Electronic Supplementary Information

Characterization of Xenobiotic Reductase A (XenA): Study of Active Site Residues, Substrate Spectrum, and Stability

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Section 1. Experimental Procedures

1.1. XenA Expression and Purification

The gene of XenA from *Pseudomonas putida* was kindly provided by Prof. Brian Fox (University of Wisconsin, Madison, WI).[1] The *xenA* gene was transformed into *E. coli* BL21 for protein expression. The culture was started in a 5 mL LB_{Kan} medium inoculated from frozen glycerol stock and grown overnight at 37°C. The preculture was used for inoculation of a 1 L culture (0.1% v/v), which was gently aerated until OD600 reached 0.6 before addition of 0.1 mM of IPTG. The induced culture was incubated overnight for protein expression at 37°C. Harvested cell pellets were kept at -80°C before protein purification.

XenA was purified with a two-step column purification process: HiTrap Q-XL anion chromatography and HiPrep Sephacryl S-100 gel filtration chromatography on an ÄKTAexplorer™ system (all by GE Healthcare, Sweden). The harvested xenA pellet was first resuspended in 20 mM pH 8.0 bis-tris buffer and disrupted by sonication. The cell debris were discarded by centrifugation, and the supernatant was washed with a gradient of 0 mM to 500 mM KCl in 20 mM bis-tris buffer on a Q-XL column at a flow rate of 1 mL/min. The majority of xenA eluted at 300 mM KCl and was applied to the Sephacryl S-100 column. 50 mM sodium phosphate buffer with 300 mM NaCl at pH 7.5 was used as the running buffer with flow rate set at 0.5 mL/min, where pure xenA was collected at 60 – 70 mL elution range (total column volume is 120 mL). The protein concentration was determined by the Bradford method using Coomassie

Plus Protein assay reagent with BSA assay (Pierce Chemical) as the calibration curve standard. SDS-PAGE analysis was performed with 50 µg protein loaded into each well (Figure 1). The protein samples were mixed with 2X sample buffer (125 mM TrisHCl, pH 6.8, 4% SDS, 50% glycerol, 0.02% bromophenol blue, and 10% 2-mercaptoethanol). The mixed samples were incubated at 100°C for 5 min and loaded onto 12% PAGE^{TM} Gold precast gel and run in BioRad Mini Protean chamber (BioRad, Hercules CA) at 150 V for 45 min with Tris-HEPES-SDS running buffer (12.1 g/L Tris, 23.8 g/L HEPES, 1 g/L SDS). Biorad Precision Plus protein ladder was used as standards in the gel analysis. GelCodeR Blue Stain Reagent (Pierce, Rockford IL) was used for final gel staining. Lastly, we measured the FMN occupancy of the purified xenA (all purified xenA wild type and variants showed full FMN occupancy) and checked the enzymatic activity with 2-cyclohexenone as substrate (Table 1). The FMN occupancy study was performed by observing the changes in xenA at both 360 nm and 450 nm wavelength for native and unfolded states. The changes in absorbance were used to compute the FMN concentration with extinction coefficient of 12,500 $M^{-1}.cm^{-1}$. The occupancy of FMN was determined by comparing the calculated FMN concentration with pure enzyme concentration.

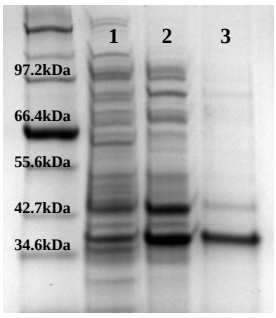

Figure 1. SDS-PAGE analysis for wild type XenA purification.
Lane #1 lysate, #2 Q-XL fraction, #3 S-100 fraction.

Table 1. XenA purification.

XenA Purification	Total Protein [mg]	% Recovery	Specific Activity [U/mg]	Total Activity [U]	% Yield
Lysate	38.4	100	2.56	98.3	100
Q-XL Fraction	13.3	34.6	2.97	39.5	40.2
S-100 Fraction	3.81	9.91	3.84	14.6	14.9

Assay: 200 mM phosphate buffer pH 7.5, 1 mM 2-cyclohexenone, 0.2 mM NADPH, 37°C, and 10 µg of enzyme.

