RESEARCH ARTICLE

Crystal structure of the 6-phosphogluconate dehydrogenase from *Gluconobacter oxydans* reveals tetrameric 6PGDHs as the crucial intermediate in the evolution of structure and cofactor preference in the 6PGDH family [version 1; peer review: 1 approved, 1 approved with reservations]

Pablo Maturana ¹, Eduardo Tobar-Calfucoy ¹, Matías Fuentealba ^{1,2}, Pietro Roversi ³, Richard Garratt⁴, Ricardo Cabrera¹

¹Laboratorio de Bioquímica y Biología Molecular, Departamento de Biología, Universidad de Chile, Santiago, Chile

²Institute of Healthy Aging, Department of Genetics, Evolution and Environment, University College London, London, England, UK

³Leicester Institute of Chemical and Structural Biology and Department of Molecular and Cell Biology, University of Leicester, Leicester, Henry Wellcome Building, England, UK

⁴Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, São Paulo, Brazil

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Abstract

Background: The enzyme 6-phosphogluconate dehydrogenase (6PGDH) is the central enzyme of the oxidative pentose phosphate pathway. Members of the 6PGDH family belong to different classes: either homodimeric enzymes assembled from long-chain subunits or homotetrameric ones assembled from short-chain subunits. Dimeric 6PGDHs bear an internal duplication absent in tetrameric 6PGDHs and distant homologues of the β -hydroxyacid dehydrogenase (β HADH) superfamily.

Methods: We use X-ray crystallography to determine the structure of the apo form of the 6PGDH from *Gluconobacter oxydans* (Go6PGDH). We carried out a structural and phylogenetic analysis of short and long-chain 6PGDHs. We put forward an evolutionary hypothesis explaining the differences seen in oligomeric state vs. dinucleotide preference of the 6PGDH family. We determined the cofactor preference of Go6PGDH at different 6-phosphogluconate concentrations, characterizing the wild-type enzyme and three-point mutants of residues in the cofactor binding site of Go6PGDH.

Results: The structural comparison suggests that the 6PG binding site initially evolved by exchanging C-terminal α -helices between subunits. An internal duplication event changed the quaternary structure of the enzyme from a tetrameric to a dimeric arrangement. The phylogenetic analysis suggests that 6PGDHs have spread from Bacteria to Archaea

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1. **José Gregório C. Gómez** , University of São Paulo, São Paulo, Brazil

2. **Paul C. Engel**, University College Dublin, Dublin, Ireland

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and Eukarya on multiple occasions by lateral gene transfer. Sequence motifs consistent with NAD⁺- and NADP⁺-specificity are found in the β 2- α 2 loop of dimeric and tetrameric 6PGDHs. Site-directed mutagenesis of Go6PGDH inspired by this analysis fully reverses dinucleotide preference. One of the mutants we engineered has the highest efficiency and specificity for NAD⁺ so far described for a 6PGDH.

Conclusions: The family 6PGDH comprises dimeric and tetrameric members whose active sites are conformed by a C-terminal α -helix contributed from adjacent subunits. Dimeric 6PGDHs have evolved from the duplication-fusion of the tetrameric C-terminal domain before independent transitions of cofactor specificity. Changes in the conserved β 2- α 2 loop are crucial to modulate the cofactor specificity in Go6PGDH.

Keywords

6PGDH, 6-phosphogluconate dehydrogenase, enzyme superfamily evolution, cofactor specificity engineering, oxidative pentose phosphate pathway, X-ray crystallography

Corresponding authors: Pablo Maturana (pmaturana.v@gmail.com), Ricardo Cabrera (ricabrer@uchile.cl)

Author roles: **Maturana P:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Tobar-Calfucoy E:** Formal Analysis, Investigation, Methodology; **Fuentealba M:** Formal Analysis, Investigation, Methodology; **Roversi P:** Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Writing – Review & Editing; **Garratt R:** Formal Analysis, Funding Acquisition, Validation, Visualization, Writing – Review & Editing; **Cabrera R:** Conceptualization, Formal Analysis, Funding Acquisition, Project Administration, Resources, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing

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Introduction

6-phosphogluconate dehydrogenases (6PGDHs) (EC 1.1.1.44) catalyse the oxidative decarboxylation of the β -hydroxyacid 6-phosphogluconate (6PG) to yield ribulose 5-phosphate¹. 6PGDHs have been characterized from bacterial, archaeal and eukaryal species. They are either homodimeric enzymes made of long-chain subunits (about 52 kDa) or homotetrameric enzymes made of short-chain subunits (about 35 kDa) - although a recent report proposed a dimeric oligomeric state for a short-chain 6PGDH². Pickl and Schonheit were the first to reveal that long- and short-chain 6PGDHs form separated clusters (subfamilies) in a phylogenetic tree³. In spite of the current available structural knowledge of short- and long-chain 6PGDHs and other homologous β -hydroxyacid dehydrogenases, the structural changes associated with the evolution of the 6PG-binding site have not been taken into consideration in order to understand the constraints substrate binding imposed on 6PGDH sequence length and oligomeric state. Moreover, evolution of cofactor specificity in 6PGDHs remains relatively understudied. To address these questions, we provide here a structural evolutionary framework that considers 6PGDH structures jointly with their phylogeny - both at the family and superfamily levels- and looks at the phylogenetic distribution of the 6PGDH residues mediating cofactor binding.

Short-chain 6PGDHs are reminiscent of their most distant relatives in the β -hydroxyacid dehydrogenases (β -HADHs) superfamily⁴, tetrameric enzymes that carry out the oxidation of a hydroxyl group in the β -position to an acidic moiety in substrates such as 3-hydroxy-isobutyrate (3HIBA) and glycerate⁵, serine⁶, 3-hydroxy-propionate⁷, tartronate⁸ and

2-hydroxy-propyl-phosphonate⁹. However, compared to β -HADHs, which generally show some substrate promiscuity^{5,7}, reports on 6PGDHs do not support oxidation of β -hydroxyacids other than 6PG.

β -HADHs generally assemble as a dimer of dimers. The tertiary structure of the archetypical β -HADH subunit is composed of two domains: an N-terminal Rossmann fold domain (with additional structural elements) that harbours the dinucleotide cofactor binding site (either NAD⁺ or NADP⁺); and a C-terminal helical domain that harbours the β -hydroxyacid (β -HA) substrate site and provides oligomerization interfaces¹⁰. In the case of dimeric, long-chain 6PGDHs enzymes, the N-terminal domain is followed by a tandem array of two helical domains¹¹ (named hemi-domains hereafter), mediating the formation of the dimer. In these 6PGDH dimers the interface between two hemi-domains within a monomer is equivalent to the interface between two β -HADH monomers in a β -HADH dimer. Likewise, the interface that holds two monomers together in a long-chain 6PGDH dimer is akin to that between dimers in the β -HADH dimer of dimers (Figure 1A and B)^{10,12,13}. These observations originated the hypothesis that the structure of one long-chain 6PGDH subunit probably evolved from a β -HADH precursor by gene duplication and fusion, followed by deletion of the N-terminal domain¹⁰.

We posit that inclusion of short-chain 6PGDHs in the phylogeny of this hypothetical evolutionary pathway can reveal a connection between the structural changes underpinning 6PG binding and the duplication of the helical domain concurrent to the change in 6PGDH oligomerization state.

Figure 1. Structural correlations among the β HADH superfamily. (A) Structural superposition (center) of quaternary structure of the tetrameric human 3HIBADH (PDBid: 2I9P, left) and the dimeric *Ec*6PGDH (PDBid: 3FWN, right). (B) Structural correlation between the monomeric form of *Ec*6PGDH and the obligate dimer of the human 3HIBADH. The N-terminal domain and repeated C-terminal hemi-domains of a monomer *Ec*6PGDH (green) align with two packeted hemi-domains from the obligate dimer of the tetramer 3HIBADH (subunits in red and blue). (C) Structure of the dimeric *Ec*6PGDH, one subunit is represented in cartoon and another subunit in surface. Both subunits contribute the active site through the exchange of the last C-terminal alpha helix.

The structural determinants of specificity for substrate and dinucleotides in 6PGDHs have been studied mainly using long-chain enzymes. When it comes to the 6PG binding site, a crystal structure of the sheep and *E. coli* dimeric 6PGDH in complex with 6PG shows that the phosphate moiety and the nearby hydroxyl groups of 6PG in one subunit are contacted by residues on the C-terminal α -helix, belonging to the other subunit in the dimer (Figure 1C)^{11,14}. Hawes *et al.* in 1996 pointed out that the presence of this α -helix distinguishes 6PGDHs from 3HIBA dehydrogenases¹⁵. Recent crystal structures of long-chain dimeric 6PGDHs complexes with 6PG^{16–18} confirm strong structural conservation of the enzyme: 6PG interactions in the binding site across the superfamily.

With regard to the dinucleotide cofactor binding site, sequence motifs consistent with NAD⁺- and NADP⁺-specificity are found in the N-terminal domain β 2- α 2 loop of both dimeric and tetrameric 6PGDHs. Crystal structures of long-chain 6PGDHs uncovered in the β 2- α 2 loop an NRX₃K motif which provides favorable interactions with NADP⁺, thereby explaining the preference for this particular dinucleotide cofactor^{17,19,20}. In particular, the Asn and Arg residues at the beginning of the NRX₃K motif bind the 2'-phosphate group of the dinucleotide with H-bonds and a salt bridge, and the guanidinium moiety of the same arginine residue also interacts with the adenine ring of the cofactor through π -stacking. Residues in the NRX₃K motif determine the cofactor preference of 6PGDHs²¹. For example, the NR motif found in NADP⁺-dependent 6PGDHs changes to D[I/L/R] in the sequences of 6PGDHs that can use NAD⁺ as the cofactor²². In particular, the sequences of 6PGDHs from acetic acid bacteria show the DR motif, which correlates with the utilization of both cofactors². Among them, the 6PGDH from *Gluconobacter oxidans* (Go6PGDH) compared to that of *Gluconoacetobacter diazotrophicus* (Gd6PGDH) possesses higher specificity constants for both cofactors and thus it might serve as a useful model to study the residues that determine the dinucleotide specificity of short-chain 6PGDHs.

