6-Phosphogluconate dehydrogenase from *Lactococcus lactis*: a role for arginine residues in binding substrate and coenzyme

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A gene encoding 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44) was identified from the homofermentative lactic acid bacterium *Lactococcus lactis*, by complementation of *Escherichia coli* mutants. The cloned gene was then expressed to high levels in *E. coli* and the protein purified for kinetic analysis. The enzyme had a K_m for 6-phosphogluconate of $15.4 \pm 1.4 \mu\text{M}$ and for NADP of $1.9 \pm 0.2 \mu\text{M}$ at pH 7.5. Sequence comparison of the *L. lactis* 6-PGDH with the corresponding enzyme derived from the pathogenic protozoan *Trypanosoma brucei* and sheep liver revealed the substrate-binding residues to be identical in all three species, although the three coenzyme-binding pockets differed slightly. A totally conserved arginine residue (Arg-447), believed to bind the 6-phosphate of substrate, was mutated to lysine, aspartate, alanine or tryptophan. In each case enzyme

activity was lost, confirming an essential role for this residue on activity. A second arginine (Arg-34), believed to be critical in binding the 2'-phosphate of cofactor NADP⁺, was mutated to a tyrosine residue, as found in one atypical isoform of the enzyme in *Bacillus subtilis*. This alteration led to decrease in affinity for NADP⁺ of nearly three orders of magnitude. A second 6-PGDH gene has been identified from the genome of *B. subtilis*. This second isoform contains an arginine (Arg-34) in this position, suggesting that *B. subtilis* has two 6-PGDHs with different coenzyme specificities.

Key words: mutagenesis, NADPH binding, pentose phosphate pathway.

INTRODUCTION

6-Phosphogluconate dehydrogenase (6-PGDH) is the third enzyme of the pentose phosphate pathway (PPP) [1]. It catalyses the oxidative decarboxylation of 6-phosphogluconate (6PG) to ribulose 5-phosphate, with the concomitant production of NADPH. The mechanism of action of the enzyme has been studied in several species [2–5] and crystal structures are known for the enzyme from various sources [6,7]. The enzyme has been proposed as a potential target for anti-microbial chemotherapy, since accumulation of its substrate, 6PG, is toxic to eukaryotic cells, causing a decrease in glycolysis due to its inhibitory effect of phosphoglucose isomerase [8]. Understanding more about the structure and function of the enzyme from many sources will assist in developing inhibitors, which may be of specific anti-microbial value. The enzyme has been subject to detailed analysis from African trypanosomes which cause the disease sleeping sickness [9,10].

The lactic acid bacteria are an artificially classified group that produce lactic acid as a major end product of sugar metabolism [11]. Homofermentative members of this group use the Embden–Meyerhof pathway to produce ATP, with lactate being the major end product, whereas heterofermentative organisms rely on a variant of the PPP known as the phosphoketolase pathway to generate ATP with the end products of lactate plus ethanol [12]. *Lactococcus lactis* is homofermentative, relying on the classical glycolytic pathway for ATP production.

In most homofermentative species, 6-PGDH uses NADP⁺ as

a cofactor, as it does in other prokaryotes and eukaryotes. In some heterofermentative species, this enzyme and the other dehydrogenase of the oxidative PPP, glucose 6-phosphate dehydrogenase, use NAD⁺ [13]. This adaptation arises from a need to generate NADH, which is consumed in the latter stages of the phosphoketolase pathway [14]. Examples of enzymes that are able to use either cofactor also exist [15,16]. Whereas structural and functional data exist for the NADP⁺-dependent enzymes, to date no information is available on the structure of the NAD⁺-dependent 6-PGDHs.

We report here the first cloning of a gene encoding an enzyme from the PPP of *L. lactis*, and compare kinetic parameters of the *L. lactis* 6-PGDH with those of the *Trypanosoma brucei* and sheep liver enzymes. Moreover, the *Lactococcus* enzyme is particularly stable and crystallized more easily than that of the trypanosome [17]. Consequently it has offered an excellent model for studying the activity of the enzyme through site-directed mutagenesis studies of the residues believed to bind substrate and participate in catalytic activity.

