

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

Lee W. Tremblay,[†] Guofeng Zhang,[‡] Jianying Dai,[‡] Debra Dunaway-Mariano,^{*,‡} and Karen N. Allen^{*,†}

Department of Physiology and Biophysics, Boston University School of Medicine,
Boston, Massachusetts 02118-2394 and Department of Chemistry, University of New Mexico,
Albuquerque, New Mexico 87131

Received February 11, 2005; E-mail: dd39@unm.edu; allen@med-xtal.bu.edu

The catalytic power of enzymes originates from their ability to stabilize high-energy transition states and intermediates. In a recent article,¹ we reported the 1.2 Å resolution X-ray crystal structure of *Lactococcus lactis* β -phosphoglucosmutase (β -PGM) cocrystallized from an equilibrated mixture containing substrate (β -glucose 1-phosphate (β -G1P)), intermediate (β -glucose 1,6-(bis)phosphate (β -G16P)), and product (β -glucose 6-phosphate (β -G6P)), which revealed a covalent adduct formed between the active-site aspartate nucleophile (Asp8) and the C(1) phosphoryl group of the β -G16P ligand (Figure 1). The pentavalent phosphorane thus observed reflects nucleophilic substitution via an associative pathway and the ability of the enzyme to stabilize the phosphorane intermediate along that pathway. An alternative interpretation of the X-ray diffraction data has been offered by Blackburn and co-workers, who in their commentary to *Science* argued that the structure is of the β -PGM-glucose-6-phosphate-1-MgF₃ complex (with Mg²⁺ in the place of phosphorus and F⁻ replacing the equatorial oxygens).² This structure would represent a transition-state analogue rather than a true reaction intermediate. Because the crystallization solutions contained high concentrations of MgCl₂ (10 mM) and NH₄F (100 mM) and the apical bond lengths (2.0 and 2.1 ± 0.11 Å) observed in the structure were longer than predicted based on those observed in crystal structures of pentaalkoxyphosphoranes (1.66–1.76 Å),³ the Blackburn hypothesis was sufficiently credible to create a controversy.⁴ Because of the significance of the proposed phosphorane intermediate, it is imperative that this controversy be resolved.

Herein we report experimental evidence that unambiguously supports the assignment of the β -PGM- β -D-glucose-6-phosphate-1-phosphorane structure and rules out the β -PGM- β -D-glucose-6-phosphate-1-MgF₃ structure.⁵ An indisputable difference in these two complexes is that the phosphorane complex contains two phosphorus atoms per one enzyme molecule, while the MgF₃ complex would contain one phosphorus atom per one enzyme molecule. Thus, the two complexes could be distinguished based on Malachite green phosphate⁶ and Bradford protein⁷ analyses of the protein crystal provided that the proper standard could be applied for accurate calibration of the respective assays. The standard used was crystals of the β -PGM- α -D-galactose-1-phosphate complex. α -D-Galactose-1-phosphate (α -Gal1P) was found to be a linear competitive inhibitor of β -PGM ($K_i = 30 \mu\text{M}$; see Supporting Information). Crystals of the β -PGM- α -Gal1P complex were grown from a solution of 100 mM Tris pH 8.5, 200 mM sodium acetate, 30% PEG 4000 also containing the 10 mM MgCl₂, and 100 mM NH₄F used to grow the original “phosphorane” crystals.¹ Control crystals were grown under these conditions but omitted NH₄F. The structures in the presence and absence of NH₄F refined to 1.97 Å

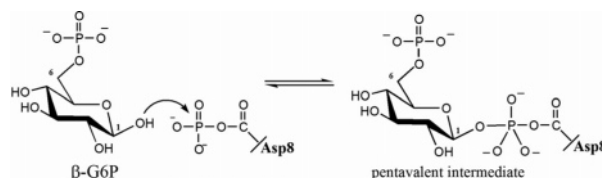


Figure 1. Formation of intermediate in β -PGM.

resolution with an $R_{\text{work}} = 20.8\%$ and $R_{\text{free}} = 23.9\%$ and to 1.90 Å resolution with an $R_{\text{work}} = 19.9\%$ and $R_{\text{free}} = 25.4\%$ were indistinguishable (see Supporting Information). As pictured in Figure 2, the active site contains α -Gal1P bound with its C(1)-phosphate group in the “nontransferring” phosphate binding site distal to the nucleophilic Asp8.

Protein and phosphate assays were performed in duplicate and triplicate, respectively, on protein solutions generated from rinsed crystals of the β -PGM- α -Gal1P complex (control crystals) using a α -Gal1P standard and β -PGM- β -D-glucose-6-phosphate-1-phosphorane complex (test crystals). The phosphate determination (in triplicate) was repeated for the β -PGM- β -D-glucose-6-phosphate-1-phosphorane complex (test crystals) four times using standards of either phosphate or G1P and G6P. The results (see Table 1) show that the control crystals contain the anticipated 1:1 ratio of enzyme-to-labile phosphorus in contrast to the test crystals that contain a 1:2 ratio. This finding is direct evidence for the β -PGM- β -D-glucose-6-phosphate-1-phosphorane structure, for which a 1:2 ratio is required and direct evidence against the β -PGM- β -D-glucose-6-phosphate-1-MgF₃ structure for which a 1:1 ratio is required.