Phylogenetic studies of 6PGDHs have revealed Lateral Gene Transfer (LGT) events and suggested a correlation between cofactor preference and subunit size. So far, dimeric 6PGDHs have been characterized from eukaryotes and eubacteria only. Krepinsky *et al.*²³ showed that animal and fungal cytosolic 6PGDH sequences cluster with their orthologues from γ -proteobacteria. However, these authors speculated that once α -proteobacteria sequences become available, their 6PGDH sequences would be expected (as mitochondrial ancestors) to be even closer to eukaryotic ones than those of γ -proteobacteria. On the other hand, tetrameric 6PGDHs have been found only in prokaryotes^{24–26}. Pickl and Schönheit³ found a phylogenetic cluster of haloarchaeal 6PGDH sequences flanked by bacterial sequences and concluded they were likely the result of an LGT event. They also noted that, while the kinetically characterized tetrameric 6PGDHs were NAD⁺-dependent, the dimeric ones were NADP⁺-dependent. A more recent study also recognized that sequence patterns in the nucleotide binding site of short-chain 6PGDHs suggest that they would be NAD⁺-dependent². However, a tetrameric 6PGDH from a thermophilic bacterium has been reported whose catalytic efficiency with NADP⁺ was

far higher than with NAD⁺²⁷. Therefore, at the family level, it is important to clarify the main phylogenetic relationships and the evolution of cofactor specificity of 6PGDHs.

In this work, we describe the crystallographic structure of the tetrameric Go6PGDH and use it to study the structural evolution of the β -HADHs fold. We find that the active site remodeling required to establish the 6PG binding site implies that short-chain 6PGDHs must conserve a tetrameric structure for full activity. Our rooted phylogenetic tree of the 6PGDH family indicates that while long-chain 6PGDHs have been transferred from Bacteria to Eukarya, short-chain 6PGDHs were transferred from Bacteria to Archaea, and provide evidence that NAD⁺ and NADP⁺-dependent groups could be found in both short and long-chain subfamilies. Finally, enzyme kinetics of Go6PGDH and site-directed mutagenesis of the conserved DR motif allowed us to obtain a mutant with the highest activity and selectivity towards NAD⁺ so far described for 6PGDHs.

Methods

Molecular cloning and site-directed mutagenesis

The gene *gox1705* from *Gluconobacter oxidans* present in the plasmid pASK-IBA3plus (plasmid pASK-IBA3plus-*gox1705* was granted by Uwe Deppenmeier from the Institute für Mikrobiologie und Biotechnologie, Bonn University, Germany in 2012²⁸), was subcloned into the pET-TEV-28a plasmid. The pET-TEV plasmid was derived from pET28-a (Millipore Sigma #69864-3) by insertion of the TEV cleavage site and removing the thrombin cleavage site and T7 tag. This manipulation was important to avoid the presence of a streptavidin tag fusion in the C-terminal region of the expressed protein in the pASK-IBA3plus plasmid, because of its role in 6PG binding. The pET-TEV plasmid was a kind donation of Dr. Adriana Krapp to R. Cabrera (research stay 2010 at the Laboratory of Dr. Néstor Carrillo, Instituto de Biología Molecular y Celular de Rosario, Argentina). Primers were designed to introduce a site for NdeI in frame with the start codon of *gox1705* (Forward primer: CATATGCGGATCGGAAT-CATCGGACTG), and a site for XhoI downstream of its stop codon (Reverse primer: CTCGAGTAATTTTCTTTTC-GACGTGTCCGCCG). The PCR product and pET-TEV plasmid were digested with one enzymatic unit (U) of NdeI (ThermoScientific #FD0583) and XhoI (ThermoScientific # FD0694) in FastDigest® buffer (ThermoFisher) at 37 °C for one hour and ligated with 5 U of T4 DNA Ligase (ThermoScientific #EL0011) at 20°C overnight. Colonies were evaluated for protein expression. The resulting plasmid (named pET-TEV-Go6PGDH) expressed the full Go6PGDH coding sequence preceded by 21 amino acids, including a 6xHis tag at the N-terminal end and a cleavage site for the TEV protease before the first Met of Go6PGDH. After treatment with TEV protease, a dipeptide (Gly-His) remains at the N-terminus of Go6PGDH immediately preceding its first Met. Site-directed mutagenesis was performed by PCR amplification of pET-TEV-Go6PGDH, using mutagenic primers, according to the Genetaylor™ site-directed mutagenesis system (Invitrogen #A13282). To ensure that no spurious mutations had been introduced, the entire ORF was subsequently sequenced (Macrogen USA).

Expression and protein purification

Go6PGDH and *Ec6PGDH* were expressed in *E. coli* strain BL21(DE3) as described by Olavarria *et al.*²⁹. Briefly, cells of *E. coli* strain BL21(DE3) carrying the pET-TEV-28a-*Go6PGDH* wild-type, site-directed mutants or pET-TEV-28a-*Ec6PGDH* plasmids were inoculated in Luria-Bertani culture medium at 37 °C. When the attenuation reached 0.4–0.5 AU at 600 nm, over-expression was induced by adding 1 mM isopropyl- β -D-thiogalactoside (US Biological Swampscott, MA, USA). After 4 h of induction at 37 °C in the case of *Ec6PGDH* expression and 16 h at 28 °C in the case of *Go6PGDH* wild-type and mutants, biomass were collected by centrifugation (10 min, 4500 g, 4 °C in a Thermo Scientific™ Sorvall™ RC 6 Plus Centrifuge), and in each case, the cell extract was loaded onto a 5 ml HisTrap HP column (GE Healthcare, Little Chalfont, UK), equilibrated with the resuspension buffer: 50 mM Tris-HCl, 500 mM NaCl, 40 mM Imidazole, pH 8.0. For elution, a continuous flow of the resuspension buffer with a linear gradient of imidazole from 40 mM to 500 mM was used. The enzyme activity was measured using 0.4 mM NAD⁺ (Sigma-Aldrich # N1511) and 2 mM 6PG (Sigma-Aldrich # P7877) in the same resuspension buffer (without imidazole), and the Bradford assay was used to monitor the protein concentration. The fractions with activity were pooled. Overnight TEV protease digestion removed the 6xHis tail at room temperature in buffer Tris-HCl 50 mM, 1 mM TCEP (Sigma-Aldrich # 646547), 0.5 mM EDTA, pH 8.0 using a molar ratio protease:enzyme of 1:10. After protein digestion, the mixture was centrifuged to remove the aggregate and reloaded onto a HisTrap column to remove the TEV protease (which also contained a His tail). The eluted enzyme was dialyzed and concentrated in buffer, 50 mM Tris-HCl, 100 mM NaCl, pH 8.0. Finally, the pure protein solution was concentrated and supplemented with 50% glycerol and stored at -20°C. SDS-PAGE was used to follow all the purification steps.

Enzyme kinetics

Enzyme activity was determined at 25°C by following the formation of NAD(P)H at 340 nm using a UV/vis Synergy 2 spectrophotometer (BioTek Winooski, VT, United States). An extinction coefficient of 5186 AU·M⁻¹ for NAD(P)H was used, this value due to considering our enzymatic experiment conditions pH 8.0, 100 mM NaCl and 25 °C, as described by Olavarria *et al.*²⁹. The substrates were titrated, after been neutralized to pH 7.0, using the 6PGDH from *Gluconobacter oxydans* in the case of NAD⁺ and 6PG and the G6PDH from *Escherichia coli* in the case of NADP⁺.

Progress curve analysis for *Go6PGDH* was performed for both NAD⁺ and NADP⁺ under initial velocity conditions, using different concentrations of the co-substrate 6PG. Our measurements were made before 5% of the initial substrate was consumed. To determine the kinetic parameters for NADP⁺, we used a concentration range of the cofactor between 33 μ M and 20 mM at different fixed 6PG concentrations, ranging between 0.25 and 7 mM. To determine the kinetic parameters for NAD⁺, we used concentrations of the cofactor between 6 μ M to 5 mM at fixed 6PG concentrations between 0.065 and 4 mM. The reaction buffer contained Tris-HCl 50 mM, 10 mM MgCl₂ and 100 mM NaCl at pH 8.0. The enzyme concentrations varied

according to the substrate range used in the kinetic experiments. For low and high 6PG concentrations respectively, 20 nM and 5 nM of the enzyme was used. In the case of the mutants R31V, R31L and D33N, the kinetic parameters for NAD⁺ and NADP⁺ were determined using 2 mM of 6PG. The effect of the 3HIBA on the *Ec6PGDH* and *Go6PGDH* initial velocity was measured at saturating concentrations of substrates using NADP⁺ and NAD⁺ as cofactors, respectively. The 3HIBA was synthesized by basic hydrolysis of the ester Methyl (S)-(+)-3-hydroxy-2-methylpropionate in 5% of NaOH as excess. The product was frozen and lyophilised. The 3HIBADH salt was neutralised with HCl and purity corroborated by ¹H-NMR.

X-Ray Crystallography

After size-exclusion chromatography, *Go6PGDH* was concentrated to 15 mg/ml in buffer Tris-HCl 50 mM pH 8.0, 100 mM NaCl and 2 mM DTT. Sitting drop vapor diffusion experiments were performed by mixing 0.8 μ L protein solution and 0.8 μ L of crystallization condition and equilibrating against over 70 μ L mother solution at 18 °C. After optimization, the crystallization condition contained 2 M (NH₄)₂SO₄ and 5% (v/v) 2-propanol and crystals appeared after 48 hours. For cryoprotection the same solution was supplemented with 25% (v/v) glycerol and the sample cooled by plunging it into liquid nitrogen. Diffraction data were collected at the MX2 beamline at the UVS National Laboratory Light Synchrotron (Campinas, SP, Brazil)³⁰. Data were indexed and integrated with XDS software (<http://xds.mpimf-heidelberg.mpg.de/>, version March 15, 2019)³¹ and scaled and merged with Aimless³², included in the CCP4 suite version 7.1 (<https://www.ccp4.ac.uk/>)³³. Phasing was carried out by molecular replacement with MOLREP (included in CCP4 suite version 7.1)³⁴ using the homologous structure from *Geobacter metallireducens* (PDBid: 4E21). The model was iteratively refined using Coot version 0.8.9.3 (<https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/>)³⁵ and Phenix Refine from the PHENIX suite (version 1.18.2, <https://www.phenix-online.org/>)³⁶. The geometry and stereochemistry of the structure was validated with MolProbity (<http://molprobity.biochem.duke.edu/>)¹⁴. The structure refinement statistics are shown in Table 1.