1.2. QuikChange® Mutagenesis Protocol

The mutations on *xenA* were accomplished with the QuikChange® mutagenesis protocol with the following PCR primers design (*red italic* letters indicate the changes to the wild type):
C25G: Forward: 5' CGCCATTCCGCCCATG*G*GCCAGTACATGGCCGAGGAT 3'
C25V: Forward: 5' CATCGCCATTCCGCCCATG*GTG*CAGTACATGGCCGAGGATGG 3'
A59V: Forward: 5' TGGTGGTCGAAGCCACGG*T*GGTGGCACCGGAAGGGC 3'

The *xenA* gene from *Pseudomonas putida* in pET27 vector was used as the template for mutations. Failsafe buffer J (EPICENTER® Biotechnology, Madison, WI) was used in the standard PCR protocol with Pfu polymerase from New England Biolabs (Marlborough, MA). PCR assay: 25 μL of Failsafe buffer J, 125 ng *xenA* DNA plasmid template, 0.25 μM each of both forward and reverse primers, and sterile water to 50 μL in total volume. The mixed sample was first denatured at 98°C for 5 minutes, then supplemented with 1 μL of Pfu buffer, followed by denaturation at 98°C for another 30 seconds. The annealing temperature was set at 55°C for 1 minute, extension at 72°C for 16 minutes, and repeated for total of 18 cycles. The final extension was set at 72°C for 25 minutes, and finished with a temperature hold at 4°C. All mutants were sequenced with Eurofins MWG Operon (Birmingham, AL) service and transformed into *E. coli* BL21 strain for protein expression and purification as described in Section 1.1.

1.3 Gas Chromatography Analytical Procedures

GC-FID analyses were performed on a Shimadzu GC-2010 instrument with helium (ultra high purity grade, Airgas South, Atlanta, GA) as carrier gas. Conversions of compounds **1–14** and e.e. values of the corresponding products were determined with the columns and methods described below. All the substrates and the corresponding products were purchased from Sigma Aldrich unless stated otherwise with literature resources provided.

2-Cyclohexen-1-one (**1**), *trans*-β-nitrostyrene (**7**), *trans*-β-methyl-β-nitrostyrene (**8**), 1-nitro-1-cyclohexene (**9**), 2,4-dinitrotoluene (**10**), nitrobenzene (**11**), 2-nitrofuran (**12**), 2-methyl-5-nitrofuran (**13**), and 2-(2-nitrovinyl)-furan (**14**) were analyzed using a SHRX5 column (15 m, 0.25 mm, 0.25 μm) (Shimadzu, Kyoto, Japan). Temperature program for **1**: injector and detector

temperature at 220°C; split ratio 25:1; start at 100°C, hold 2 min, 10°C.min^{-1} to 160°C, hold 2 min. Retention time: 2-cyclohexen-1-one 1.4 min, cyclohexanone 1.3 min. Temperature program for **7**: injector and detector temperature at 320°C; split ratio 25:1; start at 100°C, hold 2 min, 10°C.min^{-1} to 180°C, 20°C.min^{-1} to 180°C, hold 1 min. Retention time: *trans*-β-nitrostyrene 6.5 min. Temperature program for **8**: injector and detector temperature at 320°C; split ratio 25:1; start at 100°C, hold 2 min, 10°C.min^{-1} to 180°C, 20°C.min^{-1} to 180°C, hold 1 min. Retention time: *trans*-β-methyl-β-nitrostyrene 6.5 min. Temperature program for **9**: injector and detector temperature at 220°C; split ratio 25:1; starting at 100°C, hold 1 min, 20°C.min^{-1} to 220°C, hold 1 min. Retention time: 1-nitro-1-cyclohexene 8.2 min, nitrocyclohexane 16.3 min. Temperature program for **10**: injector and detector temperature at 300°C; split ratio 25:1; starting at 80°C, hold 2 min, 15°C.min^{-1} to 290°C, hold 4 min. Retention time: 2,4-dinitrotoluene 8.1 min. Temperature program for **11**: injector and detector temperature at 300°C; split ratio 25:1; starting at 80°C, hold 5 min, 10°C.min^{-1} to 290°C, hold 5 min. Retention time: nitrobenzene 5.6 min, aniline 3.2 min, nitrosobenzene 2.2 min, azobenzene 14.1 min, phenylhydroxylamine 8.0 min. Temperature program for **12**: injector temperature at 150°C and detector temperature at 220°C; split ratio 25:1; starting at 50°C, hold 3 min, 30°C.min^{-1} to 200°C, hold 2 min. Retention time: 2-nitrofuran 4.6 min. Temperature program for **13**: injector temperature at 150°C and detector temperature at 220°C; split ratio 25:1; starting at 50°C, hold 3 min, 30°C.min^{-1} to 200°C, hold 2 min. Retention time: 2-methyl-5-nitrofuran 6.1 min. Temperature program for **14**: injector temperature at 150°C and detector temperature at 220°C; split ratio 25:1; starting at 50°C, hold 3 min, 30°C.min^{-1} to 200°C, hold 2 min. Retention time: 2-(2'-nitrovinyl)-furan 6.4 min.

