**Kinetic characterization of over 100 glycoside hydrolase mutants enables the discovery of structural features correlated with kinetic constants**

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**ABSTRACT**

The use of computational modeling algorithms to guide the design of novel enzyme catalysts is a rapidly growing field. Force-field based methods have now been used to engineer both enzyme specificity and activity. However, the proportion of designed mutants with the intended function is often less than ten percent. One potential reason for this is that current force-field based approaches are trained on indirect measures of function rather than direct correlation to experimentally-determined functional effects of mutations. We hypothesize that this is partially due to the lack of data sets for which a large panel of enzyme variants has been produced, purified, and kinetically characterized. Here we report the *k*cat and KM values of over 100 purified mutants of a glycoside hydrolase enzyme. We demonstrate the utility of this data set by using machine learning to train a new algorithm that enables prediction of each kinetic parameter based on readily modeled and calculated structural features. The generated dataset and analyses carried out in this study not only provide insight into how this enzyme functions, they also provide a clear path forward for the improvement of computational enzyme redesign algorithms.

**INTRODUCTION**

The ability to rationally reengineer enzyme function using computational approaches has the potential to enable rapid development of highly efficient and specific catalysts tailored for needs beyond those selected for during natural evolution. (1) A growing route for engineering enzyme catalysts is the use of computational tools to evaluate potential mutations *in silico* prior to experimental characterization. Using the Rosetta Molecular Modeling Suite, reengineering of both specificity and chemistry has been accomplished. (2) (3) (4) (5) (6) However, often less than ten percent of designs engineered using this force-field based approach are found to have the intended functional effect. Furthermore, there have been no reports evaluating the predictive power of the Rosetta Molecular Modeling Suite on the functional effects of enzyme mutations. Therefore efforts to both evaluate and improve the predictive power of this computationally inexpensive and widely accessible algorithm are necessary.

The use of large datasets to train and evaluate force-field based algorithms for protein function has been previously validated in the context of protein thermostability. For example, the ProTherm database has over twenty thousand measured effects of mutations on thermostability, and serves as the gold standard for the development of numerous algorithms developed to predict effects of mutations on thermostability. (7) (8) (9) Current algorithms for protein redesign are not directly trained on experimentally measured effects, but rather indirect measures such as sequence recovery (*i.e.* the ability to recapitulate a known active site after running a design simulation). While there have been several previous efforts to construct large families of functionally-characterized mutants, none have produced, purified, and measured the kinetic constants of more than twenty mutants. (10) (11) (12) (13) (14) In order to develop algorithms for the rational modulation of kinetic parameters we hypothesize that it will be necessary to explicitly develop libraries of mutant enzymes for which the functional effects of mutations on catalytic efficiency (*k*cat/KM), apparent substrate affinity (estimated by KM), and turnover rate (*k*cat) have been explicitly measured.

Here, we take the first step towards developing a data set of enzyme mutants with measured effects on kinetic constants that is both large enough and has a wide enough dynamic range to enable training of computational protein design algorithms. The initial enzyme of focus is a family 1 glycoside hydrolase: ß-glucosidase B (BglB) from *Paenibacillus polymyxa*. The family 1 glycoside hydrolases have been the subject of numerous structural and kinetic studies due to their importance as the penultimate step in cellular ligno-cellulose utilization. (15) The structure of BglB indicates that it follows a classical Koshland double-displacement mechanism in which E353 performs a nucleophilic attack on the anomeric carbon of the substrate’s glucose moiety. The leaving group is protonated by E164. A third active site residue, Y295, orients E353 for catalysis with a hydrogen bond. (15) The protein structure and reaction scheme are provided in Figure 1.

**Figure 1. Structure and catalyzed reaction of BglB**

(A) Structure of BglB in complex with the modeled *p*-nitrophenyl-ß-D-glucoside (pNPG) used for design. Alpha carbons of residues mutated shown as blue spheres. The image was generated with PyMOL. (16) (B) The BglB–catalyzed reaction on pNPG used to evaluate kinetic constants of designed mutants

In this study we report the largest data set of its kind, in which 103 mutants of BglB are produced, purified, and kinetically characterized (*i.e.,* kinetic constants *k*cat, KM, Ki measured) using the reporter substrate *p*-nitrophenyl-ß-D-glucoside (pNPG). The production of this dataset revealed several mutations to non-catalytic residues (*i.e.* those not directly involved in the proposed reaction chemistry) that are as important to the enzyme-catalyzed reaction as catalytic residues. In addition, we demonstrate the ability to use this dataset to train computational algorithms for the prediction of *k*cat, KM, and *k*cat/KM using readily calculated metrics derived from molecular modeling. Finally, we illustrate how machine learning can be used to identify structural features from the molecular models that significantly improve the predictive accuracy of the molecular modeling. These analyses provide insight into the factors important for catalysis in BglB as well as a path forward for the development and evaluation of next-generation enzyme reengineering algorithms.