Small Angle X-ray Scattering

Small Angle X-ray Scattering data were collected at the beamline SAXS2 at the National Synchrotron Light Laboratory (Campinas, SP, Brazil), using the photon-counting Pilatus detector. A wavelength $\lambda = 1.488$ Å was used with a sample-detector distance of 1000 mm. A sample of *Go6PGDH* 3.2 mg/ml in Tris-HCl 50 mM pH 8.0, 100 mM NaCl and 2 mM DTT was irradiated for 3 min at room temperature. The SAXS Data were analyzed with the software ATSAS 3.0.1.1³⁷. The scattering of the corresponding buffer was subtracted to that of the protein solution and the resulting curve was analyzed with PRIMUS. The Radius of gyration was determined using the Guinier approximation and the pair distance distribution function $p(r)$ was evaluated using the program GNOM. *Ab initio* shape determination was performed using DAMMIN without any symmetry restriction. The structural superposition of the crystallographic structure of *Go6PGDH* and the SAXS envelope was performed using SUPCOMB.

Table 1. Crystallography Statistic and refinement for apo Go6PGDH (6XEQ).
Statistics for the hisghest resolution shell are shown in parenthesis.

	6XEQ
X-Ray source	LNLS MX2, Brazil
Detector	PILATUS 2M
Wavelength	1.458660 Å
Resolution range Å	100.3 - 3.2 (3.37 - 3.2)
Space group	P 2 ₁ 2 ₁ 2 ₁
Unit cell	87.061Å 125.675Å 166.273Å 90° 90° 90°
Unique reflections	30273 (2601)
Completeness (%)	97.65 (85.54)
Mean I/sigma(I)	6.6 (2.3)
CC(1/2)	0.978 (0.757)
Wilson B-factor	38.3
R-merge	0.092 (0.327)
R-meas	0.130 (0.462)
R-pim	0.092 (0.327)
Reflections used in refinement	30098 (2596)
Reflections used for R-free	1495 (124)
R-work	0.246
R-free	0.286
Number of non-hydrogen atoms	9789
Number of macromolecules atoms	9634
Numbers of solvents atoms	155
Protein residues	1271
RMS(bonds)	0.004
RMS(angles)	0.64
Ramachandran favored (%)	98.09
Ramachandran allowed (%)	1.83
Ramachandran outliers (%)	0.08
Rotamer outliers (%)	0.00
Clashscore	7.62
Average B-factor	37.61
Average B-factor for macromolecules	37.76
Average B-factor for Solvent	28.05

Structural alignment and analysis

We searched in the Protein Data Bank for structures classified within the SCOPe family a.100.1.1, which contains 6PGDHs, 3HIBADHs and others β HADH. To include members not assigned to the SCOPe classification, we used the 3D similarity

function in the PDB, with a cut-off of 30% sequence identity³⁸. The structures found are listen in Table 2 and we also incorporated propanoate dehydrogenase from the related SCOPe family a.100.1 to serve as outgroup (PDBid: 3QSG and 1I36). After 3D superposition using MultiSeq³⁹, an evolutionary tree

Table 2. Structures identified as part of the β HADH superfamily. The bold PDB codes were used to inference the structure-based phylogenetic tree of Figure 5.

Reported Function in the PDB	PDB	Organism	Uniprot	Ligand
6-phosphogluconate dehydrogenase	6FQX	<i>Plasmodium falciparum</i>	Q8IKT2	
6-phosphogluconate dehydrogenase	6FQZ	<i>Plasmodium falciparum</i>	Q8IKT2	6PG
6-phosphogluconate dehydrogenase	6FQY	<i>Plasmodium falciparum</i>	Q8IKT2	NADP ⁺
6-phosphogluconate dehydrogenase	4GWK	<i>Homo sapiens</i>	P52209	3PG
6-phosphogluconate dehydrogenase	2JKV	<i>Homo sapiens</i>	P52209	NADP ⁺
6-phosphogluconate dehydrogenase	4GWG	<i>Homo sapiens</i>	P52209	
6-phosphogluconate dehydrogenase	5UQ9	<i>Homo sapiens</i>	P52209	8HS
6-phosphogluconate dehydrogenase	2ZYG	<i>Klebsiella pneumoniae</i>	C4XAX8	
6-phosphogluconate dehydrogenase	3FWN	<i>Escherichia coli</i>	P00350	ATR/6PG
6-phosphogluconate dehydrogenase	2ZYA	<i>Escherichia coli</i>	P00350	6PG
6-phosphogluconate dehydrogenase	2ZYD	<i>Escherichia coli</i>	P00350	Glucose
6-phosphogluconate dehydrogenase	1PGJ	<i>Trypanosoma brucei</i>	P31072	
6-phosphogluconate dehydrogenase	2IYO	<i>Lactococcus lactis</i>	P96789	6PG
6-phosphogluconate dehydrogenase	2IZ1	<i>Lactococcus lactis</i>	P96789	ATR
6-phosphogluconate dehydrogenase	2IZO	<i>Lactococcus lactis</i>	P96789	NADP ⁺
6-phosphogluconate dehydrogenase	2IYP	<i>Lactococcus lactis</i>	P96789	NADP/5RP
6-phosphogluconate dehydrogenase	2W90	<i>Geobacillus stearothermophilus</i>	I3NI58	6PG
6-phosphogluconate dehydrogenase	2W8Z	<i>Geobacillus stearothermophilus</i>	I3NI58	6PG
6-phosphogluconate dehydrogenase	2P4Q	<i>Saccharomyces cerevisiae</i>	P38720	Citrate
6-phosphogluconate dehydrogenase	1PGO	<i>Ovis aries</i>	P00349	NADPH
6-phosphogluconate dehydrogenase	1PGP	<i>Ovis aries</i>	P00349	6PG
6-phosphogluconate dehydrogenase	2PGD	<i>Ovis aries</i>	P00349	
6-phosphogluconate dehydrogenase	1PGQ	<i>Ovis aries</i>	P00349	
6-phosphogluconate dehydrogenase	1PGN	<i>Ovis aries</i>	P00349	POP/NBP
6-phosphogluconate dehydrogenase	4E21	<i>Geobacter metallireducens</i>	Q39SD5	
6-phosphogluconate dehydrogenase	6VPB	<i>Gluconacetobacter diazotrophicus</i>	A9H324	
6-phosphogluconate dehydrogenase	6XEQ	<i>Gluconobacter oxydans</i>	G5EBD7	
6-phosphogluconate dehydrogenase	4GBJ	<i>Dyadobacter fermentans</i>	C6VW20	
3-hydroxyisobutyrate dehydrogenase	3G0O	<i>Salmonella typhimurium</i>	Q8ZMG4	L(+)Tartaric acid
Succinic semialdehyde reductase	3PDU	<i>Geobacter sulfurreducens</i>	Q74DE4	NADP ⁺
3-Hydroxyisobutyrate dehydrogenase	2I9P	<i>Homo sapiens</i>	P31937	NAD
3-Hydroxyisobutyrate dehydrogenase	2CVZ	<i>Thermus thermophilus HB8</i>	Q5SLQ6	NADPH
3-Hydroxyisobutyrate dehydrogenase	1WP4	<i>Thermus thermophilus HB8</i>	Q5SLQ6	NADPH
3-Hydroxyisobutyrate dehydrogenase	5JE8	<i>Bacillus cereus</i>	Q81DR6	NAD ⁺
Tartronate semialdehyde reductase	1VPD	<i>Salmonella typhimurium</i>	Q8ZLV8	L(+)Tartaric acid
Hydroxymethylglutarate dehydrogenase	3CKY	<i>Eubacterium barkeri</i>	Q0QLF5	
L-Serine dehydrogenase	3OBB	<i>Pseudomonas aeruginosa</i>	Q9I5I6	
L-Serine dehydrogenase	3WS7	<i>Pyrobaculum calidifontis</i>	A3MU08	NADP ⁺
L-Serine dehydrogenase	5XVH	<i>Pyrobaculum calidifontis</i>	A3MU08	NADP ⁺ /L(+)Tartaric acid
2-hydroxypropylphosphonate reductase	5U5G	<i>Pseudomonas syringae</i>	I6R485	NADP ⁺

was built from atomic distances (RMSDs) and the fraction of equivalent residues from the superimposed tertiary structures, using the method of Johnson *et al.*⁴⁰. From the RMSD values obtained for each pair of structures in the prior structural alignment, a matrix of SRMS was built:

$$SRSM = 1 - RMSD / 3.5 \quad (1)$$

For that, the number of topological equivalences for two structures was determined, given by the number of overlays per residue whose homologous positions along the structural alignment had an RMSD value of less than 3.5 Å. In this way the fractional topological equivalence by pairs (PFTE) was calculated, dividing the number of topological equivalences by the length of the minor structure compared. Considering these two values, the structural evolutive distance (D) was calculated by the following equation:

$$D = -100 \ln(w_1 PFTE + w_2 SRM) \quad (2)$$

where $w_1 + w_2 = 1$ and these values are calculated as follows:

$$w_1 = (1 - PFTE) + (1 - SRMS) / 2 \quad (3)$$

$$w_2 = (PFTE + SRMS) / 2 \quad (4)$$

Finally, with the D values represented in a distance matrix obtained for each pair of structures, a dendrogram was represented using the neighbor-joining method available in the T-rex web server⁴¹.

For the analysis of quaternary structure in short-chain 6PGDHs we used the Protein interfaces, surfaces and assemblies' service PISA at the European Bioinformatics Institute: <https://www.ebi.ac.uk/pdbe/pisa/>⁴².

Multiple sequences alignment and phylogenetic analysis

We first searched the TrEMBL (UniProtKB) database for *Ec*6PGDH and *Go*6PGDH homologs that have been kinetically characterized using NAD⁺ or NADP⁺ as the cofactor⁴³. Then, we performed an exhaustive search of sequences with one or two hemi-domains that possess the 6PGDH catalytic motif (identified in the superfamily structural tree) but containing different motifs in the β 2- α 2 loop. We retrieved a total of 83 6PGDH sequences. To align these sequences using ClustalX⁴⁴, we created a structural profile based on an alignment of 6PGDHs from *E. coli* and *G. metallireducens*, (PDB identifiers 3FWN and 4E21 respectively). Using this structural profile, 10 sequences with one or two hemi-domains in the C-terminal region were aligned, generating a second profile. The remaining sequences were aligned against the second profile, using the BLOSUM62 substitution matrix and iterating each step of the alignment. Misaligned positions were manually corrected.

