The University of Oklahoma

6-Phosphogluconate Dehydrogenase

Term Paper

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Introduction

Pentose Phosphate Pathway

For reductive biosyntheses the living cell requires besides adenosine triphosphate (ATP) also a "reductive power" in form of either NADH or NADPH. Also both pyridine nucleotides are very similar (they differ only in one phosphate group), they are used for different purposes in the cell and are not interchangeable. NADH is utilized mainly in the generation of ATP, NADPH is used for other reductive biosyntheses, as for example fatty acid biosynthesis and steroid biosynthesis. To make this difference possible, there must be a high cofactor specificity for enzymes involved in generation and utilization of NADPH and NADH.

To favor both metabolite oxidation and reduction cells normally keep their $[NAD^+]/[NADH]$ and $[NADP^+]/[NADPH]$ ratios near 1000 and 0.01, respectively.

The pentose phosphate pathway is an important source of NADPH and R5P. It also provides the cell with ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids. It transforms glucose-6-phosphate (G6P) into fructose-6-phosphate (F6P) and glyceraldehydes-3-phosphate (GAP) under generation of 2x NADPH and CO_2. Its overall reaction is

$3\ G6P + 6\ NADP^+ + 3\ H_2O \rightleftharpoons 6\ NADPH + 6\ H^+ + 3\ CO_2 + 2\ F6P + GAP$

It consists of eight steps and can be divided into three stages:

1. Oxidative reactions. This stage consists of three steps, yielding two NADPH and ribulose-5-phosphate (Ru5P).

   $3\ G6P + 6\ NADP^+ + 3\ H_2O \rightarrow 6\ NADPH + 6H^+ + 3\ CO_2 + 3\ Ru5P$

2. Isomerization and epimerization reactions. The two steps involved in this stage transform Ru5P either to ribose-5-phosphat (R5P) or to xylulose-5-phosphate (Xu5P).

   $3\ Ru5P \rightleftharpoons R5P + 2\ Xu5P$

3. C-C bond reactions. In three steps fructose-6-phosphate (F6P) and glyceraldehydes-3-phosphate (GAP) are formed via several C-C bond cleavages and reformations.

   $R5P + 2\ Xu5P \rightleftharpoons 2\ F6P + GAP$

Stage 2 and 3 are also considered the non-oxidative branch and stage 1 the oxidative branch of the pentose phosphate pathway.

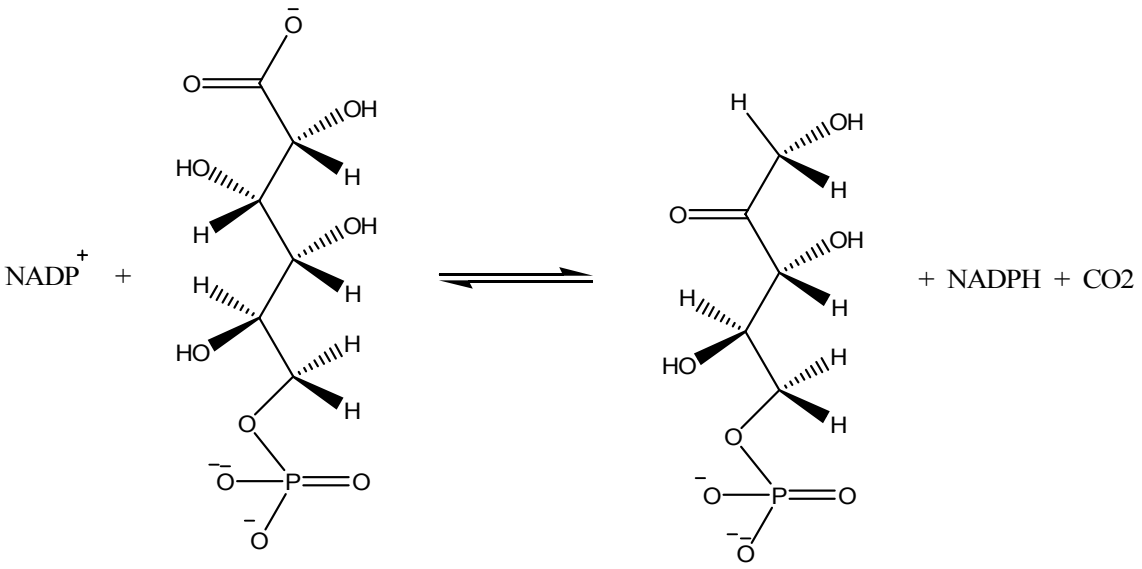
Stage 3 represents a possibility to convert excessive R5P to products used by other pathways and therefore forming inter-pathway branch points. Due to the reversible nature of stage 2 and 3 the main product of the pathway is R5P. (Voet and Voet, 1995)