**RESULTS**

**Computationally-directed engineering of BglB**

A crystal structure (PDB 2JIE) of recombinant BglB in complex with the substrate analog 2-deoxy-2-fluoro--D-glucopyranose was used to identify the substrate binding pocket and the catalytic residues. To generate a molecular model representative of a proposed transition state in the hydrolysis of pNPG, an SN2-like transition state structure was built and minimized in Spartan based on a 3D conformer of PubChem CID 92930. Functional constraints were used to define catalytic distances, angles, and dihedrals between pNPG, the acid-base E164, the nucleophile E353, and Y295, which is proposed to orient the nucleophilic glutamate. The angle between the attacking oxygen from E353, the anomeric carbon, and the phenolic oxygen was constrained to 180˚, in accordance with an SN2-like mechanism. (17) A complete set of files that were used for modeling are provided in S10.

Two approaches were used to establish a set of mutants to generate and kinetically characterize. The first approach was a systematic alanine scan of the BglB active site where each residue within 12 Å of the ligand in our model was individually mutated to alanine. In the second approach, mutations predicted to be compatible with the modeled pNPG transition state in BglB structure were selected by students learning about molecular modeling through the program Foldit, a graphical user interface to the Rosetta Molecular Modeling Suite. (4) (18) Mutations were modeled and scored in Foldit and a selection of mutations that were either favorable or did not increase the energy of the overall system by greater than 5 Rosetta energy units were chosen to synthesize and experimentally characterize. Figure 1A illustrates the positions in the protein where mutations were introduced, and the complete set of mutations selected is listed in S1. A total of 69 positions were covered over the 103 mutants made.

**Protein production and purification**

Each of the 103 mutants was made via Kunkel mutagenesis (19) using the Transcriptic cloud laboratory platform and sequence-verified. Plasmids containing the mutant genes were transformed into *Escherichia coli* BL21(DE3), 5 mL cultures grown in Terrific Broth and expression induced with IPTG. Proteins purified via immobilized metal affinity chromatography and eluted in 200 µL HEPES buffer, as described in detail in S8. The absorbance at 280 nm of eluted protein was used to quantify protein yield and SDS-PAGE was used to evaluate purity (S2). All proteins used in this study were greater than 80% pure, and fresh resin was used for each mutant to prevent wild type contamination.

A total of ten biological replicates of the native BglB were used to assess expression and purification. The average concentration of proteins after purification was found to be 1.2 ± 0.4 mg/mL. Of the 103 mutants synthesized, 89 express and purify as soluble protein (Figure 2). The final concentrations for all 103 mutants are included in S1. Greater than 35% maintained the yields obtained for native BglB, and 15% did not express and purify as a soluble protein above our limit of detection (0.1 mg/mL) for protein yield after purification based on A280 and SDS-PAGE.

**Kinetic characterization of mutants**

Michaelis-Menten kinetic constants for each of the 103 mutants were determined using the colorimetric assay of pNPG hydrolysis and the results are represented as a heatmap in Figure 2. Ten biological replicates of the wild type enzyme have an average *k*cat of 880 ± 10 min–1, KM of 5 ± 0.2 mM, and *k*cat/KM of 171,000 ± 8000 M–1 min–1. To determine kinetic constants, observed rates at 8 substrate concentrations were fit to the Michaelis-Menten equation. If no clear saturation was observed then a linear equation was used to determine *k*cat/KM. Experimentally measured kinetic constants and nonlinear regression analysis for each mutant can be found in S1 and S5, respectively.

**Figure 2. Log scale relative kinetic constants of 103 BglB mutants**

The heatmap depicts the effect of each mutation on each kinetic constant relative to native BglB, normalized at 0. As indicated in the color legend, gold is for higher value and blue for a lower value. The metric 1/KM is used so a higher value is consistently corresponding to a “better” kinetic constant (assuming a lower KM is better) when evaluating *k*cat, *k*cat/KM, and KM. If the kinetic constant was not measurable, an X is depicted in the box. Proteins that were expressed as soluble protein with a final purification concentration of >0.1 mg/mL and validated by SDS-PAGE are labeled with a black box in the first column. Those below our limit of detection of 0.1 mg/mL are labeled with an empty box. Values are on a log scale and the ranges are as follows: 10–11,000 min-1 (*k*cat), 0.6–85 mM (KM), and 10–560,000 M-1min-1 (*k*cat/KM) with wild type constants of 880 ± 10 min-1, 5.0 ± 0.2 mM, and 171,000 ± 8000 M-1 min-1 for *k*cat, KM, and *k*cat/KM respectively. A full table of kinetic constants and substrate versus velocity curves for each are provided in S1 and S5.

