Directed Evolution of a Ring-cleaving Dioxygenase for Polychlorinated Biphenyl Degradation*

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DoxG, an extradiol dioxygenase involved in the aerobic catabolism of naphthalene, possesses a weak ability to cleave 3,4-dihydroxybiphenyls (3,4-DHB), critical polychlorinated biphenyl metabolites. A directed evolution strategy combining error-prone PCR, saturation mutagenesis, and DNA shuffling was used to improve the polychlorinated biphenyl-degrading potential of DoxG. Screening was facilitated through analysis of filtered, digital imaging of plated colonies. A simple scheme, which is readily adaptable to other activities, enabled the screening of >10^8 colonies/h. The best variant, designated DoxG_M2A2, cleaved 3,4-DHB with an apparent specificity constant of 2.0 ± 0.3 × 10^6 M^-1 s^-1, which is 770 times that of wild-type (WT) DoxG. The specificities of DoxG_M2A2 for 1,2-DHN and 2,3-DHB were increased by 6.7-fold and reduced by 2-fold, respectively, compared with the WT enzyme. DoxG_M2A2 contained three substituted residues with respect to the WT enzyme: L190M, S191W, and L242S. Structural data indicate that the side chains of residues 190 and 242 occur on opposite walls of the substrate binding pocket and may interact directly with the distal ring of 3,4-DHB or influence contacts between this substrate and other residues. Thus, the introduction of two bulkier residues on one side of the substrate binding pocket and a smaller residue on the other may reshape the binding pocket and alter the catalytically relevant interactions of 3,4-DHB with the enzyme and dioxygen. Kinetic analyses reveal that the substitutions are anti-cooperative.

Polychlorinated biphenyls (PCBs) are the most widely distributed chlorinated pollutants in the environment. Microbial catabolic activities have been investigated as a means of remediating PCB-contaminated sites. Many PCB congeners, of which there are ~100 in typical commercial formulations, are transformed via the pathway (Fig. 1A), which aerobically catabolizes biphenyl to benzoate and 2-hydroxy-...
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In the present study, a directed evolution strategy combining error-prone PCR, saturation mutagenesis and DNA shuffling was used to improve the 3,4-DHB-cleaving capabilities of DoxG. A simple and versatile high-throughput colorimetric screening method was developed. The specificity of DoxG and DoxGSMA2 for 1,2-DHN, 2,3-DHB, and 3,4-DHB was investigated. The effects of the observed mutations are discussed with respect to crystal structures of enzyme-substrate complexes.

MATERIALS AND METHODS

Chemicals—2,3-DHB was a kind gift from Dr. Victor Snieckus (14). 3,4-DHB was from ULTRA Scientific (North Kingstown, RI). 1,2-DHN was from Sigma-Aldrich. Ferene S was from ICN Biomedicals, Inc. (Costa Mesa, CA). All other chemicals were of analytical grade and were used without further purification.

Strains, Media, and Growth—Escherichia coli strain DH5α was used for DNA propagation and was cultured at 37 °C and 200 rpm in Luria-Bertani (LB) broth with the appropriate antibiotics. Pseudomonas putida KT2442 (15) transformed with pVLT31 (16) derivatives were used for overexpression and was cultured at 30 °C and 250 rpm in LB medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside and 100 mg/liter ampicillin at a colony density of 15–20 colonies/cm².

DNA Manipulation—Plasmid DNA was propagated, purified, digested, and amplified according to standard procedures (19). Sequencing was performed using an ABI 373 Stretch instrument (Applied Biosystems, Foster City, CA) using Big-Dye 3.1 terminators. For directed evolution experiments, doxG was cloned into pT7-7. Briefly, the gene was amplified from pMPVDG (13) using two oligonucleotides: 5′-doxG (5′-GAGATTCCATATGAGTAGCAAGCTGCAGT-CAGT-3′; an Ndel site is underlined) and 3′-doxG (5′-Cggatcct-TACACATCCACGTGAC-3′; a BamHI site is underlined). The PCR reaction was performed using Pwo DNA polymerase (Roche Applied Science) according to the manufacturer’s instructions and at an annealing temperature of 45 °C. The resulting amplicon was digested with Ndel and BamHI and cloned in pT7-7, yielding pT7doxG. The sequence of the cloned gene was verified. To obtain the L242S variant, the 595-bp Ndel/BclI fragment of pT7doxG was ligated to the 2776-bp Ndel/BclI fragment of the vector encoding DoxGSMA2 (see “Results”). The L190M/S191W double variant was similarly obtained using the 2776-bp Ndel/BclI fragment of pT7doxG and the 595-bp Ndel/BclI fragment of the vector carrying DoxGSMA2.