6-Phosphogluconate dehydrogenase

6-Phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) is a homodimer with a 51,000Da molecular weight for each subunit in most species. Only in *Schizosaccharomyces pombe* a tetrameric 6PGDH with a 38,000Da subunit mass was found (Tsai and Chen, 1998). 6-phosphogluconate dehydrogenase is involved as the third step in the pentose phosphate pathway. There it catalyses the generation of ribulose-5-phosphat (Ru5P) and CO_2 from 6-phosphogluconate under reduction of $NADP^+$ to NADPH. The enzyme belongs to the class of pyridine nucleotide linked oxidative decarboxylases. This class of enzymes generally utilizes one of the pyridine nucleotides NAD^+ or $NADP^+$ as a cofactor to catalyze the oxidative decarboxylation of a β-hydroxyacid, yielding a ketone and CO_2. Most of the enzymes in this class, like for example malic enzyme (Hsu and Lardy, 1967), require the presence of a divalent metal ion in order to catalyze their specific reactions. It was proposed that the metal ion acts as a Lewis acid involved in the decarboxylation of the keto intermediate. 6PGDH therefore does not need any metal ion in its reaction, even if some 6-phosphogluconate dehydrogenases were observed to show dependence on metal ions either with inhibition or activation (Niehuas et al., 1996; Tsai and Chen,1998). Although studies have been carried out for 6-phosphogluconate dehydrogenases from several sources like sheep liver, *Candita utilis*, *Trypanosama brucei*, human erythrocyte, *Haemophilus influenzae*,

Crypthococcus neoformans, *Lactococcus lactis* and *Schizosaccharomyces pombe* (Zhang, 2000b; Price and Cook, 1996; Berdis and Cook,1993; Hanau et al.,1996; Dallocchio et al., 1985; Yoon et al., 1989; Niehuas et al., 1996; Tetaud et al., 1999; Tsai and Chen, 1998), this paper will mainly focus on the well studied 6PGDHs from sheep liver and *Candita utilis*, both metal ion independent.

The overall reaction catalyzed by 6-phosphogluconate dehydrogenase is shown below:



Purification of 6-phosphogluconate dehydrogenase

After several failed attempts to isolate cDNA encoding sheep liver 6PGDH it was finally obtained utilizing reverse-transcript PCR. It was cloned into pBluescript

and then subcloned into pKK223-3 and expressed in *E. coli*. Unfortunately the native *E. coli* 6-phosphogluconate dehydrogenase was also expressed. This was not a problem when the wild-type enzyme was expressed, but due to the high decrease in activity for most mutant enzymes, the *E. coli* 6PGDH was contaminating the protein when expressing mutants of the sheep liver 6PGDH. Due to the similar properties of both enzymes it was not possible to separate them. To perform site-directed mutagenesis studies another expression system was needed.

In the attempt to produce a glutathione-S-transferase fusion protein, the cDNA was subcloned into PGEX-4T-1. The fusion protein was purified using a glutathione-S-transferase affinity resin. This attempt failed due to the impossibility to cleave the fusion protein using thrombin protease. It was assumed that the cleavages site was inaccessible because of protein folding. Surprisingly the fusion protein still showed activity. This led to the assumption, that the N-terminus of 6-phosphogluconate dehydrogenase is not involved in enzyme activity. So a histidine-tag at this end of the protein should not interfere with the activity of the enzyme.