Based on the maximum concentration of enzyme used in our assays and colorimetric absorbance changes at the highest substrate concentration used, we estimate our limit of detection for *k*cat/KM to be 10 M-1min-1. Of the 89 solubly purified mutants, 6 are below the limit of detection. The highest catalytic efficiency observed is 560,000 M-1min-1 for mutation R240A. In addition, while no substrate inhibition is observed for the wild type BglB, four mutants exhibit measurable substrate inhibition (the inhibition parameter Ki for only these mutants is reported in S1 as it was not measurable for most mutants).

**Observed sequence–structure–function relationships in BglB**

In agreement with previous studies, our results demonstrate the importance of E164, E353, and Y295 for catalysis. Mutating any of these residues to alanine results in a >85,000-fold reduction in catalytic efficiency (*k*cat/KM). However, beyond the catalytic residues, the systematic alanine scan of every residue within 12 Å of the ligand revealed mutations which have an equivalent functional effect to mutating the established catalytic residues to alanine.

For example, the Q19A mutant showed a dramatic effect on function: catalytic efficiency decreased by 57,000-fold. Analysis of the crystal structure of BglB suggests that both the nitrogen and oxygen of the amide sidechain interact with hydroxyl groups on the substrate (Figure 3A). A multiple sequence alignment of the BglB enzyme family in the Pfam database (comprising 1,554 non-redundant proteins), revealed that Q19 is 95% conserved in this family (Figure 3B). Unlike E353, the nucleophilic glutamate directly involved in the reaction chemistry, Q19 is not directly involved in the reaction. This is consistent with the theory that orientation of the substrate is a critical aspect of catalysis ("orbital steering") for which Q19 is likely crucial. (20) A crystal structure of BglB Q19A in complex with the 2-deoxy-2-fluoro--D-glucopyranose inhibitor may help elucidate the structural effect of this mutation. Based on molecular modeling, no major structural change for this mutant is predicted (S3).

**Figure 3. Active site model and conservation analysis of BglB**

(A) Docked model of pNPG in the active site of BglB showing established catalytic residues (navy) and a selection of residues mutated (gold). A multiple sequence alignment of the Pfam database’s collection of 1,554 family 1 glycoside hydrolases was made and the sequence logo for (B) selected regions around specific residues discussed in the text and (C) over the entire BglB coding sequence is represented. The height for each amino acid indicates the sequence conservation at that position.

A novel finding was a tenfold increase of *k*cat by a single point mutant, R240A. The BglB crystal structure reveals that R240 forms two hydrogen bonds with E222 (Figure 3A). Molecular modeling of the R240A mutant predicts that E222 would adopt an alternative conformation in which the acid functional group of the glutamate is 2 Å closer to the active site (S3). This would likely result in a significant change of the electrostatic environment around the active site, and indicates that the electronegative environment enhances catalysis of pNPG hydrolysis. Consistent with this hypothesis is the observation that the mutation E222A decreases *k*cat by ten fold. Both observations support previous evidence that the electrostatic environment of an enzyme's active site is of primary importance to catalysis. (21)

**Conservation analysis of the BglB active site**

Of the 44 positions in the active site systematically mutated to alanine, 11 are conserved by >85% in amino acid identity with respect to 1,554 homologues in the Pfam database. When any one of these amino acids is mutated to alanine, catalytic efficiency decreases >100-fold (S7). This supports the widely held assumption that highly conserved residues within an enzyme active site are functionally important. However, only 11 of the 44 residues within 12 Å of the active site are >85% conserved. Of the 33 remaining residues within 12 Å of the active site, only 8 alanine mutations resulted in a decrease in catalytic efficiency of greater than 100-fold, and 10 of these 33 mutations were not found to significantly affect catalytic efficiency.

Based on these findings, there does not appear to be a strong correlation between residue identity and function if a particular residue is <85% conserved. This observation supports the hypothesis that native sequence recovery is not a good metric for training design algorithms. In addition, the mutation R240A, which is not observed in any natural variant in the glycoside hydrolase 1 family, resulted in a 10-fold increase in *k*cat on pNPG. This emphasizes the importance of not limiting design efforts to changes previously observed in nature when engineering function towards a new substrate.

**Computational modeling and evaluation of predictive ability**

In order to evaluate the Rosetta Molecular Modeling Suite’s ability to predict the functional effects of mutations on BglB kinetic properties, molecular models were generated for each of the 103 BglB mutants. For each mutant, the modeled pNPG previously described was docked into the active site by a Monte Carlo simulation with random perturbation of the ligand followed by functional constraint optimization through rigid body minimization of the ligand, sidechain and ligand conformational sampling, and finally ligand, sidechain, and backbone minimization. This protocol was used to mimic protocols used in successful enzyme reengineering efforts. (2) An example set of input files for wild type BglB are provided in S10.