Protein Purification—DoxGSMA2 was purified from E. coli Nova Blue DE3 freshly transformed with a pT7-7 vector carrying the doxG variant. The cell pellet originating from a 4-liter culture was resuspended in 10 mM Tris, 10% glycerol, pH 7.5. Subsequent manipulations were performed anaerobically as described previously (17). The cells were disrupted by three successive passages through a French press (Spectronic Instruments Inc., Rochester, NY) operated at a pressure of 20,000 p.s.i. The cell debris was removed by ultracentrifugation in gas-tight tubes at 37,000 rpm for 40 min in a T1250 rotor (DuPont Instruments). The clear supernatant was carefully decanted and filtered using a 0.45-µm filter (Sartorius AG, Göttingen, Germany). This fluid was referred to as the raw extract. The raw extract was divided into two equal portions (~15 ml each). Each portion was loaded onto an HR16/10 Mono Q column equilibrated with 10 mM Tris, 10% glycerol, pH 7.5. The enzyme was eluted at a flow of 6 ml/min using a 320-ml linear gradient from 150 to 350 mM NaCl in buffer A. Fractions of 8 ml were collected. Activity-containing fractions were pooled, concentrated to 3 ml by ultrafiltration, and loaded onto a HiLoad 26/60 Superdex 200 column equilibrated with 20 µg/ml tetracycline supplemented with phosphate buffer and mineral salts as previously described (17). E. coli Nova Blue DE3 (EMD Biosciences, Inc.) and pT7-7 (18) derivatives were used to screen libraries of enzyme variants and for overexpression. The libraries were grown on 1/10 LB agar plates (1 g of Tryptone extract, 0.5 g of yeast extract, 10 g of NaCl, and 15 g of agar/liter) containing 0.5 mM isopropyl-β-D-thiogalactopyranoside and 100 mg/liter ampicillin at a colony density of 15–20 colonies/cm².

FIGURE 1. A, the upper bph pathway consists of four enzymes that degrade biphenyl to benzoate and hydroxynaphthalene. The 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphH) catalyzes the third step of the pathway. B, generation of the “dead-end” metabolite 2,2,5,5′-tetrachloro-3,4-dihydroxybiphenyl from 2,2,5,5′-tetrachlorobiphenyl.
with buffer A containing 33 mM ammonium sulfate and 0.25 mM ferrous ammonium sulfate. The protein was eluted at a flow rate of 2.5 ml/min. Activity-containing fractions (5 ml) were pooled, concentrated to greater than 25 mg/ml protein, and frozen as beads in liquid N₂. Purified DoxG<sub>SMA2</sub> was stored at −80 °C for up to 6 months without any significant loss of activity. DoxG was overexpressed in <i>P. putida</i> KT2442 freshly transformed with pHPVDG and purified essentially as described for DoxG<sub>SMA2</sub>.

**Directed Evolution**—Directed evolution experiments involved three successive steps: 1) error-prone PCR, 2) saturation mutagenesis of select positions, and 3) DNA shuffling. Libraries generated from each step were transformed into <i>E. coli</i> Nova Blue DE3 and screened for increased 3,4-DHB cleavage activity following the protocol described below. Error-prone PCR was carried out according to published protocols (21) using T7For (5′-GACTCACTATAGGGAG-3′) and oT7-7rev2 (5′-CTCATGTTGACGCTTATC-3′) as primers. The resulting amplicon was digested with NdeI and BamHI and cloned in pT7-7 to yield a library of doxG variants. Saturation mutagenesis was performed using the QuikChange<sup>®</sup> multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) and oligonucleotides containing 32-fold degenerated codons (NN(G/T)) at the target positions.

DNA shuffling was performed using the Expand High Fidelity<sup>®</sup> polymerase mixture (Roche Applied Science) and the T7For and oT7-7rev2 primers according to published protocols (22). After reassembly, the shuffled variants were amplified using the 5′ doxG and 3′ doxG primers as described above. The obtained amplicon was digested using Ndel and C.BamHI and cloned in pT7-7.

**Activity Screening**—Libraries of DoxG variants (pT7-7 derivatives) were transformed into <i>E. coli</i> Nova Blue DE3, plated on 15-cm Petri dishes, and grown for 16 h. Colonies were then sprayed with a 5 mM solution of 3,4-DHB; those containing an extradiol 3,4-DHB cleaving activity developed a yellow coloration.

