

Directed evolution of aniline dioxygenase for enhanced bioremediation of aromatic amines

Ee Lui Ang · Jeffrey P. Obbard · Huimin Zhao

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Abstract The objective of this study was to enhance the activity of aniline dioxygenase (AtdA), a multi-component Rieske non-heme iron dioxygenase enzyme isolated from *Acinetobacter* sp. strain YAA, so as to create an enhanced biocatalyst for the bioremediation of aromatic amines. Previously, the mutation V205A was found to widen the substrate specificity of AtdA to accept 2-isopropylaniline

(2IPA) for which the wild-type enzyme has no activity (Ang EL, Obbard JP, Zhao HM, FEBS J, 274:928–939, 2007). Using mutant V205A as the parent and applying one round of saturation mutagenesis followed by a round of random mutagenesis, the activity of the final mutant, 3-R21, was increased by 8.9-, 98.0-, and 2.0-fold for aniline, 2,4-dimethylaniline (24DMA), and 2-isopropylaniline (2IPA), respectively, over the mutant V205A. In particular, the activity of the mutant 3-R21 for 24DMA, which is a carcinogenic aromatic amine pollutant, was increased by 3.5-fold over the wild-type AtdA, while the AN activity was restored to the wild-type level, thus yielding a mutant aniline dioxygenase with enhanced activity and capable of hydroxylating a wider range of aromatic amines than the wild type.

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E. L. Ang · H. Zhao (✉)
Department of Chemical Engineering and Biomolecular Engineering, University of Illinois at Urbana-Champaign,
600 South Mathews Avenue,
Urbana, IL 61801, USA
e-mail: zhao5@uiuc.edu

H. Zhao
Center for Biophysics and Computational Biology,
University of Illinois at Urbana-Champaign,
600 South Mathews Avenue,
Urbana, IL 61801, USA

H. Zhao
Department of Chemistry,
University of Illinois at Urbana-Champaign,
600 South Mathews Avenue,
Urbana, IL 61801, USA

E. L. Ang
Department of Chemical and Biomolecular Engineering,
National University of Singapore,
21 Lower Kent Ridge Road,
Singapore 119077, Singapore

J. P. Obbard
Division of Environmental Science and Engineering,
National University of Singapore,
21 Lower Kent Ridge Road,
Singapore 119077, Singapore

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Introduction

Aniline and its derivatives are highly toxic compounds, with numerous reports on their carcinogenic effects (Bomhard and Herbold 2005; Markowitz and Levin 2004; Nohmi et al. 1983; Przybojewska 1999; Shardonofsky and Krishnan 1997; Weisburger et al. 1978). These compounds may be released to the environment through effluent streams from the pharmaceutical and azo-dye manufacturing industries (Essington 1994; Michaels and Lewis 1985, 1986; Rai et al. 2005) where they are commonly used as intermediates (Grayson et al. 1984; Radomski 1979). As biodegradation has been found to be the main route for the removal of aromatic amine pollutants from the natural

environment (Lyons et al. 1984), with the hydroxylation of the aromatic ring often constituting the first step of biodegradation (Bugg and Winfield 1998), an enzyme with the ability to hydroxylate a wide range of aniline homologues would be a practical and valuable biocatalyst for the remediation of harmful aromatic amine contaminants.

The aniline dioxygenase (AtdA) is a multi-component enzyme isolated from *Acinetobacter* sp. strain YAA, which carries out the simultaneous deamination and oxygenation of aniline and *o*-toluidine to produce catechol and 3-methylcatechol, respectively (Takeo et al. 1998a, b). The aniline dioxygenase is a Rieske non-heme iron dioxygenase (Wackett 2002) encoded by five genes (*atdA1-A5*) that constitute four putative components: AtdA1 which is a glutamine synthetase-like protein, AtdA2 which is a glutamine amidotransferase-like protein, AtdA3 and AtdA4 which resemble the large (α) and small (β) subunits of the terminal class dioxygenase, as well as AtdA5 which is a reductase component (Takeo et al. 1998a, b).