MATERIALS AND METHODS

Organisms, complementation and reagents

L. lactis strain MG1363 was used throughout [18]. It was grown in M17 broth (Difco, West Molesey, Surrey, U.K.) supplemented with 0.5% (w/v) glucose. DNA was purified according to standard procedures [19]. Routine DNA manipulations were

Abbreviations used: PPP, pentose phosphate pathway; 6PG, 6-phosphogluconate; 6-PGDH, 6-phosphogluconate dehydrogenase; ORF, open reading frame.

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performed in *Escherichia coli* strain JM109 and overexpression in *E. coli* BL21(DE3). Complementation experiments were performed in *E. coli* strain DF1070 [20] (*tonA22 ompF627 edd⁻¹ gnd⁻¹ relA1 SpoT1 pit⁻¹⁰ T₃R*), which was obtained from Dr. B. Bachmann (Yale University, New Haven, CT, U.S.A.). *E. coli* strain GB23152 [21] [W3110 *trpR lacZ(Am) trp* A9605 Kdg Rc Δ (*edd-zwf*)] was a kind gift from Dr. R. Wolf, Jr. (University of Maryland, Baltimore, MD, U.S.A.). *E. coli* DF1070 and GB23152 strains were maintained on minimal media containing 6% (w/v) K₂HPO₄, 0.2% (w/v) KH₂PO₄, 0.1% (w/v) (NH₄)₂SO₄, 0.1% (w/v) trisodium citrate, 0.01% (w/v) MgSO₄ (pH 7.2) plus 0.2% (w/v) glucose. Complementation experiments used the same minimal media, except that D-gluconate was used in place of D-glucose and ampicillin was added to a final concentration of 100 µg·ml⁻¹. All chemicals were of the highest grade available and from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.). Restriction enzymes and DNA modifying enzymes were from Promega (Southampton, Hants., U.K.).

Genomic library and DNA manipulations

A genomic DNA library from *L. lactis* strain MG1363 was produced by partial digestion (5 µg) of *L. lactis* DNA with restriction endonuclease *Sau3A*. DNA fragments were separated by agarose-gel electrophoresis and DNA in the size range 3–4 kb was isolated. The fragments were cloned into the *Bam*HI site of pUC18, and subsequently transformed into *E. coli* JM109. Recombinant plasmids (10000) were obtained and the colonies pooled to produce an amplified library by preparing plasmid according to standard procedures [22].

DNA sequencing

Sequencing of double-stranded DNA was accomplished by the dideoxynucleotide chain-termination method as described in the T7 sequencing kit from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). DNA and amino acid sequences were analysed with the DNA Strider program and multiple alignment was performed with the Pileup program from the GCG Wisconsin Sequence Analysis Software Package [23].

Expression of the *L. lactis* 6-PGDH in *E. coli*

E. coli strain BL21(DE3) expressing the *L. lactis* 6-PGDH gene were obtained by transformation with the gene cloned in the expression vector pET-3a or pET-16b, which adds an N-terminal His10 tag to facilitate purification (Novagen, Cambridge, U.K.). The gene was obtained by PCR amplification of the 6-PGDH open reading frame (ORF) in plasmid pC7 using oligonucleotide LL5', 5'-GGAGGATTTTCATATGGCACAAGCA-3', for the sense strand. This also contained an *Nde*I cloning site (underlined) incorporating an initiation codon (ATG). For the antisense strand, oligonucleotide LL3', 5'-CCCTGAGGATCCTTAAT-CTTCTGT-3', was designed. This oligonucleotide includes a stop codon with a *Bam*HI restriction site (underlined) just downstream. The PCR product was blunt-end ligated into the *Sma*I site of pUC18 (part of the SureClone kit from Amersham Pharmacia Biotech). The insert was removed from this subclone by digestion with *Nde*I-*Bam*HI and ligated into the pET-3a or pET-16b vectors digested by *Nde*I-*Bam*HI, creating plasmids pLL6-PGDH and pHisLL6-PGDH, respectively.

Site-directed mutagenesis

The oligonucleotides used for mutagenesis to modify arginine residues (Arg-34 and Arg-447) of the *L. lactis* 6-PGDH are

Table 1 Oligonucleotides used for site-directed mutagenesis of the *L. lactis* 6-PGDH genes

The mutagenesis sequences are shown in bold.