For each mutant, 100 models were generated as described above and the lowest 10 in overall system energy for each mutant were selected for subsequent structural analysis. A value for each of 59 potentially informative features (such as predicted interface energy, number of hydrogen bonds between protein and ligand, and change in solvent accessible surface area upon ligand binding) was calculated for each model. Correlation of the average calculated structural features to each kinetic constant was assessed using Pearson Correlation Coefficient (PCC) and Spearman Rank Correlation (SRC). For both *k*cat/KM and *k*cat, the strongest correlation observed is to the total number of non-local contacts (count of residues separated by more than 8 sequence positions that interact with each other), with a PCC of 0.56 (p-value 0.009; Wilcoxon test) and 0.43 (p-value 0.004; Wilcoxon test), respectively. For 1/KM, the highest PCC is 0.29 (p-value 0.0005; Wilcoxon test) to the total number of hydrogen bonds in each BglB model. The SRC follows similar trends to PCC for all three predicted constants (SRC of 0.55, 0.42 and 0.38 for *k*cat/KM, *k*cat and 1/KM respectively). The PCC and SRC values for all features are available in S4.

**Machine learning prediction of kinetic constants**

Because no single structural feature predicts *k*cat, 1/KM, or *k*cat/KM with high accuracy, machine learning techniques were used to identify a subset of calculated features correlated to observed kinetic constants. Elastic net regularization, a constraint regression technique that uses both l1 and l2 regularization for feature selection, was used to identify structural features that could be combined in order to predict each kinetic constant. To increase robustness to sample size and remove bias, we used a bootstrapping aggregating technique, where the predicted value was an average of 1000 elastic net models, each trained on a different subset of the data.

The final prediction from this ensemble learning regression method outperformed single feature selection for each kinetic constant. For *k*cat/KM, the PCC increased to 0.76 from 0.56, in the case of *k*cat to 0.60 from 0.56, and for 1/KM to 0.71 from 0.29. Figure 4 (top panel) illustrates the correlations between machine learning predictions and experimentally-measured values. Figure 4 (bottom panel) depicts the histogram of samples with respect to their measured kinetic constant value and the observed error between predicted and measured value.

**Figure 4. Correlation between machine learning predictions and experimentally-determined kinetic constants**

*Top panels*: predicted versus experimentally-measured values for kinetic constants *k*cat/KM (A), *k*cat (B), and 1/KM (C). All values are relative to the wild type enzyme and on a log scale. The standard deviation (error bars) of the predicted values was calculated based on the prediction by 1000-fold cross validation for each point. The red line corresponds to linear regression and has been added for visualization purposes. *Bottom panels*: Histograms of experimentally-determined values in the data set (90, 80 and 80 samples for *k*cat/KM, *k*cat, and KM, respectively), along with the residual errors (scatter plot) between predicted and measured kinetic values.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ***k*cat/KM** | ***k*cat** | **1/KM** | **Description** | **Min.** | **Max.** |
| -1.00 | ns | ns | Hydrogen bonding energy of pNPG | -4.53 | -1.8 |
| -0.63 | 1.00 | -0.03 | Total number of polar contacts | 144 | 155 |
| -0.43 | ns | ns | Count of hydrogen bonds to pNPG | 4 | 9 |
| -0.03 | ns | ns | Hydrogen bonding energy of E164 | -0.93 | -0.21 |
| 0.29 | ns | -0.27 | Lennard-Jones repulsion of Y295 | 0.54 | 0.99 |
| 0.39 | 0.92 | ns | Change in pNPG solvent-accessible surface upon binding | 0.86 | 0.96 |
| 0.44 | 0.15 | 1.00 | Packing of the system without pNPG | 0.67 | 0.72 |
| 0.44 | 0.53 | 0.46 | Packing of the system with pNPG | 0.67 | 0.73 |
| 0.98 | 0.09 | ns | Hydrogen bonding energy of Y295 | -1.28 | -0.5 |
| ns | -0.51 | ns | Packing with pNPG around E353 | 0.19 | 1 |
| ns | -0.10 | ns | Total system energy | -636.44 | -621.6 |
| ns | -0.01 | ns | Hydrogen bond energy of the total system | -76.7 | -67.63 |
| ns | ns | 0.11 | Lennard-Jones repulsion around E353 | 0.67 | 1.41 |
| ns | ns | 0.27 | Average hydrophobic surface area without pNPG | 0.51 | 1.75 |
| ns | ns | 0.32 | Packing around E353 without pNPG | 0.37 | 0.99 |
| ns | ns | 0.34 | Packing around E164 without pNPG | 0.37 | 0.99 |
| ns | ns | 0.38 | Packing around Y295 without pNPG | 0.34 | 0.99 |
| ns | ns | 0.51 | Lennard-Jones repulsion of E164 | 0.83 | 1.53 |