Previously, the AtdA3 subunit of the AtdA was found to play a key role in controlling the substrate range and activity of the enzyme (Ang et al. 2007). The mutant 1-K31, which had a V205A mutation in the AtdA3 subunit, was able to accept 2-isopropylaniline (2IPA), a substrate not accepted by the wild-type (WT) enzyme. However, the widened substrate range of the enzyme came at the expense of its activity. Compared to the WT enzyme, the V205A mutant had an 8.4- and 28-fold decrease in activity for aniline (AN) and 2,4-dimethylaniline (24DMA), respectively. The objective of this work was to enhance V205A mutant as a biocatalyst suitable for the bioremediation of aniline and its derivatives by improving its activity on AN, 24DMA, 2IPA, as well as to introduce novel activity for 2-secbutylaniline (2SBA). Chemical structures of these substrates are shown in Fig. 1.

A round of saturation mutagenesis on active site residues followed by error-prone polymerase chain reaction (epPCR) yielded the mutant 3-R21. This mutant had three mutations—V205A, I248L, and S404C. The activ-

ities of 3-R21 for AN, 24DMA, and 2IPA were improved by 8.9-, 98.0-, and 2.0-fold, respectively, over its parent V205A. In particular, the specific activity of the final mutant 3-R21 was improved by 3.5-fold for 24DMA over the WT. The biocatalyst successfully engineered in this work will hold exciting possibilities for the bioremediation of aromatic amines.

Experimental procedures

Materials

AN, 24DMA, 2IPA, 2SBA, catechol, isopropyl- β -D-thiogalactopyranoside (IPTG), dimethylformamide, and ampicillin were purchased from Sigma (St. Louis, MO, USA). 3-Isopropylcatechol (3IPC) was purchased from Chem Service (West Chester, PA, USA). Gibbs' reagent was purchased from MP Biomedicals (Solon, OH, USA). Quikchange XL site-directed mutagenesis kit and *Pfu Turbo* DNA polymerase were purchased from Stratagene (La Jolla, CA, USA). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and 1st Base (Singapore). All DNA gel purifications were carried out using the QIAEX II gel purification kit from Qiagen (Valencia, CA, USA). All plasmid isolations were carried out using the QIAprep Miniprep kit from Qiagen. *Escherichia coli* JM109 was purchased from Novagen (Madison, WI, USA). The plasmid pTrc99A was from Amersham Pharmacia (Piscataway, NJ, USA). The GeneMorph II EZClone domain mutagenesis kit used for epPCR library creation was purchased from Stratagene (La Jolla, CA).

Saturation mutagenesis

A saturation mutagenesis library at each substrate binding pocket residue was created using the Quikchange XL site-directed mutagenesis kit (Stratagene), with the mutant 1-K31 and 2-A21 as parental templates for the first and second rounds, respectively. The primers used are listed in Table S1 of the Electronic Supplementary Material. The PCR and transformation protocol recommended in the manufacturer's manual were used. Transformants were plated on Luria–Bertani (LB) agar plates containing 100 mg/L ampicillin and incubated overnight at 37°C.

Random mutagenesis by error-prone PCR

The random mutagenesis library was created using the GeneMorph II EZClone domain mutagenesis kit from Stratagene. For the mutant megaprimer synthesis, the primers used were A3 *EcoRI* F (5'-CTCGGTGGGTGATGTATAGAATTCAGGAACAGACCATGAAAACC-3') and

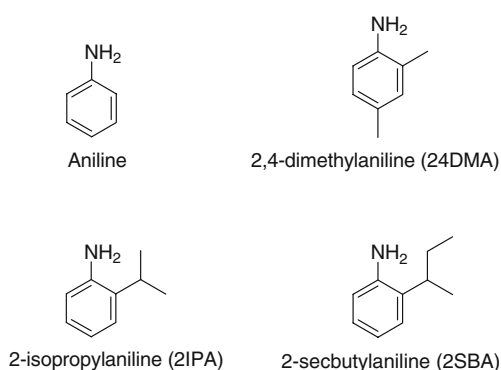


Fig. 1 Aniline and analogue substrates used in this study

A3 lib R2 (5'-CTTTATTATTATTCATGGCGTACCTCAACCGGT-3'). Seven hundred nanograms of template DNA was used. Twenty-five amplification cycles were used in the PCR reaction with the annealing temperature set at 54°C. All other components and conditions were as recommended by the manufacturer's manual. The PCR product was gel-purified using the QIAEX II gel purification kit from Qiagen. The EZClone reaction was carried out using the protocol recommended in the manufacturer's manual. Transformants were plated on LB agar plates containing 100mg/L ampicillin and incubated overnight at 37°C.