Position	Mutation	Oligonucleotide sequence (5' → 3')
Arg-34	Arg → Tyr	TTT TGA AGT CGT ATA GTT GTA GAT AGC
Arg-447	Arg → Lys	GAA ATA GTC TTT TTG AGC TTG
Arg-447	Arg → Asp	GAA ATA GTC GTC TTG AGC TTG
Arg-447	Arg → Ala	GAA ATA GTC AGC TTG AGC TTG
Arg-447	Arg → Trp	GAA ATA GTC CCA TTG AGC TTG

shown in Table 1. Mutagenesis was performed by the method described by Deng and Nickoloff [24] using the Chameleon Kit (Stratagene, Heidelberg, Germany) and the plasmid pHisLL6-PGDH as the matrix. All mutants were sequenced to confirm that the desired mutation was introduced and that other mutations had not arisen.

Purification of the recombinant *L. lactis* 6-PGDH

E. coli BL21(DE3) containing pLL6-PGDH were grown at 37 °C with vigorous aeration in Luria-Bertani-broth medium containing 100 µg·ml⁻¹ ampicillin. When cultures reached a *D*₆₀₀ of ≈ 0.6, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.4 mM to induce expression of the recombinant LL6-PGDH. Cultures were grown for an additional 3–4 h and then harvested by centrifugation. The pellet was resuspended in 20 ml of buffer containing 50 mM triethanolamine/HCl, 1 mM EDTA (pH 7.5). Cells were lysed by flash-freezing in liquid nitrogen. Cell debris was separated by centrifugation at 12000 g. The supernatant was applied to a 15 ml DEAE-Sephacel column equilibrated in 50 mM triethanolamine/HCl/1 mM EDTA (pH 7.5). The same buffer was flushed through the column until no protein eluted. The enzyme was then eluted in the same buffer plus 0.2 M NaCl, dialysed overnight against 5 mM triethanolamine with 0.1 mM EDTA (pH 7.5), and then concentrated using a centriflo membrane cone (Amicon-30). The extract was then loaded on to a column of 2',5'-ADP-Sephacel, which had been equilibrated with 5 mM triethanolamine/0.1 mM EDTA (pH 7.5). The enzyme with a specific activity of 35 units·mg⁻¹ was eluted with 1 mM NaCl/50 mM triethanolamine/HCl/0.5 mM EDTA. Purity was checked by SDS/PAGE. The protein was more than 95% pure.

Purification of recombinant His-tagged 6-PGDH by affinity chromatography

E. coli BL21(DE3)/pHisLL6-PGDH and all of the various mutant species which were present in the same vector were grown at 37 °C with vigorous aeration in Luria-Bertani-broth medium containing 100 µg·ml⁻¹ ampicillin. The enzyme was purified as above, with an additional chromatography step: Ni²⁺-resin column chromatography was performed according to the manufacturer's instructions (Novagen), prior to passing the enzyme over the 2',5'-ADP-Sephacel column. The R34Y mutant bound the 2',5'-ADP-Sephacel with markedly reduced affinity compared with the wild-type enzyme and was released using washing conditions similar to those applied to other enzymes. Consequently, this enzyme was assayed after the Ni²⁺-chromatography step. Yields of enzyme were determined by the absorbance at *A*₂₈₀ after each step. Yield and purity were confirmed by Coomassie Brilliant Blue staining of SDS/PAGE gels. Although variability in different batch preparations was evident, yield and

purity for all of the mutants were similar to the wild-type enzyme.

Enzyme assays

All assays were performed at 20–25 °C, measuring the initial rate in the direction of oxidative decarboxylation of 6PG. To calculate the specific activity, NADPH production was recorded spectrophotometrically at 340 nm. The buffer used was 120 mM triethanolamine (pH 7.5) with 0.1 mM EDTA and the concentrations of 6PG and NADP were saturating (1.4 mM and 0.5 mM, respectively).

