Improving alternate lignin catabolite utilization of LigAB from *Sphingobium* sp. strain SYK-6 through site directed mutagenesis

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ABSTRACT

Protocatechuate 4,5-dioxygenase (LigAB) catalyzes dioxygenation of multiple lignin derived aromatic compounds—such as protocatechuate (PCA), gallate (GA) and 3-*O*-methyl gallate (3OMG)—with decreasing proficiency as the molecule size increases. We predicted that phenylalanine-103 of the α subunit (Phe103 α) controls substrate specificity through interaction with the C5-funtionality of bound substrates, and mutagenesis would enhance GA and 3OMG catalysis. LigAB with Phe103 α mutations (F103V, F103T and F103H) displayed enhanced catalytic efficiency for dioxygenation of 3OMG, with mutants displaying 12- to 31-fold increases in k/K , making these mutant enzymes more active with 3OMG than its native dioxygenase (DesZ). The F103T and F103V point mutants also exhibited allosteric activation for the dioxygenation of PCA and GA, respectively, in the presence of vanillin, as previously observed for LigAB. The enhanced utilization of substrates by these mutants makes them potentially useful for efforts to develop engineered organisms that catabolize lignin into biofuels or fine chemicals.

KEYWORDS

Protocatechuate 4,5-dioxygenase; Dioxygenase; Gallate; 3-*O*-methyl gallate; Lignin; PCAD superfamily

INTRODUCTION

As concerns about fossil fuel consumption increase, developing methods to produce fuels and fine chemicals from renewable biomass is more important than ever. Many scientists are working to develop methods for the conversion of lignocellulosic biomass (which includes switchgrasses, trees and other terrestrial plants) into these important commodities, as assessments of this biomass reveal high sustainability and low environmental impact as compared to other sources of carbon mass [1-5]. With the intention of enabling improvement of the enzymatic conversion of lignin derived aromatic compounds (LDACs) into common metabolites, which could improve the efficiency of fermentative lignin catabolism, we investigated the substrate utilization of the ring-cleaving dioxygenase LigAB from *Sphingobium* sp. strain SYK-6 (Figure 1) [6, 7]. Previously in our lab we identified a library of compounds that function as substrates, inhibitors, or activators of LigAB [6, 8]. Therein, we identified a pocket adjacent to the active site that is the hypothesized binding site of vanillin, an allosteric activator of LigAB. We performed mutagenesis of various residues to test the impact of those mutants on the enzymatic activation, including residue Phe103 α which is at the interface of the allosteric pocket and the active site. Further examination of the LigAB active site lead us to hypothesize that mutants of Phe103 α (Histidine, Leucine, Valine and Threonine), which enlarge the volume of the active site, could potentially enhance the turnover of the LDACs gallate (GA) and 3-*O*-methyl gallate (3OMG), two previously identified substrates that were found to be less catalytically efficient than protocatechuate (PCA; the native substrate of LigAB). We had initially sought to also investigate Serine and Alanine mutants, but neither of these mutants

1 allowed for the LigA and LigB proteins to co-purify, suggesting the importance of Phe103 α for
2 enabling protein-protein interactions in the LigAB complex [8].

3 Here we report the *in silico* docking of PCA, GA and 3OMG to this series of Phe103 α
4 mutants, the kinetic characterization of these mutants with PCA, the assessment of the impact of
5 mutagenesis on the catalytic efficiency with the alternate substrates of LigAB (GA and 3OMG),
6 and the corresponding activation kinetics for these mutants with all three substrates. Multiple
7 mutants enhanced catalysis for the C5-substituted derivatives of PCA (Figure 1). Some mutants
8 also display catalytic activation in the presence of vanillin when either PCA or alternate
9 substrates were being dioxygenated (vanillin is the pre-pre substrate of LigAB which in our prior
10 study enhanced turnover for the wild-type enzyme with PCA but none of the alternate substrates)
11 [8]. These studies provide interesting insight into elements through which LigAB controls
12 substrate specificity. This work suggests that scientists attempting to engineer microbes to utilize
13 LDACs could take advantage of the results herein to simplify their efforts through using one
14 promiscuous ring-cleaving dioxygenase in the place of the three enzymes found in nature.

