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Communication

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A Single Point Mutation Converts GH84 *O*-GlcNAc Hydrolases into Phosphorylases. Experimental and Theoretical Evidence.

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Supporting Information Placeholder

ABSTRACT: Glycoside hydrolases and phosphorylases are two major classes of enzymes responsible for the cleavage of glycosidic bonds. Here we show that two GH84 *O*-GlcNAcase enzymes can be converted to efficient phosphorylases by a single point mutation. Noteworthy, the mutated enzymes are over ten-fold more active than naturally occurring glucosaminide phosphorylases. We rationalize this novel transformation using molecular dynamics and QM/MM metadynamics methods, showing that the mutation changes the electrostatic potential at the active site and reduces the energy barrier for phosphorolysis by 10 kcal·mol⁻¹. In addition, the simulations unambiguously reveal the nature of the intermediate as a glucose oxazolinium ion, clarifying the debate on the nature of such reaction intermediate in glycoside hydrolases operating via substrate-assisted catalysis.

Glycoside hydrolases (GHs) are enzymes catalyzing the hydrolysis of glycosidic bonds. They are classified into families according to sequence similarity (<http://www.cazy.org>).¹ Members of a given GH family typically share common reaction mechanism and active site architecture.²⁻⁴ Family GH84 enzymes catalyze the cleavage of the *N*-acetylglucosamine (GlcNAc) attached to Ser/Thr residues on proteins. There is a well-established interplay between protein *O*-GlcNAcylation and phosphorylation,⁵⁻⁶ with *O*-GlcNAcylation modulating both protein phosphorylation and expression in essential signaling pathways.⁶ Animals, plants and some bacteria⁷ possess *O*-GlcNAc transferases and GH84 *O*-GlcNAcases (OGA) for GlcNAc cycling on selected Ser/Thr. These enzymes are thus involved in dynamic protein regulation, an unusual function for carbohydrate-active enzymes.

Given their crucial role in biology, GH84 enzymes have been extensively studied.⁸⁻¹² They follow a mechanism that has been described as *substrate-assisted* or *neighbouring group participation*,¹²⁻¹⁴ which is operated by two consecutive Asp residues (Figure 1a). In the first reaction step, D119 takes a proton from the substrate *N*-acetamido group (NAc), while the oxygen atom of NAc attacks the anomeric carbon to form an oxazoline (Glc-ox) reaction intermediate.¹¹ D120 acts as a general acid/base, assisting leaving group departure by protonating the glycosidic bond oxygen.⁸⁻¹² In the second step, a water molecule, activated by the general acid/base residue, attacks on the anomeric carbon to release the product, GlcNAc.

Figure 1. (a) Substrate-assisted mechanism in GH84 (*TiOGA* numbering). (b) The same mechanism considering an oxazolinium ion intermediate. (c) Hydrolysis and phosphorylation reactions catalyzed by *SpOGA*-D245N and *TiOGA*-D120N.

Glycoside phosphorylases (GPs) catalyze the transfer of an inorganic phosphate to a glycoside acceptor, producing energetically valuable compounds. From mechanistic and structural standpoints, most GPs are similar to hydrolytic GHs.¹⁵ Notably, GPs are found to mingle with glycosyltransferases (families GT4 and 35) and GHs (families GH3, 13, 65, 94, 112, 130, 149)¹⁶⁻¹⁷ rather than forming separate families. The retaining β -GPs reported to date are either β -*N*-acetylglucosaminide phosphorylases¹⁸ or β -glucoside phosphorylase,¹⁹ both of which belong to family GH3 and catalyze phosphorolysis through a glycosyl-enzyme intermediate.¹⁸ Whereas conversion of hydrolytic

GHs into synthetic enzymes is well established,²⁰⁻²¹ including for GHs performing substrate-assisted catalysis,²²⁻²³ there is so far no report of the conversion of a genuine hydrolytic GH into a GP. Here we report such transformation for two different GHs from family GH84.

