Enzymatic production of β-glucose 1,6-bisphosphate through manipulation of catalytic magnesium coordination

Henry P. Wood¹, Nicola J. Baxter¹,², Clare R. Trevitt¹, F. Aaron Cruz-Navarrete¹, Andrea M. Hounslow¹, Jonathan P. Waltho¹,²,a

¹Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, S10 2TN, United Kingdom; ²Manchester Institute of Biotechnology and School of Chemistry, The University of Manchester, Manchester, M1 7DN, United Kingdom

To whom correspondence may be addressed: Prof. Jonathan Waltho, Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, S10 2TN, +44 114 22717, j.waltho@sheffield.ac.uk, ORCID 0000-0002-7402-5492

Keywords: glucose bisphosphate | enzymatic production | NMR spectroscopy
Manipulation of enzyme behaviour represents a sustainable technology that can be harnessed to enhance the production of valuable metabolites and chemical precursors. β-glucose 1,6-bisphosphate (βG16BP) is a native reaction intermediate in the catalytic cycle of β-phosphoglucomutase (βPGM) that has been proposed as a treatment for human congenital disorder of glycosylation involving phosphomannomutase 2 (PMM2). Studies of both βPGM and PMM2 could benefit from a green and high-yielding method for βG16BP production. Three strategies have been reported previously for the synthesis of βG16BP; however, each of these methods either delivers low yields or uses chemicals and procedures with significant environmental impacts. Herein, we report the efficient enzymatic synthesis of anomer-specific βG16BP using a variant of βPGM. Further purification, employing a simple environmentally considerate precipitation procedure requiring only a standard biochemical toolset, results in a product with high purity and yield. Moreover, this synthesis strategy illustrates how manipulation of the catalytic magnesium coordination of an enzyme can be utilised to generate large quantities of a valuable metabolite.
Enzyme engineering represents an emerging technology with the potential to deliver solutions to many sustainable development problems [1, 2]. Biofuel production, plastic degradation and the clean generation of industrial reagents and precursors are three examples of areas where enzymes already make a significant contribution [3–6]. With a comprehensive understanding of enzyme mechanism still lacking, research into the fundamentals of enzyme catalysis is of great interest. Phosphoryl transfer enzymes are at the forefront of research models for investigating the origins of enzyme catalysis because they exhibit some of the largest enzymatic rate enhancements known [7–8]. In addition, phosphate esters are often covalently incorporated into pharmaceutical products to improve bioavailability [9–10].

β-phosphoglucomutase (βPGM; EC 5.4.2.6) has emerged as an archetypal enzyme in the study of phosphoryl transfer, and substantial progress has been made in understanding its mechanism of catalysis [11–16]. This magnesium-dependent enzyme from Lactococcus lactis (subspecies lactis IL1403) catalyses the isomerisation between β-glucose 1-phosphate (βG1P) and glucose 6-phosphate (G6P) via a β-glucose 1,6-phosphate (βG16BP) intermediate, which is released to solution before rebinding in the alternate orientation (Figure 1) [11, 17]. The βG1P substrate of βPGM is commercially unavailable, but appropriate quantities for research have been produced enzymatically from maltose using a simple method involving maltose phosphorylase [18]. To initiate the catalytic cycle, βPGM requires priming with a phosphorylating agent to generate the active phospho-enzyme (βPGM\(^\text{p}\), phosphorylated on residue D8) and βG16BP performs this role primarily in vivo. Since βG16BP is also commercially unavailable, alternative phosphorylating agents such as acetyl phosphate (AcP), fructose 1,6-bisphosphate (F16BP) and α-glucose 1,6-bisphosphate (αG16BP) have been used to generate βPGM\(^\text{p}\) in vitro, but these compounds are less effective and produce complicated kinetic behaviour [12, 19].

βG16BP has also been identified as a potential pharmacological chaperone for the management of a human congenital disorder of glycosylation involving phosphomannomutase 2 [20]. Acting as a weakly binding competitive inhibitor, βG16BP is able to rescue the compromised activity of pathological variants of phosphomannomutase 2 by stabilising the protein fold. Therefore, further investigations of
phosphomannomutase 2 and of βPGM are reliant on the availability of substantial quantities of βG16BP. Three strategies have been reported previously for the synthesis of βG16BP; however, each of these methods either delivers low yields or uses chemicals and procedures with significant environmental impacts. Firstly, the chemical synthesis of βG16BP from α-glucose involves an eight step protocol [12], requiring considerable time and technical expertise, together with the use of harmful and environmentally hazardous reagents. Low yields are obtained, since the β-anomer must be selected carefully on the basis of solubility from a racemic mixture of glucosaccharide products. Secondly, an enzymatic production method utilises a non-native reaction of phosphofructokinase to generate βG16BP from βG1P using adenosine triphosphate as the phosphoryl donor [17, 20]. Purification of the product, though, cannot be achieved simply using precipitation procedures, since contaminating adenosine diphosphate co-precipitates with βG16BP [21] and therefore ion-exchange HPLC purification is required. The use of HPLC columns is inherently damaging to the environment owing to the use of triethylammonium bicarbonate as a volatile buffer mobile phase, which during its production results in enormous quantities of carbon dioxide being released into the atmosphere as a greenhouse gas [22]. Thirdly, an extraction method involves the removal of βG16BP from a variant of βPGM that co-purifies with a stoichiometric quantity of the molecule [18]. This method suffers from low yields, since it relies on very high recombinant βPGM production levels, and requires a week-long protein growth and purification procedure for each new batch of βG16BP. The limited availability of βG16BP therefore represents a significant barrier to the structural, kinetic and therapeutic investigations of phosphomutase enzymes. Herein, we describe a room-temperature, enzymatic method using the D170N variant of βPGM (βPGM_D170N) for the production of 100% anomer-specific βG16BP, which requires only micromolar quantities of enzyme and a simple environmentally considerate purification procedure that can be performed easily by a non-chemist over the course of two days. More generally, this enzymatic synthesis strategy illustrates how manipulation of catalytic magnesium coordination can be utilised to generate large quantities of a valuable metabolite.
βPGM has two phosphoryl transfer steps in its catalytic cycle: Step 1 comprises phosphoryl transfer from βPGM\(^p\) to the βG1P substrate forming the βG16BP reaction intermediate, whereas Step 2 involves phosphoryl transfer from βG16BP to βPGM forming the G6P product and regeneration of βPGM\(^p\) (Figure 1). When wild-type βPGM (βPGM\(_{WT}\)) is incubated in the presence of Mg\(^{2+}\) ions, with 20 mM AcP as the phosphorylating agent and 10 mM βG1P as a substrate, βG16BP generated in the catalytic cycle does not accumulate to detectable levels when monitored using \(^{31}\)P NMR experiments [18]. Instead, βG16BP rebinds the enzyme with micromolar affinity in the alternate orientation, for the Step 2 reaction. Thus, the tight binding and high reactivity of βG16BP maintains a low steady state concentration, which precludes the harvesting of this species in useful quantities. The crystal structures of substrate-free βPGM\(_{WT}\) (PDB: 6YDL; [23]) and of the βPGM\(_{WT}\)^p analogue complex (βPGM\(_{WT}\):BeF\(_3\) complex, PDB: 2WFA; [15]) indicate that the catalytic magnesium ion (Mg\(_{cat}\)) is coordinated through three enzyme atoms in the former and four phospho-enzyme atoms in the latter (Figure 2). Therefore, the differential coordination and affinity of Mg\(_{cat}\) provides an appropriate target with which to manipulate βPGM to shift the balance in the rates of Step 1 and Step 2 so that βG16BP will accumulate to a greater extent. Two potential strategies emerged where the rate of Step 2 could be retarded with respect to the rate of Step 1, which involved either performing the reactions of the catalytic cycle under Mg\(^{2+}\)-free conditions or perturbing Mg\(_{cat}\) coordination through point mutation to alter its binding properties. In either scenario, it was hypothesised that βPGM with a compromised Mg\(_{cat}\) site could be phosphorylated efficiently by reactive phosphorylating agents such as AcP, thereby generating βPGM\(^p\) and subsequent reaction with βG1P to produce βG16BP in Step 1 (Figure 1). In contrast, phosphorylation of βPGM by βG16BP in Step 2 is less likely under these circumstances, which would lead to an accumulation of the reaction intermediate that could be harvested.