It is noteworthy that the β -PGM- α -Gal1P complex provides the ideal binding partner for MgF₃⁻ if such a chemical species were to form from MgCl₂ and NH₄F. Indeed, the β -PGM-Mg²⁺- α -Gal1P structures (crystals grown with and without NH₄F) contain three ordered water molecules (temperature factors of 11.6, 15.9, and 14.8 Å² compared to the average of 33.8 Å² for solvent) overlapping with the positions occupied by the equatorial oxygen atoms of the C(1)-phosphorane group in the β -PGM-glucose-6-phosphate-1-phosphorane complex. The three equatorial oxygen atoms of the C(1)-phosphorane group observed in the phosphorane complex are engaged in hydrogen bonds with Val9, Asp10, Ser114, Ala115, Lys145, and coordinated to the Mg²⁺ cofactor.¹ If MgF₃⁻ were present, would it not bind in place of the three water molecules? Indeed it should.

The fact that α -Gal1P forms a catalytically competent complex with β -PGM is shown by its activity with the phosphorylated enzyme. β -PGM phosphorylated by the β -G16P cofactor reacts with α -Gal1P to form α -galactose 1,6-(bis)phosphate as product ($k_{\text{cat}} = 33.4 \pm 0.6 \text{ s}^{-1}$, $K_m = 530 \pm 20 \mu\text{M}$; see Supporting Information). Thus, during catalytic turnover the C(6)OH of the bound α -Gal1P

[†] Boston University School of Medicine.

[‡] University of New Mexico.

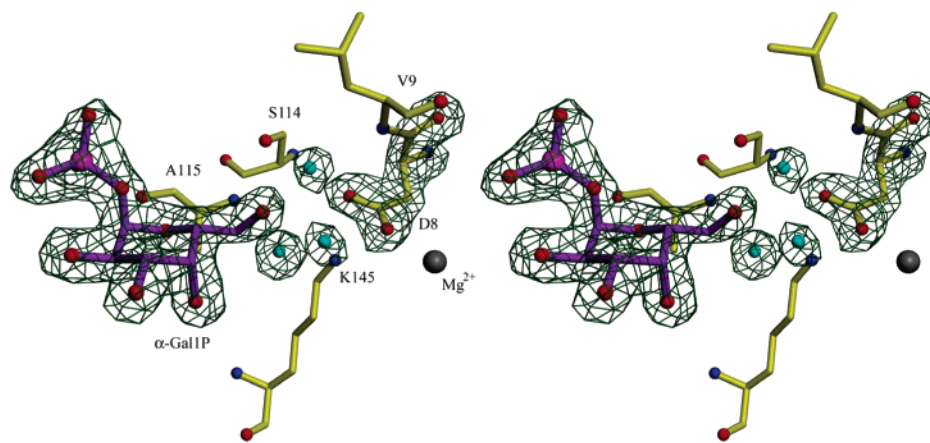


Figure 2. Stereoview of α -Gal1P (purple) bound in the active site of β -PGM. The simulated annealing $F_o - F_c$ electron density map contoured at 4σ with α -Gal1P, selected waters, and Asp8 omitted is depicted (dark blue cages). The Mg^{2+} cofactor is gray, and water molecules are cyan.

Table 1. Results for Bradford Protein and Malachite Green Phosphate Assays of 0.40 ± 0.02 mg/mL and $0.32\text{--}0.45 \pm 0.01$ mg/mL Protein Solutions Prepared from 1.7 mg of Test Crystals of the Putative β -PGM- β -D-glucose-6-phosphate-1-phosphorane Complex and 44 μ g of Control Crystals of the β -PGM- α -Gal1P Complex^a

standard	dephosphorylation method	phosphate/ β -PGM
Test		
phosphate	HCl	2.12 ± 0.06
G1P, G6P	HCl and alkaline phosphatase	2.0 ± 0.3
G1P, G6P	HCl and alkaline phosphatase	1.9 ± 0.3
phosphate	alkaline phosphatase	2.0 ± 0.4
Control		
α -Gal1P	alkaline phosphatase	1.29 ± 0.08

^a The phosphate determinations were first calibrated using a standard curve generated from serial dilution of an inorganic phosphate standard, then checked against phosphate determinations carried out with solutions of G1P, G6P, or α -Gal1P, hydrolyzed in the same manner as the protein sample.