The kinetic parameters of wild-type enzyme were determined by varying the concentration of each substrate (in the range 2–35 μ M for NADP; 10–150 μ M for 6PG) for five fixed concentrations of the other. Measurements were performed fluorometrically (excitation light, 340 nm; emission light, 450 nm) with a Perkin-Elmer MPF spectrophotometer. Arbitrary units of fluorescence intensity were converted to molar amounts of NADPH, calibrating the instrument with solutions of known NADPH concentration. Concentrations of 6PG and NADP were determined enzymically and assays initiated by the addition of enzyme. Values were then calculated from Lineweaver–Burk plots with the respective slope and intercept replots. The slopes of the lines were drawn as the best visual fit to the experimental points. The agreement with calculations by non-linear regression using the Graft software was within the standard error, which was less than 10 % of the calculated value. For the active R34Y mutant, kinetics were performed spectrophotometrically, measuring production of NADPH or NADH at 340 nm, varying the concentration of either coenzyme (up to 50 mM) with a fixed concentration of 6PG (1 mM), and the values determined using non-linear regression.

RESULTS

Identification of the *L. lactis* 6-PGDH gene by complementation in *E. coli*

E. coli strain DF1070 was transformed with a *Sau*3A size-selected genomic DNA library of *L. lactis*. From 5×10^5 plated transfectants 56 colonies were found growing on gluconate minimal medium 3 days after transformation. Plasmid DNA purified from one of these colonies, designated pC7, was transformed into the *E. coli* deletion mutant GB23152 and the complementing phenotype was verified. The specific activity of the *L. lactis* 6-PGDH expressed in *E. coli* GB23152 was 0.18 units $\cdot$ mg of protein⁻¹.

The insert in plasmid pC7 was sequenced on both strands and three putative ORFs were found (Figure 1) of which one (ORF2, 1416 bp), translated to give high-level amino acid identity to 6-PGDH from other sources. The nucleotide sequence of the insert in pC7 was AT-rich, in accordance with other *L. lactis* DNA [18]. Amino acid sequences of various 6-PGDHs are aligned in Figure

2. Residues shown to be present in the pockets which bind substrate and coenzyme are shown in bold (Figure 2).

Kinetic analysis of the *L. lactis* 6-PGDH

In order to determine the nature of the cofactor used by the *L. lactis* 6-PGDH, an *L. lactis* MG1363 cell-free extract was produced and assayed for 6-PGDH activity using NAD or NADP as a cofactor. Assaying over 30 min, no activity could be detected using NAD at 1 mM, whereas a specific activity of 0.117 units $\cdot$ mg of cell protein⁻¹ could be detected using NADP as a cofactor, classifying this enzyme as NADP-dependent.

The *L. lactis* 6-PGDH gene was cloned into an expression vector (pET-3a) for overexpression in *E. coli* and purified to near homogeneity (Table 2). It is unlikely that this purification procedure would separate the endogenous *E. coli* enzyme. However, control experiments using the strain BL21(DE3) show that recombinant enzyme contributes more than 98 % of the total 6-PGDH activity; hence, kinetic measurements represent the *L. lactis* rather than endogenous *E. coli* enzyme. An optimum pH for activity was found to be between pH 7.5 and 8.3, with the enzyme activity dying at pH 4.5 as a lower limit and pH 10 as an upper limit (results not shown). From the secondary plots of Figures 3A (6PG) and 3B (NADP), kinetic parameters were calculated and compared with values determined by non-linear regression of the primary plots. The K_m for 6PG and NADP was 15.4 ± 1.4 and 1.9 ± 0.2 μ M respectively, with a V_{max} of 38.3 ± 3.5 μ mol $\cdot$ min⁻¹ mg⁻¹. Although ordered or random mechanisms can be distinguished only by inhibition patterns, the common intercept on the axis of secondary plots in both cases makes it probable that a rapid equilibrium mechanism operates for the *L. lactis* enzyme, as for other 6-PGDHs [2,3,9].

In order to generate enzyme with no contaminants, including the endogenous *E. coli* variant, an N-terminal histidine tag was added to the *L. lactis* 6-PGDH protein (HisLL6-PGDH). This allowed for rapid purification of enzyme, which retained the kinetic parameters of the unmodified enzyme (results not shown), and has been crystallized [17].

