

Original Article

Site-directed mutagenesis of a family 42 β -galactosidase from an antarctic bacterium

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Abstract: Site directed mutagenesis was used to modify the active site of a cold active beta-galactosidase taken from an Antarctic psychrotolerant *Planococcus* Bacterial isolate. The goal was to modify the active site such that there would be an increase in activity on certain substrates which showed little to no activity with the wild type enzyme. A total of 5 mutant enzymes were constructed with amino acid changes based on an analysis done via homology modeling. All 5 modified enzymes were assayed using 14 different nitrophenol substrates. In most cases there was a loss of activity on substrates that showed activity with the wild type enzymes. None of the expected activity was observed with any of the mutants, possibly in part due to a decrease in hydrogen bonding between the active site and the substrates. With the substrates p-nitrophenyl- β -d-galacturonide and p-nitrophenyl- α -d-glucopyranoside we saw increased activity. With one of the mutants we measured a 320% increase in activity on p-nitrophenyl- β -d-galacturonide. Two other mutants showed activity on p-nitrophenyl- α -d-glucopyranoside, which showed no activity at all with the wild type enzyme.

Keywords: Directed evolution, cold active, beta-galactosidase, site directed mutagenesis

Introduction

Extremozymes are enzymes that retain some function in extreme environments. Environments with very high or very low temperatures ($>55^{\circ}\text{C}$, $<15^{\circ}\text{C}$), high salinity (2-5 M NaCl), or extremes in pH (>8 , <4) can mostly be considered outside of the range of human habitation (with some exceptions), and therefore, relatively extreme [1]. The organisms that inhabit these environments and produce these enzymes were dubbed extremophiles by Macelroy [2]. Extremozymes have become the target of much investigation for a number of reasons. First, they have allowed scientists to study the relationships between the sequence, structure, and function of enzymes in ways not previously possible. Being able to compare a mesophilic enzyme and its extremophilic homologue has given rise to many new insights into the mechanisms of enzyme structure, stability, and function [3-10]. Secondly, there is a lot of interest in the use of extremozymes in industrial processes [11-13]. Because of the oftentimes harsh environments of these industrial processes, enzymes that re-

tain some function in those environments are greatly desired. The biofuels industry is one such industry that has taken a great interest in both extremophilic organisms and extremozymes [14-18]. Bioethanol [14, 17], biodiesel [15], and methane, or biogas [19, 20] are all examples of biofuels in production right now. Extremozymes could facilitate the production of these biofuels [21]. Other industries looking at extremozymes include the detergent, agriculture, food, and textile industries [13, 22]. The scientific industry has been using extremozymes for decades now in the form of thermophilic DNA polymerases used in PCR [23].

While extremozymes often have properties useful in various industrial processes, the enzymes in their wild type form are often not ideal for the job due to other properties such as low catalytic efficiency, stability, or selectivity [24]. Directed evolution or *in vitro* evolution is a process that can be used to modify the enzyme in a way that could allow the enzyme to retain its desirable traits, while improving it in other ways. Directed evolution is a powerful technique used for both

exploring the sequence, structure, and function relationships of proteins, as well as making modifications to the function or activity of proteins [25]. In its simplest form, it involves making changes to a gene, then examining the phenotype of the resulting enzyme. Directed evolution can be approached by either a rational design method, involving site directed mutagenesis, or a random mutagenesis method, involving mutagenic PCR and/or gene shuffling [24, 25]. Directed evolution has frequently been used as a method to improve enzymes for various uses. As has been mentioned, biofuels represent a promising industry for the use of extremozymes, and these enzymes have frequently been the target of directed evolution projects [26-28].

A cold active beta-galactosidase (*bgaA*) from a *Planococcus* isolate called SOS Orange was selected for use in this experiment [29]. Beta-galactosidases are classified as exoglycosidases, which refer to their function of breaking glycosidic bonds at the terminal residue of a carbohydrate polymer. Specifically, beta-galactosidases hydrolyze the 1-4 beta linkage between galactose and some other moiety. The SOS Orange beta-galactosidase has been determined to be an extremozyme, due to its activity at very cold temperatures (over 10% activity at 0°C) and very high salt concentrations (40% activity at 4M KCL and 20% activity at 4M NaCl). It has been classified as a family-42 beta-galactosidase, according to the Hennrisat classification scheme [30]. The SOS Orange beta-galactosidase shows the greatest activity with the substrate o-nitrophenyl- β -d-galactopyranoside (ONPG). A crystal structure of the enzyme has not been solved although it was determined to be a dimer. However there is another family 42 beta-galactosidase (A4- β -Gal) that has had its crystal structure solved and published [31], allowing us to use homology modeling to make predictions about sequence/function relationships. A4- β -Gal comes from the organism *Thermus thermophilus* A4. It should be noted that not only is it a heat tolerant enzyme from a thermophilic organism, but it is also a trimer.