aligns with the aspartyl phosphate group. In the β -PGM- Mg^{2+} - α -Gal1P complex, the C(6)OH aligns with the aspartate carboxylate, generating the ideal binding site for the putative MgF_3^- ion. However, in the crystal structure, three highly ordered and triangularly arranged water molecules are observed at this site rather than MgF_3^- .⁸

These results validate our original assignment of the β -PGM- β -D-glucose-6-phosphate-1-phosphorane complex to the previously reported X-ray diffraction data. Moreover, an anomalous-difference electron density map calculated using a single-wavelength 1.2 Å dataset and protein model phases only (excluding cofactor and ligand) shows electron density of identical magnitude (contoured at 3.5σ) for both atoms assigned as phosphorus. This result is not consistent with the β -PGM- MgF_3 structure because the anomalous scattering from the Mg^{2+} ion is less than half that of the C(1)P at the wavelength of data collection (0.9000 Å). In addition, when a protein electron density map was calculated on an absolute scale (number of electrons) using only observed amplitudes and protein model phases (excluding ligand), the same number of electrons was found at peaks corresponding to the C(1)P and C(6)P positions. Also, we note that the controversial,⁴ long apical bond length observed in the β -PGM- β -D-glucose-6-phosphate-1-phosphorane structure has since been predicted by high level computation, which shows elongation of the apical bonds (to 1.9 Å) as a result of hydrogen-bond formation to a phosphorane oxygen atom.⁹ Finally,

in a very recent article, the structure lipase-D complexed with its own phosphorane intermediate has been reported.¹⁰ Thus, although the β -PGM-phosphorane intermediate was the first to be trapped in a crystal, there is now a second example, and we expect with time others will be discovered.

Acknowledgment. We thank Mr. Liangbing Wang for carrying out the experiments that ruled out F^- inhibition of Mg^{2+} -activated β -PGM. We acknowledge NIH GM61099 (to K.N.A. and D.D.-M.) and NIH Training Grant HL07291 (to L.W.T.). Coordinates have been submitted to the Protein Data Bank under accession codes 1Z4N and 1Z4O for the structures with and without NH_4F , respectively.

Supporting Information Available: Crystallization and structure determination, summarized crystallographic data collection and refinement statistics, phosphate assays, substrate kinetics of α -Gal1P, inhibition kinetics of α -Gal1P, and inhibition by F^- . This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Lahiri, S. D.; Zhang, G.; Dunaway-Mariano, D.; Allen, K. N. *Science* **2003**, *299*, 2067–2071.
- (2) (a) Blackburn, G. M.; Williams, N. H.; Gamblin, S. J.; Smerdon, S. J. *Science* **2003**, *301*, 1184. (b) Response to comment: Allen, K. N.; Dunaway-Mariano, D. *Science* **2003**, *301*, 1184.
- (3) (a) Holmes, R. R. *Pentacoordinated Phosphorous*; Holmes, R. R., Ed.; ACS Monograph 176; Washington, DC, 1980; Vol. II, p 9. (b) Corbridge, D. E. C. *The Structural Chemistry of Phosphorus*; Elsevier Scientific Publishing Co.: Amsterdam, 1974; p 296.
- (4) Yarnell, A. *Chem. Eng. News* **2003**, *81*, 30.
- (5) Crystals of the β -PGM-glucose-6-phosphate-1-phosphorane complex were grown from β -PGM and G1P (or G6P) from 100 mM NH_4F , 10 mM $MgCl_2$, and 16% PEG 3350. The obvious experiment was to crystallize the protein without $MgCl_2$ or without NH_4F . The Mg^{2+} is, however, a required cofactor and an essential component of the active site. Attempts to crystallize the protein in the absence of NH_4F yielded crystals, which diffracted poorly. Stable crystals were, however, grown at low concentrations of NH_4F (1 mM). These crystals were shown by X-ray structure determination to contain the β -PGM-glucose-6-phosphate-1-phosphorane complex.
- (6) (a) Martin, B.; Pallen, C. J.; Wang, J. H.; Graves, D. J. *J. Biol. Chem.* **1985**, *260*, 14932. (b) Harder, K. W.; Owen, P.; Wong, L. K.; Aebersold, R.; Clark-Lewis, I.; Jirik, F. R. *Biochem. J.* **1994**, *298*, 395.
- (7) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
- (8) We note in proof the paradox that crystals of the β -PGM- β -D-glucose-6-phosphate-1-phosphorane complex formed at 100 mM NH_4F are also formed at 1 mM NH_4F ,⁵ yet we found that the $MgCl_2$ -activated β -PGM is not inhibited even at 20 times this concentration of NH_4F (see Supporting Information).
- (9) Range, K.; McGrath, M. J.; Lopez, X.; York, D. M. *J. Am. Chem. Soc.* **2004**, *126*, 1654.
- (10) Leiros, I.; McSweeney, S.; Hough, E. *J. Mol. Biol.* **2004**, *339*, 805.

JA0509073