Supporting Information for

Chemical Confirmation of a Pentavalent Phosphorane in Complex with β-Phosphoglucomutase

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Crystallization and Structure Determination

Crystallization conditions for the β-PGM-α-D-galactose-1-phosphate complex were obtained by sparse matrix screening[1] with Crystal screen Kits I and II (Hampton Research). A number of different crystallization conditions were obtained which were further screened for diffraction and for unit-cell dimensions (from 1-4 frames of data). Those unit-cell dimensions compatible with the cap-closed conformation (indicating the presence of ligand in the active site) were optimized[2,3]. Notably, the β-PGM-α-D-galactose-1-phosphate complex crystallizes in the absence of F^- (see below) while the β-PGM-glucose-6-phosphate-1-phosphorane complex does not. The unit-cell dimensions of the β-PGM-α-D-galactose-1-phosphate complex and the β-PGM-glucose-6-phosphate-1-phosphorane complex differ from one another by only a few Ås as a result of small differences between the two monomers in the asymmetric unit of β-PGM in the β-PGM-α-Gal1P complex (space group $P2_1$), causing a loss of symmetry from the phosphorane complex with one monomer per asymmetric unit (space group $P2_12_12_1$). The crystal forms observed for the phosphorane complex and for the β-PGM-α-Gal1P complex differ only slightly, but in such a way that F^- might be needed to stabilize the crystal contacts of the former complex but not the latter complex.

For crystallization, the purified protein[4] (~25 mg/ml), was dissolved in 1 mM Hepes buffer (pH 7.5), 10 mM $MgCl_2$ plus 50 mM α-D-Galactose-1-phosphate (α-Gal1P) (Sigma). Crystals grew at 18°C as orthorhombic rhomboids with overall dimensions of 0.5mm×0.4 mm×0.4mm in 7-14 days from solutions of 100 mM Tris HCl pH 8.0, 200 mM sodium acetate, and 30% PEG 4000. Addition of 100 mM NH_4F to the crystallization conditions did not significantly alter the crystal growth or morphology. Crystals were frozen in 100% Paratone-N (Hampton Research) for data collection directly in a stream of nitrogen gas cooled by liquid nitrogen. Diffraction data were collected at -180°C to 1.97 Å resolution using CuKα radiation from a Rigaku RU-300 generator equipped with an on the R-Axis IV^{++} image plate (Boston University School of Medicine). Data were indexed and scaled using DENZO and SCALEPACK[5]. Data collection statistics are summarized in Supporting Information Table S1. Phases were solved via the molecular replacement program Molrep[6] using the model of β-PGM cocrystallized with glucose-1-phosphate (G1P)[3] (pdb accession code 1O08). The program yielded a solution with correlation coefficient of 27.0% and an R-factor of 52.7%. The model was refined using successive rounds of manual rebuilding and the molecular graphic program O[7] followed by minimization and simulated annealing in CNS[8] with data to 1.97. Å. To avoid model bias, ligand and waters were added when R_{free}[9] was less than 30%. Refinement and final model statistics are summarized in Table S1.

Table S1. Summarized Crystallographic Data Collection and Refinement Statistics[a]