The phylogenetic tree of the aligned 6PGDH sequences was inferred using MrBayes version 3.1.2⁴⁵, using 3HIBADHs as the outgroup. We used WAG as the evolutionary model of amino acid replacement for globular proteins⁴⁶, and a

gamma-shaped rate variation across sites was assumed with a proportion of invariable sites. The number of generations was adjusted to 10 million trees with a sampling frequency every 1000 generations and 20% of generated trees burned. The average standard deviation of split frequencies was less than 0.01 upon convergence.

Statistical analysis

The statistical analysis was performed for the enzyme kinetic experiments using the GraphPad Prism version 8 (<https://www.graphpad.com/>). To the different enzymatic progress curves, a Michaelis-Menten model was fitted to the data. To the tertiary graphs (kinetic parameters versus substrate concentration), a hyperbolic model was fitted to the k_{cat} dependence with 6PG concentration. A model of hyperbolic decay with an intersection in the y-axis was fitted to the K_M behaviour with 6PG concentration. We use a confidence level of 95% computed with an asymmetrical model (profile-likelihood) for all the cases. The GraphPad Prism software provided the statistical analysis.

Results

The crystal structure of *Go*6PGDH: features of a tetrameric assembly

The asymmetric unit of the crystal contains four monomers of *Go*6PGDH. The overall fold of each shows an elongated monomer, as illustrated in Figure 2A. Three regions can be distinguished: The N-terminal domain, which adopts the Rossmann-fold, followed by an extension made of a three-stranded β sheet topped by three helices (we name this extension the $3\beta 3\alpha$ unit). The central part of the protein folds as a helical domain, with a long helix ($\alpha 9$) surrounded by pairs of shorter β -helices, two of them ($\alpha 10$ and $\alpha 11$) distal to the N-terminal domain and two of them ($\alpha 12$ and $\alpha 13$) proximal to it. Distal and proximal α -helices are connected by a connecting loop (residues 265-276) that is disordered in the crystal. The third region of the *Go*6PGDH monomer is the C-terminal α -helix ($\alpha 14$), protruding from the body of the helical domain. The *Go*6PGDH tetramer assembles as a dimer of dimers, in which all four subunits contact each other, with the largest interface formed by the helical domains within the dimer (Figure 2B). In this interface, two $\alpha 9$ helices contact each other in a nearly antiparallel configuration, with the loops between the $\alpha 10$ and $\alpha 11$ proximal helices are flanking each other. Within the dimer, the $\alpha 10$ and $\alpha 11$ pair of distal helices of one monomer slot in a cavity on the surface of the $3\beta 3\alpha$ unit of the other monomer (Figure 2A). The active sites of the two subunits in the dimer face outwards. Two of these elongated dimers interact through a smaller interface in which the pairs of proximal helices $\alpha 12$ and $\alpha 13$ form an anti-parallel 2+2 helix-bundle and the C-terminal helix $\alpha 14$ reaches across the $\alpha 11$ and $\alpha 12$ helices of a third monomer (Figure 2C). According to SAXS data, a tetrameric arrangement is present in solution as well (Figure 3). The scattering curve and distance distribution function (Figure 3A and Figure 3B) revealed a radius of gyration and maximum distance distribution of 3.8 and 11.9 nm, respectively. The superposition of our X-ray structure and the *ab initio* model built from the $P(r)$ distribution curve shows that the dimer-dimer interface seen in the crystal is preserved in solution (Figure 3C).

The first structure of the tetrameric 6PGDH to be reported in the PDB was the 6PGDH from *Geobacter metallireducens* (*Gm*6PGDH, PDB: 4E21, rmsdC_α = over 1.41 C_{αs} compared with *Go*6PGDH), which we used as the search model for Molecular Replacement. Although 4E21 shows a dimer in the asymmetric unit, a tetramer equivalent to our *Go*6PGDH can be generated by crystallographic symmetry. Furthermore, while we were preparing this report, the X-ray structure of *Gd*6PGDH was disclosed (PDBid, rmsdC_α = 1.02 compared with *Go*6PGDH) also showing a dimer in the asymmetric unit. Likewise, a tetramer that conserves the same features described for *Go*6PGDH can be generated by applying a crystallographic two-fold. In fact, analysis by PISA indicated a buried surface area compatible with tetrameric association for all three short-chain 6PGDHs (Figure 3D). However, *Gd*6PGDH was reported to behave as a dimer in solution, according to native gel electrophoresis². Structural superposition of *Gm*6PGDH and

*Go*6PGDH (Figure 4A, left) shows slight changes in the relative orientation of the N-terminal domain respect to the helical domain, and of the C-terminal α -helix with respect to the latter.

Superfamily-level relationships: architectural changes of 6PGDHs

We compared the structures of the tetrameric enzymes *Gm*6PGDH, *Go*6PGDH and *Hs*3HIBADH and the dimeric *Ec*6PGDH (Figure 4). In the N-terminal domain, we found differences that affect subunit interactions. In the $3\beta 3\alpha$ unit, the loop formed by the residues 160 to 182 of *Go*6PGDH is absent in *Hs*3HIBADH and shorter in *Ec*6PGDH (Figure 4A and Figure 4C). This loop covers most of the surface of the distal helices from the partner subunit within the dimer. On the other hand, the loop $\beta 2-\alpha 2$ is a short connector whose sequence features are related to cofactor specificity (see below). The alignment (Figure 4C) showed that it harbors an NR motif in

Figure 2. Tertiary and quaternary structure of *Go*6PGDH. (A) the monomeric structure. The N-terminal domain is colored violet and the $3\beta 3\alpha$ unit is purple. The helical domain is colored light gray and the C-terminal α -helix in yellow; (B) Two monomers packed through the more extended interface; (C) The tetrameric structure. The second dimer is represented as surface.

Figure 3. SAXS structure of the apo GoPGDH and quaternary structures of short-chain 6PGDHs. (A) SAXS data of apo Go6PGDH. The inset shows the linear fitting of q values $< 1.3/R_g$ according to the Guinier approximation. (B) Interatomic distance distribution function $p(r)$ of Go6PGDH. (C) Different side views of the *Ab initio* shape model of Go6PGDH superimposed to the crystal structure of Go6PGDH. (D) (Above) Tetrameric structure of Go6PGDH, Gm6PGDH and Gd6PGDH. For each protein dimers are represented in cartoon and surface. (Below) Surface, buried area and ΔG for interaction and dissociation for dimer and tetramer for each protein, determined by PISA are shown.

the case of the NADP⁺-dependent *Ec*6PGDH, a DV motif in the NAD⁺-dependent *Hs*3HIBADH, and the mixed sequence DR, in *Go*6PGDH.

However, the main architectural differences are found in the helical domain and are associated with a remodeling of the active site. In the case of *Hs*3HIBADH, the C-terminal α -helix packs against the long helix (structurally equivalent to *Go*6PGDH $\alpha 9$), while the segment connecting distal and proximal helices forms part of the substrate binding site (Figure 4D). In the case of the dimeric *Ec*6PGDH, the C-terminal α -helix of hemidomain 1 moves away from the long helix allowing the linkage with hemidomain 2, while the segment connecting distal and proximal helices is displaced away from

the active site permitting the insertion of the C-terminal α -helix to the neighboring subunit (Figure 4E). In hemidomain 2, which does not contribute to the active site, the C-terminal α -helix is swung away by a further 65° with respect to the position seen in hemidomain 1, enabling the orientation required for insertion into the neighboring subunit, while the segment connecting distal and proximal helices is packed against the long helix (Figure 4F). Comparing *Go*6PGDH to *Ec*6PGDH, while the C-terminal α -helix superposes well with that from hemidomain 2, the segment connecting distal and proximal helices takes on a role equivalent to the one seen in hemidomain 1 (Figure 4B and Figure 4G). Comparing *Go*6PGDH to *Hs*3HIBADH, it is noteworthy that the C-terminal α -helix in the former occupies a location in the neighboring subunit equivalent to that

Figure 4. Topology, structural superposition and sequence alignment of tetrameric and dimeric 6PGDHs. (A) Monomers superposition of *Go6PGDH* (orange) with *Gm6PGDH* (left) and *Ec6PGDH* (right). The last two structures are colored following the segmentation depicted in Figure 2A (hemidomain 2 of *Ec6PGDH* is represented transparent). (B) Topology diagrams of *Go6PGDH* (left) and *Ec6PGDH* (right), colored following the segmentation depicted in Figure 2A. Note that the first strand in the $\beta_3\alpha_3$ unit results from the elongation of the 6th canonical Rossmann strand. (C) The loop encircled shows variable length when comparing different. Identical residues are colored in red. Blue dotted rectangles denote the β_1 - α_1 and β_2 - α_2 loops that interact with the adenine-ribose region of the cofactor. Blue and red rectangles indicate the zones of the interaction with the rest of the cofactor and 6PG, respectively. In a black rectangle is showing the catalytic motif. The secondary structure of *Go6PGDH* and *Ec6PGDH* is shown as a reference. Note that the helical domains of the tetrameric representatives aligned with *Ec6PGDH* hemidomain 1, except for the C-terminal α -helix, which aligned with that of hemidomain 2. (D) *Hs3HIBADH*. (E) Hemi-domain 1 (F) 2 from *Ec6PGDH* and (G) *Go6PGDH*. In each structure, the segment connecting distal and proximal helices and the C-terminal α -helix are labeled and colored to highlight their different orientations. Note that the penetrating α -helix (yellow) occupy an equivalent location as the C-terminal α -helix of *Hs3HIBADH* but in the opposite orientation.

of the C-terminal α -helix in the helical domain of the latter. However, the C-terminal α -helices of these proteins adopt opposite orientations to compound the tertiary structure, and thus it cannot be considered a true case of a minimalist domain swapping. These results reveal that the tetrameric structure of *Go6PGDH* is indispensable for full interactions at the 6PG binding site.