15 **MATERIALS AND METHODS**

16 Commercially available reagents and solvents were purchased from Sigma-Aldrich or Alfa
17 Aesar and used without purification unless otherwise noted. 3-*O*-methyl gallate was purchased
18 from ChromaDex. Bradford assays, standardized against BSA according to the methods in the
19 Bio-Rad Quick Start Bradford Protein Assay Kit (Hercules, CA), were conducted in 96-well
20 plate format and absorption values were recorded on a SpectraMax M5 plate reader (Molecular
21 Devices, Sunnyvale, CA). MAX Efficiency DH5 α and One Shot BL21 Star chemically
22 competent *E. coli* cells were purchased from Life Technologies (Carlsbad, CA). QuikChange
23 Lightning Site Directed Mutagenesis kit was purchased from Agilent (Santa Clara, CA).

Centrifugation and ultracentrifugation were performed on a DuPont Instruments (Wilmington, DE) Sorvall RC-5B centrifuge and Beckman (Brea, CA) L7-80 Ultracentrifuge with type 60-Ti rotor, respectively. All cells were lysed using a SIMO-Aminco Industry Inc. (Rochester, NY) French press. Wild-type LigAB and the Phe103 α mutants were prepared as described previously [8].

Kinetic Analyses. Kinetic parameters for LigAB and all mutants were determined by measuring the rate of O₂ consumption in the presence of varying concentrations of organic substrate in air-saturated solutions containing 50 mM buffer (specified below) with 3-10 nM enzyme (exact concentration determined for each experiment using a Bradford assay) using a Clark-type electrode as previously described [6, 8]. The effect of pH on the dioxygenation rate of each mutant was determined using 250 μ M PCA and buffers over the range of pH 6 – 10 at 25 °C. The buffer solutions (50 mM) used to span this pH range were: phosphate (pH 6 to 8), Tris (pH 7.5 to 9), BICINE (pH 8 to 9), and CAPSO (pH 9 to 10). Steady-state kinetics were determined for PCA, GA or 3OMG (1 μ M–5000 μ M) in a Tris buffer, with data obtained at least in triplicate. Aqueous stock solutions (25 mM) of the desired organic substrate (PCA, GA, or 3OMG) were prepared immediately prior to use in a 10 mL volumetric flask. 3OMG (25 mM) was prepared in a 10 % DMSO water solution. Control experiments to determine the impact of DMSO on catalysis were performed by assessing the reaction rates for LigAB-WT with PCA dissolved in a 10 % DMSO solution versus PCA prepared in water alone at varying concentrations (1 μ M–5000 μ M); these experiments showed that DMSO had minimal impact on catalysis (Supplemental Figure S1). Analogous experiments were performed on the LigAB mutant enzymes, with little impact on catalysis being observed (data not shown). Kinetic parameters for all steady-state data were determined analogously to our prior work by fitting the

data to either the Michaelis–Menten equation or the Haldane equation for substrate inhibition using KaleidaGraph (Synergy). Substrate induced enzyme inactivation was determined for each mutant-substrate combination as previously described [6, 8].

Iron Binding. Thawed anaerobically purified enzyme was buffer exchanged using Sephadex G-25 desalting gel as above into degassed 50 mM Tris (pH 7.5), 1:9 *t*-butanol/H₂O to remove excess iron from storage. A 30 μ L aliquot of each enzyme sample was removed for use in a Bradford assay to determine the enzyme concentration. Each protein sample was further analyzed using a Ferene-S assay adapted for the 96-well plate format was performed, as described by Capyk, *et al* [9], to determine the iron content of all samples. The iron concentration was divided by the concentration of enzyme determined from the Bradford assay to give the relative iron-enzyme ratios.