**Table 1. Most informative structural features predicting each kinetic constant**

For each mutant, 10 out of 100 models were selected based on the lowest total system energy. Fifty-nine structural features were calculated for the selected models and the most informative features were selected based on a constrained regularization technique (elastic net with bagging; see Methods). The table contains features that have been assigned non-zero weights during training (9 for *k*cat/KM, 8 for *k*cat, 10 for KM). The weights are multiplied by a normalized form of the value (not shown), and can therefore indicate both a positive or negative relationship. For example, a negative weight for hydrogen bonding is consistent with a positive correlation to hydrogen bonding where a smaller number indicates more hydrogen bonding is occurring. Inversely, a positive weight for packing would indicate a positive correlation since a larger value indicates a system with fewer voids. The relative contribution of each feature in determining the kinetic constant is given as a normalized weight (columns 1-3). Column 4 provides a description of each feature, and columns 5 and 6 show the range of observed values in the training dataset. The full feature table is available in S4. *ns=feature not selected by the algorithm*

The primary features found to correlate to 1/KM are metrics of protein packing without the ligand present (*i.e.* a minimal number of voids). All of these packing features are positively correlated to 1/KM, meaning that, in BglB, a decrease in structural packing (*i.e.* a higher packing value) around the catalytic residues and protein results in a lower KM. A tightly packed enzyme without voids would likely result in pre-ordering of the active site. Therefore this correlation is consistent with BglB requiring a pre-ordered active site for efficient substrate binding, and potentially catalysis. (20) To further support this proposed classical lock-and-key mechanism, the observed root mean square deviation (RMSD) between the crystal structures of the apo (2O9P) and transition state analogue–bound (2JIE) forms of BglB is < 0.2 Å. It is important to note that KM is a complex kinetic constant and not necessarily correlated to substrate binding (i.e. Kd). Furthermore, the relationship between KM and Kd may change between mutants. However, when modulating enzyme activity KM is a primary parameter of functional interest, and therefore the ability to predictively model KM is of significant importance. Future efforts including mechanistic and structural studies for a diverse set of mutants from this data set will be needed in order to elucidate the detailed molecular mechanism of the discovered relationship between KM, Kd, and structural packing in BglB.

The features selected by the algorithm as predictive of *k*cat include a count of polar contacts, consistent with mechanistic studies that indicate BglB stabilizes the positive charge on the oxocarbenium ion in the proposed transition state. (22) Another primary featured selected as a predictor of *k*cat by the elastic net algorithm is a ligand burial term (change in solvent accessible surface area on binding) which is consistent with the stabilization of the transition state and catalysis through tight interface packing and shape complementarity. In addition, these features will all have a significant effect on the electrostatic environment of the enzyme active site, and are consistent with effects observed for R240A and E222A on catalysis. However, similar to the analysis of KM, the rate-limiting step for BglB under these experimental conditions with this substrate is not known. As the rate limiting step may change between mutants, further studies on the detailed kinetic parameters of the native enzyme and mutants will likely provide further insight into the determinants of function and key structural factors required for high turnover rates.

In BglB, the most informative feature predicting *k*cat/KM is the calculated hydrogen bonding energy of the substrate. The identification of this feature by the machine learning algorithm indicates the importance of protein-ligand hydrogen bond interactions. Hydrogen-bonding interactions are exceptionally important for the enzyme-catalyzed reaction, as noted for the Q19A mutation. Strong hydrogen bonding interactions between the protein and substrate are likely of the utmost importance for optimally positioning the substrate and the protein sidechains to enable catalysis ("orbital steering"). (23) This is consistent with the hydrogen bonding energy being selected by machine learning as a feature of primary importance for catalytic efficiency.

While many of the selected features are consistent with well-established mechanisms of enzyme catalysis, there were several unexpected observations. One unexpected trend is that several features are selected as predictive of *k*cat/KM but not either *k*cat or KM. Further analysis of *k*cat and KM revealed that there is no significant correlation between two parameters in this dataset (S6). This suggests that *k*cat and KM are independent parameters for BglB, and it is therefore not unexpected that features found to be predictive of *k*cat/KM are not predictive of either *k*cat or KM independently.

A second unexpected observation is that the most common metric used for evaluating designs, interface energy, (2) (3) (4) (5) (6) is not selected by the algorithm to be predictive of any kinetic constant. Ideally, this would be the single metric optimally correlated with either *k*cat or *k*cat/KM. This likely stems from training the enzyme design algorithm on indirect measures of function, further supporting the need to train force-field based algorithms on direct experimental measurements.

**DISCUSSION**

The Rosetta Molecular Modeling Suite has been successfully used to guide the engineering of a wide range of enzyme functions. However, there has been a limited ability to benchmark its predictive power for enzyme reengineering due to the lack of a large, quantitative dataset correlating the effects of mutations to kinetic parameters over a large dynamic range. Here, we construct the first such dataset and report statistically significant evaluation of our ability to predict the functional effects of enzyme mutations.