The role of the *L. lactis* 6-PGDH Arg-447 in binding substrate

Figure 4 shows the orientation of substrate within the substrate-binding pocket of the sheep liver enzyme, as determined by X-ray crystallography [6]. Arg-446, which is conserved in all species (Arg-447 for *L. lactis* 6-PGDH), is sufficiently close to make hydrogen-bond contacts with the 6-phosphate of substrate, and the structural data suggested that it probably plays a role in anchoring substrate while it is oxidatively decarboxylated to ribulose 5-phosphate. In order to test this, the same residue from *L. lactis* (Arg-447) was mutated to a lysine (conservation of charge but decreased size), alanine (loss of charge), aspartate (reversal of charge) or tryptophan (addition of aromatic group). Despite the fact that the mutated enzymes were all soluble and could be purified by Ni²⁺ chromatography, none had any activity whatsoever, even when adding substrate up to 1 mM and using large quantities of enzyme, confirming that this residue plays a critical role in activity.

Bacillus subtilis contains two 6-PGDH isoforms

Figure 5 shows residues comprising the coenzyme pocket of the sheep liver enzyme. The arginine residue (Arg-33) believed to contact the 2'-phosphate of NADP is conserved in all enzymes except that encoded by a gene from *Bacillus subtilis*, which sits within a gluconate-utilizing operon (Bs-gntz, Y33) [25]. Recently, the *B. subtilis* genome was sequenced in its entirety, and two additional putative 6-PGDH sequences were deposited in the

Figure 1 Restriction map of plasmid pC7

The different ORFs are represented by black boxes and numbered 1–3. ORF2 encodes the *L. lactis* 6-PGDH.

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Figure 3 Double reciprocal plot of the initial rate of reaction

(A) Fixed 6PG concentration and varying NADP concentration; (B) fixed NADP concentration and varying 6PG concentration. Reactions were followed fluorometrically, initiated by the addition of 0.023 μg of *L. lactis* enzyme in 1 ml of reaction mixture. Inset: secondary plots of intercepts and slopes of lines from the main plot, from which the maximal rate of reaction and the K_m of NADP or 6PG were determined.

A frameshift appears to exist in the deposited sequence of the second putative novel 6-PGDH (Bs-vqjI), since this gene appears to have neither initiator methionine nor homology to the N-terminus, including the coenzyme-binding pocket. However, insertion of two nucleotides towards the N-terminus (thus shifting the frame) in the region underlined in the sequence (Figure 2), generates a full-length, typical 6-PGDH, including a coenzyme-binding pocket which contains the arginine residue Arg-34 (Figure 2). This second gene (Bs-vqjI) is adjacent to that encoding the other PPP dehydrogenase, glucose- 6-phosphate dehydrogenase. Whereas individual activities of the two different isoforms encoded by these genes have not been measured in isolation, it seems likely that this bacterium contains two different 6-PGDHs. One of these has a typical NADP-binding pocket and

Figure 4 Substrate-binding site of 6-PGDH from sheep liver

All residues are numbered. Black ovals represent bound water molecules. Zigzag lines and dotted lines represent actual and potential hydrogen bonds respectively.

Figure 5 Coenzyme-binding site of 6-PGDH from sheep liver

Residues are numbered. Zigzag lines represent hydrogen bonds.

is involved in the PPP whereas the second has an atypical pocket and is involved in gluconate metabolism.

The role of the *L. lactis* 6-PGDH Arg-34 in binding coenzyme

The 2'-phosphate which distinguishes NADP⁺ from NAD⁺ is critical in the binding of the former cofactor to 6-PGDH [27]. In order to test the effect of altering the 2'-phosphate-binding arginine to a tyrosine residue, as found in the atypical *B. subtilis* enzyme (Bs-gntz), the arginine of the *L. lactis* enzyme (Arg-34)

was mutated to tyrosine. The enzyme lost much of its affinity for NADP (K_m of 1.4 ± 0.3 mM; 737-fold reduced affinity) when measured at pH 7.5. In order to determine if this mutation changed the cofactor of the enzyme, the activity was tested using NAD⁺. A K_m for NAD⁺ was not measured for either wild-type or mutant enzyme, since initial rates were linear for both enzymes using this dinucleotide, even at concentrations as high as 50 mM. The mutant enzyme had a measurable activity of 0.007 units $\cdot$ mg⁻¹ at 1 mM NAD⁺, whereas for the wild-type enzyme no activity could be detected at this level.