As the next possibly system the cDNA was cloned into pQE-30, adding an N-terminal 6xHis-tag to the protein sequence. Metal ions show a high affinity to this 6xHis- tag. By immobilizing metal ions on a resin the protein will be tightly bound

to it and all other contaminants can be washed of easily. Using a Ni-NTA column to purify the protein it now was possible to get pure sheep liver 6-phosphogluconate dehydrogenase and perform site directed mutagenesis experiments. To ensure this assumption was right, some mutations replacing the general base in the active site of sheep liver 6PGDH were expressed and characterized. The purified protein was inactive, showing no *E. coli* 6-phosphogluconate dehydrogenase was present (Chooback et al., 1998).

Kinetic Mechanism

Although there have been numerous kinetic studies for 6-phosphogluconate dehydrogenases from several sources there are very few systematic studies including initial velocity and isotope effects to determine the overall kinetic mechanism. This was done for the *Candida utilis* (Berdis and Cook, 1993a) and the sheep liver enzyme (Price and Cook, 1996).

Initial velocity studies performed for the *Candida utilis* 6-phosphogluconate dehydrogenase by varying NADP at fixed concentrations suggest a sequential kinetic mechanism.

Inhibition studies showed that both E:NADP:(ribuloses-5-phosphate) and E:NADPH:(6-phospho gluconate) are dead-end complexes. Also product

inhibition patterns for NADPH vs. 6PG, Ru5P vs. NADP and 6PG vs. NADPH are noncompetitive leading to the assumption that product binds to either the free enzyme or the enzyme:substrate complex, indicating a random mechanism. Isotope effects indicate that the chemical step in the reaction is rate limiting.

Considering all obtained data, a rapid equilibrium random mechanism was suggested.

The complete kinetic characterization for the sheep liver enzyme are almost identical to those obtained for the *Candida utilis* form of 6PGDH. Isotope effects showed that the hydride transfer seams partially rate limiting.

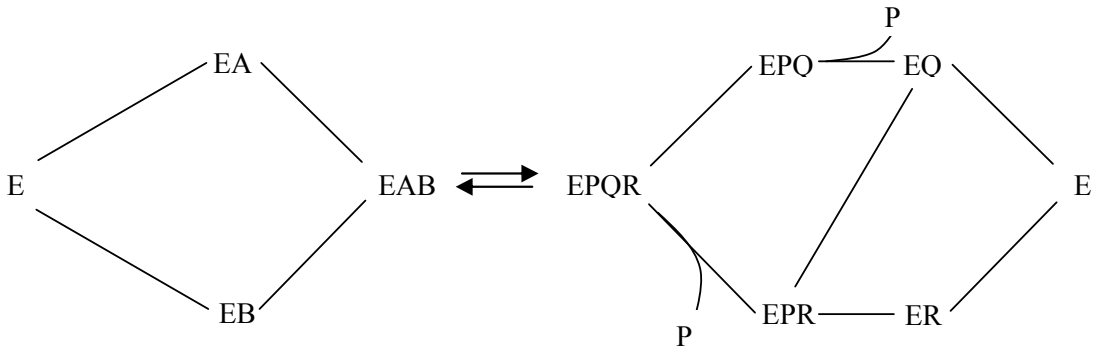


Figure 2: A: NADP, B: 6PG, P: CO_2, Q: Ru5P, R: NADPH

Structure

Recent X-ray crystallography studies solved the structure of 6-phosphogluconate dehydrogenase as the apo-enzyme as well as for the binary enzyme:substrate complexes.

As mentioned before, most 6-phosphogluconate dehydrogenases are homodimers with the exception of the *Schizosaccharomyces pombe* form. Each monomer was found to consist of three domains. Beginning from the N-terminus the first domain is a βαβ domain, spreading from residues 1 to 176. This folding is typical for dinucleotide binding proteins. The second domain is mainly helical (177-434), followed by a tail without obvious secondary structure at the C-terminus (435-482). This tail contributes to the active site of the opposite subunit, reaching through its helical domain.