To facilitate the visualization and quantification of the resulting degradation products (λ<sub>max</sub> ≈ 400 nm), images of the plates were captured through a 400-nm optical filter at different time points using a Canon Powershot A80 digital camera (Canon Canada, Mississauga, Ontario, Canada) (Fig. 3A). In the resulting images the colonies producing yellow products appeared as dark spots against a light background (Fig. 3B). These spots were quantified using ImageQuant 5.2 (Amersham Biosciences; Fig. 3C). The colonies showing the greatest volume, determined by integration using ImageQuant software, were picked and grown in 96 deep-well plates in 1 ml of LB medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside and 100 mg/liter ampicillin.

The cells were harvested, frozen at −80 °C for at least 15 min, and lysed using BugBuster<sup>™</sup> (Novagen, San Diego, CA). Activities were measured in the cleared cell lysates using 100 μM 3,4-DHB and phosphate buffer (I = 0.1 M, pH 7.0). Reactions were performed at 25 °C, and ring cleavage was monitored at 405 nm using a Victor<sup>®</sup> microplate reader (PerkinElmer Life Sciences). The cleavage of 2,3-DHB was monitored under similar conditions at 450 nm. Activities were normalized according to the expression level of each doxG variant. Expression levels were assessed using SyproRuby-stained SDS-PAGE gels imaged using a Typhoon 9410 Imager (Amersham Biosciences).

**Steady-state Kinetic Measurements and Data Analysis**—Ring-cleaving activity was measured by following the consumption of dioxygen using a Clark-type polarographic electrode (Yellow Springs Instrument Co. model 5301, Yellow Springs, OH) as previously described (17). All experiments were performed using phosphate buffer, pH 7.0, I = 0.1 M, 25.0 ± 0.1 °C (290 μM dissolved O₂) unless otherwise stated. The standard activity assay was performed using 80 μM substrate. The coupling of DHB and O₂ consumption was assessed by measuring the amount of O₂ consumed after the addition of 110 nmol of either 2,3- or 3,4-DHB. In addition, the yield of HOPDA from the cleavage of 2,3-DHB was assessed spectrophotometrically at 434 nm (ε = 11,300 M<sup>−1</sup> cm<sup>−1</sup>) (9).

For kinetic experiments, concentrations of active enzyme in the assay were defined by the iron content of the injected purified enzyme solution and were used in calculating specificity and catalytic constants. Steady-state rate equations were fit to data using the least squares and dynamic weighting options of LEONORA (23). For experiments performed using cell extracts, activities were normalized according to the expression level of DoxG, determined by SDS-PAGE analyses as described above. One unit of enzymatic activity was defined as the quantity of enzyme required to consume 1 μmol of O₂ per min.

**HPLC Analysis of Cleavage Products**—Reaction products were separated by reverse-phase chromatography using a Waters 2695 separation module equipped with a Waters 2996 photodiode array detector and a C<sub>18</sub> symmetry 3.5-μm column (4.6 × 75 mm) (Waters, Mississauga,
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Ontario, Canada). Products were eluted using a 20–90% acetonitrile gradient in an aqueous solution of 0.5% phosphoric acid.

RESULTS

A library of DoxG variants was generated by random mutagenesis using error-prone PCR. Sequence analysis of 6 randomly selected clones indicated that the mutation rate was 0.7% at the nucleotide level, corresponding to an average of 2 amino acid changes per enzyme variant. This analysis further indicated that the rates of transition and transversion mutation were equivalent. Libraries of more than 200,000 clones were routinely obtained from a single ligation reaction containing 0.15 μg of DNA.

A high throughput colorimetric screen was designed to identify DoxG variants with improved specificity for 3,4-DHB. Accordingly, libraries of mutated DoxG were plated on solid medium and sprayed with 5 mM 3,4-DHB. Colonies containing a 3,4-DHB cleavage activity developed the yellow color characteristic of the meta cleavage products (enolate anions). Images were captured through a 400-nm optical filter 2 min after exposure to 3,4-DHB and processed to extract the blue channel (Fig. 3, A–B). In the resulting image the colonies producing yellow products appeared as dark spots against a light background (Fig. 3B). The intensity of these spots was quantified using ImageQuant 5.2 software (Fig. 3C). Analysis of the processed images obtained using a library of randomly mutated DoxG revealed that a small number of colonies (<0.5%) developed more intense color than colonies containing the wild-type enzyme. Attempts to follow the development of the yellow color with time (30-s intervals) were not successful. Colonies associated with spots having the highest pixel intensity were selected for further screening using a 96-well microplate assay.