To explore whether AcP is able to phosphorylate Mg\(_{cat}\)-free βPGM\(_{WT}\), \(^{31}\)P NMR experiments were acquired to measure the change in AcP concentration over time in the presence and absence of 300 μM βPGM\(_{WT}\). The addition of βPGM\(_{WT}\) resulted in a 25% increase in the rate of AcP hydrolysis (Figure 3A), implying that βPGM\(_{WT}\)^p is generated in the absence of Mg\(_{cat}\). Consequently, the Step 1 reaction
between Mg\textsubscript{cat}-free βPGM\textsubscript{WT} (300 μM) and 10 mM βG1P in the presence of 20 mM AcP together with the Step 2 production of G6P was monitored using \(^{31}\)P NMR time-course experiments. However, there was no detectable accumulation of βG16BP (Figure 4A–C) and the appearance of G6P product proceeded with a rate constant of 6.7 × 10^{-3} s\(^{-1}\), which is 4 orders of magnitude slower than the rate constant observed in the presence of 5 mM MgCl\(_2\) [18]. Hence, the observed enzymatic activity appears to arise simply due to the presence of very low levels of residual Mg\(^{2+}\) ions associated with the reagents. Taken together, these results indicate that both Mg\textsubscript{cat}-bound βPGM\textsubscript{WT}\(^p\) and Mg\textsubscript{cat}-free βPGM\textsubscript{WT}\(^p\) can be generated by AcP, but both the Step 1 and Step 2 phosphoryl transfer reactions are seriously impaired by the absence of Mg\textsubscript{cat}.

Given the low rate of βG16BP production in the absence of Mg\(^{2+}\) ions in the reaction buffer, a more subtle modification of the enzyme Mg\textsubscript{cat} site was engineered. In βPGM\textsubscript{WT}, Mg\textsubscript{cat} is coordinated octahedrally by a carboxylate oxygen atom of residue D8, a carboxylate oxygen atom of residue D170 and the carbonyl oxygen atom of residue D10, together with three water molecules. In βPGM\textsubscript{WT}\(^p\), one of the water molecules (water 3) is displaced by a phosphate oxygen atom of the D8 aspartyl phosphate moiety, creating bidentate coordination of Mg\textsubscript{cat} in a six-membered ring of atoms (Figure 2). Point mutations involving residue D8 have been reported to result in the complete loss of measurable catalytic activity [19]. Therefore, perturbation of Mg\textsubscript{cat} was achieved through the generation of the D170N variant (βPGM\textsubscript{D170N}), where the carboxamide group of residue N170 retains an oxygen atom with which to coordinate Mg\textsubscript{cat}, but the sidechain is not charged. Accordingly, the reaction of βPGM\textsubscript{D170N} with 10 mM βG1P and 20 mM AcP in the presence of 100 mM MgCl\(_2\) was monitored using \(^{31}\)P NMR time-course experiments and in contrast to βPGM\textsubscript{WT}, the βG16BP intermediate was observed to accumulate to a level comparable with the initial βG1P concentration (Figure 4D–E). The G6P product was only generated to a measurable extent once the AcP concentration had reduced significantly (Figure 4F). Hence, perturbation of Mg\textsubscript{cat} in βPGM\textsubscript{D170N} (in the presence of excess AcP) results in a retardation in the rate of phosphorylation of βPGM\textsubscript{D170N} by βG16BP (Step 2) with respect to the rate of phosphorylation of βG1P by βPGM\textsubscript{D170N}\(^p\) (Step 1), therefore allowing βG16BP to accumulate.
Further NMR time-course experiments were conducted to assess whether the βG16BP accumulation could be enhanced through changes in the concentration of Mg$^{2+}$ ions. At 5 mM MgCl$_2$ the accumulated βG16BP was converted to G6P more rapidly than at 100 mM MgCl$_2$ (Figure 4G–H). The combined G6P peak intensities (αG6P and βG6P peaks) at maximum βG16BP accumulation, although a relatively crude metric, corresponded to 174% of the βG16BP peak intensity at 5 mM MgCl$_2$ and only 15% at 100 mM MgCl$_2$. The observation that βPGM$_{D170N}$ is active at similar MgCl$_2$ concentrations to βPGM$_{WT}$ is supported by analysis of the initial rates of reaction for the conversion of βG1P to G6P by βPGM$_{D170N}$ at increasing concentrations of MgCl$_2$ (Figure 3B). This experiment resulted in an apparent $K_m$ for Mg$^{2+}$ of 690 ± 110 µM, which is only 4 times higher than that determined for βPGM$_{WT}$ (apparent $K_m$ = 180 ± 40 µM). Hence, for the enzyme form involved in the rate-limiting step, the Mg$_{cat}$ binding affinity has not been disrupted substantially by the removal of the negative charge from the coordinating sidechain of residue D170, although the observed catalytic rate of βPGM$_{D170N}$ ($k_{cat} = 0.0086$ s$^{-1}$) is reduced 30,000-fold with respect to βPGM$_{WT}$ ($k_{cat} = 285$ s$^{-1}$) under the same conditions. The accumulation of βG16BP at higher concentrations of MgCl$_2$ therefore suggests that the affinity of βPGM$_{D170N}$ for Mg$_{cat}$ is not the predominant factor influencing this observation. To ascertain whether the effect is instead caused by Cl$^{-}$ ions, experiments were conducted in which the reaction of βPGM$_{D170N}$ with βG1P and AcP was monitored in the presence of 200 mM sodium chloride and 5 mM magnesium acetate. The conversion of βG16BP to G6P progressed at a similar rate to the experiment performed in the absence of NaCl (Figure 4I), indicating that the accumulation of βG16BP is a MgCl$_2$-dependent phenomenon.