Screening method

The screening method was adapted from (Sakamoto et al. 2001) with modifications. Each colony of a library was picked into 200 μ L of LB containing ampicillin (100 mg/L) in separate wells of a 96-well microplate, with three WT clones included as positive controls in each plate. The plates were incubated overnight at 37°C with shaking at 250 rpm. Ten microliters of the overnight culture was inoculated into new wells containing 90 μ L of M9 minimal media supplemented with 100 mg/L ampicillin and 1 mM IPTG. Two replicates of each plate were made. The plates were incubated at 30°C with 250 rpm shaking for 4 h. Then, 100 μ L of M9 media with 100 mg/L ampicillin, 1 mM IPTG, and 2 mM substrate was added into each well of a plate. A different substrate was added to each plate, either 24DMA or 2SBA. The plates were then incubated at 30°C with 250 rpm shaking for 4 h. Optical density at 600 nm (OD_{600}) was measured after incubation. Ten microliters of 0.32% Gibbs' reagent was added directly to each well, and absorbance at wavelength 620 nm was measured after 5 min. The activity of each mutant, as indicated by the absorbance at 620 nm, was then normalized to its cell density (OD_{600}). Positive mutants from each screen were subjected to a second screen carried out in larger volumes using culture tubes instead of 96-well microplates.

Whole cell activity assay

An overnight LB culture of JM109 with WT or mutant plasmid was inoculated into 150 mL LB to an OD_{600} of 0.02 and incubated in a 37°C shaker at 250 rpm. JM109 transformed with the empty vector, pTrc99A, was used as the negative control. When the OD_{600} reached 0.50 to 0.55, IPTG was added to a final concentration of 1 mM. The culture was then incubated in a 30°C shaker at 250 rpm for 3 h. The induced culture was then centrifuged at 4,000 \times g for 10 min. The supernatant was discarded and the cell pellet was resuspended with 150 mL of modified M9 buffer (M9 minimal media with 0.1% glucose). The resuspended

cells were centrifuged using the same conditions again. The supernatant was discarded and the cell pellet was resuspended with modified M9 buffer to a final OD_{600} of about 10. Then, 5 mL of the resuspended cells was aliquoted into a 50-mL centrifuge tube, and 5 μ L of 1 M substrate, dissolved in *N,N*-dimethylformamide, was added to a final concentration of 1 mM. The cells were then incubated at 30°C with 250 rpm shaking. Samples (0.5 mL) were drawn at various time points. The samples were centrifuged at 16,000 \times g in a benchtop centrifuge for 3 min, and the supernatant was stored in -20°C until it was analyzed.

The substrate and products were separated and quantified using high-pressure liquid chromatography (HPLC) with a 250 \times 4.60-mm Synergi 4 μ Polar-RP 80A column from Phenomenex (Torrance, CA, USA). All HPLC methods used were isocratic with a flow rate of 1 mL/min. Aniline was analyzed using 90% 20 mM potassium phosphate buffer (pH7.0) and 10% acetonitrile as mobile phase. 2IPA was analyzed using 60% 20 mM potassium phosphate buffer (pH7.0) and 40% acetonitrile as mobile phase. 24DMA was analyzed using 70% 20 mM potassium phosphate buffer (pH7.0) and 30% acetonitrile as mobile phase.

For each culture, 1 mL of the resuspended cells was centrifuged at 6,000 \times g in a benchtop centrifuge for 3 min and the supernatant discarded. The cell pellet was resuspended in 50 mM Tris-HCl (pH7.5) and disrupted using a single pass through the Constant Systems cell disruptor (Warwick, UK) at 20.3 kpsi. The disrupted cells were centrifuged at 16,000 \times g in a benchtop centrifuge for 5 min at 4°C, and the supernatant was assayed for protein concentration using the BCA protein assay kit from Pierce (Rockford, IL, USA). The whole cell activity was calculated by normalizing the initial rate of substrate conversion or product formation to the protein concentration.