In an attempt to modify the preferred substrate of the enzyme, 5 mutants were designed that were predicted to have some activity on certain chromogenic substrates similar to ONPG. Each mutation was designed based on examination of a three dimensional model which was pre-

dicted using the Phyre 2 web servers [32]. Site directed mutagenesis via whole plasmid PCR was performed to introduce the DNA changes for each mutant. Mutants were cloned using the PCR product into *E. coli*, then selected, grown up, and induced to produce the enzyme. The 5 mutants and the substrates they were designed for are as follows: 1) Mutant P changed Phe347 to Tyr with the intent of introducing a hydrogen bond between the hydroxyl group of the tyrosine and the 4' hydroxyl group of substrates in which this hydroxyl group is in the down position. 2) Mutant A changed Asn150 to Gly in order to allow room for an amide group at the 2' carbon. 3) Mutant G changed Glu 357 to Gly to allow room for a carboxyl group at the 5' carbon. 4) Two double mutants (PA and PG) were also created that combined the modifications listed above.

Materials and methods

Addition of a 6 HIS tag and beta-galactosidase gene cloning

Primers containing a 6 HIS tag for the 3' end of the gene were constructed (Planococcus Forward pBad 5'-TAA GAG GAA TAA TAA ATG ATT AAC GAT AAA TTG CCG-3', Planococcus Reverse pBad 5'-TTA ATG GTG ATG GTG ATG ATG CAC TTT CGC CAA AAT CAA CAC GCC-3'), and the gene was amplified (95°C for 10 minutes, 35 cycles of (95°C for 1 minute, 53°C for 1 minute, 72°C for 4 minutes), then a final extension step at 72°C for 10 minutes). PCR amplicons were used directly in the ligation reaction without cleanup. Ligation of the amplified gene and cloning into a TOP10 *E. coli* host was performed using the pBad TOPO TA cloning kit (Invitrogen). Transformants were selected based on their resistance to ampicillin (100mg/liter), and screened based on their ability to hydrolyze X-Gal. A transformant was selected, grown overnight in 5 ml LB broth at 37°C, and its plasmid purified using the Wizard Plus SV Miniprep kit (Promega). The gene was sequenced at the ISU Molecular Research Core Facility.

Creation and cloning of mutants

The amino acid sequence of the protein was uploaded to the Phyre 2 web servers [32]. The three dimensional model that was returned was shown to have a 100% homology to a previously solved and published three dimensional model

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Table 1. The three mutations along with the mutant names and the primers used.

Mutation (Mutant Name)	Forward Primer	Reverse Primer
Phe347 -> Tyr TTC -> TAT (P Mutant)	GATACCATTTTGTACTATCAGTTGCGTCTCGCTCA	TGAGCGACGCAACTGATAGTACAAAATGGTATC
Asn150 -> Gly AAC -> GGC (A Mutant)	ATTTGGCACGTCTCCGGCGAATACGGCGGCTAC	GTAGCCGCCGTATTTCGCCGGAGACGTGCCAAAT
Glu357 -> Gly GAG -> GGC (G Mutant)	TCAGTCGGCGCTTGTGGCAAATACCACGGAGCG	CGCTCCGTGGTATTTGCCACAAGCGCCGACTGA

of a beta-galactosidase from *Thermus thermophilus* [31]. The sequences of the SOS β -Galactosidase and the *Thermus thermophilus* β -Galactosidase were aligned using ClustalW [33] running under Bioedit [34] in order to identify the active site residues of the SOS β -gal. The active site was analyzed using the Swiss-PdbViewer software [35], and the decisions on which residues to modify were based on this analysis. Three pairs of mutagenic primers were designed (Table 1). Whole plasmid PCR amplification was performed as follows: 1 μ l Vent_R DNA polymerase (New England Biolabs), 5 μ l 10X buffer, 5 μ l (65ng) template DNA, 5 μ l each forward and reverse primers (100ng each), 8 μ l DNTP mix, 21 μ l H₂O. PCR conditions were as follows: 95°C for 10 minutes, 20 cycles of (95°C for 30 seconds, 55°C for 1 minute, 72°C for 13 minutes), then a final extension step at 72°C for 10 minutes. 1 μ l of Dpn I restriction enzyme was added following amplification, and the solution was allowed to incubate for 1 hour at 37°C. Cloning was performed using TOP10 *E. coli* following the protocol in the pBad TOPO TA cloning kit (Invitrogen). Transformants were selected based on their resistance to ampicillin (100mg/liter). Because we saw both blue and white colonies with some mutations, 10 colonies of each color were picked, restreaked on ampicillin plates, and isolated colonies were chosen from each plate. These were grown overnight in 5 ml LB broth at 37°C, and plasmid purification was done with the Wizard Plus SV Miniprep kit (Promega). Sequencing was done at the ISU Molecular Research Core Facility. The P mutant plasmid was then used as the template for two more mutants, each of which combined the P mutation with one of the other two mutations, resulting in a PA mutant and a PG mutant. Double mutants were amplified, cloned, and sequenced as described above.