Activity Enhancement Assays. The kinetic values were determined by methods analogous to those previously reported [8], by measuring the initial rate of O₂ consumption with varying concentrations of vanillin (10 μ M - 5 mM) for each mutant-substrate combination in 50 mM Tris buffer (pH 7.5, 25 °C). The concentration of organic substrate (PCA, Gallate, or 3OMG) was set as 10 $\times$ K of the enzyme for the respective substrate as determined by the least-squares fitting of steady-state kinetics data (measured in the absence of vanillin) to the Michaelis–Menten equation. For enzyme-substrate combinations where the value of 10 $\times$ K exceeded 5 mM, reactions were performed using 5 mM organic substrate. Each reaction was initiated by the addition of enzyme (using LigAB-WT or the Phe103 α mutants at concentrations from 3–7 nM that were specifically determined after each experiment using a Bradford assay). The baseline rate corresponding to 100 % of the native (un-stimulated) LigAB-WT catalysis rate (v_0) of PCA was obtained at 500 μ M PCA in the absence of vanillin. The percent increase or decrease in reaction rate over v_0 was calculated for substrate-vanillin combination at each concentration of

vanillin, and kinetic parameters were determined by a least-squares fitting of the data to the Michaelis–Menten equation modified to describe non-competitive inhibition and allosteric activation, as previously described [8].

Computational Docking. Computational dockings were performed using AutoDock v4.2 (AD4) and the LigAB structure PDB: 1B4U (2.20 Å) with methods identical to those previously reported [8], except as indicated below. Each of the LigAB substrates described herein (PCA, GA, and 3OMG) was docked to the active sites of LigAB-WT and LigAB with computationally mutated Phe103 α (F103H, F103L, F103T, and F103V). In all cases, the 103 α residue was left to be flexible during the docking. These dockings were performed in a search grid containing the active site of 40 (x) by 40 (y) by 40 (z) with a step spacing of 0.375 Å, and centered near the crystal structure coordinates of the active-site non-heme iron (x = 55.198, y = 44.339, z = 47.175). The docking search parameters generated 25 docked conformations for PCA and GA and 200 docked conformations for 3OMG using a genetic algorithm with a population size of 150 individuals, a maximum generation number of 2.7×10^4 , and 2.5×10^6 energy evaluations (all other parameters left at their default values). Two hundred (as opposed to 25) conformations were generated for 3OMG because of an absence of what is believed to be the physiological conformation of binding in the initial generation of 25 docked conformations.

RESULTS AND DISCUSSION

Altered Substrate Utilization of LigAB Mutants. Our previous investigation of LigAB identified its ability to catalyze 4,5-dioxygenation reactions on seven different substrates with rates ranging over four orders of magnitude [6]. This makes LigAB the most omnivorous of the protocatechuate 4,5-dioxygenases that have been investigated to date [10-16]. Analysis of the LigAB crystal structure (PDB: 1B4U) shows that Phe103 α , in addition to being part of the

previously identified allosteric pocket, is positioned within 5 Å of the C5 position of PCA. We hypothesized that steric interaction of the C5 functional groups of substrates with this residue imparted the trend in catalytic efficiency (k/K) observed for the natural substrates PCA, GA, and 3OMG; that is, as the C5 functional group increases in size, the observed k/K decreases [6]. To investigate this, we generated a series of Phe103α mutants, including valine (F103V), leucine (F103L), threonine (F103T), and histidine (F103H). The pH rate profiles of each mutant (Supplemental Figure S2) and the iron binding of each mutant (Supplemental Table 1) were assessed, confirming our prediction that the mutants would maintain the general catalytic features previously observed for wild-type LigAB, since we were not directly changing any of the residues involved in acid-base catalysis or metal binding. Each of the mutants were then tested for their ability to utilize PCA, GA and 3OMG (Table 1) and the partition ratios for each enzyme-substrate combination was assessed (Table 2). Additionally, computational docking of PCA, GA and 3OMG was performed with LigAB-WT and with each of the mutants to allow examination of potential active site interactions (Figure 2).

Not surprisingly, none of the mutants had greater activity for PCA (measured by k and k/K) than the wild-type enzyme (Table 1; Figure 2; Supplemental Figure S3). The reduction in turnover and the increases in K of the Phe mutants can be explained by the increase in the active site volume (from replacing Phe103α with smaller amino acids) which allows PCA to explore a larger conformational space. This is exemplified in Figure 2B, where the F103H mutant allows the non-productive binding of PCA, where only one hydroxyl group is coordinating to the Fe(II), as compared to the productive binding geometry reflected in Figure 2A. At the onset, we recognized that other dioxygenase enzymes have been mutated in attempts to improve substrate scope; however, these studies often did not yield significant improvement in catalytic activity

[17, 18]. We had hypothesized that the valine and leucine mutants would enable favorable interactions with the methyl of 3OMG, while the histidine and threonine mutants would enhance the binding and catalysis with GA. While these hypotheses were not fully supported by our data, encouragingly, multiple Phe103 α mutants were identified in which k and/or k/K values were greater for gallate or 3OMG than LigAB-WT.