Sample preparation for SDS-PAGE analysis

Overnight cell cultures in LB with 100 mg/L of ampicillin were inoculated into fresh LB with ampicillin (100 mg/L) at a volume ratio of 1:100 and incubated in a 37°C shaker at a speed of 250 rpm. When OD_{600} reached 0.5, the cultures were induced with 1 mM IPTG and incubated in a 30°C shaker at a speed of 250 rpm for 3 h. After induction, the OD_{600} of the cells was measured and the cells were then centrifuged at 6,000 \times g in a Beckman J2-21M induction drive centrifuge at 4°C. The supernatant was discarded and the cell pellet resuspended in 50 mM Tris-HCl (pH7.5) to a final OD_{600} of the cells of about 15. Then, the cells were disrupted using a single pass through the Constant Systems cell disruptor at 20.3 kpsi. The disrupted cells were centrifuged at 16,000 \times g in a benchtop centrifuge for 5 min. The supernatant was stored at -20°C until ready

for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Results

Saturation mutagenesis of mutant V205A

A recent study on directed evolution suggested that mutations closer to the active site of enzymes may be more effective at creating novel activity (Morley and Kazlauskas 2005). Hence, saturation mutagenesis was first used to explore the active site residues for mutants that can further widen the substrate range of the 1-K31 mutant or improve its activity. Thirteen active site residues in 1-K31 were identified using the homology model of AtdA3 developed previously (Ang et al. 2007) based on the crystal structures of the α -subunits of naphthalene dioxygenase (Kauppi et al. 1998), biphenyl dioxygenase (Furusawa et al. 2004), and cumene dioxygenase (Dong et al. 2005). These residues were N198, G202, L213, I248, Q250, K256, E257, W260, A293, G294, N296, L304, and F348. A total of 186 clones from each saturation mutagenesis library were screened for novel activity toward 2SBA and improved activity against 24DMA using the Gibbs' reagent-based screening method described in "Experimental procedures". 24DMA was chosen as the screening substrate since 1-K31 had the lowest activity for this substrate (Table 1). Although 2SBA and 2IPA differs only by a methyl group in the alkyl side chain, 2IPA was accepted by 1-K31, but not 2SBA (Ang et al. 2007). As such, 2SBA was chosen as the screening substrate for novel activity.

From the saturation mutagenesis studies, five mutants (2-A21, 2-A22, 2-A23, 2-A24, and 2-A25) with improved activity for 24DMA were isolated from the I248 library. All the mutants were found to be more active than the parent 1-K31 when assayed in a second tier assay in culture tubes to measure their activity. Sequencing of the mutants revealed that all had an isoleucine to leucine mutation at residue 248. The mutant 2-A21, which has the V205A/I248L double mutation, was transformed into *E. coli* JM109 strain for

further analysis. Although mutants that have improved activity toward 24DMA were discovered, no active mutants for 2SBA were isolated.

Using mutant 2-A21 as the parent, another round of saturation mutagenesis was conducted on the remaining active site residues (N198, G202, L213, Q250, K256, E257, W260, A293, G294, N296, L304, and F348) of AtdA3. The target substrates were again 24DMA and 2SBA. However, no additional beneficial mutation was found.

SDS-PAGE analysis of mutant 2-A21

The expression level of AtdA in the V205A/I248L double mutant (mutant 2-A21) was compared to those of the parent and the mutant V205A using SDS-PAGE analysis (Fig. S1 in Electronic Supplementary Material). The SDS-PAGE gel showed no observable difference between the concentrations of the AtdA1 (56.8 kDa), AtdA2 (28.5 kDa), AtdA3 (50.3 kDa), AtdA4 (24.0 kDa), and AtdA5 (37.2 kDa) subunits in the mutant compared to their corresponding subunits in the parent. Thus, the improved activity of the mutant was most likely not a result of altered expression.

Whole cell activity of mutant 2-A21

The activity of the mutant 2-A21, which had the V205A/I248L double mutations, was characterized using the whole cell activity assay. The substrates used in the assay were AN, 24DMA, and 2IPA. The results are presented in Table 1. The activities of 2-A21 for these three substrates were compared to that of its parent, the mutant V205A, to gauge the impact on activity of the I248L mutation.