In order to have a wider landscape of the structural similarity at the superfamily level, we generated a phylogenetic tree based on the structural superposition of the monomeric unit of superfamily members with available crystal structures (Table 2). As shown in Figure 5, dimeric and tetrameric 6PGDHs constitute a clade, having the same catalytic motif KMXHNXXE (Figure 5B)⁴⁷, while the rest of β HADHs form a paraphyletic

Figure 5. Structure-based phylogenetic tree of the β HADH superfamily. (A) Left, neighbor-joining dendrogram based on RMSD and residue conservation after 3D alignment of selected PDBs⁴⁰, listed in Table 2. Abbreviations: L-SDH (L-serine dehydrogenase), TSR (tartronate semialdehyde reductase), SSR (succinic semialdehyde reductase), HMGDH (hydroxymethylglutarate dehydrogenase), 2OPPR (2-hydroxypropylphosphate reductase), OXR (putative oxidoreductase). Branches of the tree colored according to dimeric 6PGDHs (green), tetrameric 6PGDHs (orange) and other β HADHs (red). Middle, correlated sequence alignment obtained from the structural superposition used to build the tree of Figure 5. Orange and green branch possess the conserved motif KMXHNGXE. Right, simplified schematization of the three oligomerization kinds observed in the superfamily. Domains are represented by squares (N-terminal domain) and semicircles (helical domain). The yellow arrows represent the C-terminal α -helix that is exchanged between neighboring active sites. Entire subunits are colored in orange and dark gray. (B) 3D view of the segment of the α -helix bearing the catalytic motif in the PDB structures 2I9P, 6XEQ and 3FWN. Residues involved in the interaction with 6PG of *Ec*6PGDH, and the homologous residues in the rest of the structures are shown.

group. The exchange of the C-terminal α -helix between subunits is the synapomorphic feature that distinguished 6PGDHs, while the tetrameric state with no structural elements shared between subunits is the symplesiomorphic trait shown by the rest of β HADHs. Thus, the short-chain 6PGDHs can be viewed as intermediate in the former evolutionary pathway proposed by Andreeva and Murzin 2006, from β HADHs towards dimeric 6PGDHs (Figure 5A, schematic representation)¹⁰.

The hypothesis that tetrameric 6PGDHs were intermediate in the evolutionary pathway towards dimeric 6PGDHs would imply increasing substrate selectivity at play in the β HA binding site. To test this idea, we evaluated the activity of *Go* and *Ec*6PGDH against the substrate 3HIBA⁴⁸. Neither enzyme is active against 3HIBA, but *Go*6PGDH and not *Ec*6PGDH is inhibited by it, as illustrated in Figure 6. Although the effect occurs in a concentration range that is not physiological, it suggests that the 6PG binding site is more promiscuous in tetrameric 6PGDHs than in the dimeric ones.

Family-level relationships: phylogeny and signatures of dinucleotide specificity

A phylogenetic reconstruction was generated by using a broad sample of sequences from tetrameric and dimeric 6PGDHs from a diverse range of eukaryotic, bacterial and archaeal lineages. Short-chain and long-chain 6PGDHs form basally separated clusters. In the branch of dimeric 6PGDHs (Figure 7, green

Figure 6. Effect of the 3HIBA on the activity of *Go*6PGDH and *Ec*6PGDH. Data is available in 48.

subdivision) we found mainly bacteria and eukaryotes from different phyla. Among the bacterial lineages three different clades stand out: b_1 , cluster mainly made of firmicutes; b_2 , cluster containing mainly cyanobacteria; and b_3 , α -proteobacteria. Among the eukaryotic representatives, we also found three monophyletic groups: e_1 , opisthokonta: fungi (ascomycetes and basidiomycetes) and animals; e_2 , cluster mainly made of plants; and e_3 , a second ascomycetes group plus euglenozoa. The clades b_1 and e_1 constitute a bigger group, named *FO*

Figure 7. Bayesian phylogenetic tree of the 6PGDH family. The root was set by using 3HIBADHs sequences as the outgroup. The reported oligomeric state is consistent with the most basal division in tetrameric and dimeric branches. The sequences in the tetrameric branch have an average extension of 339 amino acids (indicating a single hemi-domain), and in the dimeric branch the average is 472 (indicating a duplicated hemi-domain). Nodes are labelled with the posterior probability whenever this value was lower than 1. Clades labeled by arrows are defined in Results. At the left side of the species name, white circles indicate that cofactor specificity has been reported, and black circles that the structure is known. At the right side, sequence logos are used to show the sequence conservation of the $\beta 2$ - $\alpha 2$ loop (actual sequences of these segments are available in Zenodo depository⁴³). The sequence logo starts from the aminoacid 29 to 35, considering the position in Go6PGDH. Note that in each subfamily, a monophyletic group showing a changed pattern was found.

(firmicutes + opisthokonta). Likewise, the b_2 and e_2 clades compose the group *CP* (cyanobacteria + plant). Remarkably, although the α -proteobacteria clade is a sister group to the superclade *FO* + *CP*, there is a eukaryotic clade lying closer to the root, namely e_3 . Meanwhile, in the branch of tetrameric 6PGDHs (Figure 7, orange subdivision) we found sequences from bacteria and archaea. Among the bacterial representatives, three groups were the most clearly differentiated: a cluster mainly made of α -proteobacteria, a cluster mainly made of chloroflexi, and a cluster mainly made of firmicutes (lying closer to the root). Although several archaeal sequences from different phyla were interspersed in different branches, only one group congregated a coherent lineage: the order Halobacteriales.

In the branch of dimeric 6PGDHs (Figure 7, green subdivision), reports on kinetic characterization and crystal structures were found mostly for representatives of the *FO* clade. For the *CP* clade, kinetic information has been reported for several members, but a crystal structure was available only for *Plasmodium* 6PGDH (a close relative of cluster e_2). However, for α -proteobacteria

and the ascomycota from clade e_3 , neither kinetic nor crystal structures reports were found. Considering cofactor specificity, the dimeric 6PGDHs have been mostly characterized for the use of NADP⁺, showing K_M values between 2 and 258 μ M (Table 3). In the cases of NAD⁺ characterization the K_M values varied between about 4 and 9 mM, indicating that they have preference or selectivity towards NADP⁺. This trend is consistent with the presence of a strongly conserved NRX₃K motif in the β_2 - α_2 loop of most dimeric 6PGDHs (Figure 7). Interesting exceptions were found, especially in the case of the ascomycota 6PGDHs from clade e_3 , which possessed a DVX₃N motif⁴³. The change of NR motif to DV suggested a preference for NAD⁺.

Among the tetrameric 6PGDHs (Figure 7, orange subdivision), information on kinetics and structure is more limited. Enzymes from firmicutes, proteobacteria and archaea have been kinetically characterized, showing that in general, NAD⁺ is the preferred cofactor (Table 3). Interestingly, only the α -proteobacteria clade, to which *Go*6PGDH belong, showed a conserved DR

Table 3. Reported kinetic parameters of members of the 6-phosphogluconate dehydrogenase family. *: Specific activity is reported.

Protein	NADP ⁺		NAD ⁺		NAD ⁺ /NADP ⁺	
	K _M (μM)	k _{cat} (s ⁻¹)	K _M (μM)	k _{cat} (s ⁻¹)	[k _{cat} /K _M] ^{NAD⁺} /[k _{cat} /K _M] ^{NADP⁺}	Reference
Dimeric 6PGDH						
<i>Enterococcus faecalis</i>	15.0					49
<i>Thermotoga maritima</i>	5.0	17.6	4,300	23.3	6.7 × 10 ⁻⁴	22
<i>Trypanosoma cruzi</i>	5.9	98.0				50
<i>Trypanosoma brucei</i>	1.5	27.0				50
<i>Ovis aries</i>	2.0	9.3				19
<i>Geobacillus stearothermophilus</i>	3.4	22.1				51
<i>Klebsiella pneumoniae</i>	118.0	13.0 U/mg*				17
<i>Escherichia coli</i>	49.0	21.0				17
<i>Homo sapiens</i>	6.9	21.4				18
<i>Plasmodium falciparum</i>	9.0	7.6				18
<i>Synechocystis</i> PCC 6803	58.0	51.0	4,460	4.89	1.3 × 10 ⁻³	52
<i>Arthrospira platensis</i>	19.0	16.6				52
<i>Spinacia oleracea</i> chloroplastic	6.0					23
<i>Saccharomyces cerevisiae</i>	35.0					53
<i>Ricinus communis</i> (plastidial)	5.4					54
<i>Ricinus comunis</i> (cytosolic)	4.1					54
<i>Brevibacterium flavum</i>	36.0					55
<i>Rattus norvegicus</i>	258.0	10.8 U/mg*				56
<i>Lactococcus lactis</i>	1.9	38.3 U/mg*				21

Protein	NADP ⁺		NAD ⁺		NAD ⁺ /NADP ⁺	Reference
	$K_M(\mu\text{M})$	$k_{cat}(\text{s}^{-1})$	$K_M(\mu\text{M})$	$k_{cat}(\text{s}^{-1})$	$[k_{cat}/K_M]^{\text{NAD}^+}/[k_{cat}/K_M]^{\text{NADP}^+}$	
<i>Dicentrarchus labrax</i>	0.9					57
<i>Ctenopharyngodon idella</i>	5.2					58
<i>Catharanthus roseus</i> (cytosolic)	8.0					59
<i>Catharanthus roseus</i> (plastidial)	11.0					59
<i>Candida utilis</i>	8	11				60
<i>Pisum sativum</i>	11.0					59
<i>Pisum sativum</i> (plastidial)	23.0					59
<i>Arthrobacter globiformis</i>	35.0					
<i>Phormidium</i> sp.	13.0					61
<i>Synechococcus</i> 6307	25.0					62
<i>Synechococcus</i> 6716	10.0					62
<i>Candida utilis</i> type I	20.0					63
<i>Candida utilis</i> type II	20.0					63
<i>Methylophilus methylotrophus</i>	25.0	5 U/mg*	9,050	2.5 U/mg*		25
Tetrameric 6PGDH						
<i>Bradyrhizobium</i> sp.				31 U/mg*		64
<i>Gluconobacter oxydans</i>	440	41.5	64	41.7	6.9	28
<i>Haloferax volcanii</i>	2900	0.42 U/mg*	33	10 U/mg*		3
<i>Leuconostoc lactis</i>			320			65
<i>Methylophilus methylotrophus</i>			105	2.72 U/mg		25
<i>Moorella thermoacetica</i>	13.9	8.7	1397		1.4x10 ⁻²	27
<i>Pseudomonas fluorescens</i>	23 U/mg*		121 U/mg*			66
Uncharacterized oligomeric state						
<i>Pseudomonas multivorans</i> I	20					24
<i>Pseudomonas multivorans</i> II	20		200			24

motif in the $\beta 2$ - $\alpha 2$ loop⁴³. In the rest of sequences, the $\beta 2$ - $\alpha 2$ loop showed moderate conservation of D and non-polar residues are slightly more frequent than R in the next position. Remarkably, the chloroflexi group did show the NR motif, suggesting preference for NADP⁺, although it was not possible to find reports of the enzymatic characterization for any representative in this clade.