The structures of the enzyme:substrate complexes suggest several possible residues involved in the proposed chemical mechanism. The complex E:6PG helps to determine residues involved in binding of 6PG as well as in the chemical reaction. There are two candidates for the general base. Lys 183 and Asn 187 are both in hydrogen bonding distance from the 3-hydroxyl group of 6PG. Because Asn 187 is not ionizable, the properly positioned Lys 183 is the best candidate for the general base. For the general acid Glu 190 is a good candidate, although Adams proposed a water molecule bound to Gly 130 to play that role. (Zheng, 2000b; Adams et. al., 1994)

Chemical Mechanism

Due to the difference between 6-phosphogluconate dehydrogenase and other oxidative decarboxylates in the lack of a divalent metal ion, there must be a difference in the chemical mechanism, too.

In a first assumption, Topham and Dalziel (1986) tested the possible reaction involing a Schiff-base intermediate. Therefore they monitored the reductive carboxylation using [2-^{18}O]ribulose-5-phophate as substrate. Finding a complete retention of the isotope indicated that there is no Schiff-base intermediate involved in the reaction. If there was a Schiff-base intermediate a solvent exchange at the C-2 oxygen would have been observed.

If the alternate substrate 2-deoxy-6-phosphogluconate is used for the reaction, it is firs oxidized in the presents of $NADP^+$. The product, 3-keto-2-deoxy-6-phosphogluconate is released and concentrated in the solution, where it will be decarboxylated to 1-deoxyribulose-5-phosphate by the same enzyme when the concentration of 6PG is low. Using this intermediate as substrate yields either 2-deoxy-6-phosphogluconate or 1-deoxyribulose-5-phosphate, which indicates a two-step mechanism consisting of first the oxidation followed by the decarboxylation (Rippa et al., 1973).

Therefore a general acid – general base mechanism was proposed (see figure 2 below). In this mechanism the 3-hydroxyl group of 6PG is deprotonated by a