One plausible explanation for these observations is that in substrate-free βPGM$_{D170N}$, the loss of the negative charge from the sidechain of residue D170 could be mitigated by the binding of a Cl$^{-}$ ion in the active site of the enzyme, with the displacement of a water molecule (water 3 in Figure 2B) to confer charge balance in the presence of Mg$_{cat}$. In this scenario, the Cl$^{-}$ ion in turn can bind a separate Mg$^{2+}$ ion (with attendant counter ions) occupying the position adjacent to the nucleophilic oxygen of residue D8 and analogous to the position of the phosphorus atom of βPGM$^P$ (Figure 2A). In this arrangement a six membered ring is formed consisting of both carboxylate oxygen atoms of residue D8, Mg$_{cat}$, a
Cl\(^{-}\) ion and a Mg\(^{2+}\) ion (a Mg\(_{\text{cat}}\)–Cl–Mg moiety), mimicking the to the BeF\(_3\)\(^{-}\) and AlF\(_4\)\(^{-}\) moieties, which are representative of the ground state and transition state of \(\beta\)PGM\(^{\text{P}}\) hydrolysis, respectively [13, 15]. This hypothesis is supported by the observation of an analogous enzyme-bound fluoride species in \(^{19}\)F NMR experiments conducted with \(\beta\)PGM\(_{\text{WT}}\) (Figure 4J–K), in which the chemical shift difference between free F\(^{-}\) and MgF\(^{+}\) is similar to that between MgF\(^{+}\) and the putative enzyme-bound Mg\(_{\text{cat}}\)–F–Mg moiety, the chemical shift of which is comparable to that of the bridging F\(^{-}\) atom in the \(\beta\)PGM\(_{\text{WT}}\)-MgF\(_3\)-G6P complex [24]. A weakly bound Mg\(_{\text{cat}}\)–Cl–Mg moiety would impede phosphorylation of \(\beta\)PGM\(_{\text{D170N}}\) by \(\beta\)G16BP in Step 2. In contrast, the reaction of \(\beta\)PGM\(_{\text{WT}}\) with AcP proceeds in the absence of Mg\(_{\text{cat}}\), generating \(\beta\)PGM\(_{\text{WT}}\)\(^{\text{P}}\) (Figure 3A). The presence of the D8 aspartyl phosphate moiety will prevent the formation of the Mg\(_{\text{cat}}\)–Cl–Mg moiety and will itself facilitate the coordination of Mg\(_{\text{cat}}\), thus enabling reaction with \(\beta\)G1P in Step 1. The binding of a Mg\(_{\text{cat}}\)–Cl–Mg moiety by \(\beta\)PGM\(_{\text{D170N}}\) therefore represents a mechanism by which the rate of Step 2 of the catalytic cycle can be retarded compared to the rate of Step 1, and result in the accumulation of the \(\beta\)G16BP intermediate. The generation of \(\beta\)G16BP by the \(\beta\)PGM\(_{\text{D170N}}\)-catalysed reaction at high concentrations of MgCl\(_2\) therefore presented an opportunity for harvesting significant quantities of this valuable compound and a purification protocol was devised.

Recombinant \(\beta\)PGM\(_{\text{D170N}}\) is overexpressed in high yields from *Escherichia coli* BL21(DE3) cells (>100 mg/L) using routine culture techniques and is readily purified using a two-step protocol involving ion-exchange chromatography followed by a size-exclusion chromatography step (see materials and methods). \(\beta\)PGM\(_{\text{D170N}}\) can be stored at −20 \(^{\circ}\)C for long periods and responds well to multiple freeze-thaw cycles, meaning that once purified, a batch of enzyme can be used for numerous \(\beta\)G16BP preparations. \(^{31}\)P NMR time-course experiments were used to monitor the reaction between \(\beta\)PGM\(_{\text{D170N}}\) and \(\beta\)G1P to determine the optimal point at which to harvest \(\beta\)G16BP. In a representative reaction (15 mL) containing 20 \(\mu\)M \(\beta\)PGM\(_{\text{D170N}}\), 20 mM \(\beta\)G1P, 40 mM AcP, 100 mM MgCl\(_2\) in 200 mM K\(^{+}\) HEPES buffer, a maximum concentration of \(\beta\)G16BP was reached after 265 min at 25 \(^{\circ}\)C. The reaction was quenched by heat-denaturation of \(\beta\)PGM\(_{\text{D170N}}\) at 90 \(^{\circ}\)C for 10 min. Precipitated enzyme was pelleted
using centrifugation (Sigma Model 3-15) and the βG16BP-rich supernatant was collected and filtered with a Vivaspin (5 kDa molecular weight cut off; Sartorius) using a benchtop centrifuge (Thermo Scientific Heraeus Labofuge 400 R).

The resulting enzyme-free solution contained βG16BP alongside contaminants that included significant amounts of βG1P, G6P and inorganic phosphate (Pᵢ), in a ratio of 1 : 0.07 : 0.2 : 3.9, respectively. As substrates of βPGM, these phosphorylated impurities are undesirable, therefore the solution was subjected to a barium salt precipitation protocol to obtain βG16BP with high purity. Barium salts of phosphate species are relatively insoluble [25], and the difference in relative solubility of the βG16BP barium salt compared with those of βG1P and G6P was exploited to enable further purification [26–28]. The solution was passed through a 20 x 10 mm column packed with IR120 (H⁺) ion-exchange resin, which had been washed with 15 mL of milliQ water. This step acidified the solution, which was then neutralised using 0.2 M barium hydroxide solution, resulting in significant precipitation. The solution was kept on ice during neutralisation to increase the solubility of the mono-phosphorylated glucosaccharide barium salts [29]. Fractions obtained along the course of the barium salt formation were analysed using ³¹P NMR experiments, which indicated that the βG16BP barium salt was contained mainly in the precipitate, and that the βG1P and G6P barium salts remained in solution. The precipitate was pelleted using centrifugation at 4 °C (4,500 rpm, Thermo Scientific Heraeus Labofuge 400 R) and the supernatant was discarded. Conversion of the βG16BP barium salt to the more soluble sodium salt involved resolubilising the pellet in a large volume (~1 L) of cold milliQ water and passing the resulting solution through a 20 x 10 mm column packed with IR120 (Na⁺) ion-exchange resin. The flow-through was then frozen at −80 °C and lyophilised to leave a fine powder as the final βG16BP product.