The specific activity of the mutant 2-A21 for AN was 9.3 nmol min⁻¹ mg⁻¹ protein, while that of the parent 1-K31 was 3.1 nmol min⁻¹ mg⁻¹ protein. Thus, the introduction of the I248L mutation to mutant 1-K31 resulted in a threefold increase in specific activity for AN. The sole product of AN conversion was catechol, as evidenced by the product's HPLC retention time compared to that of the authentic standard, and the rate of catechol

Table 1 Conversion rates of aniline, 24DMA, and 2IPA by *E. coli* JM109 expressing the AtdA mutants 1-K31, 2-A21, and 3-R21

| Mutant | Mutation | AN | | 24DMA | | 2IPA | |
|--------|-------------------|--|---------------|--|---------------|--|---------------|
| | | Rate (nmol min ⁻¹ mg ⁻¹ protein) | Relative rate | Rate (nmol min ⁻¹ mg ⁻¹ protein) | Relative rate | Rate (nmol min ⁻¹ mg ⁻¹ protein) | Relative rate |
| 1-K31 | V205A | 3.1±0.1 | 1.0 | 0.1±0.02 | 1.0 | 1.1±0.2 | 1.0 |
| 2-A21 | V205A/I248L | 9.3±2.4 | 3.0 | 1.7±0.1 | 17.0 | 1.6±0.5 | 1.5 |
| 3-R21 | V205A/I248L/S404C | 27.7±1.5 | 9.0 | 9.8±0.4 | 98.0 | 2.2±0.1 | 2.0 |

Relative rates are calculated with respect to mutant 1-K31, the parent in the second round of active site residue saturation mutagenesis. Rates listed are averages of the triplicate experiments and errors are represented by the standard deviation of the triplicates.

formation was the same as that of AN conversion (data not shown). The I248L mutation in this case resulted in a 17-fold improvement in activity for 24DMA, with the mutant 2-A21 converting 24DMA at a rate of $1.7 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein and the parent V205A only converting the same substrate at a rate of $0.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. The mutant 2-A21 converted 24DMA to 3,5-dimethylcatechol (35DMC), as confirmed by co-elution with the compound purified as described in previous work (Ang et al. 2007). For the substrate 2IPA, the mutant 2-A21 displayed an activity of $1.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, a 1.5-fold improvement in activity over the mutant 1-K31, which had an activity of $1.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. 3IPC was the sole product of 2IPA conversion by the mutant 2-A21. Again, HPLC co-elution with authentic 3IPC was the means of identifying the product.

Despite the improvement of overall activity of the enzyme in this round of saturation mutagenesis, the AN and 24DMA activity of the mutant 2-A21 was still lower than that of the WT, which displayed a rate of 26.0 and $2.8 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein for AN and 24DMA, respectively (Ang et al. 2007). Further engineering would be required to increase the activity of the enzyme.

Random mutagenesis of mutant 2-A21

As a further round of saturation mutagenesis of the AtdA3 active site residues did not improve AtdA activity, the focus was shifted to residues further away from the active site of the enzyme. To enhance the activity and substrate range of AtdA3, the mutant 2-A21 was subjected to random mutagenesis using epPCR to generate the mutant library.

The epPCR library of the 1.3-kb *atdA3* gene was generated using the GeneMorph II EZClone domain mutagenesis kit as described in “Experimental procedures”. Sequencing of randomly selected clones from the epPCR library of *atdA3* revealed that the mutation frequency of the library was ~ 2.7 nucleotide per gene. Approximately 2,500 clones were screened for improved activity against 24DMA and novel activity for 2SBA. From the screen, one mutant, 3-R21, which exhibited significantly improved 24DMA activity over the parent 2-A21, was isolated. The 3-R21 mutant was then subjected to a secondary screening to ascertain its activity. Sequencing of the *atdA3* gene revealed a serine to cysteine mutation at residue 404 (S404C mutation). No mutant with 2SBA activity was isolated.

SDS-PAGE analysis of mutant 3-R21

The expression level of AtdA in *E. coli* XL10 from mutant 3-R21 was compared to that of its parent, 2-A21. The SDS-PAGE gel is shown in Fig. S2 in the Electronic Supplementary Material. The SDS-PAGE gel showed that the

AtdA3, A4, and A5 subunits in mutant 3-R21 were expressed at a slightly higher level compared to their corresponding subunits in the 2-A21.

Whole cell activity of mutant 3-R21

The activity of mutant 3-R21 for 24DMA was compared to its parent 2-A21 using the whole cell activity assay. To each resting cell culture, 1 mM of 24DMA was added, and the fraction of 24DMA remaining with time is shown in Fig. 2. Mutant 3-R21 was able to convert all of the 24DMA within 90 min, while the parent 2-A21 was much slower, with less than 30% of DMA converted after 210 min.