	+NH_4F	**-NH_4F**
Data Collection statistics		
Space group	$P2_1$	$P2_1$
Unit-cell dimensions	a= 57.143 Å b=36.451 Å c=105.119 Å β=94.353°	a= 55.069 Å b=36.351 Å c=104.883 Å β=101.77°
X-ray source	CuKα	CuKα
Resolution range	100-1.97	20-1.9
Highest resolution shell	(2.04-1.97)	(1.97-1.90)
No. of total/unique reflections	160,938/31,017	95,896/32,403
Completeness (%)[a]	94.3 (93.0)	99.4(99.2)
I/σ(I)	21.3 (5.6)	14.5(4.2)
R_{merge} (%)	4.9 (33.6)	7.6(39.4)
Volume fraction of protein	55.5%	60.2%
Data Redundancy	5.2(5.2)	3.0(2.9)
Refinement statistics		
Resolution Range (Å)	20.4-1.97	25.0-1.90
No. of protein atoms/asymmetric unit	3254	3397
No. of waters/asymmetric unit	415	453
No. of Mg(II) ion/asymmetric unit	2	2
No. of total reflections(working set/free set)	26,391/2,942	28,604/3,166
R_{work}/R_{free} (%)	20.8/23.9	19.9/25.4
Ramachandran Plot		
residues in most favored regions	92.4%	96.9%
residues in additionally allowed regions	7.6%	3.1%
Average B-factor ($Å^2$)	31.0	25.0
Average of all amino acids	30.8	24.7
Average of Mg^{2+} atoms	22.8	18.4
Average of Solvent	33.8	27.8
Average of main chain atoms	28.2	23.2
Average of side chain atoms	33.8	26.4
Average of Ligand atoms	15.1	13.6
Luzzati coordinate error (Å)	0.27	0.28
Rmsd		
Bond length (Å)	0.012	0.016
Dihedrals (deg)	23.70	23.40
Angles (deg)	1.60	1.80
Impropers (deg)	1.69	1.98

[a]The numbers in parenthesis refer to the highest resolution shells, (2.04-1.97) and (1.97-1.90) for the structures with and without NH_4F, respectively.

Phosphate Assays

Crystals were harvested by the addition of 10 µl wash solution consisting of 100 mM Tris pH 8.5, 200 mM sodium acetate, 40% PEG 4000 to each 2 µl hanging drop and removal with a pipette. Crystals from multiple wells were combined and rinsed with 5 mL wash solution via repeated addition and removal of the solution from the crystals in a siliconized crystallization dish. All washes were performed within 15 min to prevent diffusion of substrates. The combined, washed crystals were dissolved in 240 µl of alkaline phosphatase buffer consisting of 5 mM Tris pH 7.0, 5 mM $MgCl_2$, 100 mM $ZnCl_2$ and the protein concentration determined via Bradford assay (in triplicate). The protein solution was boiled for 10 min to release bound substrates and phosphate from the active site, and then centrifuged at 13,000 rpm for 10 min to remove denatured protein. The supernatant was incubated with 7.5 units of alkaline phosphatase (Biozyme Co.) for 25 min to insure complete dephosphorylation of hexose-phosphate sugars. Alternatively, the phosphate was liberated by boiling in 4M HCl for 10 min followed by neutralization with NaOH. The hydrolysate was then divided into three portions for triplicate phosphate determination using a Malachite Green phosphate assays (BioMol Co.)[10].

Substrate Kinetics of Gal1P

α-Gal1P acts as a substrate in the presence of the phosphorylated enzyme. Initial velocity data were measured as a function of α-Gal1P concentration (0.2-5.0 mM) in solutions initially containing 25 µM β-D-glucose 1,6-(bis)phosphate (β-G16P), 0.005 µM β-PGM, 200 µM NADP, 2 mM $MgCl_2$, 10 U/ml glucose 6-phosphate dehydrogenase (type IX: from Bakers yeast) in 50 mM Hepes (pH 7.0, 25°C). NADPH formation via glucose-6-phosphate oxidation was used to monitor the reaction velocities ($\Delta\varepsilon$ = 62000 M^{-1} cm^{-1} at 340 nm). In this reaction β-G16P phosphorylates the β-PGM to form glucose-6-phosphate (G6P). The phosphorylated β-PGM phosphorylates α-Gal1P, and the cycle is repeated until the β-G16P is depleted. ^{1}H-NMR (Bruker 500 mHz; reaction sample filtered through a 5 kDa membrane to remove enzyme, lyophilized, then dissolved in 0.5 ml D_2O) analysis of the products formed in 1 ml reactions of 10 mM β-G1P or α-Gal1P, 100 µM β-G16P, 11 µM β-PGM, 1 mM $MgCl_2$, and 20 phosphate (pH 7.0, 25°C) showed that the β-G1P had been converted to G6P in one reaction while the α-Gal1P had not been converted to Gal6P in the other reaction. β-G1P, α-Gal1P, G6P and α-galactose-6-sulphate served as standards.