Determinants of dinucleotide specificity in Go6PGDH

Given that Go6PGDH can use both NADP⁺ and NAD⁺ cofactors, we evaluated the effect of saturation of the 6PG binding site on the dinucleotide preference. We determined

the kinetic parameters for the cofactors at a variety of 6PG concentrations, ranging from 0.25 to 7.2 mM (in the case of NADP⁺) and 0.1 to 4 mM (in the case of NAD⁺). A simple Michaelis-Menten model can be fitted to the data. Tertiary plots (Figure 8A and Figure 8B) showed that, for both dinucleotides the apparent k_{cat} increases hyperbolically with the 6PG concentration, while the apparent K_M decreases hyperbolically. At the asymptotes, we obtained the real kinetic parameters, for NAD⁺ the k_{cat} and K_M was $47.4 \pm 1.2 \text{ s}^{-1}$ and $20.1 \pm 3.2 \mu\text{M}$, respectively. For NADP⁺ instead, the k_{cat} and K_M was $41.4 \pm 2.8 \text{ s}^{-1}$ and $285 \pm 25 \mu\text{M}$, respectively (Figure 8A and 8B, Table 4). The resulting real specificity constants at 6PG saturation

Figure 8. Effect of the 6PG concentration on the apparent kinetic parameters and consequence of mutations of conserved residues of the dinucleotide binding site of *Go6PGDH*. Kinetic parameter versus dinucleotide concentration (**A**, **B**). The best values for k_{cat} (white triangles) and K_M (black circles) were obtained from hyperbolic fitting of individual curves of V_0 versus the NAD^+ (**A**) and $NADP^+$ (**B**) concentration. Symbols indicate the average of three independent trials conducted at each 6PG concentration and the bars show the standard deviation. (**C**) Effect of 6PG concentration on the cofactor preference, as estimated from the quotient of specificity constants for NAD^+ over $NADP^+$. A hyperbolic decay model was fitted to this behavior. From **D** to **F**, NAD^+ -dependent activity (black circles) and NAD^+ -dependent activity (white symbols) of (**A**) *Go6PGDH^{NR}*, (**B**) *Go6PGDH^{DV}* and (**C**) *Go6PGDH^{DL}* variants were measured at a saturating concentration of 6PG. In all cases x-axis is shown as log-scale and the Michaelis-Menten equation was fitted to the data.

Table 4. Kinetic parameters of wild type *Go6PGDH* and mutants of the $\beta 2$ - $\alpha 2$ loop. Estimates were obtained using either $NADP^+$ or NAD^+ as a substrate. The saturation curves of the mutant enzymes were carried out with 8 mM 6PG. Values correspond to the mean $\pm$ standard deviation of three independent replicates.

Protein	$K_M^{NAD^+}$ (μM)	$k_{cat}^{NAD^+}$ (s ⁻¹)	$[k_{cat}/K_M]^{NAD^+}$ (s ⁻¹ /μM)	$K_M^{NADP^+}$ (μM)	$k_{cat}^{NADP^+}$ (s ⁻¹)	$[k_{cat}/K_M]^{NADP^+}$ (s ⁻¹ /μM)	$[k_{cat}/K_M]^{NAD^+}/[k_{cat}/K_M]^{NADP^+}$
<i>Go6PGDH</i>	20.1 $\pm$ 3.2	47.4 $\pm$ 1.2	2.4	285 $\pm$ 25	41.4 $\pm$ 2.8	0.14	16
<i>Go6PGDH^{DV}</i>	58 $\pm$ 7	42.5 $\pm$ 3	0.73	37160 $\pm$ 6700	42.2 $\pm$ 4.1	1.14 $\times$ 10 ⁻³	640
<i>Go6PGDH^{DL}</i>	59 $\pm$ 5	44.5 $\pm$ 2.6	0.75	10134 $\pm$ 3200	4.7 $\pm$ 0.8	4.64 $\times$ 10 ⁻⁴	1617
<i>Go6PGDH^{NR}</i>	7503 $\pm$ 860	18.5 $\pm$ 1.5	2.4 $\times$ 10 ⁻³	62.2 $\pm$ 7.1	48 $\pm$ 3	0.77	3.31 $\times$ 10 ⁻³

(Table 4), are 0.14 and 2.4 s⁻¹μM⁻¹ for $NADP^+$ and NAD^+ , respectively. More importantly, the dependence of the specificity constant k_{cat}^{app}/K_M^{app} with 6PG saturation differed for the two cofactors: at low 6PG concentrations the preference for NAD^+ was more pronounced, reaching a value of 200 at 0.45 mM 6PG and decreasing to 20.5 at 4 mM (Figure 8C).

For the dinucleotide binding site, we focused on the DR motif of the $\beta 2$ - $\alpha 2$ loop. These residues were replaced, in the case of D30 for N (*Go6PGDH^{NR}*), and in the case of R31 for V or L (*Go6PGDH^{DV}* and *Go6PGDH^{DL}*, respectively). We determined the kinetic parameters for NAD^+ and $NADP^+$ at 8 mM 6PG, a saturating concentration for the wild-type enzyme. The

mutant *Go6PGDH^{NR}* showed a striking reversal of the cofactor preference (Figure 8D). The K_M for NAD^+ increased more than 370 times over wild-type and the k_{cat} decreased almost 3 times. For NADP^+ , the K_M decreased almost 5 times and the k_{cat} slightly increased with respect to wild type. The ratio of specificity constants changed from 16 favoring NAD^+ to 302 favoring NADP^+ (Table 4). On the other hand, R31 mutants showed a notable increase in the preference for NAD^+ compared to wild type: for the mutant *Go6PGDH^{PV}* the K_M for NADP^+ increased 130 times (Figure 8E) while the k_{cat} remained unchanged (Table 4), and the K_M for NAD^+ increased almost 3 times while the k_{cat} remained unchanged. Hence, the ratio of the specificity constant for NAD^+ over NADP^+ showed an enzyme 640-fold more specific for NAD^+ upon R31V mutation. Likewise, the mutant *Go6PGDH^{PL}* (Figure 8F) exhibits similar kinetic constants for NAD^+ as *Go6PGDH^{PV}*, but for NADP^+ the K_M was more than 10 mM and the k_{cat} was 4.7 s^{-1} (Table 4), leading to a ratio of specificity constants for NAD^+ over NADP^+ of 1617 upon R31L mutation (more than 100-fold more specific for NAD^+ than the wild type).

Discussion

Architectural remodeling for the establishment of the 6PG binding site

Our comparative analysis showed that major fold-level changes occurred concomitantly with the remodeling of the substrate binding site. The interchange of α -helices between subunits preceded hemi-domain duplication and is the earliest hallmark in the evolution of the 6PGDH family. It is well known that recombination events can add or remove whole domains constituting long-scale mechanisms for domain architectural change⁶⁷. In this context, tandem domain duplication provides a particular mechanism for modifying the oligomeric state of an enzyme without altering its function^{68–71}. This occurred in the evolution of dimeric 6PGDHs, with the additional consideration that the duplication lead to the loss of two active sites in the oligomeric assembly. The described architectural remodeling could have been consequence of a destabilization of the interactions around the C-terminal α -helix, together with an extension of the C-terminal end. In fact, the number of residues after the C-terminal helix increases from β HADHs to tetrameric 6PGDHs and finally to dimeric 6PGDHs (not shown). Perhaps the kind of genetic instability that favored an increase in the number of residues also affected the tendency of recombination at that genetic locus.

In this context, it is expected that the tetrameric structure would be needed to provide the full set of interactions with 6PG. Surprisingly, Sarmiento-Pavía *et al.*² observed a dimeric state of *Gd6PGDH* in native gel electrophoresis, and proposed that the last C-terminal alpha helix could be a mean for association of this enzyme to the membrane. In this regard, first; we are not convinced that the sequence AEKVSMRFGF is mainly hydrophobic to interact with the membrane, and second; supporting the dimeric state only by native gel and omitting the tetrameric form of *Gd6PGDH* built by crystallographic symmetry corresponds to a monumental error that does not contribute to understanding the structural evolution of the 6PGDH

family. While *Gd6PGDH* might not form obligate tetramers and the rest of its residues at the binding pocket could still sustain 6PG binding, the cost would be paid by a decreased catalytic efficiency. Perhaps that could be the reason why the observed k_{cat} and K_M values of *Gd6PGDH* were lower and higher, respectively, than the corresponding values of *Go6PGDH*. We encourage the exploration of conditions for tetramer formation of *Gd6PGDH*, mostly as the equivalent tetramer observed here can be generated by crystallographic symmetry (Figure 3D), to determine its full activity and cofactor preference.

A clue about the substrate specificity of the ancestral form of β HADHs could be drawn from the structural tree of the superfamily (Figure 5). One of the proteins that emerged closer to the root correspond to Hydroxymethyl-glutarate dehydrogenase (HMGDH; PDBid: 3CKY). It is noteworthy that the substrate of HMGDH is 6C long and bears multiple negatively charged groups, as does 6PG. It is plausible to think that the specificity towards 6PG could have originated from a similar substrate, whereas on the path leading to 3HIBADHs, a preference evolved favoring smaller substrates (2–4C⁷²). In fact, HMGDH showed some promiscuity towards 3C substrates, including 3HIBAs. In this context, the sensitivity to 3HIBA inhibition displayed by *Go6PGDH* agrees with the idea that the tetrameric 6PGDHs are less differentiated than the dimeric forms in a pathway of increasing selectivity towards 6PG.