The 2-A21 mutant had a specific activity of $1.4 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, calculated based on the initial rate of 24DMA conversion normalized to total protein concentration. In contrast, the 3-R21 mutant displayed an activity of $9.8 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, which is a sevenfold increase in activity compared to 2-A21. The regiospecificity of mutant 3-R21 was unchanged, as the only product formed was 35DMC as confirmed by HPLC co-elution with the standard.

Similarly, the mutant 3-R21 was tested for its activity on AN and 2IPA. The mutant 3-R21 had an activity of $27.7 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. This is a threefold increase in activity over mutant 2-A21 ($9.3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) obtained from the previous round. Catechol is the sole product of aniline conversion by 3-R21. For 2IPA, mutant 3-R21 exhibited a conversion rate of $2.2 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, which is a 1.4-fold increase over mutant 2-A21 which displayed an activity of $1.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. The product of 2IPA conversion by 3-R21 was 3IPC. The activities of 3-R21 for AN, 24DMA, and 2IPA are shown in Table 1.

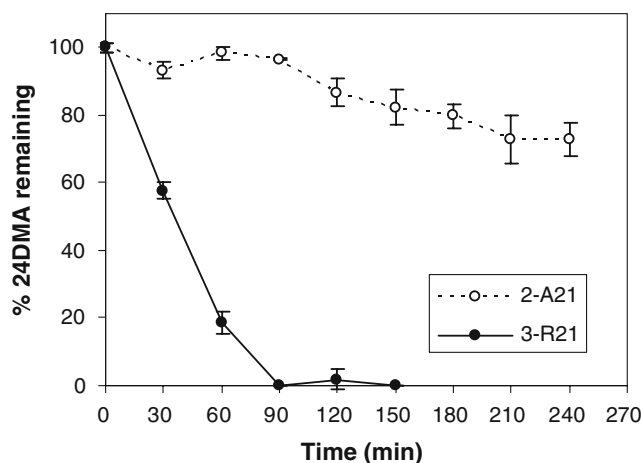


Fig. 2 Percentage of 24DMA remaining with time when added to resting cell cultures of mutant 3-R21 and its parent 2-A21. Error bars are standard deviation of triplicate experiments

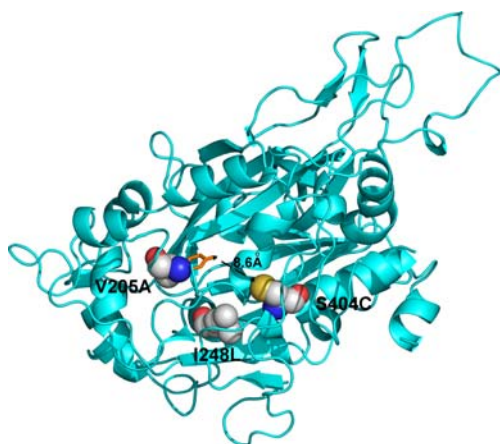


Fig. 3 Location of V205A, I248L, and S404C mutations in the AtdA3 subunit. The orange molecule represents 24DMA bound in the substrate binding pocket of the subunit

Discussion

From the first round of saturation mutagenesis of these residues, two mutants, 1-K31 and 1-A21, were identified. The 1-K31 mutant had a V205A mutation, which introduced activity for 2IPA but lowered the overall activity of AtdA for other substrates. The 1-A21 mutant, which had a I248L mutation, increased the AtdA activities for aniline and 24DMA but did not widen the substrate range of AtdA. Mutant 1-K31 was chosen over mutant 1-A21 as the parental template because creating activity for novel substrates was deemed more challenging than improving the activity for substrates already accepted by the WT enzyme (Zhao 2007).

The introduction of the I248L mutation had a greater effect on the activity of mutant 1-K31 than it had on the WT, for which the activity for AN and 24DMA was increased by 1.7- and 2.0-fold, respectively (Ang et al. 2007). From the analysis of the homology model of AtdA3, residue 248 and residue 205 are about 9.7 Å apart from each other and do not come into direct contact (Fig. S3 in the Electronic Supplementary Material). Hence, the effects of the two mutations on increased activity are likely to be independent. It was postulated that the I248L mutation improved activity of the enzyme by enlarging the entrance of the substrate binding pocket. The low activity of 1-K31 for AN and 24DMA would mean that the same increase in the ease of substrate entry and product exit from the substrate binding pocket would bring about a greater improvement in activity relative to the WT.