Interestingly, hydrolases and phosphorylases from GH3 display similar active sites but for their acid/base catalyst (Glu in hydrolases, His in some phosphorylases),^{18-19, 24} suggesting that this position might determine enzyme specificity. This prompted us to mutate the acid/base catalytic residue (D245) in *SpOGA*, a bacterial GH84 from *Streptococcus pyogenes*. However, the D245H mutation led to an enzyme with no phosphorylolytic activity, and severely reduced hydrolysis in 0.1 M phosphate buffer pH 7.8 ($k_{\text{cat}} < 10^{-3} \text{ s}^{-1}$ for *pNP*-GlcNAc cleavage). Conversely, replacement of D245 by Asn led to an enzyme with an unusually high catalytic rate for a catalytic mutant ($k_{\text{cat}} = 2.7 \pm 0.2 \text{ s}^{-1}$). Whereas the wild-type enzyme released exclusively GlcNAc, thin layer chromatography and capillary electrophoresis (CE) indicated the formation of a more polar compound by the *SpOGA*-D245N mutant in phosphate buffer. This product was absent for the catalyzed reactions occurring in HEPES or Tris buffer, with only GlcNAc release (Figure S1). β -GlcNAc-1-phosphate (GlcNAc-1-P) was identified by ¹H NMR as the predominant product of the action of *SpOGA*-D245N on *pNP*-GlcNAc in 50 mM phosphate buffer (Figure 2). At 30 and 100 mM, GlcNAc-1-P represented 55% and 76% of the final mixture, respectively (Figures S3 and S4). At 0.5 M phosphate, GlcNAc formation was not observed by CE, and only GlcNAc-1-P was produced by *SpOGA*-D245N (Figure 2b). Therefore, the D245N mutation changed enzyme activity from hydrolysis to phosphorylolytic.

It is common that GHs with a mutated catalytic residue can partially recover their activity upon addition of an exogenous nucleophile.²⁵ However, phosphate has never been described to act as such, despite being a common buffer component in enzyme reaction mixtures¹⁸ and an intrinsically better nucleophile than water.²⁶ Addition of 0.1 M acetate (the most common anion used for chemical rescue) to a HEPES buffer pH 7.8 did not enhance *pNP*-GlcNAc consumption by *SpOGA*-D245N. Interestingly, the substrate-assisted mechanism in *SpOGA*-D245N is preserved despite the modification of the catalytic residue. Indeed, *SpOGA*-D245N was also able to form GlcNAc-1-P from the reaction intermediate, 2-methyl-(1,2-dideoxy- α -D-glucopyranosyl)[2,1-d]- Δ 2'-oxazoline (Glc-ox), and hydrolyze the formed GlcNAc-1-P (Figure S5). Moreover, it catalyzed phosphorylation of both Glc-ox and *pNP*-GlcNAc at the same rate (Figure S5). Together with the rate enhancement observed upon phosphate addition (Figures S6 and S7, also showing no phosphate saturation for *SpOGA*-D245N phosphorylolytic), this indicates that phosphorylation of Glc-ox is rate-limiting. Therefore, the D245N mutation creates a retaining glycoside phosphorylase (GP) that catalyzes phosphorylolytic through a substrate-assisted mechanism (Figure 1c). To the best of our knowledge, this is the first example of a conversion of a GH into a GP by mutagenesis.

Figure 2. (a) NMR spectrum of phosphorylolytic and hydrolytic products of 7.5 mM *pNP*GlcNAc by 1 $\mu\text{g}\cdot\text{mL}^{-1}$ *SpOGA*-D245N in 50 mM phosphate pH 7.8, after 180 min. (b) Time course of phosphorylolytic and hydrolytic reactions of 7.5 mM *pNP*GlcNAc by 1 $\mu\text{g}\cdot\text{mL}^{-1}$ *SpOGA*-D245N in 50 or 500 mM phosphate pH 7.8, monitored by NMR or CE.

Strikingly, the observed catalytic constant of *SpOGA*-D245N towards *pNP*-GlcNAc in presence of 0.1 M phosphate ($k_{\text{cat}} = 2.7 \text{ s}^{-1}$) is higher than those reported for the natural β -GlcNAc phosphorylases Nag3 and BglP of family GH3 ($k_{\text{cat}}[\text{Nag3}] = 0.15 \text{ s}^{-1}$; $k_{\text{cat}}[\text{BglP}] = 0.03 \text{ s}^{-1}$).¹⁸⁻¹⁹ *SpOGA*-D245N is thus an efficient catalyst superior to the reported natural GPs for the synthesis of GlcNAc-1-P.