Compounds 2-methyl-2-cyclopentenone (**2**), 3-methyl-2-cyclohexenone (**3**), ketoisophorone (**4**), and citral (**5**) were analyzed using Rt-BDEXcst chiral column (30 m, 0.32 mm, 0.25 μm) (Restek, Bellefonte, PA). Temperature program for **2**: injector and detector temperature at 200°C; split ratio 25:1; start at 70°C, hold 8 min, 10°C.min^{-1} to 80°C, hold 2 min, 30°C.min^{-1} to 180°C, hold 5 min. Retention time: 2-methyl-2-cyclopentenone 16.3 min, (*R*)-2-methylcyclopentanone 15.5 min, (*S*)-2-methylcyclopentanone 15.4 min. The absolute configurations of the enantiomers were determined via CD method as described in literature.[2] Temperature program for **3**: injector and detector temperature at 200°C; split ratio 20:1; start at 90°C, 1°C.min^{-1} to 130°C, 5°C.min^{-1} to 180°C, hold 10 min. Retention time: 3-methyl-2-cyclohexenone 50.7 min, (*R*)-2-methylcyclohexanone 37.4 min, (*S*)-3-methylcyclohexanone 37.6 min. Temperature program for

4: injector and detector temperature at 210°C; split ratio 25:1; start at 100°C, 3°C.min^{-1} to 130°C, 20°C.min^{-1} to 200°C, hold 3 min. Retention time: ketoisophorone 15.4 min, (*R*)-levodione 15.9 min, (*S*)-levodione 15.7 min. Temperature program for **5**: injector and detector temperature at 210°C; split ratio 6.4:1; start at 80°C, 2.5°C.min^{-1} to 130°C, hold 10 min, 5°C.min^{-1} to 180°C, hold 5 min. Retention time: citral 31.2 min, (*R*)-citronellal 22.1 min, (*S*)-citronellal 21.9 min.

1-Nitro-2-phenylpropene (**6**) was analyzed using Cyclosil-B chiral column (30 m, 0.32 mm, 0.25 μm) (Agilent J&W Scientific, Santa Clara, CA). Temperature program for **6**: injector and detector temperature at 210°C; split ratio 25:1; start at 100°C, hold 10 min, 5°C.min^{-1} to 130°C, hold 15 min, 5°C.min^{-1} to 220°C, hold 2 min. Retention time: 1-nitro-2-phenylpropene 28.7 min and 35.3 min (isomers), (*R*)-1-nitro-2-phenylpropane 25.0 min, (*S*)-1-nitro-2-phenylpropane 24.7 min. The substrate synthesis and product identification were accomplished with the reported method.[3]

Section 2. Additional Data

Kinetic and thermal deactivation: XenA kinetic measurement was performed by monitoring the oxidation of cofactor NADPH at 340 nm using spectrophotometer (Beckman Coulter DU800, Brea, CA), with extinction coefficient ε_{340nm} of 6.22 $mM^{-1}.cm^{-1}$. Assay condition: 200 mM phosphate buffer pH 7.5, 1 mM substrate, 0.2 mM cofactor NADPH, 10 $\mu g.mL^{-1}$ enzyme, at 37°C. Enzyme thermostability study (half-life $t_{1/2}$) was performed with incubation of purified enzyme at 37°C, and monitoring the residual activity with 2-cyclohexenone kinetic assay over time. The collected data was fitted to a 1st order deactivation kinetic to determine the observed deactivation constant, $k_{d,obs}$.

$$1^{st} \text{ order deactivation equation: } [A] = [A]_0 e^{-k_{d,obs}t}$$

$$\text{Linearization: } \ln\left(\frac{[A]}{[A]_0}\right) = -k_{d,obs}t$$

$$\text{Half life equation: } t_{1/2} = \frac{\ln(2)}{k_{d,obs}}$$

Note: [A] is the enzyme concentration at time t, $[A]_0$ is the original enzyme concentration t time zero, $t_{1/2}$ is the half-life, and $k_{d,obs}$ is the observed 1st order deactivation rate constant.