Induction and purification of protein

5 ml LB broth cultures of each mutant were started from a glycerol stock. 25 μ l ampicillin was added (100mg/liter), and the culture was grown overnight at 37°C. 5 ml of overnight culture was then added to 1.5 liters of LB, plus ampicillin (100mg/liter) and arabinose (.02% final concentration), and allowed to grow overnight with shaking at 37°C. The culture was pelleted and resuspended in 12 ml Z buffer [36]. 120 μ l of a 100mM solution of PMSF was added, and the cells were disrupted using a French Press (2 treatments at 20,000 lb/in²). The lysate was centrifuged at 14k RPM for 15 minutes, and the supernatant was collected. 5 ml of the supernatant was then added to a nickel column packed with Pro Bond resin (Invitrogen) and primed with wash solution (100mM HEPES, 10mM imidazole). Both ends of the column were stopped and the column was shaken horizontally for 15 minutes. The column was then placed upright and washed with 25 ml of the wash solution. 5 ml of elution buffer was then added (100mM HEPES, 500mM imidazole), and the column was again shaken for 15 minutes. The eluent was then collected and protein gel electrophoresis (10% acrylamide, run at 140V) was used to check for purity.

Protein assays

Solutions (2mg/ml) of 14 different substrates were made with Z buffer. The substrates used were: o-nitrophenyl- β -d-galactopyranoside (ONPG), p-nitrophenyl- α -d-galactopyranoside (PNP- α -galactose), p-nitrophenyl- β -d-galactopyranoside (PNPG), o-Nitrophenyl N-acetyl- α -D-galactosaminide (ONP-acetyl- α -galactose), p-nitrophenyl- β -d-galacturonide

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Table 2. Specific activity of all mutants on all substrates.

Substrate	Wild Type	Mutants				
		P Mutant	A Mutant	G Mutant	PG Mutant	PA Mutant
ONPG	16.8157	8.7787	3.2209	0	0	0.0790
PNP- α -galactose	0	0	0	0	0	0
PNPG	14.7740	8.8372	5.3413	0	0	0.0875
ONP-acetyl- α -galactose	0	0	0	0	0	0
PNPgalacturonide	0.0502	0.1621	0.0118	0	0	0
PNPglucose	0	0	0	0	0	0
PNP- α -glucose	0	0	0	0	0.0205	0.0110
ONPglucose	0	0	0	0	0	0
PNP-acetamido-glucose	0	0	0	0	0	0
PNPglucuronide	0	0	0	0	0	0
ONPF	0.9160	0.1982	0	0	0	0
PNPF	1.2157	0.6501	0.1933	0	0	0
ONPX	0	0	0	0	0	0
PNPX	0	0	0	0	0	0

Units are in $\mu\text{mol min}^{-1} \text{mg}^{-1}$. o-nitrophenyl- β -d-galactopyranoside (ONPG), p-nitrophenyl- α -d-galactopyranoside (PNP- α -galactose), p-nitrophenyl- β -d-galactopyranoside (PNPG), o-Nitrophenyl N-acetyl- α -D-galactosaminide (ONP-acetyl- α -galactose), p-nitrophenyl- β -d-galacturonide (PNPgalacturonide), p-nitrophenyl- β -d-glucopyranoside (PNPglucose), p-nitrophenyl- α -d-glucopyranoside (PNP- α -glucose), o-nitrophenyl- β -d-glucopyranoside (ONPglucose), p-nitrophenyl-2-acetamido-2-deoxy- β -d-glucopyranoside (PNP-acetamido-glucose), p-Nitrophenyl β -D-glucuronide (PNPglucuronide), o-nitrophenyl- β -d-fucopyranoside (ONPF), p-nitrophenyl- β -d-fucopyranoside (PNPF), o-nitrophenyl- β -d-xylopyranoside (ONPX), p-nitrophenyl- β -d-xylopyranoside (PNPX).

(PNPgalacturonide), p-nitrophenyl- β -d-glucopyranoside (PNPglucose), p-nitrophenyl- α -d-glucopyranoside (PNP- α -glucose), o-nitrophenyl- β -d-glucopyranoside (ONPglucose), p-nitrophenyl-2-acetamido-2-deoxy- β -d-glucopyranoside (PNP-acetamido-glucose), p-Nitrophenyl β -D-glucuronide (PNPglucuronide), o-nitrophenyl- β -d-fucopyranoside (ONPF), p-nitrophenyl- β -d-fucopyranoside (PNPF), o-nitrophenyl- β -d-xylopyranoside (ONPX), p-nitrophenyl- β -d-xylopyranoside (PNPX) (all substrates obtained from Sigma-Aldrich except p-nitrophenyl-2-acetamido-2-deoxy- β -d-glucopyranoside which was obtained from Acros Organics). Protein concentrations were determined with the Bradford method [37]. For 1 assay, 200 μl of substrate solution was added to 990 μl Z buffer in a 2 ml microcentrifuge tube. The substrate temperature was equilibrated in a 37 $^{\circ}\text{C}$ water bath for 10 minutes. 10 μl of protein solution was added, and the tube was allowed to incubate for 5 minutes at 37 $^{\circ}\text{C}$. 500 μl of Na_2CO_3 was added to stop the reaction, and the absorbance was measured at 420 nm. This was performed 3 times for each substrate/enzyme combination, and the absorbance values were averaged. Specific activity was calculated from these averages. The entire assay experiment was then repeated 2 more times and the three specific