The presence of dimeric and tetrameric 6PGDHs across species

Our phylogenetic results are consistent with 6PGDHs having appeared first in Bacteria and from them the different forms were transferred to Eukarya and Archaea (Figure 9). Lateral Gene Transfer (LGT) to Eukaryotes was apparent from at least three associations seen in the dimeric branch of 6PGDHs. The case of plants and cyanobacteria has been studied previously²³. The close relationship of Opisthokonta with β - and γ -proteobacteria (members of the clade *b*₁) has also been highlighted before^{23,73}, but our analysis made clear that firmicutes was the most conspicuous bacterial group associated to these eukaryotes. Contrary to expectations based on their status as ancestors of mitochondria⁷⁴, α -proteobacterial sequences were not closely related to those of opisthokonta; ruling out that, like plants, the animal and fungal lineages would have acquired their nuclear genes for 6PGDH through endosymbiotic gene transfer. The most puzzling observation is that the clade *e*₃ (ascomycota+euglenozoa) is more ancient than plants and opisthokonta and yet it does not show a bacterial sister group (Figure 7). This might be due to either the loss of the 6PGDH gene in the bacterial lineage from which the gene was transferred, or an unsolved relationship with some of the close bacterial neighbours, perhaps α -proteobacteria. On the other hand, although our reconstruction supports the hypothesis that haloarchaeal 6PGDHs were derived from bacterial homologs via LGT⁷⁵, it also suggests that transference was not restricted to haloarchaea. It is noteworthy that the branching pattern of the bacterial lineages of dimeric and tetrameric 6PGDHs do not coincide, and particularly the main

Figure 9. Overview of the evolutionary history of 6PGDHs. It is proposed that the family was originated in the Bacteria domain, where the homologous are more widespread, after the architectural changes occurred in the C-terminal hemi-domain. Only the clear monophyletic clades seen in Figure 7 are shown here. In the case of dimeric 6PGDHs, the branching order does not follow the phylogenetic tree of Figure 7, but the conventional view of bacterial phylogeny. Lateral Gene Transfer is indicated by vertical connectors between Domains. The dotted line raises the possibility that the 6PGDHs from the amorpha + excavata group could have evolved from α -proteobacteria. In the Chloroflexi and Ascomycota branches sequence conservation suggest a transition of cofactor specificity.

clades of dimeric 6PGDHs did not follow the conventional view of bacterial evolution⁷⁶, probably because of extensive LGT and gene loss events.

The presence of multiple 6PGDH forms in single species was observed in different lineages (Figure 7 and Table 5). Plants possess plastidial and cytosolic variants of dimeric 6PGDH, both bearing an NR motif, which are derived from the duplication of an ancestral plastidial gene^{23,73}. In addition, we observed that Ascomycetes carry two dimeric 6PGDHs, one with the NR motif and the other with the D ϕ motif (with ϕ = V or L), thus suggesting preference for NADP⁺ and NAD⁺, respectively. Unlike plants, these fungi do not carry products of a recent gene duplication and their 6PGDH could even represent independent LGTs from different bacterial species. Interestingly, the presence of both tetrameric and dimeric 6PGDHs – possessing the D ϕ and the NR motifs, respectively – was found only among bacteria such as firmicutes (*Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis* and *Bacillus subtilis*), actinobacteria (*Streptomyces coelicolor*) and β -proteobacteria (*Methylobacillus flagellatus*). Also, 6PGDHs that differ in size and cofactor specificity were reported for other bacteria in early biochemical studies^{26,66,77,78}, that, unfortunately, had no access to the sequence features of the β 2- α 2 loop. It is noteworthy that α -proteobacteria and cyanobacteria possess either tetrameric or dimeric 6PGDHs, but individual species in these groups bearing both forms were not found.

Dinucleotide specificity in 6PGDHs, from engineering to evolution

The substrate saturation level of the 6PG binding site modulates the cofactor preference of *Go*6PGDH (Figure 8C). This feature could be physiologically relevant for dual dehydrogenases. Interestingly, the conservation of the DR motif in the clade of tetrameric 6PGDHs of α -proteobacteria suggests dual cofactor specificity⁴³, and hence the possibility that the ratio of NADH to NADPH they produce could vary according to the intracellular 6PG concentration. On the other hand, the substrate-concentration dependence of k_{cat} and K_M for either cofactor indicates the formation of a ternary complex with 6PG and *Go*6PGDH, ruling out a ping-pong mechanism⁷⁹. Similar kinetic results have been obtained for the dimeric 6PGDHs from *Candida utilis*⁸⁰ and human erythrocytes⁸¹.

By changing the DR motif of *Go*6PGDH to D ϕ ; the specificity constant for NADP⁺ dropped by at least 2 orders of magnitude (Table 4). This is explained by the absence of the guanidinium group of the R side chain that provides favorable interactions with the 2'-phosphate and the adenine ring of the cofactor, and also by the electrostatic repulsion with the negative charge of the aspartate carboxylate group. On the other hand, when changing the motif to NR, it was the specificity constant for NAD⁺ which spectacularly dropped 1000-fold (Table 4). An explanation could be looked for in the crystal structures of dinucleotide-complexes of orthologues. In most of 6PGDH structures N₂ from the N residue is an H-bond donor to the

Table 5. Organism bearing 6PGDH isoforms present in the phylogenetic tree (see Figure 7.)

Isoform	Uniprot	Kingdom	Class	Sequence in the $\beta 2$ - $\alpha 2$ motif	C-terminal Hemi-domain
<i>Streptomyces coelicolor</i> (1)	O88014	Bacteria	Actinobacteria	YDTNPR	Single
<i>Streptomyces coelicolor</i> (2)	Q7AKL1			YDRNPDL	Single
<i>Streptomyces coelicolor</i> (3)	Q93J68			HNRTASR	Duplicated
<i>Heimdallarchaeota archaeon</i> (1)	A0A2U3CDI6	Archaea	Asgard group	KARSQKP	Single
<i>Heimdallarchaeota archaeon</i> (2)	A0A2U3CCV3			YNRTSKS	Duplicated
<i>Leuconostoc mesenteroides</i> (1)	Q03YQ7	Bacteria	Firmicutes	FDLNADF	Single
<i>Leuconostoc mesenteroides</i> (2)	Q03XP2			YNRSRER	Duplicated
<i>Lactococcus lactis</i> (1)	A2RNY8	Bacteria	Firmicutes	YDVNAEA	Single
<i>Lactococcus lactis</i> (2)	P96789			YNRTTSK	Duplicated
<i>Lactobacillus plantarum</i> (1)	D7VB15	Bacteria	Firmicutes	MDLNPTN	Single
<i>Lactobacillus plantarum</i> (2)	D7V8V6			FNRTGAK	Duplicated
<i>Bacillus subtilis</i> (1)	P54448	Bacteria	Firmicutes	YDVNQAA	Single
<i>Bacillus subtilis</i> (2)	P80859			YNRSSSK	Duplicated
<i>Bacillus subtilis</i> (3)	P12013			YNYTRDL	Duplicated
<i>Enterococcus faecalis</i> (1)	E0HFQ9	Bacteria	Firmicutes	FDVTKEA	Single
<i>Enterococcus faecalis</i> (2)	E0H9P8			YNRTGSK	Duplicated
<i>Methylobacillus flagellatus</i> (1)	Q1H2F7	Bacteria	Betaproteobacteria	FDFNQDA	Single
<i>Methylobacillus flagellatus</i> (2)	Q1GY23			YNRDPEK	Duplicated
<i>Coniosporium apollinis</i> (1)	R7YPD3	Fungi	Ascomycota	FNRTVSK	Duplicated
<i>Coniosporium apollinis</i> (2)	R7YWE2			WDISGEN	Duplicated
<i>Coniosporium apollinis</i> (3)	R7YZH7			QDPSVEA	Duplicated
<i>Macrophomina phaseolina</i> (1)	K2S8M9	Fungi	Ascomycota	YNRTVSK	Duplicated
<i>Coniosporium apollinis</i> (2)	R7YWE2			WDISGEN	Duplicated
<i>Trichophyton tonsurans</i> (1)	F2S4G0	Fungi	Ascomycota	FNRTVEK	Duplicated
<i>Trichophyton tonsurans</i> (2)	F2S8U3			WDAKADN	Duplicated
<i>Rasamsonia emersoni</i> (1)	A0A0F4YZ37	Fungi	Ascomycota	YNRTVSK	Duplicated
<i>Rasamsonia emersoni</i> (2)	A0A0F4YQX6			WDVSGPN	Duplicated
<i>Emericella nidulans</i> (1)	Q5B676	Fungi	Ascomycota	YNRTTSK	Duplicated
<i>Emericella nidulans</i> (2)	C8VP36			WDVKYDN	Duplicated
<i>Aspergillus oryzae</i> (1)	Q8NKA5	Fungi	Ascomycota	YNRTTSK	Duplicated
<i>Aspergillus oryzae</i> (2)	Q8NKA4			WDVKKEN	Duplicated
<i>Penicillium rubens</i> (1)	B6GWT7	Fungi	Ascomycota	YNRTTAK	Duplicated
<i>Penicillium rubens</i> (2)	B6GYV9			WDVVKKN	Duplicated
<i>Ricinus communis</i> (1) chloroplastic	B9RVA7	Plantae	Magnoliopsida	YNRSTSK	Duplicated
<i>Ricinus communis</i> (2) chloroplastic	B9RCL8			YNRSTSK	Duplicated
<i>Ricinus communis</i> (3)	B9SXT4			YNRTTSK	Duplicated
<i>Spinacia oleracea</i> (1) chloroplastic	Q94KU2	Plantae	Magnoliopsida	YNRTASK	Duplicated
<i>Spinacia oleracea</i> (2)	Q94KU1			YNRTTSK	Duplicated

backbone carbonyl of the residue i+2, which would otherwise be fully engaged in the H-bonding pattern of helix $\alpha 2$. In *Go6PGDH*, *Gm6PGDH* and *Go6PGDH*, an Asx turn⁸² is formed between the $O_{\delta 1}$ of the D sidechain and the backbone amide of i+2, allowing the carbonyl to integrate the first turn of $\alpha 2$ (Figure 10). An Asx turn is also observed in the NAD^+ - complex of *Hs3HIBADH*, where the carboxylate of D performed as a bidentate H-bond acceptor for the ribose hydroxyls. Therefore, we could hypothesize that upon the mutation from D to N, the loss of the Asx turn induces a reordering of the H-bond network and weakens the interactions with the ribose hydroxyls. This will be tested in planned structural work on dinucleotide-complexes of *Go6PGDH* and its mutants.