The S404C mutation was mapped onto the homology model of AtdA3 to analyze the molecular basis of its effect. The S404C mutation is located on an α -helix close to the C

terminus of the protein, more than 8 Å away from the substrate binding pocket (Fig. 3). Residue 404 is not exposed to the surface of the protein, and the orientation of the S404 side chain is similar to that of C404 (Fig. 4). The closest residues to residue 404 in both mutants are I376, H403, and E401. The serine to cysteine mutation shifts the residue 404 side chain slightly towards H403. The C404 residue, which has a slight negative charge, may be attracted to the positively charged H403, and being more hydrophobic than S404, may have enhanced hydrophobic interactions with I376 and F408. Both of these factors can help to stabilize the α -helix structure near the C terminus of the protein and help to improve its overall activity. It should

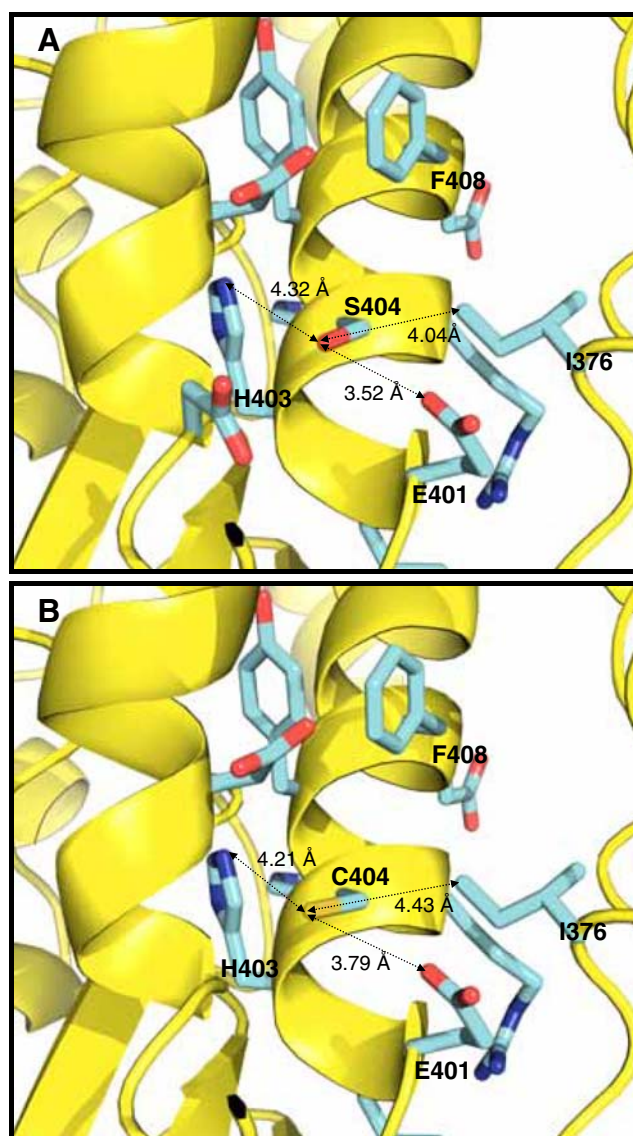


Fig. 4 Residue 404 and its neighboring residues in mutant 2-A21 (a) and mutant 3-R21 (b)

be noted that the structural and molecular interpretations of the mutations presented above are based on the homology model of the AtdA3 subunit, and a crystal structure of AtdA3 would help to confirm these interpretations. Purification and characterization of the k_{cat} and K_{m} AtdA1–A5 subunits would also help to better evaluate the effects of each mutation on the enzyme.

The authors do not rule out the possibility that the subunits AtdA1 and A2 may have effects on the enzyme's substrate specificity and activity as well, and their sequence space can be probed in a similar way as AtdA3 to widen the substrate range and activity of AtdA. As shuffling of the α -subunits of homologous dioxygenases have shown to enhance the degradation capacities of the enzymes as well as introduce novel substrate activity (Kumamaru et al. 1998), another possible strategy is to shuffle the *atdA* genes with homologous aniline dioxygenases such as those from *Frateriia* sp. ANA-18 (Murakami et al. 2003), *Delftia acidovorans* strain 7N (Urata et al. 2004), and *Delftia tsuruhatensis* AD9 (Liang et al. 2005). In addition, shuffling of the entire dioxygenase operons instead of just the α -subunit may yield interesting mutants with enhanced substrate range, as was reported from the multigene shuffling of the chlorobenzene dioxygenases and toluene dioxygenases (Beil et al. 1998).