To confirm the identity and assess the purity of the final βG16BP product, a sample of the fine powder was resolubilised in 100% ²H₂O containing 1 mM sodium trimethylsilyl propionate (TSP) as a reference, and analysed using ¹H, ¹³C and ³¹P NMR experiments (Figure 5). The identity of the resulting compound was established to be βG16BP by comparison of ¹H and ¹³C chemical shifts with previously reported values [12]. Glucose and maltose contaminants were identified in the sample using ¹H chemical
shifts and scalar coupling constants (BMRB: bmse000015, BMRB: bmse000017). Based on integral values of the anomic proton signals and of the phosphorus signals in quantitative $^1$H and $^{31}$P NMR spectra, the $\beta$G16BP concentration was determined to be 67 mM, which represented 98% of the total phosphorylated glucosaccharide components and 72% of the total glucosaccharide components present in the final sample. $\beta$G1P, G6P and glucose comprised <1%, 1% and 3%, respectively, of the total glucosaccharide content. Maltose was present at a greater concentration in the sample (24%), but as a bystander in the reactions of $\beta$PGM, and not known to bind to phosphomannomutase 2, this contamination is unlikely to be problematic for users. $P_i$ was also present at a concentration 2.9 times higher than that of $\beta$G16BP. The glucose, maltose and $P_i$ components, which were carried through into the final $\beta$G16BP product are contaminants derived from the enzymatic synthesis of $\beta$G1P and would otherwise not be present if a purer source of $\beta$G1P were used. Residual HEPES buffer and acetate were also present as minor contaminants. The final yield for the $\beta$PGM$_{D170N}$-catalysed conversion of $\beta$G1P to $\beta$G16BP was 33.6% and the yield for the overall conversion of maltose to $\beta$G16BP was 7.7%. Since the equilibrium for the enzymatic conversion of maltose to $\beta$G1P lies in favour of maltose, conducting the reactions for the maltose phosphorylase synthesis of $\beta$G1P and the $\beta$PGM$_{D170N}$ synthesis of $\beta$G16BP in a one-pot system is likely to lead to higher $\beta$G16BP yields. The removal of $\beta$G1P by $\beta$PGM$_{D170N}$ would drive the maltose reaction to produce more $\beta$G1P, which in turn would result in a greater overall yield of $\beta$G16BP. This approach has been demonstrated previously for the protocol involving maltose phosphorylase and phosphofructokinase [17]. The removal of $\beta$G1P by $\beta$PGM$_{D170N}$ drives the maltose reaction to produce more $\beta$G1P, which in turn results in a greater overall yield of $\beta$G16BP. To demonstrate the biochemical effectiveness of the final $\beta$G16BP product at activating $\beta$PGM$_{WT}$, a kinetic experiment was conducted using a glucose 6-phosphate dehydrogenase coupled assay (see Materials and Methods). $\beta$PGM$_{WT}$ was mixed with the $\beta$G1P substrate and activated using either 1 $\mu$M $\beta$G16BP or 8 mM AcP as the phosphorylating agent. The kinetic profile obtained was linear for the $\beta$G16BP-containing reaction, but exhibited a lag phase when AcP was used (Figure 3C). As $\beta$G16BP is the only
phosphorylating agent known to induce linear initial kinetics in βPGM [23], this experiment provided a clear demonstration of the activity of the final βG16BP product.

The successful manipulation of βPGM behaviour to facilitate βG16BP production is a demonstration of how detailed structural and mechanistic knowledge of an enzyme can lead to novel engineering strategies. Specifically, the modification of the metal binding site of the enzyme dramatically increases the steady state concentration of its reactive metabolite. This highlights the transformative potential that enzymes have within chemical industries and vindicates the intensive study of these useful biomolecules.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We would like to thank Prof. Nicholas Williams for useful insight into βG16BP purification. This research was supported by the Biotechnology and Biological Sciences Research Council (BBSRC; H.P.W. – Grant Number X/009906-20-26, N.J.B. – Grant Number BB/M021637/1 and BB/S007965/1, C.R.T – Grant Number BB/P007066/1), Consejo Nacional de Ciencia y Tecnologia, Mexico (CONACYT; F.A.C.N. – Grant Number 472448).
Materials and Methods

Reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich, Fischer Scientific, Alfa Aesar and VWR. Isotopically enriched $^{15}\text{NH}_4\text{Cl}$ was purchased from CortecNet.

Gene sequence for $\beta$PGM$_{D170N}$.

1-CATATGTTTAAGCAGTATTGTTTAGATGGTGTAATTACAGATACCGCAGAGTATCATTTTAGAGCTTGG-75
76-AAAGCTTTTGCTGAAGAAATTTGCCCATTAATGGTGACCCCAAATTTAATGAGCAATTAAAAGGGGTCACAAG-150
151-GAAGACTCGCTTCAGAAAAATTCTAGATTTAGCTGATAAAAAGTAGAATCTGAGGAAATTTTTATTAGAG-225
226-AGAAAAATGATAACTATGTGAAAAATGATTCAGGATGTGTCGCCAGCCGATGCTATCCTGGAATTTTACAATA-300
301-CTCAAAAGATTTACGTTCAAAATAAATAAAAATCCAAATTTAGCTTTAGCCTTTGCTTTGCTCTAAGATGTTGCGC-375
376-AGAAATGATTTAAGTGAATTGTCGATACTGCGATCCGGCTGAAAGTTGCAGCATCAAAACCAGCACCAGAT-450
451-ATTTTTTATTTGAGCAGACATGCAGTTGGTGGTTGCCCTCTCTGATATTCAATGTTAGGATTTTATTAGAG-525
526-ATTCAACGCTATCAAAGATTCAGGGGCTTTACCAATTGGTGTAGGGCGCCCAGAAGATTTGGGAGATGATATCGTC-600
601-ATTGTGCTGTACACTCTCAGACTATACATTAGAATTAGTTTGGCTTCAAAGCAAAAAATAACTCGAG-675