DISCUSSION

6-PGDH is the third enzyme of the PPP. The facts that the loss of this enzyme is toxic to eukaryotic cells, and that the enzyme from the protozoan pathogen *T. brucei* has diverged very significantly from its counterpart in other systems, have led to suggestions that it may represent a target for chemotherapy against African trypanosomiasis [28]. Kinetic and chemical mechanisms for its action have been proposed [2–5] and X-ray crystal structures are known for both the sheep liver [6] and trypanosome [7] enzymes; however, a more detailed understanding of the structure and function of this enzyme from other sources will facilitate the design of specific inhibitors which may have therapeutic potential.

In this study it has been shown that the Gram-positive homofermentative lactic acid bacterium *L. lactis* possesses a typical NADP⁺-dependent version of the enzyme related to other 6-PGDHs, for which the primary amino acid sequence is known.

Detailed kinetic analyses for the 6-PGDHs from *T. brucei* and sheep liver have previously been performed [9]. The K_m for 6PG for these enzymes was similar to the *L. lactis* enzyme, and all residues involved in the substrate-binding pocket are conserved in the three species. Lys-183 has been proposed as an enzymic general base which accepts the 3'-OH of substrate [3,6]. The substrate-binding site, however, consists of not only residues which take part directly in catalysis, but also residues which orient the substrate while catalysis proceeds. The crystal structure of the sheep liver enzyme bound to substrate resolved, at 2.3 Å, showed that Arg-446 was crucial in binding to the 6-phosphate of substrate. This arginine is conserved in the enzyme from all species and mutagenesis of the cognate residue in the *L. lactis* enzyme (Arg-447) abolished activity, supporting a role for this entirely conserved arginine in binding 6PG. The fact that conservation of charge was not sufficient to retain activity if the side chain was reduced in length (arginine to lysine) also points to the necessity of orienting the substrate correctly with respect to other residues in the pocket involved in binding and catalytic activity.

Alteration of another arginine residue, proposed to play a role in binding to the 2'-phosphate of the coenzyme from structural analysis, also had a pronounced effect on activity. Mutating Arg-34 to a tyrosine, as found in an atypical sequence from *B. subtilis* (Bs-gntz), yielded an enzyme with nearly 1000-fold reduced affinity for NADP⁺. These data, together with the observation that *B. subtilis* possesses a second putative 6-PGDH, containing a typical coenzyme-pocket arginine, suggest that this organism contains two 6-PGDH isoforms. One is probably involved in the PPP (the gene is proximal to that encoding the other PPP enzyme glucose 6-phosphate dehydrogenase) and contains a typical NADP-binding pocket (Bs-vqjI), whereas the second probably encodes an enzyme with different coenzyme specificity acting as part of a gluconate-metabolizing pathway (Bs-gntz) [27].

Since several Gram-positive bacteria have been shown to contain NAD⁺-dependent 6-PGDH, a candidate cofactor for this isoform could be NAD⁺. The arginine residue believed to play a critical role in binding the 2'-phosphate of NADP⁺ is replaced by tyrosine in the atypical *B. subtilis* enzyme (Bs-gntz). Whereas alteration of the arginine in the *L. lactis* NADP⁺-specific enzyme did reduce affinity for NADP⁺, its increase in affinity for NAD⁺ was modest. This indicates some increased ability to use the non-phosphorylated dinucleotide, and that the arginine does play a critical role in binding the 2'-phosphate of NADP⁺. A tyrosine in place of arginine alone, however, is insufficient to produce an enzyme with high affinity for NAD⁺. Whether other differences which also distinguish the atypical *B. subtilis* enzyme might act along with the tyrosine to confer specificity for NAD⁺, or whether a different cofactor is involved in activity of this enzyme, remains to be investigated. Future experiments will focus on directly measuring the activities of the two *B. subtilis* enzymes and mutating other residues in the coenzyme-binding pockets of *L. lactis* 6-PGDH, which differ between the different enzymes.

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