The data generated here uncovered new structure-function relationships in BglB, and provides the quantitative contribution towards catalysis of each amino acid in the active site. This systematic analysis revealed that several amino acids within the active site which are not directly involved in the reaction chemistry are almost as important to catalysis as the three residues which are directly involved in the chemistry. This highlights the importance of the entire active site in catalysis. This is consistent with a recent report exploring the interconnectedness of a network of five residues in alkaline phosphatase. (24)

Furthermore, the large dataset of kinetic constants reported here enabled the use of machine learning to select structural features that are predictive of function. It was unexpected to observe that the calculated interface energy is not found to be predictive of any kinetic parameter, and was not a feature selected by machine learning as predictive of function. This has significant implications for future design strategies since interface energy is one of the most common metrics currently used to evaluate redesigned enzyme-ligand interfaces. It may be pertinent to develop additional training datasets, such as we have done for BglB, in order to further quantify the appropriate metrics to be used for selecting designed mutants to functionally characterize in other enzyme systems. Similarly, the development and quantitative characterization of mutant datasets in the case of other enzymes will show which features are general and which are specific to different enzymatic classes.

From the machine learning analysis, an interesting non-linear relationship between predicted and experimental rates is revealed as the residual error increases with the measured kinetic value (Figure 4, bottom panel). There are two factors that contribute to this effect. First, as is evident from the histogram, mutants with lower activity have been sampled more in all cases, and the sampling size per bin tends to bias the error distribution. Second, there are no features or feature combinations in the regression model that correlate well with the observed non-linearity. Non-linear regression methods (second-order polynomial and Poisson kernels) achieved similar performance (data not shown). As such, there is room for improvement in future studies by uniformly sampling the parameter space (which is difficult to predict *a priori* but can be rectified by increasing the sample size). This could be achieved by building on recent high throughput experiments that systematically screen the phenotypic effect of every possible enzyme point mutant. (14) A combination of high throughput screening with molecular modeling could be used to identify a subset of mutants to purify and kinetically characterize in order to maximize the information content when training new algorithms. In addition, introducing informative features that capture different aspects of the variation observed and exploring other non-linear regression methods that balance the bias-variance trade-off could be used to address the non-linear relationship between predicted and experimental kinetic constants.

This work demonstrates how constrained statistical learning can be integrated with measured functional effects of a mutation on enzyme kinetic constants in order to build predictive models. As more datasets of kinetically characterized mutant variants become available for a variety of enzymes, our understanding of how these systems function and our ability to identify the most informative features will increase. Integration of these data-driven methods with enzyme redesign algorithms has the potential to significantly increase the predictive performance of the computational tools that are currently available, with far-reaching applications.

**CONCLUSION**

In this work, over 100 computationally-designed mutants of a family 1 glycoside hydrolase were produced, purified, and kinetically characterized. This dataset revealed new insights into structure-function relationships in BglB. Machine learning protocols were employed to select a subset of readily calculated structural features that are highly predictive of each measured kinetic parameter. The development of this large data set allowed a statistically significant assessment of the Rosetta Molecular Modeling Suite’s ability to predict functional effects of mutations on this enzyme’s kinetic properties. This data set will be invaluable for the development of computational enzyme engineering algorithms and providing insight into the physical basis of enzyme sequence-structure-function relationships.

**METHODS**

**Molecular modeling for mutant selection**

The crystal structure of recombinant BglB in complex with the substrate analog 2-deoxy-2-fluoro--D-glucopyranose was used to identify the substrate binding pocket and the catalytic residues. Functional constraints were used to define catalytic distances, angles, and dihedrals among 4-nitrophenyl-ß-D-glucoside, E164, E353, and Y295. The structure was then loaded into Foldit, a graphical user interface to Rosetta. Point mutations to the protein were modeled, and a subset were chosen by students learning about molecular modeling. Generally the designs had energies no more than 5 Rosetta energy units higher than the native structure.

**Mutagenesis, expression, and purification**

The BglB gene was codon-optimized for *E. coli*, synthesized as a DNA String by Life Technologies, and cloned into a pET29b+ vector using Gibson assembly. (25) Site-directed mutagenesis performed according to the method developed by Kunkel was used to generate mutations to BglB via the Transcriptic cloud laboratory platform. Variants were expressed and purified via immobilized metal ion affinity chromatography and assessed using 4-20% gradient SDS-PAGE Bolt Gels from Life Technologies. More details are provided in S8.

**Kinetic characterization**

The activity of the computationally designed enzyme variants was measured by monitoring the production of 4-nitrophenol. Mutant proteins ranging in concentration from 0.1 to 1.7 mg/mL were aliquotted in triplicate in 25 µL volumes and 75 µL of *p*-nitrophenyl-ß-D-glucoside (100 mM, 25 mM, 6.25 mM, 1.6 mM, 0.4 mM, 0.1 mM, or 0.02 mM) in enzyme storage buffer was added. Absorbance at 420 nm was measured every minute for 30-60 min and the rate of product production in M/min was calculated using a standard curve (see Supplemental Materials). A total of 2944 observed rates for 119 individual proteins (including biological replicates) were fit to the Michaelis-Menten equation using SciPy.