$\beta$PGM expression and purification

The $\beta$PGM$_{D170N}$ gene sequence was created by modifying the pgmB gene (encoding the $\beta$PGM$_{WT}$ enzyme) from Lactococcus lactis (NCBI: 1114041). The $\beta$PGM$_{D170N}$ gene was generated and cloned by GenScript into a pET22b(+) vector. The $\beta$PGM$_{WT}$ and $\beta$PGM$_{D170N}$ plasmids were transformed into Escherichia coli BL21(DE3) cells and grown using $^{15}$N isotopically enriched M9 minimal media [30]. Cells were grown to an OD$_{600}$ of 0.6 at 37 °C and overexpression was induced with IPTG (0.5 mM final concentration) before a 16-hour incubation at 25 °C and centrifugation (Sigma Model 3-15; 9,000 rpm for 10 min) to harvest the cells. The $\beta$PGM$_{WT}$ and $\beta$PGM$_{D170N}$ proteins were purified using the following protocol. The cell pellet was resuspended in ice cold standard buffer (50 mM K$^+$ HEPES buffer (pH 7.2), 5 mM MgCl$_2$, 2 mM NaN$_3$, 1 mM EDTA) containing a cOmplete protease inhibitor cocktail. The cell suspension was sonicated on ice for 6 × 20 s pulses separated by 60 s intervals. The cell lysate was separated from the insoluble cell debris using centrifugation (Beckman Coulter Avanti centrifuge,
Rotor: JA-25-50) at 20,000 rpm for 30 min at 4 °C. The soluble fraction was loaded onto a DEAE-Sepharose anion-exchange column connected to an ÄKTA Prime purification system, which had been washed previously with 1 M NaOH and 6 M guanidinium chloride and equilibrated with 5 column volumes of standard buffer. Bound proteins were eluted using a gradient of 0 to 50% standard buffer supplemented with 1 M NaCl over 300 mL. Fractions containing βPGM were identified using SDS-PAGE and were concentrated to a 5–10 mL volume using centrifugation in a Vivaspin (10 kDa molecular weight cut off; Sartorius) at 4,500 rpm and 4 °C (Thermo Scientific Heraeus Labofuge 400 R). The concentrated protein sample was loaded onto a pre-packed HiLoad 26/60 Superdex 75 size-exclusion column connected to an ÄKTA Prime purification system, which had been washed previously with degassed 1 M NaOH and equilibrated with 3 column volumes of degassed standard buffer supplemented with 1 M NaCl. Following elution, the fractions containing βPGM were checked for purity and were pooled and buffer-exchanged into standard buffer for βPGM<sub>WT</sub> and standard buffer containing 50 mM MgCl<sub>2</sub> for βPGM<sub>D170N</sub>. Mg<sup>2+</sup>-free βPGM<sub>WT</sub> was prepared by buffer-exchanging into standard buffer without MgCl<sub>2</sub>. The final protein samples were concentrated using a Vivaspin (10 kDa molecular weight cut off; Sartorius) to a 1 mM concentration, as measured by Nanodrop OneC (Thermo Scientific) (βPGM molecular weight = 24.2 kDa, ε<sub>280</sub> = 19,940 M<sup>-1</sup> cm<sup>1</sup>), and were stored at −20 °C.

NMR Spectroscopy

All NMR spectra were acquired at 298 K, unless otherwise stated. The AcP hydrolysis experiments and the reactions involving the βPGM-catalysed conversion of βG1P to G6P were monitored with <sup>31</sup>P time-course experiments acquired, apart from where stated otherwise, without proton-phosphorus decoupling using a Bruker 500 MHz Avance II spectrometer (operating at 202.456 MHz for <sup>31</sup>P) equipped with a 5-mm room-temperature broadband probe and running TopSpin version 3.5. Samples were prepared in 200 mM K<sup>+</sup> HEPES buffer (pH 7.2) containing 10% <sup>2</sup>H<sub>2</sub>O (v/v) and 1 mM TSP. <sup>19</sup>F experiments were recorded at 278 K using a Bruker 500 MHz Avance I spectrometer (operating at 470.59 MHz for <sup>19</sup>F) equipped with a 5-mm dual <sup>1</sup>H/<sup>19</sup>F probe. Samples were prepared in 50 mM K<sup>+</sup> HEPES buffer (pH 7.2)
containing 5 mM MgCl₂, 10 mM NH₄F, 10% ²H₂O (v/v) and 1 mM TSP. For the final βG16BP product prepared in 100% ²H₂O containing 1 mM TSP, ¹H and natural abundance ¹H/¹³C-HSQC experiments were recorded using standard Bruker pulse sequences on an 800 MHz Bruker Neo spectrometer with a 5-mm TCI cryoprobe equipped with z-axis gradients and running TopSpin (Bruker) version 4.0. ³¹P experiments were also recorded for this sample, as described above. ¹H and ¹³C chemical shifts were referenced to TSP resonating at 0.0 ppm. ³¹P experiments were either referenced to 1 M HPO₃ resonating at 0.0 ppm, sealed inside a glass capillary and inserted into the sample NMR tube or were referenced indirectly to TSP using the gyromagnetic ratios of the ¹H and ³¹P nuclei. ¹⁹F experiments were referenced indirectly to TSP using the gyromagnetic ratios of the ¹H and ¹⁹F nuclei. NMR data were processed with baseline correction and Lorentzian apodisation using either FELIX (Felix NMR, Inc.) or TopSpin version 4.0 (Bruker). Quantitative NMR experiments were performed using a recycle delay of 60 s.