Three of the mutant enzymes (F103V, F103H and F103T) had k/K values for the dioxygenation of 3OMG that were not only greater than those of LigAB-WT, but also greater than those reported for DesZ, the physiological catalyst for ring opening 3OMG (DesZ $k/K = 1.03 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) [19, 20]. Substitution of Phe with either the smaller, hydrophobic valine or the hydroxyl-containing threonine yielded mutant enzymes that were unchanged for GA dioxygenation but had greater turnover and lower K values for the dioxygenation of 3OMG (k/K was greater in both cases by over an order of magnitude; Table 1). The similar catalytic profiles for F103V and F103T are probably due to the increased size of their active site pockets. With any of the substrates tested, F103T has smaller K values as compared to F103V, but these gains are offset by lower k values. We had hypothesized that the difference might be due to the ability of the Thr hydroxyl to hydrogen bond with the substrate C5 hydroxyl or methyl ether; however, computational docking of GA and 3OMG into the F103T mutant revealed that the Thr hydroxyl group hydrogen bonds to the C1 carboxylate of GA and 3OMG (Figure 2d and 2f, respectively). Since Valine cannot hydrogen bond, this likely explains the relative differences in the kinetics for these isosteric substitutions. Based upon the comparison of the K for the F103L mutant (all of which are above 1 mM) with LigAB-WT, F103T or F103V, we are lead to believe that the size and conformational flexibility of Leucine must make formation of the E-S complex difficult.

1 The Phe to His mutant, which preserves the side chain aromaticity but introduces the
2 potential for hydrogen bonding interactions in the active site, exhibited the smallest decrease in
3 catalytic efficiency for PCA and improved catalysis for both alternate substrates (GA and
4 3OMG). The F103H mutant has a 4.2-fold decrease in catalytic efficiency as compared to
5 LigAB-WT, due to ~2-fold changes in both k and K . We hypothesize that the hydrogen bonding
6 ability of the F103H mutation allows non-productive binding of PCA, where the C3 hydroxyl
7 group faces the 103 α residue leading to a hydrogen bond forming with the introduced histidine
8 residue (observed in 24 % of docked conformations of PCA to the F103H mutant; Figure 2b). A
9 similar non-productive ‘flipped’ binding mode of PCA occurred in 16 % of docked
10 conformations to LigAB-WT; however, hydrogen bonding to the native Phe103 α side chain is
11 chemically not possible, and so this conformation is inherently less stable than in F103H. While
12 the F103H mutant had a smaller K for both GA and 3OMG (2.3- and 2.5-fold differences,
13 respectively) as compared to the wild-type enzyme, the k of F103H is smaller for GA but greater
14 for 3OMG. Therefore the gain in catalytic efficiency for 3OMG catalysis is much larger (31.4-
15 fold) than for GA (1.8-fold). This large increase in catalytic efficiency for 3OMG can likely be
16 explained by docking studies performed with LigAB-WT in which 3OMG failed to adopt a
17 geometry with bidentate-hydroxyl coordination of iron within the wild-type enzyme (likely due
18 to steric conflict between the methyl ether and Phe103 α), preferring instead to bind with the –
19 OCH₃ facing away from Phe103 α (Figure 2e). However, when 3OMG was docked onto any of
20 the LigAB mutants (including F103L where little kinetic change was observed), it was able to
21 find the physiologically relevant binding conformation (Figure 2f), suggesting that the steric bulk
22 of Phe is indeed a contributor to the substrate specificity of LigAB. While Phe103 α clearly plays
23 a role in substrate recognition and selectivity for functional group variation at position C5, there

are very likely other residues that contribute to substrate selectivity that could be altered to lead to changes in selectivity for other positions on the catechol frame.