**Predictive modeling**

One hundred molecular models of each mutant enzyme were generated using the Rosetta Molecular Modeling Suite by Monte Carlo optimization of total system energy and the lowest 10 selected for feature generation. Elastic net regularization was used to select the most informative features. To evaluate the prediction performance of the method, stratified 10-fold cross-validation together with bootstrap aggregating (bagging) was used. Bagging was used to improve the stability and robustness of the predictor and entail in training 1,000 elastic net models with randomly drawn but stratified 10-fold cross-validation samples. The final three feature sets (one of each parameter to be estimated) were selected according to the averaged weight of each feature in all the 10,000 elastic net models (10 models per cross-validation, randomized 1,000 times). The weight of each selected feature in table 1 was normalized with respect to the weight with the largest absolute value. P-values were calculated based on the Wilcoxon signed-rank test after features and kinetic constants were normalized in the [0,1] interval. More information about the optimization and statistical procedure followed is available in supplemental materials.

**SUPPORTING INFORMATION**

**S1 Table. Kinetic constants for 103 computationally-designed BglB mutants.** Included are columns (1) the mutation (2) protein yield as assessed by absorbance at 280 nm (3, 4, 5, 6) kinetic constants and nonlinear regression analysis for each of *k*cat, KM, KI, and *k*cat /KM.

**S2 Fig: SDS-PAGE images for 119 variants of BglB.** SDS-PAGE gels showing all proteins used in this study, including replicates of wild type assayed with each batch of mutants. Gels were stained overnight with Coomassie Blue. Protein ladder used was SeeBlue® Plus2 Pre-stained Protein Standard (Life Technologies). Gels were imaged on a BioRad Gel Doc EZ system.

**S3 Fig: Active site models of mutants Q19A, R240A, and wild type BglB.** The lowest energy of 100 models generated for each mutant is depicted. In panel A, mutation of the glutamine at position 19 to an alanine removes two hydrogen bonds (black) to the substrate compared to wild type (C). In panel B, mutation of the arginine at position 240 to an alanine is predicted to stabilize an alternate conformation of E222A, bringing the carboxylate group to 4.2 Å of the substrate's nitro group. Distances and between the substrate, *p*-nitrophenyl-ß-D-glucoside, and the BglB molecule are indicated by black lines.

**S4 Table. Correlations between individual structural features and each of *k*cat, KM, and *k*cat/KM.** PCC and SRC values for each individual structural feature, given by Rosetta short name. For explanation of each short name, see reference (26).

**S5 Fig.** Diagnostic plots showing Michaelis-Menten, Michaelis-Menten with substrate inhibition, or linear fit for each of 103 mutants. For each mutant, 8 observed rates (in triplicate) were fit to the Michaelis-Menten equation using SciPy and plots were generated using Matplotlib. Plots were used to visually confirm statistical analysis of the fits.

**S6 Fig.** Plot of the values of log *k*cat versus log 1/KM for 103 mutants relative to wild type BglB, showing the statistical independence of *k*cat and KM in the BglB system.

**S7 Table. Conservation analysis of BglB active site residues.** A multiple sequence alignment of 1,554 family 1 glycoside hydrolases from the Pfam database aligned to the BglB wild type sequence was used for this analysis. Column 1 is the relative *k*cat/KM compared to wild type on a log scale. Column two gives the position and native BglB residue at that position. Column three is the percentage of the 1,554 aligned sequences that have the same residue as BglB.

**S8 Text: Supplemental materials and methods**

Mutants were designed using the Foldit, a graphical user interface to the Rosetta Molecular Modeling Suite. Mutants were chosen based on proximity to the active site as well as Foldit’s predicted energy. Mutations within 12 Å of the active site, and those that did not increase the total system energy by more than 5 Rosetta energy units, were selected for experimental characterization. No other limitations were placed on designed mutations.

A sequence coding for BglB (Uniprot P22505) was codon-optimized for *Escherichia coli* and manufactured as a DNA String by Life Technologies. Using Gibson assembly, gene was inserted between the NdeI and XhoI sites of pET29b, adding a C-terminal His tag onto the protein sequence. Kunkel mutagenesis was used to create site-specific mutations, and all plasmids were sequence-verified.

For protein production, 20 μL of chemically-competent *Escherichia coli* BL21(DE3) (Novagen) were transformed on ice with 1 μL of plasmid in buffer at a concentration of 90 to 130 ng/μL. The competent cell-plasmid mixture was temperature shocked to induce plasmid uptake by heating at 42°C for one minute and then chilling on ice for one minute. Cells were recovered in 200 μL Terrific Broth (TB) media at 37 °C for one hour. They were then plated onto an LB agarose plate containing 50 mg/mL kanamycin, and incubated for 24 hours at 37 °C.