Kinetic Assays

Kinetic assays for βPGMWT were conducted using a glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) coupled assay. Here, the G6P product of βPGMWT activity is oxidised to 6-phospholactone by G6PDH, while the concomitant reduction of NAD⁺ to NADH is monitored by measuring absorbance at 340 nm (ε₃₄₀ = 6220 M⁻¹ cm⁻¹). Reactions were run at 25 °C using a FLUOstar OMEGA microplate reader (BMG Labtech). To measure the MgCl₂ dependence of the βPGMWT- and βPGMD170N-catalysed conversion of βG1P to G6P, reactions (160 µL) were conducted in 200 mM K⁺ HEPES buffer (pH 7.2) containing different concentrations of MgCl₂ (0, 0.1, 0.3, 0.6, 1.0, 1.5, 2.5, 5, 10, 20, 50 and 100 mM), 1 mM NAD⁺, 5 U/mL G6PDH, 1 mM βG1P and either 1 nM βPGMWT with 100 µM βG16BP, or 10 µM βPGMD170N with 1250 µM βG16BP. Initial rates of the reactions were obtained from the linear steady-state region of the kinetic profiles and were fit to Equation 1 using an in-house non-linear least-squares fitting program, which uses bootstrap error estimation.
\[
v_0 = \frac{V_{\text{max}}[\text{MgCl}_2]}{K_{m(\text{app})} + [\text{MgCl}_2]\left(1 + \frac{[\text{MgCl}_2]}{K_i}\right)} \tag{1}
\]

Where \(K_{m(\text{app})}\) represents the apparent Michaelis constant for activation of the enzyme by \(\text{Mg}^{\text{cat}}\), and \(K_i\) represents the inhibition constant of the enzyme for \(\text{MgCl}_2\). To measure the effect of different phosphorylating agents on the \(\beta\text{PGM}_{\text{WT}}\)-catalysed conversion of \(\beta\text{G1P}\) to \(\text{G6P}\), reactions (160 \(\mu\)L) were conducted in 200 mM \(\text{K}^+\) HEPES buffer (pH 7.2) containing 5 nM \(\beta\text{PGM}_{\text{WT}}\), 50 \(\mu\)M \(\beta\text{G1P}\), 5 mM \(\text{MgCl}_2\), 1 mM NAD\(^+\) and 5 U/mL \(\text{G6PDH}\) with either 1 \(\mu\)M of the final \(\beta\text{G16BP}\) product or 8 mM \(\text{AcP}\) as the phosphorylating agent. Blank absorbance measurements were taken on solutions identical to the reaction mixtures, without the addition of \(\beta\text{PGM}_{\text{WT}}\).

\(\beta\text{G1P}\) preparation

\(\beta\text{G1P}\) was prepared enzymatically from maltose using maltose phosphorylase (EC 2.4.1.8). A solution of 611 mM maltose was incubated overnight with 1.2 U/mL maltose phosphorylase in 0.5 M sodium phosphate buffer (pH 7.0) at 30 °C. The production of \(\beta\text{G1P}\) was confirmed using \(^{31}\text{P}\) NMR spectroscopy. Maltose phosphorylase (molecular weight = 90 kDa) was removed from the solution by centrifugation using a Vivaspin (5 kDa molecular weight cut off; Sartorius). The concentration of \(\beta\text{G1P}\) in the flow-through was measured to be 148.6 mM using quantitative \(^{31}\text{P}\) NMR experiments in which a known amount of G6P had been added to a sample of the \(\beta\text{G1P}\) product, along with 10\% \(^2\text{H}_2\text{O} (v/v)\) and 1 mM TSP. This concentration represents a yield of 24.3\%. The \(\beta\text{G1P}\) product was contaminated with glucose, maltose and \(\text{P}_i\) (estimated concentrations were 150 mM, 850 mM and 350 mM, respectively), and was not purified further since these compounds are bystanders in the reaction catalysed by \(\beta\text{PGM}\).
References

1. Huang, P.-S., Boyken, S. E., Baker, D. (2016) The coming of age of de novo protein design. *Nature* 357, 320–327.

2. Chen, K., Arnold, F.H. (2020) Engineering new catalytic activities in enzymes. *Nat. Catal.* 3, 203–213.

3. Bolon, D. N., Mayo, S. L. (2001) Enzyme-like proteins by computational design. *Proc. Natl Acad. Sci. U. S. A.* 98, 14274–14279.

4. Wen, F., Nair, N. U., Zhao, H. (2009) Protein engineering in designing tailored enzymes and microorganisms for biofuels production. *Curr. Opin. Biotechnol.* 20, 412–419.

5. Zanghellini, A. (2014) De novo computational enzyme design. *Curr. Opin. Biotechnol.* 29, 132–138.

6. Yoshida, S., Hiraga, K., Takehana T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., Oda, K. (2016) A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science* 351, 1196–1199.

7. Wolfenden, R., Snider, M. J. (2001) The depth of chemical time and the power of enzymes as catalysts. *Acc. Chem. Res.* 34, 938–945.

8. Lad, C., Williams, N. H., Wolfenden, R. (2003) The rate of hydrolysis of phosphomonoester dianions and the exceptional catalytic proficiencies of protein and inositol phosphatases. *Proc. Natl Acad. Sci. U. S. A.* 100, 5607–5610.

9. Jones, S., Selitsianos, D. (2002) A simple and effective method for phosphoryl transfer using TiCl₄ catalysis. *Org. Lett.* 4, 3671–3673.

10. Jones, S., Selitsianos, D., Thompson K. J., Toms, S. M. (2003) An improved method for Lewis acid catalyzed phosphoryl transfer with Ti(t-BuO)₄. *J. Org. Chem.* 68, 5211–5216.
11. Dai, J., Finci, L., Zhang, C., Lahiri, S., Zhang, G., Peisach, E., Allen, K. N., Dunaway-Mariano, D. (2009) Analysis of the structural determinants underlying discrimination between substrate and solvent in β-phosphoglucomutase catalysis. *Biochemistry* 48, 1984–1995.

12. Goličnik, M., Olguin, L. F., Feng, G., Baxter, N. J., Waltho, J. P., Williams, N. H., Hollfelder, F. (2009) Kinetic analysis of β-phosphoglucomutase and its inhibition by magnesium fluoride. *J. Am. Chem. Soc.* 131, 1575–1588.

13. Baxter, N. J., Bowler, M. W., Alizadeh, T., Cliff, M. J., Hounslow, A. M., Wu, B., Berkowitz, D. B., Williams, N. H., Blackburn, G. M., Waltho, J. P. (2010) Atomic details of near-transition state conformers for enzyme phosphoryl transfer revealed by MgF$_3^-$ rather than by phosphoranes. *Proc. Natl Acad. Sci. U. S. A.* 107, 4555–4560.

14. Elsässer, B., Dohmeier-Fischer, S., Fels, G. (2012) Theoretical investigation of the enzymatic phosphoryl transfer of β-phosphoglucomutase: revisiting both steps of the catalytic cycle. *J. Mol. Model.* 18, 3169–3179.

15. Griffin, J. L., Bowler, M. W., Baxter, N. J., Leigh, K. N., Dannatt, H. R., Hounslow, A. H., Blackburn, G. M., Webster, C. E., Cliff, M. J., Waltho, J. P. (2012) Near attack conformers dominate β-phosphoglucomutase complexes where geometry and charge distribution reflect those of substrate. *Proc. Natl. Acad. Sci. U. S. A.* 109, 6910–6915.