The molecular determinants of catalysis of the first *substrate-assisted* phosphorylase, *SpOGA*-D245N, were uncovered by means of computer simulation. As no crystal structure is available for *SpOGA*, we examined its closest structurally determined analogue, *TiOGA* from *Thermobaculum terrenum*,⁷ which shares 33% sequence identity with *SpOGA*. NMR spectroscopy and anion-exchange chromatography product analysis showed that its general acid/base mutant *TiOGA*-D120N was also able to catalyze phosphorylolytic, while wild-type *TiOGA* (*TiOGA*-WT) was a genuine hydrolase (Figures S2 and S8). *TiOGA*-D120N presented a 41% rate enhancement of *pNP* release observed upon addition of 20 mM phosphate into 50 mM HEPES buffer pH 7.8 ($k_{\text{cat}} = 4.4 \pm 0.2 \text{ s}^{-1}$; $K_{\text{M}} = 16 \pm 1.3 \mu\text{M}$), catalyzing both phosphorylolytic (30%) and hydrolytic (70%) (Figure S8). Hydrolysis was predominant at higher phosphate concentrations as well.

The structure of *TiOGA*-D120N in complex with GlcNAc (pdb 5DIY) solved at 2.06 Å resolution⁷ was taken as initial structure for the simulations. To reconstruct the reaction intermediate, molecular dynamics (MD) simulations were performed after replacing the active site GlcNAc by a glucose oxazolinium ion (Glc-ox⁺) for both *TiOGA*-D120N and *TiOGA*-WT (Figures S9 and S10). $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ anions were used to model a phosphate concentration of 20 mM at pH 7.8. The MD simulations showed that phosphate anions do not reach the active site of *TiOGA*-WT (Figure 3a), due to electrostatic repulsion from the D120 carboxylate, as reflected in the enzyme electrostatic potential at the active site (Figure 3a). However, the more positive *TiOGA*-D120N active site displays $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ anions within ≈ 3 Å of the anomeric carbon, in a favorable position for nucleophilic attack.

The mechanisms of hydrolysis and phosphorylation in *Tt*OGA-WT were modelled by QM(DFT)/MM metadynamics.²⁷⁻²⁸ This methodology has successfully been used to model catalysis in carbohydrate-active enzymes.²⁹⁻³² First of all, we evaluated the reactivity of the Glc-oxazoline/oxazolinium ion intermediate in *Tt*OGA-WT, using two collective variables (CVs, see definition in Figure S11). One collective variable (CV1) takes into account the nucleophilic attack on C1 (from either water or HPO_4^{2-}), while the other one (CV2) assesses the proton transfer from the protonated nucleophile (water or phosphate anion) to the acid/base catalytic residue. The hydrolysis reaction was found to be exergonic and dissociative (the C1-O_x bond is already broken at the oxocarbenium ion-like TS; Supplementary Table S4). The computed reaction free energy barrier (13.2 kcal·mol⁻¹) is in good agreement with experiments ($k_{\text{cat}} = 106 \text{ s}^{-1}$; $\Delta G^\ddagger = 14.7 \text{ kcal}\cdot\text{mol}^{-1}$).

To model the phosphorylation reaction in *Tt*OGA-WT, and given the absence of phosphate molecules close to the anomeric carbon, we manually placed a H_2PO_4^- anion in the active site and performed QM/MM metadynamics simulations. However, phosphorylation resulted in a high energy barrier (26.3 kcal·mol⁻¹, Figure 3b) and the reaction turned out to be endergonic, thus the reaction appears to be unfavorable both kinetically and thermodynamically. This indicates that would a phosphate ion visit the active site, which does not happen according to our classical MD simulation, it would not react. This is consistent with the lack of phosphorolytic activity for the *Tt*OGA-WT.

The same methodology was used to understand the phosphorylation reaction catalyzed by *Tt*OGA-D120N. In this case, phosphate ions were found to be stable near the Glc-oxazoline/oxazolinium ion during the classical MD simulations (Figure 3a). The QM/MM reaction free energy landscape (Figure 3b) reveals that, in contrast to the simulations for *Tt*OGA-WT, phosphorylation is exergonic, with a low reaction free energy barrier (14.8 kcal·mol⁻¹) that is in good agreement with experiments ($k_{\text{cat}} = 4.4 \text{ s}^{-1}$; $\Delta G^\ddagger = 16.3 \text{ kcal}\cdot\text{mol}^{-1}$). Therefore, both experiments and simulations evidence that *Tt*OGA-D120N is an efficient β -GlcNAc phosphorylase, with an activity comparable to the hydrolytic one from *Tt*OGA-WT ($k_{\text{cat}} = 106 \text{ s}^{-1}$).