For each mutant, a 50 mL Falcon tube containing 5 mL TB with 50 mg/mL kanamycin was inoculated with one colony from a kanamycin selection plate. Tubes were covered with breathable seals and incubated with shaking for 24 hours at 37 °C.

Growth cultures were pelleted by centrifugation at 4700 RPM for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 5 mL of induction medium (TB with 1 mM isopropyl-β-D-thiogalactopyranoside and 50 mg/mL kanamycin). The tubes were covered again with breathable seals and incubated with shaking at 18 °C for 24 hours.

The 5 mL expression culture was pelleted by centrifugation at 4700 for 10 minutes and the supernatant was discarded. The resulting pellet was suspended in 500 μL wash buffer (50 mM HEPES, 150 mM sodium chloride, 15 mM imidazole, pH 7.50) and lysed with BugBuster protein extraction reagent (Millipore) and 1 mg lysozyme, 0.1 mg DNase, and 0.1 mg phenylmethylsulfonyl fluoride per sample.

After 20 min, lysate was centrifuged at 14,700 RPM for ten minutes. The supernatant was loaded on to protein purification columns (BioSpin 732-6008) prepared with 100 μL of 50% Ni-NTA resin slurry. After equilibration with 500 μL wash buffer, two 500 μL aliquots of supernatant were added to the columns. Six rounds of 500 μL wash buffer were then allowed to drip through the columns. Resulting protein micro-columns were then transferred to 2 mL tubes for elution. Protein was eluted in 2x100 μL elution buffer (50 mM HEPES, 150 mM sodium chloride, and 25 mM EDTA, pH 7.50). A brief centrifugation at 4000 RPM ensured all protein was collected.

Protein yield was then determined via ratio of absorbance at 260 and 280 nm and SDS-PAGE.

Each enzyme variant was assayed in triplicate at 8 substrate concentrations ranging from 0 to 75 mM. Diluted protein solution was dispensed in 25 μL aliquots into a 96-well plate (Corning Costar #3885). Separately, in another plate, 100 uL elution buffer with 8 different concentrations of pNPG (1 per row) were prepared. The assay was initiated by multi-channel pipetting 75 μL substrate from each row of the substrate plate into the corresponding row of the assay plate. The absorbance at 420 nm was monitored every minute for 60 minutes to determine the rate of the reaction.

Unless otherwise noted, all supplies were purchased from Sigma-Aldrich.

**S9 Figure. Prediction and feature selection via Elastic net**. A regularized linear regression model, Elastic Net (EN), was chosen to fit the dataset of the kinetic constants, each constant fitted independently. Comparing to ordinary least squares regression, an EN model is able to make a prediction and select the most informative feature set simultaneously as and penalties are applied to the regression weights. The weight of each structural feature is estimated as



Where:

: the intercept;



: the weight of structural feature i in the regression model;



p: the number of structural features generated by the BglB model;

: the kinetic constant (the dependent variable to be predicted);



: structural features generated by the BglB model (the independent variables);



,: parameters tuning the constraints on the weights.



Since the structural feature were measured in different ranges and units, we first normalized all the features to be zero-centered with variance being one by subtracting the mean and dividing by the variance of the feature value. All the features are on the same scale to compare their contribution to the kinetic constants after the normalization. The tuning parameters, are determined one by one via stratified 10-fold cross validation by searching a grid of and. Each round of cross validation generated a linear regression model. In order to build a more generalized model, cross validation was run 1,000 times with a different part of the dataset each time. The final prediction of a mutant’s kinetic constant was an average of all the predictions during the 1,000 rounds of training. The average number of non-zero weights when predicting *k*cat/KM, *k*cat and KM were 9, 8 and 10 respectively. The top features were chosen and listed in table 1 with their averaged weights among all the models (9 for *k*cat/KM, 8 for *k*cat, 10 for KM)**.** Stratified 10-fold cross validation was implemented to validate the EN model. Specifically, all the mutants were first ranked according to the experimentally-measured value of the kinetic constant to be predicted and every 10 adjacent datapoints were randomly marked with an index using integers from 1 to 10 without duplication. Finally, all the datapoints with the same index were grouped together, resulting in ten folds. Since the datapoints in each folds comes from different level of the dataset, this guarantees every fold is a good representative of the dataset. In order to build a robust prediction model, the cross validation was run 1,000 times, the dataset split into training set and testing set differently each time.



Each bootstrap set is constructed by uniformly sampling from the original database, with a coverage of 87%, which is calculated as . The following figure shows the prediction of the left-out subsets during bootstrapping.

**S10 Code. Rosetta input files.** Rosetta input files and associated scripts for generating and scoring *in silico* mutations to the BglB structure.

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