16. Jin, Y., Bhattasali, D., Pellegrini, E., Forget, S. M., Baxter, N. J., Cliff, M. J., Bowler, M. W., Jakeman, D. L., Blackburn, G. M., Waltho, J. P. (2014) α-Fluorophosphonates reveal how a phosphomutase conserves transition state conformation over hexose recognition in its two-step reaction. *Proc. Natl Acad. Sci. U. S. A.* 111, 12384–12389.

17. Dai, J., Wang, L., Allen, K. N., Rädström, P., Dunaway-Mariano, D. (2006) Conformational cycling in β-phosphoglucomutase catalysis: Reorientation of the β-D-Glucose 1,6-(bis)phosphate intermediate. *Biochemistry* 45, 7818–7824.
18. Johnson, L., Roberston, A. J., Baxter, N. J., Trevitt, C. R., Bisson, C., Jin, Y., Wood, H. P., Hounslow, A. H., Cliff, M. J., Blackburn, G. M., Bowler, M. W., Waltho, J. P. (2018) van der Waals contact between nucleophile and transferring phosphorous is insufficient to achieve enzyme transition-state architecture. *ACS Catal.* 8, 8140–8153.

19. Zhang, G., Dai, J., Wang, L., Dunaway-Mariano, D., Tremblay, L. W., Allen, K. N. (2005) Catalytic cycling in β-phosphoglucomutase: a kinetic and structural analysis. *Biochemistry* 44, 9404–9416.

20. Monticelli, M., Liguori, L., Allocca, M., Andreotti, G., Cubellis, M. V. (2019) β-Glucose-1,6-bisphosphate stabilizes pathological phophomannomutase2 mutants *in vitro* and represents a lead compound to develop pharmacological chaperones for the most common disorder of glycosylation, PMM2-CDG. *Int. J. Mol. Sci.* 20, article number: 4164.

21. Chan, P. S., Black, C. T., Williams, B. J. (1973) Separation of cyclic 3′,5′-AMP from ATP, ADP, and 5′-AMP by precipitation with inorganic compounds. *Anal. Biochem.* 55, 16–25.

22. Carlson, M., Carter, J. D., Rohloff, J. (2015) Improved preparation of 2 M triethylammonium bicarbonate. *Green Chem. Lett. Rev.* 8, 37–39.

23. Wood, H. P., Cruz-Navarrete, F. A., Baxter, N. J., Trevitt, C. R., Robertson A. J., Dix, S. R. Hounslow, A. M., Cliff, M. J. Waltho, J. P. (2020) Allomorphy as a mechanism of post-translational control of enzyme activity. *Nat. Commun.* In press.

24. Baxter, N. J., Olguin, L. F., Goličnik, M., Feng, G., Hounslow, A. M., Bermel, W., Blackburn, G. M., Hollfelder, F., Waltho, J. P., Williams, N. H. (2006) A Trojan horse transition state analogue generated by MgF$_3^-$ formation in an enzyme active site. *Proc. Natl. Acad. Sci. U.S.A.* 103, 14732–14737.

25. Holt, L. E., Pierce, J. A., Kajdi, C. N. (1954) The solubility of the phosphates of strontium, barium, and magnesium and their relation to the problem of calcification. *J. Colloid Sci.* 9, 409–426.
26. Seegmiller, J. E., Horecker, B. L. (1951) The synthesis of glucose-6-phosphate and 6-phosphogluconate. *J. Biol. Chem.* 192, 175–180.

27. Brown, D. M., Usher, D. A. (1965) Hydrolysis of hydroxyalkyl phosphate esters: The epoxide route. *J. Chem. Soc.* 6547–6558.

28. Brown, D. M., Usher, D. A. (1965) Hydrolysis of hydroxyalkyl phosphate esters: Effect of changing ester group. *J. Chem. Soc.* 6558–6564.