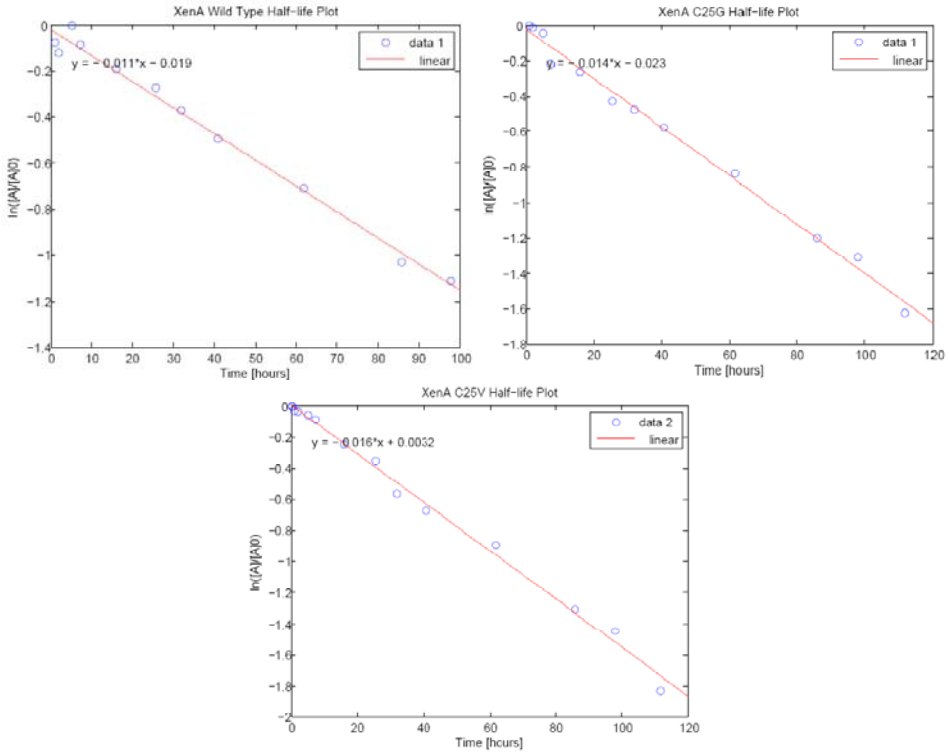

Figure 2. Thermal deactivation study of xenA wild type, C25G, and C25V mutants.
The half-life $t_{1/2}$ at 37°C results: wild type 63 h, C25G 49.5 h, C25V 44.5 h, A59V > 112 h.
The deactivation constant $k_{d,obs}$ results: wild type 0.0119 h^{-1}, C25G 0.0136 h^{-1}, C25V 0.0155 h^{-1}, A59V < 0.0062 h^{-1}.

Melting behavior: The melting temperature T_m was determined with a Circular Dichroism Spectropolarimeter (Jasco-810, Easton, MD) using 6Q quartz cuvette by analyzing the change in ellipticity at 220 nm. CD measurement assay: enzyme concentration at 50 $\mu g.mL^{-1}$ with temperature ramp of $1°C.min^{-1}$ from 20°C - 90°C. The data was interpreted employing a two-state model with a native and a denatured state.

