulose 5-phosphate (at the branching point between the Entner–Doudoroff pathway and the oxPPP). Therefore, partitioning the carbon flux through the non-essential oxPPP and the Entner–Doudoroff pathways allows an effective control of the amount of NADPH produced by the cell without direct interference in the generation of NADH.

Therefore, the results presented here expand our current interpretation of the importance of generating NADPH by EcG6PDH as a mechanism to protect the cell against oxidative stress to a view where the NADP specificity of the EcG6PDH could have also emerged from systemic metabolic constraints.

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