activity values for each substrate/enzyme combination were averaged. The extinction coefficient used for ortho nitrophenyl substrates is 4.6 $\text{mM}^{-1} \text{cm}^{-1}$ and for para nitrophenyl substrates is 18 $\text{mM}^{-1} \text{cm}^{-1}$.

Results and discussion

In total there were 5 mutant enzymes that were examined. The 3 single mutation enzymes are listed in **Table 1** along with the specific mutation that was made for each one. In addition there are two double mutants. The P mutation was combined with the G mutation to create a PG mutant, and combined with the A mutation to make the PA mutant. The P mutation, which changes Phe347 to Tyr, was done based on the hypothesis that the additional hydroxyl group of the tyrosine would be able to create a hydrogen bond with the 4' hydroxyl group of the glucose and xylose substrates which is in the down position. This hydroxyl group is in the up position on our galactose substrate (ONPG). The A mutant changes Asn150 to Gly in order to make room for an amide group at the 2' carbon of some of our substrates such as p-nitrophenyl-2-acetamido-2-deoxy- β -d-glucopyranoside. The G mutation changes Glu357 to Gly to make room for a carboxylic acid group at the 5' carbon of

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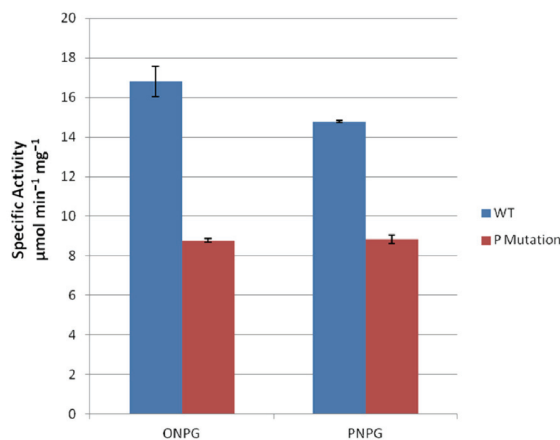


Figure 1. Specific activity of wild type and P mutant on ONPG (o-nitrophenyl- β -d-galactopyranoside) and PNPG (p-nitrophenyl- β -d-galactopyranoside).

some substrates such as p-nitrophenyl- β -d-galacturonide.

Table 2 shows all 5 mutants, along with WT, and the specific activity of each on all 14 substrates. ONPG and PNPG specific activity values for the WT enzyme are considered the standard against which all other values are compared. There is a general trend of a decrease in activity on ONPG and PNPG as we go from wild type to P to A then to G, where activity is seemingly eliminated. We then see some activity return with the PA mutant, but none on the PG mutant. This trend continues with few exceptions on all other substrates in which activity is seen on wild type. Novel activity was seen on only one substrate, p-nitrophenyl- α -d-glucopyranoside. Both the PA and PG double mutants had some activity on this substrate.

The P mutation (Phe347 to Tyr) was created in order to introduce an additional hydrogen bond between the hydroxyl group of the tyrosine and the 4' hydroxyl group of the glucose and xylose substrates which is in the down position. The P mutation caused a decrease in activity from wild type of between 40%-50% on ONPG and PNPG (**Figure 1**), and 50%-70% on o-nitrophenyl- β -d-fucopyranoside and p-nitrophenyl- β -d-fucopyranoside (**Figure 2**). This decrease in activity could be explained by the fact that changing the phenylalanine to a tyrosine does remove a reported hydrophobic interaction [31] which could cause the shifting of some residues important to hydrogen bonding. No activity was

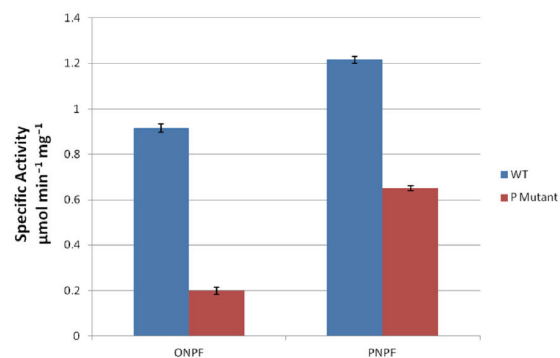


Figure 2. Specific activity of wild type and P mutant on ONPF (o-nitrophenyl- β -d-fucopyranoside) and PNPF (p-nitrophenyl- β -d-fucopyranoside).