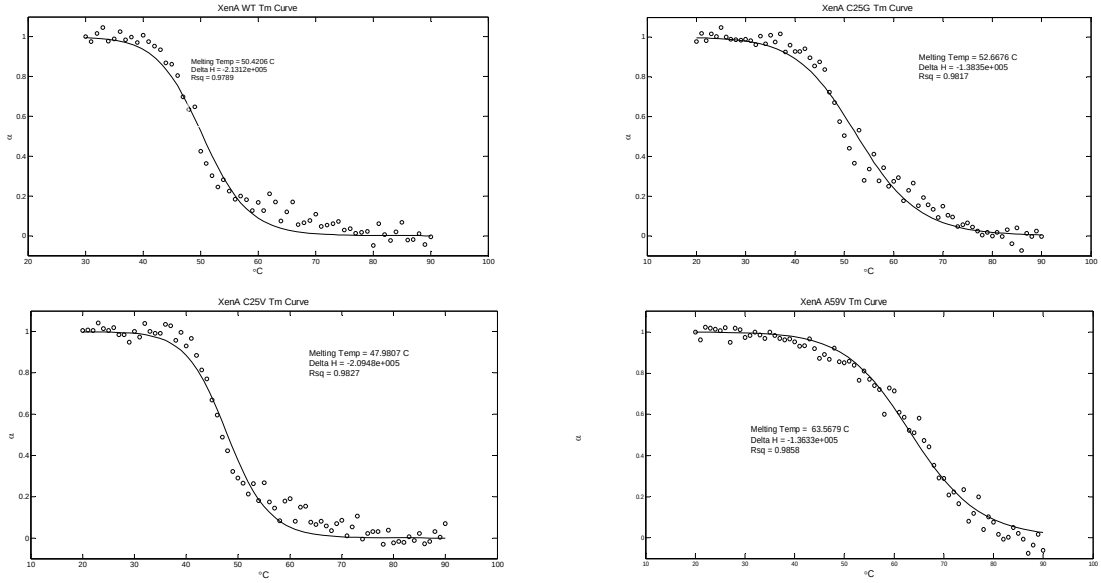

Figure 3. Tm curve for xenA WT, C25G, C25V, and A59V mutants.
α = 1 represents enzyme was at native state, α = 0 represents enzyme at denatured state.
The T_m result: wild type 50.4°C, C25G 52.7°C, C25V 48.0°C, and A59V 63.5°C.

Substrate inhibition: Substrate inhibition was observed with substrate 2-cyclohexenone with uncompetitive inhibition pattern. Concentration activity profile range (0 mM to 2.5 mM) was fitted with Matlab to determine the inhibition constant, K_I:

$$\text{Michaelis-Menten equation for uncompetitive inhibition: } v_0 = \frac{V_{max}[S]}{K_M + \alpha'[S]}$$

$$\text{where } \alpha' = 1 + \frac{[I]}{K_I}$$

Note: v_0 is the initial reaction rate at substrate concentration [S], v_{max} is the maximum rate, Michaelis constant K_M measures the substrate concentration at half v_{max}, K_I is the inhibition constant, and [I] is the inhibitor concentration.

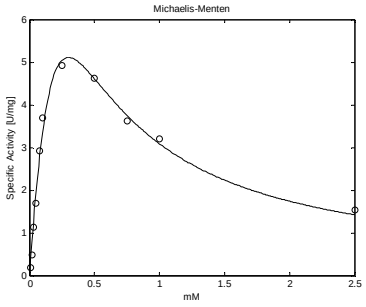

Figure 4. Fit to a Michaelis-Menten kinetics for 2-cyclohexenone (0 – 2.5 mM) substrate inhibition with xenA. The fitted parameters were: k_{cat} = 25.7 s^{-1}, K_M = 0.19 mM, K_I = 0.15 mM, R^2 = 0.9843

Total turnover: The total turnover number (TTN, dimensionless) is an indicator of lifetime biocatalyst productivity, and defined as the maximum number of molecules of substrate that an enzyme active site can convert over its life cycle.

Chemical TTN was determined by varying the enzyme concentration and observing NADPH turnover until no more turnover (i.e. change in NADPH concentration) was observed.