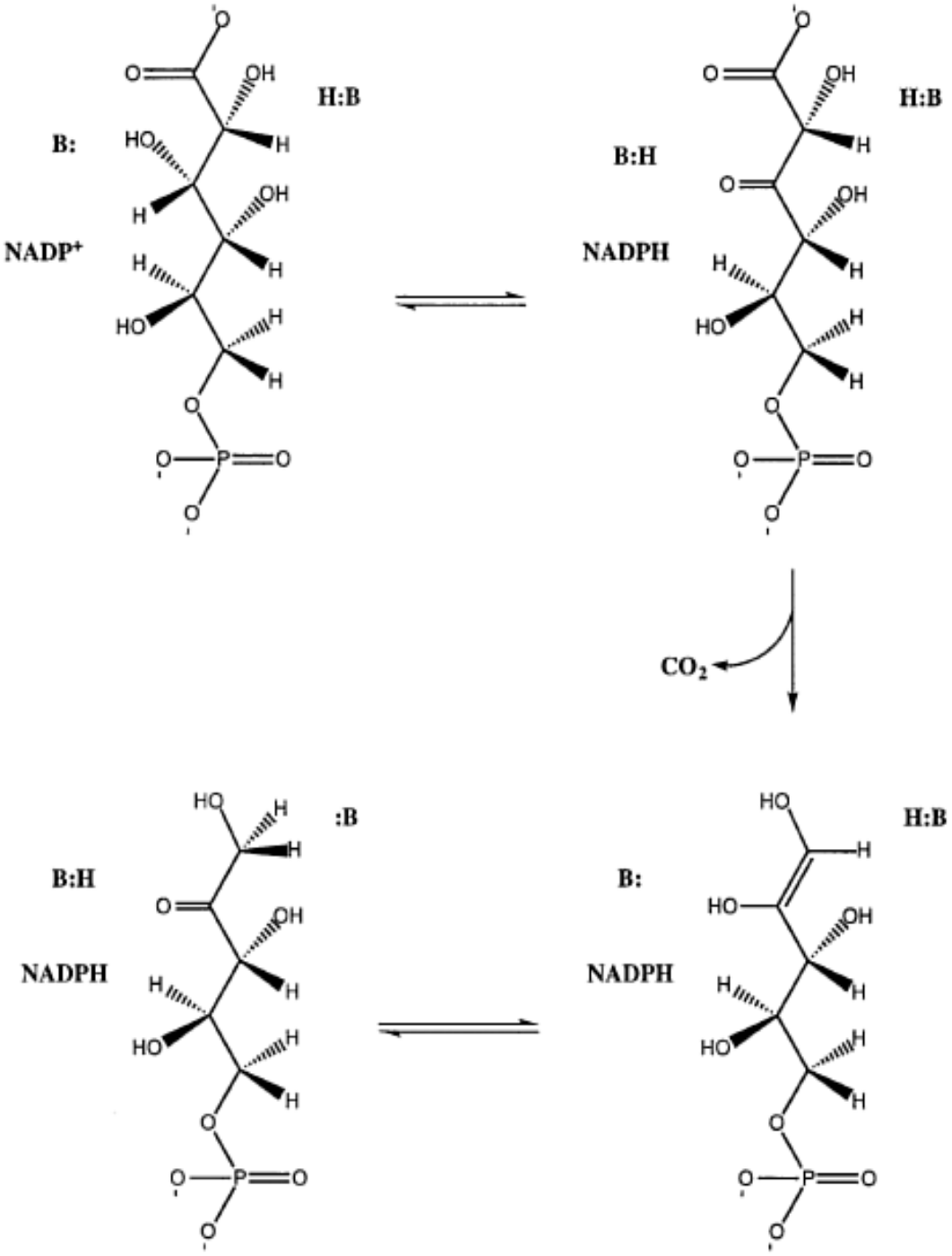


Figure 2: Proposed General Acid – General Base Mechanism

general base while the C-3 H is accepted by the $NADP^+$ giving a 3-keto intermediate as the first step. In the next step the H from the general base is transferred back to the keto group yielding a enediol during decarboxylation. The resulting CO_2 is released and the general acid facilitates the tautomerisation of the enediol, protonating the general base again.

To prove this proposal pH studies for *Candida utilis* and sheep liver 6PGDH were performed (Berdis and Cook, 1993b; Price and Cook, 1996). The optained pH rate profiles for the sheep liver enzyme are consistent with a general acid – general base mechanism, where the catalytic residues are also involved in substrate binding. The steep pH dependence for high and low pH of V_{max} also suggest that both residues must be in reversed protonation state in order to obtain maximum rates. In the binary complex E:6PG the pks of the general base and genral acid decrease/increase from 7.4-7.8 to 6.4-6.8 and from 6.2-6.9 to 8.6-8.7, respectively, fortifying the proposed mechanism (Price and Cook, 1996).

The V/K_{6PG} pH rate gives pk values of 5.6 and 8 for the general acid and general base, respectively. If the above proposed residues are correct, Lys 183 has to have a pK ~8 and Glu 190 a pK ~5.6. Since the pK for glutamate in solution is ~4.5 and the pK for lysine is ~10, the values determined seam low and high. An explanation for that is for the difference is the hydrophobic environment at the active side,

favoring the neutral forms of the amino acid residues with the observed pK values (Zhang and Cook, 2000a).

To prove this assumptions sit directed mutagenesis was performed by Zhang et. al. (1999) to change K183 to A, E, H, C, Q, R and M. The only mutant showing significant activity was the K183R enzyme. It pH dependence was characterized and a decrease in activity of 3 orders of magnitude compared to the wt enzyme was found. This proves the role of Lys 183 as a general base in the overall mechanism.

The same studies were performed for the assumed general acid, Glu 190. Site-directed mutagenesis was performed to change E190 to A, D, H, K, Q and R. Due to its role only in the last step, the tautomerization, no such drastic effect as for the general base can be expected. For the mutants removing a titratable residue from postion 190, like E190Q, E190K, E190A and E190R, there was no pH dependence on the acidic side observed anymore, resulting in a significant decrease in activity. Only E190D and E190H act in a manner similar to the wt enzyme, but also showing a significant decrease in activity. As a result, Glu 190 is most likely the general acid for the overall 6-phosphogluconate dehydrogenase reaction (Karsten et. al., 1998).

An alignment of 6-phosphogluconate dehydrogenase encoding sequences in different species also shows, that K183 and E190 are totally conserved through all

known species. This is another strong hint that the above assumptions are correct (Zhang et.al., 1999).

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