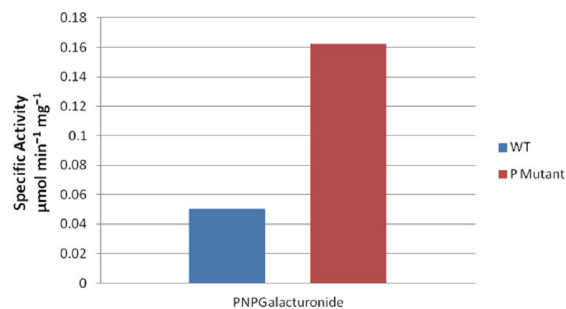


Figure 3. Specific activity of wild type and P mutant on PNPGalacturonide(p-nitrophenyl- β -d-galacturonide).

observed on any of the substrates containing a 4' hydroxyl group in the down position. The only increase in activity was on p-nitrophenyl- β -d-galacturonide where we saw approximately 3 times the activity of wild type (**Figure 3**). One possible explanation for this is the creation of a weak interaction between the hydroxyl group of the tyrosine and the oxygen of the carbonyl in the 5' carboxyl group. Because the hydroxyl group of the tyrosine in relation to the substrate is underneath the plane of the sugar, and the carboxyl group of p-nitrophenyl- β -d-galacturonide is above the plane, the carboxyl group would presumably have to swivel around to get close enough to interact. It is unknown whether any swiveling would position the carbonyl oxygen on the carboxyl group close enough for this to happen. Another possibility is that the mutation shifted other residues around enough that another weak interaction with the carboxyl group was created with an unknown residue. This also is extremely difficult to know

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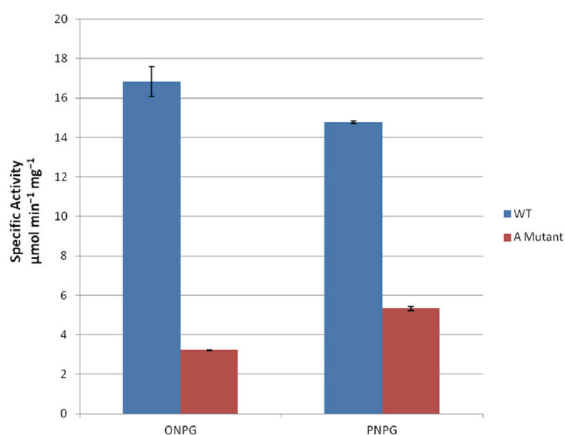


Figure 4. Specific activity of wild type and A mutant on ONPG (o-nitrophenyl- β -D-galactopyranoside) and PNPG (p-nitrophenyl- β -D-galactopyranoside).

without seeing the crystal structure of the mutant enzyme. However, we see no activity on p-nitrophenyl- β -D-glucuronide, which has the same carboxyl group, as well as the 4' hydroxyl group in the down position. This mutation was designed to interact with these groups, so neither of these explanations is fully satisfactory.

The A mutant changes Asn150 to Gly to make room for an amide group at the 2' carbon of some substrates. The A mutation resulted in a 60%-80% loss of activity on ONPG and PNPG (Figure 4), and an 80%-100% loss of activity on o-nitrophenyl- β -D-fucopyranoside and p-nitrophenyl- β -D-fucopyranoside (Figure 5). This could be explained as resulting from the loss of one direct hydrogen bond between the hydroxyl group of Asn150 and the hydroxyl group at the 2' carbon of the substrate. Another indirect hydrogen bond between the hydroxyl group of Asn150, a water molecule, and the 3' hydroxyl group of the substrate is also lost. There was no activity on any substrates with a 2' amide group, which was the purpose of this mutation. Activity on p-nitrophenyl- β -D-galacturonide also decreased to approximately 25% of the wild type (Figure 6), again presumably due to the loss of hydrogen bonds.

The G mutation changes Glu357 to Gly to make room for a carboxylic acid group at the 5' carbon of some substrates. The G mutation completely eliminated activity on all substrates. It was originally hypothesized that this mutation would allow room for the substrates with a 5'

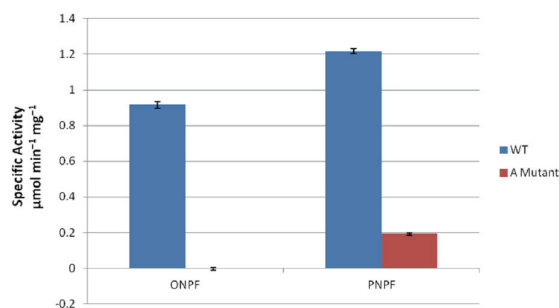


Figure 5. Specific activity of wild type and A mutant on ONPF (o-nitrophenyl- β -D-fucopyranoside) and PNPF (p-nitrophenyl- β -D-fucopyranoside).