Analysis of the atomic rearrangements during the phosphorylation reaction (Figures S12 and S14) reveals distinct changes in active site interactions upon the D120N substitution. In *Tt*OGA-WT, the carboxylate of the acid/base catalyst (D120) interacts with the phenolic hydroxyls of both Y14 and Y168 (Figure S14). However, the corresponding residue in the mutated enzyme (N120) does not interact with Y14 but with Y168 and the nucleophilic oxygen atom (O_p) of the HPO_4^{2-} (Figure S14), which in turn interacts with Y14 (Figure 3c). These small structural changes, together with the changes in electrostatic potential of the active site upon the mutation, keep the incoming HPO_4^{2-} ion above the substrate anomeric carbon in a reactive configuration, hence enabling phosphorylation.

Figure 3. (a) Computed electrostatic potential of the enzyme at the active site and radial distribution function of the distance between the C γ of the D120/N120 residue and the closest oxygen of each phosphate ion. (b) Free energy profile reconstructed from the metadynamics simulation for *Tt*OGA (left) and *Tt*OGA-D120N (right). (c) Representative structures along the reaction coordinate for *Tt*OGA hydrolysis (left) and *Tt*OGA-D120N phosphorolysis (right). Bonds being formed/broken are indicated with red dashed lines.

GH84s share some relatedness with GH25, 56 and 123, as well as with GH-K clan members (GH18, 20 and 85).³³⁻³⁴ These enzymes also catalyze hydrolysis via substrate-assisted mechanisms, and exhibit either a Cat-Cat (GH20, 84 and 123, here D119-D120 for *Tt*OGA) or a Cat-X-Cat (GH18, 25, 56, 85) sequential organization of their catalytic residues (Cat). A debated issue in these enzymes is the nature of the reaction intermediate, whether it is protonated (glucose oxazolinium ion, Glc-ox⁺) or unprotonated (Glc-ox).³⁵ It has been theoretically demonstrated that the intermediate is unprotonated (neutral oxazoline; Figure 1a) for GH18 chitinases.³⁵ All our simulations for native or mutated *O*-GlcNAcase GH84 (Figures 3 and S15) are consistent with an oxazolinium ion intermediate. In the chitinases case, the Cat-X-Cat organization allows the two catalytic residues to always share a proton,³⁵ leading to a hydrogen bond network that increases the pK_a of the assisting Cat residue. This scenario cannot take place in GH84 due to its two catalytic residues being consecutive (Cat-Cat). Moreover, the assisting Cat residue D119 is involved in a ionic interaction with K43, which lowers its pK_a and precludes D119 from deprotonating the Glc-ox⁺ species (the pK_a of Glc-ox⁺ was estimated at 7.7³⁶). Therefore, the architecture of the active site in

GH84 suggests that the intermediate is protonated, as demonstrated by our QM/MM simulations. Our work also suggests that there are at least two distinct substrate-assisted mechanisms among GHs: one in which the assisting residue plays the role of an acid/base, protonating and deprotonating the NAc group, leading to an oxazoline-type intermediate (GH18); and another one in which the assisting residue acts as a stabilizing residue, forming an ionic bond with the oxazolinium ion-type intermediate (GH84).

In conclusion, we have demonstrated that hydrolytic GHs enzymes can be turned into GPs by a single-point mutation. This small modification drastically changes the electrostatic potential of the active site, allowing the highly charged HPO_4^{2-} to accommodate and react. We also provide evidence that the intermediate of GH84-catalyzed reactions is a protonated oxazolinium ion. These insights expand our understanding both of GHs following a substrate-assisted mechanism, and of what distinguishes glycoside hydrolases from glycoside phosphorylases, providing a basis for the generation of enzymatic variants that could be amenable for the production of valuable phosphorylated glycosides.

ASSOCIATED CONTENT

Supporting Information

Experimental methods and computational details. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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