Altering the dinucleotide use of dehydrogenases has been an essential area of protein and metabolic engineering, as well as synthetic biology^{83–88}. Thus far, studies on 6PGDHs seem to indicate that the tetrameric enzymes constitute a better scaffold to achieve high performance (k_{cat}/K_M) with NAD^+ by mutation of residues in the $\beta 2$ - $\alpha 2$ loop, as well as high selectivity (ratio of k_{cat}/K_M with NAD^+ to k_{cat}/K_M with $NADP^+$). Initial studies with the dimeric 6PGDH from *Lactococcus lactis* (which bears a NR motif), showed that the mutation R33Y worsened the enzyme's K_M in presence of $NADP^+$ (increased above 700-fold)²¹. Although the mutant had measurable activity with NAD^+ , the activity level was insufficient for kinetic characterization. In the case of the hyperthermophilic dimeric

$NADP^+$ -dependent 6PGDH from *Thermotoga maritima*, a triple site-directed mutant N32E/R33I/T34I was generated based on sequence alignment and docking of substrates/cofactors. Again, an important drop of the catalytic efficiency for $NADP^+$ as a cofactor was obtained, while the performance with NAD^+ only increased 3.6-fold. As a result, the selectivity for NAD^+ was 9620. Conversely, in the case of the thermophilic tetrameric $NADP^+$ -dependent 6PGDH from *Moorella thermoacetica*, the mutant A30D/R31I/T32I, was obtained through Directed Evolution²⁷. This time, the mutant showed an outstanding increase in the performance with NAD^+ . Given the 80-fold decrease on the performance with $NADP^+$, the resulting selectivity for NAD^+ was 61. In this context, the kinetic parameters for *Go6PGDH*^{PL} mutant (Table 4) showed a performance with NAD^+ higher than that of the *Morella* mutant and a selectivity far superior to that of the *Thermotoga* mutant.

The evolution of cofactor specificity of 6PGDHs directly impacts the metabolic role of the oxPPP. Our phylogenetic analysis revealed at least two instances of transition of cofactor specificity (Figure 7 and Figure 9): one in chloroflexi (tetrameric 6PGDHs) and the other in ascomycetes (clade e_3 of dimeric 6PGDHs). On the other hand, production of reduced cofactor in the oxPPP has been studied through physiological or enzymatic characterization. In the case of *Bacillus subtilis* it has been demonstrated the involvement of a dimeric $NADP^+$ dependent 6PGDH in the carbon flux through the oxPPP⁸⁹, even though this organism possesses variants that differ in size and cofactor specificity (Figure 7, Table 3 and Table 5). In the case of *Haloferax volcanii* a $NADH$ -producing oxPPP has been described to operate, involving a NAD^+ -dependent tetrameric 6PGDH⁹⁰. In the case of *G. oxydans* the oxPPP produce both reduced cofactors, since *Go6PGDH* generates mainly $NADH$, whereas the glucose-6P dehydrogenase is engaged in $NADPH$ production²⁸. However, there remains an open question as to the metabolic constraints that determine the evolution of cofactor specificity of 6PGDHs.

Data availability

Underlying data

Protein Data Bank. X-ray data for the apo structure of the 6PGDH from *Gluconobacter oxydans* at Protein Data Bank, accession number: 6XEQ

<https://www.rcsb.org/structure/6XEQ>

Data for the analysis of the enzyme kinetics experiments. We did not save the raw data directly from the spectrophotometer (Absorbance units vs. time) for the kinetic experiments. However, we registered each slope ($\Delta A/\text{min}$) and deposited the data corresponding to specific enzyme activity (U/mg) vs. substrate concentration in the repository Zenodo (<https://zenodo.org/>). With this information and proceeding with the described methodology, it is possible to set up all the graphs and kinetic parameters reported in this work.

Zenodo: Cofactor specificity of the tetrameric 6-phosphogluconate dehydrogenase from *Gluconobacter oxydans*: Studies of wild-type and mutants forms. <https://doi.org/10.5281/zenodo.4448337>⁹¹

Figure 10. Superposition of the cofactor binding pocket of the *Gm6PGDH* and *Go6PGDH*. *Gm6PGDH* is represented in orange and *Go6PGDH* in gray, dotted lines represent H-bonds. $O_{\delta 1}$ of D30 interact with the amide of S32 and N32, in *Gm6PGDH* and *Go6PGDH*, respectively. The O_3 of the $NADP^+$ ribose interacts as an acceptor of weak H-bonds with the amide group of G7 and L8 of *Go6PGDH*. Both *Go6PGDH* and *Gm6PGDH* display the structure of the Asx turn where the $O_{\delta 2}$ of D30 interacts with the O_3 of the ribose, which acts as an H-bond donor.

This project contains the following underlying data:

-Cofactor preference vs 6PG.csv
 -D30N-NAD.csv -D30N-NADP.csv
 -Kinetic parameters NAD-6PG.csv
 -Kinetic parameters NADP-6PG.csv
 -NAD(P) efficient constant vs 6PG .csv
 -R31L-NAD.csv
 -R31L-NADP.csv
 -R31V-NAD.csv
 -R31V-NADP.csv

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Zenodo: 6-phosphogluconate dehydrogenase activity inhibition by (S)-3-Hydroxyisobutyric acid. <http://doi.org/10.5281/zenodo.4537863>⁴⁸

This project contains the following underlying data:

-Inhibition_3HIBA.csv

Protein sequence codes used in the phylogenetic inference.

Zenodo: Protein codes for tetrameric and dimeric 6-phosphogluconate dehydrogenases and their signature sequence relevant to the cofactor specificity in the 6PGDH family. <https://doi.org/10.5281/zenodo.4489052>⁴³

This project contains the following underlying data:

-Data_phylogenetic_tree.pdf
 -Data_phylogenetic_tree.rtf

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Paul C. Engel

School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin, Ireland

This is an ambitious and interesting study which attempts to draw together much information on primary, tertiary and quaternary structure in 6-phosphogluconate dehydrogenases from a wide variety of biological species, and also on related enzymes, in order to arrive at a proposed model for the sequence of evolutionary development in this enzyme family. The paper is generally clear and well written. Only time will tell how close the authors are to the truth in their interpretation, but they offer a plausible account, and papers like this do a service to everyone in having the courage to look beyond the immediate local details to survey the wider landscape. They put some of their conclusions (with regard to the basis of coenzyme specificity) to direct experimental test through mutagenesis, achieving dramatic shifts in the predicted direction in coenzyme preference.

I have only a few scientific points and a few minor language corrections.

Scientific points:

1. Discussion of Fig. 6: The authors explore their idea about the evolutionary relationship between 3-hydroxyacid dehydrogenases and 6PGDHs by looking at the response of two different 6PGDHs to 3HIBA. Neither shows any catalytic activity, but one displays significant inhibition. The interpretation here needs caution and some qualification, because the pattern, with a plateau at roughly 30% inhibition, is not what one would expect for a competitive inhibition. The experiment ideally could have done with a control using a similar compound not predicted to have any vestigial affinity for the substrate site in order to rule out (or in) a non-specific binding effect.
2. Discussion section, paragraph 2: The phrase 'monumental error' in discussing the relative merits of evidence from crystallography and from native gels is over the top. What is needed here is a fuller discussion of the relevant parameters (protein concentration, solvent components, concentration pH etc.) that might influence the predominant quaternary structure. Crystallography also offers a snapshot of behaviour under a particular set of

conditions and not necessarily absolute, unconditional and exclusive truth.

3. The final Discussion would benefit from some mention of the extent to which the means by which different coenzyme preferences are achieved in this family are or are not similar to those seen in other dehydrogenase families. This is, after all, in the wider context, a story that has been under study for different dehydrogenases for over thirty years.

Language details, etc.:

1. In Expression and Protein Purification: "...biomass was collected...".
2. Table 1 title: Crystallography.
3. Structural Alignment and analysis: "...are listed in Table 2."
4. Fig. 3 Legend last word: "shown."
5. Discussion paragraph 2: "...could be a means...".
6. Table 5 title: "Organisms bearing...".
7. Last paragraph before Data Availability: "In the case of *Bacillus subtilis* the involvement ... has been demonstrated...".

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Enzymology, kinetics, protein engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

Reviewer Report 12 May 2021

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José Gregório C. Gómez

Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

This article describes an interesting scientific work very well carried out and presenting results that are relevant to the general knowledge of cellular metabolism. The crystallographic structure of 6-phosphogluconate dehydrogenase from *Gluconobacter oxidans* (Go6PGDH) was resolved and described. Taking into account phylogenetic trees based on structure or aminoacids sequence and the inhibitory effects of 3-hydro-isobutyrate, evolutive aspects and lateral genetic transfers were proposed. Mutants of Go6PGDH were constructed and important features were revealed regards NAD or NADP specificities.

I consider this article to be of excellent quality and indicate only minor points that could improve it.

- At the beginning of the introduction, the authors could already make it clear that the reaction catalyzed by 6PGDHs is part of the oxidative part of the pentose phosphate pathway (oxPPP). This abbreviation appears only in the last paragraph of the discussion without being clarified in any previous part of the article.
- Enzyme activity data using NAD or NADP for 6PGDH from *Pseudomonas putida* KT2440 are also available from Olavarria *et al.* (2015¹). These data could be used to enrich Table 3.
- The fact that some bacteria do not have the *gnd* gene (encoding 6PGDH) could have been better discussed. Effects of *gnd* expression with different specificities (NAD or NADP) in a *Pseudomonas* sp. without the *gnd* gene in its genome have been addressed recently (Cardinali-Rezende *et al.*, 2020²).

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: bacterial metabolism; bacterial biotechnology; polyhydroxyalkanoates

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.