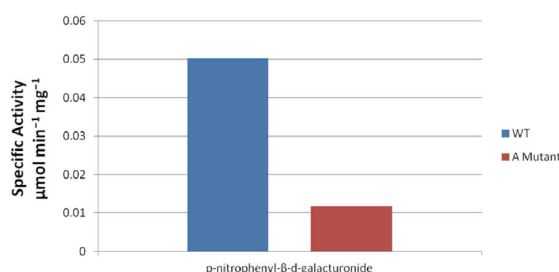


Figure 6. Specific activity of wild type and A mutant on PNPGalacturonide (p-nitrophenyl- β -D-galacturonide).

carboxyl group. However, this mutation presumably eliminated 3 hydrogen bonds, one between the carbonyl oxygen of Glu357 and the 4' hydroxyl of the substrate, and two between the hydroxyl group of Glu357 and the 3' hydroxyl group of the substrate. Whether or not the extra room for the 5' carboxyl group was critical in order to see activity is unknown, as the most likely reason for the loss of activity is the loss of the hydrogen bonds.

With the PA double mutant, activity on ONPG and PNPG was reduced to approximately 0.5% of what is seen on wild type (Figure 7). No activity was seen on p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside. This substrate contains both the 4' hydroxyl in the down position, as well as the 2' amide group, which are the two substrate features that these mutations (P and A) were designed to address. The loss of activity here seems to be the synergistic effect of the loss of activity seen in both of the single mutations that are here combined. No activity was seen on any other substrate except p-nitrophenyl- α -D-glucopyranoside, which origi-

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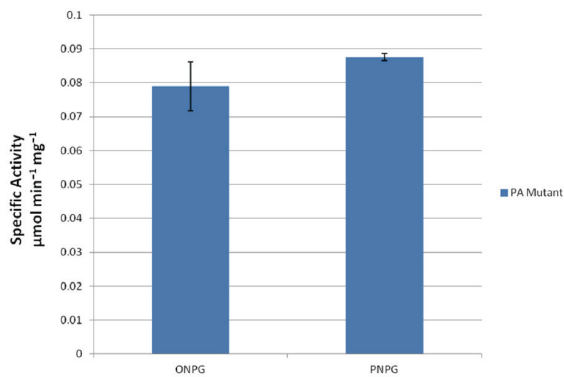


Figure 7. Specific activity of PA mutant on ONPG (o-nitrophenyl- β -d-galactopyranoside) and PNPG (p-nitrophenyl- β -d-galactopyranoside). Wild type activity on these two substrates is $16.8157 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for ONPG and $14.774 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for PNPG.

nally had no activity on the wild type enzyme. This activity was measured to be $.011 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (Figure 8).

The only activity reported for the PG double mutant was on p-nitrophenyl- α -d-glucopyranoside and was measured to be $0.02 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (Figure 8). This mutant was designed to show activity on the substrate p-Nitrophenyl β -D-glucuronide, which contains the 4' hydroxyl group in the down position, as well as a carboxyl group at the 5' position. Despite the P mutant by itself still exhibiting 40-70% activity on the two standard substrates (ONPG and PNPG), the PG double mutant loses all activity on those substrates. The addition of the G mutation and its removal of some key hydrogen bonds presumably caused the complete lack of activity in PG.

None of the proposed changes resulted in any activity on substrates they were designed for, which highlights the difficulty of predicting changes to activity based solely on analysis of the three dimensional structure of the protein. As has been shown however, we did observe novel activity on p-nitrophenyl- α -d-glucopyranoside, which was unexpected. Both alpha and beta galactosidases utilize a retaining catalytic mechanism which requires an acid/base catalyst and a nucleophile, roles generally fulfilled by glutamic acid residues. In the SOS beta-galactosidase, it is thought that Glu309 and Glu151 are these residues, based on sequence alignment with the beta-galactosidase

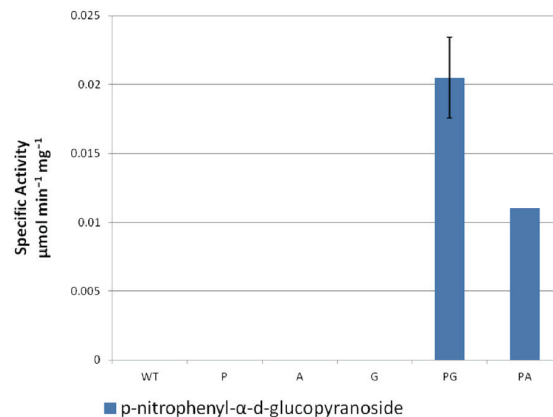


Figure 8. Specific activity of wild type and all mutants on p-nitrophenyl- α -d-glucopyranoside.