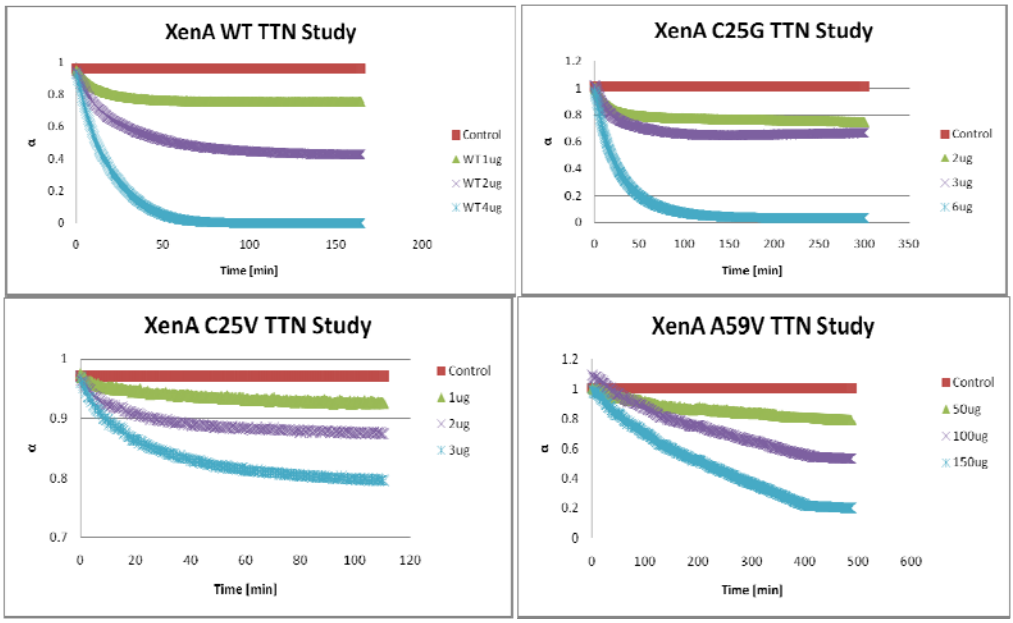

Figure 5. TTN Data for xenA WT and mutants. α defined as the amount of cofactor in the system.
Assay: 200 mM phosphate buffer pH 7.5, 0.2 mM NADPH, 2 mM 2-cyclohexenone, and varying amount of enzyme at 37°C.
The TTN_{chem} results: wild type 1959, C25G 1073, C25V 280, and A59V 38.
Note: TTN_{chem} for A59V is an approximation, this mutant still shows activity even after > 10 h of measurement.

The TTN observable in connection with unfolding at elevated temperatures, $TTN_{thermal}$, was estimated from readily measured biochemical quantities, namely the specific activity and the deactivation rate constant, measured under identical solution conditions at 37°C. This method was established and validated in recent work for enzymes whose thermal deactivation follows 1st order deactivation.[4] This estimation method requires two parameters, namely $k_{d,obs}$, the observed first-order deactivation rate constant, and $k_{cat,obs}$, the observed turnover number sufficiently far below the melting point. As T_m values here range from 48.0 to 63.5°C, no melting occurs at 37°C.

The process yield during the life time of a biocatalyst: $yield = \int_0^\infty V_{\max}(t)dt$

where we define: $V_{max} = k_{cat,obv}[E]_0 e^{-k_{d,obs}t}$

The integrated result gave: $TTN = \frac{k_{cat,obs}}{k_{d,obs}}$

where $k_{cat,obs}(s^{-1}) = \frac{specific\ activity\ (\frac{U}{mg}) \times enzyme\ MW\ (\frac{g}{mol})}{60{,}000}$

and $k_{d,obs}(s^{-1}) = \frac{\ln(2)}{t_{1/2}(s)}$

Note: $[E]_0$ is the original enzyme concentration, $k_{cat,obs}$ is the observed catalytic constant, and $k_{d,obs}$ is the observed deactivation constant.

The $TTN_{thermal}$ result: wild type 8.3×10^5, C25G 1.4×10^5, C25V 7.2×10^4, A59V 1.9×10^4.

References:

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2. J. J. Partridge, N. K. Chadha, and M. R. Uskokovic, *Journal of the American Chemical Society*, 1973, **95**, 532-540
3. M. Hall, C. Stueckler, H. Ehammer, E. Pointner, G. Oberdorfer, K. Gruber, B. Hauer, R. Stuermer, W. Kroutil, P. Macheroux and K. Faber, *Advanced Synthesis & Catalysis*, 2008, **350**, 411-418.
4. T. A. Rogers and A. S. Bommarius, *Chemical Engineering Science*, 2010, **65**, 2118-2124.