Impact of Phe103 α Mutants on Allosteric Activation of Alternate Substrate

Dioxygenation. As we described previously, LigAB-WT is capable of being allosterically activated by vanillin for the deoxygenation of PCA, but not GA or 3OMG [8]. Since mutagenesis of LigAB altered dioxygenation rates for the various substrates as compared to LigAB-WT, the mutants were also examined for alterations in allosteric activation with vanillin (Table 3; Figure S4). Interestingly, while the F103V reaction is still activated by the presence of vanillin, F103L showed no measurable change in kinetics in the presence of vanillin, while both F103T and F103H showed inhibition caused by vanillin. Additionally, like LigAB-WT, F103V and F103L also showed no activation (or inhibition) of either GA or 3OMG turnover in the presence of vanillin. F103H and F103T, however, showed a change in turnover rate for catalysis of GA and 3OMG in the presence of vanillin. For F103H, vanillin diminished the rate of turnover of all substrates, albeit to varying degrees. In addition to inhibiting F103T turnover of PCA, vanillin inhibited F103T turnover of 3OMG, though to a lesser extent (yet still maintaining a linear rate with respect to vanillin concentration). Interestingly, despite the inhibition of PCA and 3OMG turnover, vanillin elicited a turnover rate enhancement ($56 \pm 3 \%$; $K_{\text{act}} = 0.4 \pm 0.1 \text{ mM}$), of GA dioxygenation by F103T. This was the only observed enhancement with a substrate other than PCA for any of the examined mutants. Since the location of Phe103 α is at the interface between the active site and the allosteric vanillin binding site and because mutagenesis of this residue alters the activation profile, it is unsurprising that substrates with larger groups at the C5 position would also show altered activation profiles with other substrates. As we previously described, the lack of activation of LigAB-WT with GA and 3OMG makes sense because vanillin

concentrations would only impact the metabolism of PCA, but not GA or 3OMG.⁸ The involvement of Phe103 α in transmitting the activation signal is further supported by the analysis of these mutants. One could speculate that the C5 group must match in either size or polarity with the enzymatic residue at position 103 α , in order for the activation mechanism to be effective. Because the mechanism of the activation is thus far unknown, these results suggest that LigAB-WT·PCA, F103V·PCA, and F103T·GA should all be examined via experimental and computational methods to provided mechanistic insights into the activation of this enzyme.

CONCLUSIONS

The kinetic studies of LigAB-WT and its mutants have revealed that the native dioxygenation of multiple LDAC substrates can be enhanced, thereby reducing the relative specificity differences between PCA, GA, and 3OMG. These gains in catalytic efficiency for GA and 3OMG are offset with only moderate reduction in catalytic efficiency of the natural substrate. Researchers attempting to engineer a microorganism for the fermentative conversion of LDACs into biofuels would therefore be able to take advantage of the enzymatic proficiency of F103H for the simultaneous dioxygenation of PCA, GA and 3OMG, or F103V when the reaction would be occurring in reaction solutions containing high concentrations of vanillin, rather than needing to introduce multiple enzymes to perform these functions.

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FIGURE CAPTIONS

Figure 1. Abridged lignin-derived, aromatic compound metabolic pathway from *Sphingobium* sp. SYK-6 demonstrating the connection between protocatechuic acid (PCA; red), 3-*O*-methylgallate (3OMG; green), gallate (GA; blue) and LigAB (bold purple) adapted from Masai et al., 2007[19]. Enzyme abbreviations: DesA, syringate *O*-demethylase; DesZ, 3OMG 3,4-dioxygenase; DesB, gallate 3,4-dioxygenase; **LigAB**, protocatechuic acid 4,5-dioxygenase; LigC, 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) dehydrogenase; LigI, 2H-pyran-2-one-4,6-dicarboxylate (PDC) hydrolase; LigJ, 4-oxalomesaconate (OMA) hydratase; LigK, 4-carboxy-4-hydroxy-2-oxoadipate (CHA) aldolase/oxaloacetate decarboxylase; LigM, vanillin/3-*O*-methylgallate (3OMG) *O*-demethylase. Other abbreviations: CHMOD, 2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate.

Figure 2. Computational docking of substrates to LigAB-WT and F103X α mutant active sites. (A) Native substrate (PCA, brick red) docked to LigAB-WT (F103 α , red). (B) Docking of PCA to F103H (orange) showing non-productive binding of substrate, possibly contributing to experimentally observed substrate inhibition. (C) Docking of GA (light blue) to F103V (blue). (D) F103T (purple) showing an additional hydrogen bond formed between the carboxylate of a docked GA and the Thr hydroxyl. (E) LigAB-WT showing non-productive binding with docked 3OMG (green). (F) F103T with 3OMG docked in a productive binding conformation.