from *Thermus thermophilus* [31]. The only differences between the catalytic mechanisms of alpha or beta galactosidases lie simply in which direction the nucleophile is able to begin its attack on the anomeric center at the 1' carbon. For an alpha-galactosidase, the nucleophile resides above the plane of the sugar, allowing it to attack the 1' carbon of substrates which have an alpha linkage. In a beta-galactosidase, the nucleophile must be below the plane of the sugar in order to attack the 1' carbon of substrates which contain a beta linkage. Because the glutamic acid can only act as a nucleophile if it is the correct distance from the anomeric center, there are a number of different hypotheses that could partially explain this new activity. First, it is possible that our two double mutants have experienced a shift in the location of the two catalytic glutamic acid residues, such that their relative distances to the anomeric center have essentially swapped. The residue under the plane of the sugar may have moved further away, with the residue above the plane of the sugar shifting closer. Secondly, it is possible that due to changes in hydrogen bonding, the substrate itself as it sits bound to the active site has shifted upward slightly, resulting in the same distance changes between the 1' carbon and the catalytic residues. It is important to remember however that there is no measured activity on either of the other two alpha linked substrates, p-nitrophenyl- α -d-galactopyranoside and o-Nitrophenyl N-acetyl- α -D-galactosaminide, so this explanation is not complete.

Looking at each of the three mutations individu-

ally may shed some light what is happening regarding the novel α -bond hydrolyzing activity. Both the PG and PA mutants contain the P mutation, designed to accommodate a hydroxyl at the 4' carbon being in the down position. Our glucose substrates all contain this feature, including p-nitrophenyl- α -d-glucopyranoside. However, if this mutation is part of the reason we have activity on this substrate, we might expect to see some activity with our P mutant on other substrates with the same feature, yet the opposite is true. In fact there is no activity whatsoever on all glucose and xylose substrates with the P mutant, despite all of them having this feature. It seems clear that while the P mutation obviously plays some part in the activity we see in the PA and PG mutants on this substrate, it is apparently not having the effect we had predicted.

Examining the G mutation we see a slightly different situation. The G mutation was designed to accommodate a carboxyl group at the 5' carbon. The substrate p-nitrophenyl- α -d-glucopyranoside however has no such feature, which means that extra room at the 5' carbon was not an issue. This most likely means that the only effect of the G mutation here was in eliminating some hydrogen bonds. Considering that we saw no activity with G on any of the substrates that did in fact contain a carboxyl group at the 5' carbon, it's likely that a loss of hydrogen bonds was the only effect of this mutation. So once again with G we have a mutation that isn't doing what it was designed to do.

Because p-nitrophenyl- α -d-glucopyranoside shows activity with PA as well as PG (although only 50%), the A mutation also must be examined with regards to this activity. The A mutation was designed to make room for an amide group at the 2' carbon of the appropriate substrate. As with the G mutation, this feature is missing in p-nitrophenyl- α -d-glucopyranoside. The same conclusion can be arrived at here as was seen with G, that the A mutation's only effect was in getting rid of some hydrogen bonds, since making extra room for an amide group here was not needed.

The only thing that seems clear is that none of the mutations had the expected effect on p-nitrophenyl- α -d-glucopyranoside (or any of the other substrates for that matter). While it does seem likely that the appearance of activity on

this substrate is the result of some synergistic reshuffling of catalytic residues, as discussed above, there remains one last important question. How is the substrate binding to the active site in the first place? If we did, in fact, get rid of a number of hydrogen bonds that directly resulted in other substrates not being able to bind, how is this substrate binding? Did we possibly shift things enough that we introduced some hydrogen bonds from residues not previously reported as taking part in binding of the substrate? If so, why did we not see any activity on other alpha linked substrates? In order to answer these questions and to get a clearer view on what is happening, the crystal structure of both our wild type enzyme as well as each mutant will be needed.

This study highlights some of the difficulties encountered in attempting to do a rational design experiment. Making predictions of specific changes in protein function based on residue modifications is very difficult in the best of circumstances. In the absence of a crystal structure, and modeling our dimeric, cold active protein on a trimeric, heat active protein from a thermophile [31], we assumed that making accurate predictions would be difficult, and we were not incorrect.

Conversely, the fact that we did end up with over 300% activity on p-nitrophenyl- β -d-galacturonide, in addition to novel activity on p-nitrophenyl- α -d-glucopyranoside shows how important studies like these are, and why they are also so fascinating. Neither of these two phenotypes was predicted in the initial analysis, and could be considered as arising 'purely by chance'. The implication then is that if these two new phenotypes arose by chance with only a few modifications to the active site of this protein, how many other interesting phenotypes must there be among the countless possibilities?