29. Posternak, T. (1949) Synthesis of α- and β-glucose-1,6-diphosphate. *J. Biol. Chem.* 180, 1269–1278.

30. Reed, M. A. C., Hounslow, A. M., Sze, K. H., Barsukov, I. G., Hosszu, L. L. P., Clarke, A. R., Craven, C. J., Waltho, J. P. (2003) Effects of domain dissection on the folding and stability of the 43 kDa protein PGK probed by NMR. *J. Mol. Biol.* 330, 1189–1201.
Figure 1. *In vitro* phosphorylation and catalytic cycle of βPGM. AcP phosphorylates βPGM generating βPGMP (phosphorylated on residue D8) in a Mg²⁺-independent reaction (green ink). In the Mg²⁺-dependent catalytic cycle (black ink), Step 1 involves phosphoryl transfer from βPGMP to the βG1P substrate forming the βG16BP intermediate, whereas Step 2 comprises phosphoryl transfer from βG16BP (bound in the alternate orientation) to βPGM forming the G6P product and regeneration of βPGMP. In the phosphorylated glucosaccharide structures, 1-phosphate groups are coloured red and 6-phosphate groups are coloured blue. The black arrows denote the dominant direction of the corresponding reversible reactions. In the absence of the βG1P substrate, βPGMP has a half-life of 30 s [12] and hydrolyses readily to βPGM liberating inorganic phosphate (Pₐ).
Figure 2. Comparison of octahedral Mg\textsubscript{cat} coordination in $\beta$PGM\textsubscript{WT}\textsuperscript{p} (Step 1) and $\beta$PGM\textsubscript{WT} (Step 2).  
(A) A model of $\beta$PGM\textsubscript{WT}\textsuperscript{p} derived from the crystal structure of the $\beta$PGM\textsubscript{WT}:BeF\textsubscript{3} complex (PDB: 2WFA, [15]) showing Mg\textsubscript{cat} coordination. The ligands comprise a carboxylate oxygen atom of residue D170, the carbonyl oxygen atom of residue D10 and two water molecules (indicated by numbers), together with the carboxylate oxygen atom and a phosphate oxygen atom of the D8 aspartyl phosphate moiety, creating bidentate coordination of Mg\textsubscript{cat} in a six-membered ring of atoms.  
(B) The crystal structure of substrate-free $\beta$PGM\textsubscript{WT} (PDB: 6YDL, [23]) showing Mg\textsubscript{cat} coordination. The ligands involve a carboxylate oxygen atom of residue D8, a carboxylate oxygen atom of residue D170, the carbonyl oxygen atom of residue D10 and three water molecules (indicated by numbers). Mg\textsubscript{cat} is depicted as a green sphere, water molecules are illustrated as red spheres and metal ion coordination is shown as black dashes.
Figure 3. Kinetic experiments involving βPGM<sub>WT</sub> and βPGM<sub>D170N</sub>. (A) Effect of βPGM<sub>WT</sub> on the hydrolysis of AcP monitored using 31P NMR time-course experiments. AcP hydrolysis profiles were derived from normalised peak intensities obtained from reactions conducted in 200 mM K<sup>+</sup> HEPES buffer (pH 7.2), 50 mM AcP, without MgCl<sub>2</sub> in the presence (black circles) and absence (white circles) of 300 μM βPGM<sub>WT</sub>. (B) Normalised initial rate measurements for the conversion of βG1P to G6P by either βPGM<sub>WT</sub> (blue circles) or βPGM<sub>D170N</sub> (red circles) at different concentrations of MgCl<sub>2</sub> monitored using a glucose 6-phosphate dehydrogenase coupled assay. Reactions (160 μL) were conducted in 200 mM K<sup>+</sup> HEPES buffer (pH 7.2) containing different concentrations of MgCl<sub>2</sub> (0, 0.1, 0.3, 0.6, 1.0, 1.5, 2.5, 5, 10, 20, 50 and 100 mM), 1 mM NAD<sup>+</sup>, 5 U/mL G6PDH, 1 mM βG1P and either 1 nM βPGM<sub>WT</sub> with 100 μM βG16BP, or 10 μM βPGM<sub>D170N</sub> with 1250 μM βG16BP. The solid lines represent fits of the data points to Equation 1 and black vertical error bars represent the standard error of the mean of three technical replicates. (C) Kinetic profiles for the conversion of βG1P to G6P by βPGM<sub>WT</sub> monitored using a glucose 6-phosphate dehydrogenase coupled assay. Reactions were conducted in 200 mM K<sup>+</sup> HEPES buffer (pH 7.2) containing 5 nM βPGM<sub>WT</sub>, 50 μM βG1P, 5 mM MgCl<sub>2</sub>, 1 mM NAD<sup>+</sup> and 5 U/mL G6PDH with either 1 μM of the final βG16BP product (green circles) or 8 mM AcP (pink circles) as the phosphorylating agent. For clarity, only half of the acquired data points have been included in the figure.
Figure 4. $^{19}$F and $^{31}$P NMR experiments involving $\beta$PGM$_{WT}$ and $\beta$PGM$_{D170N}$. (A–C) The $\beta$PGM$_{WT}$-catalysed conversion of $\beta$G1P to G6P via $\beta$G16BP in the absence of Mg$^{2+}$ ions. (A) Reaction containing 300 $\mu$M $\beta$PGM$_{WT}$ and 10 mM $\beta$G1P in 200 mM K$^+$ HEPES buffer (pH 7.2) without MgCl$_2$, acquired 23 min after the addition of 50 mM AcP. (B) Reaction after 42 min showing the presence of $\beta$G1P and G6P. (C) Reaction after 72 min showing complete conversion of $\beta$G1P to G6P. (D–F) The $\beta$PGM$_{D170N}$-catalysed conversion of $\beta$G1P to G6P, together with the accumulation of $\beta$G16BP. (D) Reaction containing 20 $\mu$M $\beta$PGM$_{D170N}$ and 10 mM $\beta$G1P in 200 mM K$^+$ HEPES buffer (pH 7.2) with 23.6 mM P$_i$ and 100 mM MgCl$_2$, prior to the addition of 20 mM AcP. (E) Reaction after 87 min showing the generation of $\beta$G16BP. (F) Reaction after 1179 min showing complete conversion of $\beta$G1P to G6P. (G–I) The $\beta$PGM$_{D170N}$-catalysed conversion of $\beta$G1P to G6P together with the accumulation of $\beta$G16BP under variable ion concentrations. (G) Reaction containing 400 $\mu$M $\beta$PGM$_{D170N}$ and 10 mM $\beta$G1P in 200 mM K$^+$ HEPES buffer (pH 7.2) with 5 mM MgCl$_2$, 35 min after the addition of AcP. (H) Reaction containing 400 $\mu$M $\beta$PGM$_{D170N}$ and 10 mM $\beta$G1P in 200 mM K$^+$ HEPES buffer (pH 7.2) with 100 mM MgCl$_2$, 19 min after the addition of AcP. (I) Reaction containing 400 $\mu$M $\beta$PGM$_{D170N}$ and 10 mM $\beta$G1P in 200 mM K$^+$ HEPES buffer (pH 7.2) with 5 mM magnesium acetate and 200 mM NaCl, 36 min after the addition of AcP. The peak at 1.9–2.0 ppm in panels A–I corresponds to inorganic phosphate (P$_i$), which is present in the stocks of both $\beta$G1P and AcP. $^{31}$P chemical shifts were referenced to external 1 M HPO$_3$ = 0.0 ppm, which was sealed inside a glass capillary and inserted into the sample NMR tubes. The samples in G–I were recorded using experiments with proton-phosphorus decoupling in order to simplify the identification of the relevant species. (J–K) $^{19}$F NMR experiments acquired at 5 ºC involving $\beta$PGM$_{WT}$. (J) Control experiment with 5 mM MgCl$_2$ and 10 mM NH$_4$F in 50 mM K$^+$ HEPES buffer (pH 7.2). (K) 1 mM $\beta$PGM$_{WT}$ together with 5 mM MgCl$_2$ and 10 mM NH$_4$F in 50 mM K$^+$ HEPES buffer (pH 7.2). The peak at −119 ppm corresponds to free fluoride (F$^-$) in solution, the peak at −156 ppm corresponds to MgF$^-$ and the peak at −173 ppm in (K) corresponds to an enzyme-bound Mg$_{cat}$–F–Mg moiety that has been assigned on the basis of chemical shift differences between the fluoride-containing species in solution.
Figure 5. NMR experiments recorded on a sample of the final βG16BP product prepared in 100% 2H₂O. (A) ¹H spectrum showing βG16BP and other glucosaccharide species present in the sample. (B) A region of the ¹H spectrum showing the anomeric proton glucosaccharide signals, together with their assignments. (C) ³¹P spectrum showing the two phosphorus signals of βG16BP (6-phosphate, 4.76 ppm (triplet) and 1-phosphate, 2.55 ppm (doublet)) and the signal corresponding to inorganic phosphate (2.70 ppm (singlet), truncated for clarity). (D) Natural abundance ¹H¹³C-HSQC spectra comparing the final βG16BP product (orange) with chemically synthesised βG16BP (blue, [12]). Peaks are labelled with carbon ring atom assignments.
TOC Figure

β-phosphoglucomutase (D170N)

25 °C
~4 hours

β-glucose 1,6-bisphosphate