TABLES AND FIGURE

Figure 1.

1

2 **Figure 2.**

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2 **Table 1. Steady State Kinetic Constants for LigAB-WT and Mutants with PCA, Gallate,**
3 **and 3OMG.** All data were collected in air-saturated 50 mM Tris buffer (250-275 μM O_2 , pH
4 7.5, 25 $^\circ\text{C}$), in the absence of vanillin.

Enzyme	Kinetic Constant	PCA	Gallate	3OMG
LigAB-WT	K (μM)	51 ± 4	440 ± 60	2300 ± 300
	k (s^{-1})	216 ± 3	53 ± 2	7.3 ± 0.5
	k / K ($\text{M}^{-1}\text{s}^{-1}$)	4.26×10^6	1.21×10^5	3.13×10^3
F103H	K (μM)	108 ± 9	190 ± 30	930 ± 40
	k (s^{-1})	110 ± 3	41 ± 2	91 ± 1
	k / K ($\text{M}^{-1}\text{s}^{-1}$)	1.02×10^6	2.14×10^5	9.83×10^4
	Fold Change in k / K from WT	-4.2	+1.8	+31.4
F103L	K (μM)	1600 ± 200	7000 ± 3000	4000 ± 1000
	k (s^{-1})	33 ± 2	5 ± 1	7 ± 1
	k / K ($\text{M}^{-1}\text{s}^{-1}$)	2.07×10^4	7.26×10^2	1.78×10^3
	Fold Change in k / K from WT	-205.8	-166.7	-1.8
F103T	K (μM)	210 ± 20	210 ± 30	390 ± 60
	k (s^{-1})	112 ± 2	22.7 ± 0.6	15.1 ± 0.6
	k / K ($\text{M}^{-1}\text{s}^{-1}$)	5.44×10^5	1.07×10^5	3.85×10^4
	Fold Change in k / K from WT	-7.8	-1.1	+12.3
F103V	K (μM)	260 ± 20	900 ± 200	1000 ± 50
	k (s^{-1})	89 ± 2	24 ± 1	49 ± 1
	k / K ($\text{M}^{-1}\text{s}^{-1}$)	3.41×10^5	2.78×10^4	4.89×10^4
	Fold Change in k / K from WT	-12.5	-4.4	+15.6

k / K from WT

1

2 **Table 2. Partition ratios for each LigAB enzyme with each mutant.**

PCA			Gallate		3OMG	
		Partition		Partition		Partition
	(x 10 ⁻³ s ⁻¹)	Ratio	(x 10 ⁻³ s ⁻¹)	Ratio	(x 10 ⁻³ s ⁻¹)	Ratio
WT	12 ± 3 a	18000	21.6 ± 0.7	2500	1.3 ± 0.8	5600
F103V	19 ± 1	4600	32 ± 2	750	19 ± 2	2600
F103L	6 ± 0.2	5500	8 ± 2a	660	7 ± 2a	1000
F103T	10.4 ± 0.5	11000	42 ± 2	540	14 ± 3a	1100
F103H	23 ± 1	4800	70 ± 3	580	12 ± 1	7800

3

Table 3. Percentage Enhancement or Inhibition of Turnover as a Result of Co-incubating Substrate and Vanillin. All data were collected in air-saturated 50 mM Tris buffer (250-275 μ M O₂, pH 7.5, 25 °C) with variable vanillin concentrations and substrate concentrations of 10 x *K* up to 5 mM (plotted in Figure S4).

	PCA	Gal	3OMG
LigAB	+ 37 $\pm$ 3%	nmc [̄]	nmc
F103H	- 23 $\pm$ 18%	Inhib [‡]	- 20 $\pm$ 4%
F103L	nmc	nmc	nmc
F103T	Inhib [‡]	+ 56 $\pm$ 3%	Inhib [‡]
F103V	+ 20 $\pm$ 4%	nmc	nmc

[‡] accurate estimation of the percent inhibition in these cases is impaired by the linearity of the fits.

[̄]nmc: no measured change.