To broaden the substrate range of the naphthalene dioxygenase of *Ralstonia* sp. strain U2 (Fuenmayor et al. 1998) for the bioremediation of nitroaromatic compounds, Keenan et al. (2005) created a saturation mutagenesis library on position F350 of the α -subunit of the naphthalene dioxygenase (NagAc). This was found to be a key residue in determining the substrate specificity and activity of Rieske non-heme iron dioxygenases (Keenan et al. 2004; Parales et al. 2000). The F350T mutant, which had novel activity for 2,6-dinitrotoluene (26DNT) and 2-amino-4,6-dinitrotoluene (2A46DNT), was used as the parent for DNA shuffling in the next round of mutagenesis. This second round of random mutagenesis yielded the double mutant F350T/G407S, which had an activity improvement of up to threefold over its parent. However, in the course of evolution, the mutations that improved or introduced novel activity for one substrate did not negatively affect the turnover of another substrate, as was the case for the V205A mutation for WT AtdA. The evolution of the biphenyl dioxygenase from *Burholderia xenovorans* LB400 (Mondello 1989; Seeger et al. 1999) for bioremediation of chlorobiphenyls yielded variants with broader substrate ranges but reduced activity for 2,6,4'-chlorobiphenyl (2,6,4'-CB), which is a substrate accepted by the WT BphA (Zielinski et al. 2006). However, no attempts were made to recover the activity for 2,6,4'-CB. In comparison, this work clearly shows that the activity for other substrates lost by a mutation that brought about novel substrate

activity can be recovered or even improved through rounds of evolution. The result brings AtdA a step closer to its application as an agent for the bioremediation of aromatic amines.

In summary, the objective of this work was to enhance the AtdA enzyme as a biocatalyst for bioremediation by improving its activity. Overall, the activity of the parent, mutant 1-K31, for AN, 24DMA, and 2IPA was increased by 8.9-, 98.0-, and 2.0-fold, respectively, after one round of saturation mutagenesis followed by epPCR. The activity for AN was restored to the level of the WT AtdA, while the activity for 24DMA was increased by 3.5-fold. The activities of the mutants 1-K31, 2-A21, and 3-R21 for AN, 24DMA, and 2IPA are presented in Fig. 5 together with that of the WT to allow comparison of the degree of improvement resulting from each round of mutagenesis.

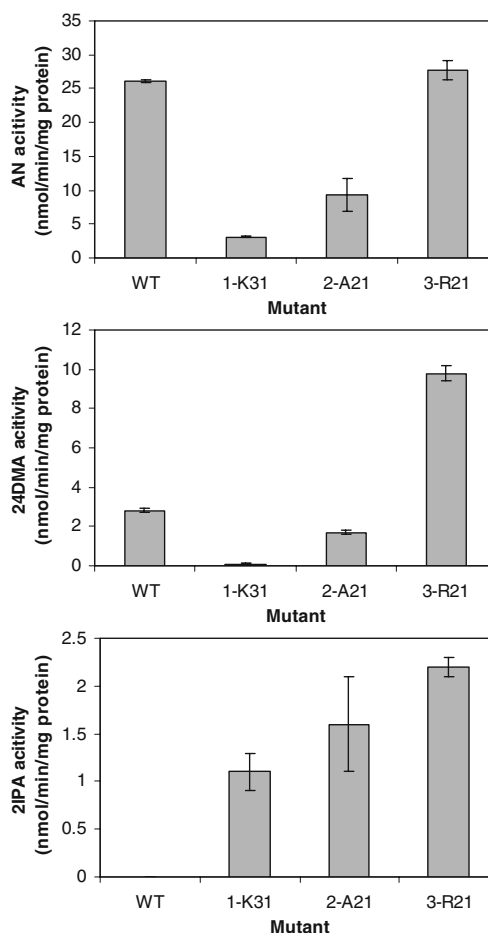


Fig. 5 Activities of WT, 1-K31 (V205A), 2-A21 (V205A/I248L), and 3-R21 (V205A/I248L/S404C) for AN (a), 24DMA (b), and 2IPA (c). Error bars are standard deviation of triplicate experiments

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