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References

- [1] Adams MWW, Perler FB, and Kelly RM. Extremozymes: Expanding the Limits of Biocatalysis. *Nat Biotech* 1995; 13: 662-668.
- [2] Macelroy RD. Some comments on the evolution of extremophiles. *Biosystems* 1974; 6: 74-75.
- [3] Spiller B, Gershenson A, Arnold FH, and Stevens RC. A structural view of evolutionary divergence. *Proceedings of the National Academy of Sciences* 1999; 96: 12305.
- [4] D'Amico S. Activity-Stability Relationships in Extremophilic Enzymes. *Journal of Biological Chemistry* 2003; 278: 7891-7896.
- [5] Wintrode PL. Cold Adaptation of a Mesophilic Subtilisin-like Protease by Laboratory Evolution. *Journal of Biological Chemistry* 2000; 275: 31635-31640.
- [6] Mavromatis K, Feller G, Kokkinidis M, and Bouriotis V. Cold adaptation of a psychrophilic chitinase: a mutagenesis study. *Protein Engineering Design and Selection* 2003; 16: 497-503.
- [7] Feller G. Molecular adaptations to cold in psychrophilic enzymes. *Cellular and Molecular Life Sciences (CMLS)* 2003; 60: 648-662.
- [8] Thorvaldsen S, Hjerde E, Fenton C, and Willassen NP. Molecular characterization of cold adaptation based on ortholog protein sequences from *Vibrionaceae* species. *Extremophiles* 2007; 11: 719-732.
- [9] Jaenicke R. Protein stability and molecular adaptation to extreme conditions. *European Journal of Biochemistry* 1991; 202: 715-728.
- [10] Feller G and Gerday C. Psychrophilic enzymes: molecular basis of cold adaptation. *Cellular and Molecular Life Sciences* 1997; 53: 830-841.
- [11] Eichler J. Biotechnological uses of archaeal extremozymes. *Biotechnology Advances* 2001; 19: 261-278.
- [12] van den Burg B. Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology* 2003; 6: 213-218.
- [13] Niehaus F, Bertoldo C, Kähler M, and Antranikian G. Extremophiles as a source of novel enzymes for industrial application. *Applied microbiology and biotechnology* 1999; 51: 711-729.
- [14] Huang R, Su R, Qi W, and He Z. Bioconversion of Lignocellulose into Bioethanol: Process Intensification and Mechanism Research. *Bio-Energy Research* 2011; 4: 225-245.
- [15] Jon Van G. Biodiesel processing and production. *Fuel Processing Technology* 2005; 86: 1097-1107.
- [16] Vasudevan PT and Briggs M. Biodiesel production-current state of the art and challenges. *Journal of Industrial Microbiology & Biotechnology* 2008; 35: 421-430.
- [17] Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, and Zacchi G. Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends in Biotechnology* 2006; 24: 549-556.
- [18] Stephanopoulos G. Challenges in Engineering Microbes for Biofuels Production. *Science* 2007; 315: 801-804.
- [19] Cuéllar AD and Webber ME. Cow power: the energy and emissions benefits of converting manure to biogas. *Environmental Research Letters* 2008; 3: 034002.
- [20] Angelidaki I and Ellegaard L. Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. *Applied Biochemistry and Biotechnology* 2003; 109: 95-105.
- [21] Barnard D, Casanueva A, Tuffin M, and Cowan D. Extremophiles in biofuel synthesis. *Environmental Technology* 2010; 31: 871-888.
- [22] Gomes J and Steiner W. The biocatalytic potential of extremophiles and extremozymes. *Food technology and Biotechnology* 2004; 42: 223-235.
- [23] Templeton NS. The polymerase chain reaction. History, methods, and applications. *Diagnostic molecular pathology: the American journal of surgical pathology, part B* 1: 58.
- [24] Bornscheuer UT and Pohl M. Improved biocatalysts by directed evolution and rational protein design. *Current Opinion in Chemical Biology* 2001; 5: 137-143.
- [25] Arnold FH. Design by directed evolution. *Accounts of chemical research* 1998; 31: 125-131.
- [26] Turner NJ. Directed evolution drives the next generation of biocatalysts. *Nat Chem Biol* 2009; 5: 567-573.
- [27] Dellomonaco C, Fava F, and Gonzalez R. The path to next generation biofuels: successes and challenges in the era of synthetic biology. *Microbial Cell Factories* 2010; 9: 3.
- [28] Wen F, Nair NU, and Zhao H. Protein engineering in designing tailored enzymes and microorganisms for biofuels production. *Current opinion in biotechnology* 2009; 20: 412-419.
- [29] Sheridan PP and Brenchley JE. Characterization of a salt-tolerant family 42 beta-galactosidase from a psychrophilic Antarctic *Planococcus* isolate. *Applied and environmental microbiology* 2000; 66: 2438.
- [30] Henrissat B. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* 1991; 280: 309-316.
- [31] Hidaka M, Fushinobu S, Ohtsu N, Motoshima H, Matsuzawa H, Shoun H, and Wakagi T. Trimeric Crystal Structure of the Glycoside Hydrolase Family 42 β -Galactosidase from *Thermus thermophilus* A4 and the Structure of its Complex with Galactose. *Journal of Molecular Biology* 2002; 322: 79-91.
- [32] Kelley LA and Sternberg MJE. Protein structure prediction on the Web: a case study using the

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- Phyre server. *Nature Protocols* 2009; 4: 363-371.
- [33] Thompson JD, Higgins DG, and Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 1994; 22: 4673-4680.
- [34] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 1999; 41: 95-98.
- [35] Guex N and Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; 18: 2714-2723.
- [36] Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Pr, 1972.
- [37] Marion MB. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976; 72: 248-254.