α-D-Galactose-1-phosphate Inhibition of β-PGM.

The 1 ml assay solutions used in the inhibition experiments initially contained 0.004 µM β-PGM, 50 µM α-G16P, 5-200 µM β-G1P, 2 mM $MgCl_2$, 200 µM β-NADP, 2.5 unit G6P dehydrogenase and varying concentrations of α-Gal1P (0, 50, 200, 500, 1000 µM) in 50 mM K^+Hepes (pH 7.0, 25°C). Reactions were monitored at 340 nm ($\Delta\varepsilon$ = 6200 M^{-1} cm^{-1}). The initial velocity data were analyzed using the equation $V_0 = V_m[A]/(K_m (1 + [I]/K_i) + [A])$ wherein [A] is the β-G1P concentration, V_0 is the initial velocity, V_m is the maximum velocity, K_m is the Michaelis constant for βG1P, [I] is the α-Gal1P concentration and K_i is the competitive inhibition constant for α-Gal1P.

Inhibition by F^-

NH_4F inhibition was tested by measuring the initial velocity of G6P formation in a reaction mixture initially containing 0.005 µM βPGM, 100 µM βG1P, 200 µM βG1,6bisP, 2 mM $MgCl_2$, 20 unit/ml G6P dehydrogenase, 1 mM NADP, 50 mM K^+Hepes pH 7.0. In the absence of NH_4F the reaction velocity was 0.89 µM/sec and in the presence of 10 mM and 20 mM NH_4F the reaction velocities were 0.87 µM/sec and 0.74 µM/sec. The experiment was repeated at 10 mM $MgCl_2$, for which the velocities were 0.83 µM/sec and 0.73 µM/sec, respectively.

If the hypothetical species, MgF_3^- were to bind as a transition-state analog and thus, with very high affinity, the absence of inhibition at 20 mM NH_4F indicates that a very low concentration of MgF_3^- is formed in the assay solution, and thus, that MgF_3^- has a very small formation constant. Therefore, it follows that at 1 mM NH_4F (at which concentration crystals also can be obtained), only a miniscule fraction of the β-PGM in the crystallization liquor could possibly be complexed with MgF_3^- and thus available for crystal formation. Equally significant is the fact that the crystalline enzyme is observed in the domain-domain closed conformation, stabilized by the crystal lattice. In this conformation the active site is closed and inaccessible to solvent and hence, to the MgF_3^- ligand[3]. In order for MgF_3^- to occupy the active site, it would have had to be bound prior to crystallization. Thus, it can not be assumed that the enzyme somehow stabilizes MgF_3^-[11] nor can one rationalize the presence of this hypothetical complex as a post-crystallization occupant of the enzyme active site.

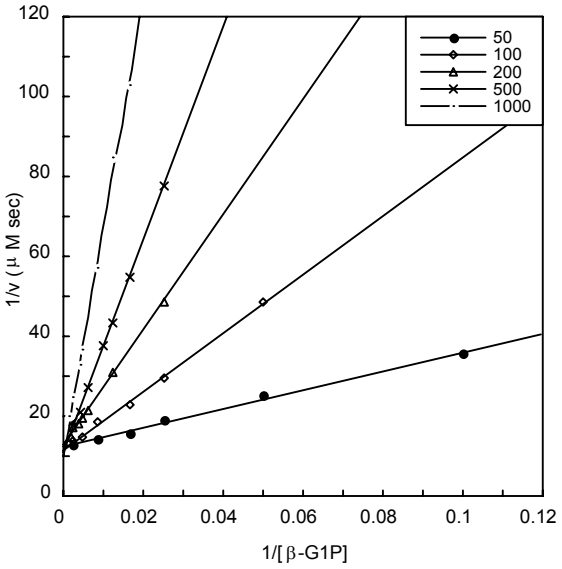

Figure S1. α-Galactose-1-phosphate inhibition of β-PGM: Plot of the inverse of the initial velocity of G6P formation (μM/s) versus inverse of the concentration of β-G1P (μM).

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