Ninety colonies were picked and grown in 96 deep-well plates in 1 ml of LB medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside and 100 mg/liter ampicillin. Upon the addition of 0.1 mM 3,4-DHB, 7 DoxG variants showed a 1.6–5-fold increase in cleavage activity versus the wild-type enzyme (TABLE ONE). To confirm that activity increases were not due to differences in expression levels, the activity of the variants was also assayed using 2,3-DHB. Those variants possessing the largest change in activity ratio were further characterized. Analyses of these clones using Sypro Ruby-stained SDS-PAGE revealed that the overall difference in expression levels of the variants was less than 20% (results not shown).

Mapping the mutations onto the structure of the DoxG:3,4-DHB complex revealed that each variant had at least one substitution in a residue that contributes to the substrate binding pocket (Fig. 4A). One notable exception occurred in variant B8. B8 contains the same two substitutions as IP33 (L242S and P267L) in addition to the Q101R substitution. Comparison of the B8 and IP33 suggests that the Q101R mutation increased substrate preference for 3,4-DHB relative to 2,3-DHB by 12-fold (TABLE ONE). This residue is 30 Å away from the enzyme active site iron, yet had a significant effect on the substrate preference of the enzyme.

For any given residue, an average of 5.7 amino acid substitutions should be accessible by random mutagenesis (24). To increase the number of substitutions sampled at key positions, four residues were subject to saturation mutagenesis: Ile-154, Leu-190, Ser-191, and Leu242. These residues are located within 14 Å of the enzyme active site iron, and the random mutagenesis results showed that their substitution had a large effect on the enzyme activity (TABLE ONE). To contain the size of the experiment, the residues were mutated pairwise: 154 and 242 in one trial and 190 and 191 in the second. The two resulting libraries consisted of variants containing all possible 20 amino acids at each target position. This strategy allowed us to explore >99.9% of the possible diversity while screening less than 10,000 clones.

TABLE ONE

Relative activities of selected DoxG variants

| Library | Variant | Mutations | Relative 3,4-DHB activity (variant/WT) | Increase in 3,4-DHB:2,3-DHB activity ratio |
|---------|---------|-----------|----------------------------------------|------------------------------------------|
| 1       | WT      | None      | 1                                      | 1                                        |
| IP27    | I154F   | 4.6       | 6.6                                    |
| IP33    | L242S   | 5.1       | 1                                      |
|         | P267L   |           |                                        |
| IP36    | D21E    | 1.8       | ND                                     |
|         | V290A   |           |                                        |
| IP37    | I154F   | 4         | 12.1                                   |
|         | L182S   |           |                                        |
|         | 1265L   |           |                                        |
|         | K300I   |           |                                        |
| IP38    | H59L    | 1.6       | ND                                     |
|         | K165R   |           |                                        |
|         | L253M   |           |                                        |
| B1      | L242S   | 3.8       | 43                                     |
|         | P267L   |           |                                        |
|         | S191T   |           |                                        |
| B8      | L242S   | 3.5       | 12                                     |
|         | P267L   |           |                                        |
|         | Q101R   |           |                                        |
| 2       | SA8     | L242V     | 1.7                                    | 10.0                                     |
| SA12    | L242T   | 2.6       | 63.0                                   |
| SB1     | L242G   | 6.4       | 18.1                                   |
| SB2     | I154W   | 9.0       | 12.3                                   |
| SB4     | I154W   | 8.8       | 12.1                                   |
| SB6     | S191W   | 3.3       | 80.2                                   |
|         | L242K   |           |                                        |
| SC1     | L190M   | 9.9       | 15.3                                   |
|         | S191Y   |           |                                        |
|         | L242I   |           |                                        |
| SG12    | L190Y   | 4.7       | 20.9                                   |
|         | L242I   |           |                                        |
| 3 SMA2  | L190M   | 9.8       | 33.6                                   |
|         | S191W   |           |                                        |
|         | L242S   |           |                                        |
DoxGSMA2 was purified anaerobically to apparent homogeneity as summarized in Table Two. Purified DoxGSMA2 contained greater than 96% of its complement of iron and had a specific activity of 1.9 units/mg using 2,3-DHB as substrate. Preparations of DoxG were of a quality similar to that obtained previously (26). WT DoxG and DoxGSMA2 were purified to apparent homogeneity, and steady-state kinetic studies were performed to investigate the combined effect of the three mutations on the specificity of the enzyme. The coupling of DHB and O2 consumption in DoxG and DoxGSMA2 was investigated using an O2 electrode that had been calibrated using 2,3-DHB and 2,3-dihydroxybiphenyl dioxygenase from \textit{B. xenovorans} LB400, a well coupled system (17). For both the WT and SMA2 variants, the amount of O2 consumed corresponded within 3% to the amount of 2,3- or 3,4-DHB added to the reaction mixture. In the case of WT DoxG, the addition of 110 nmol of 2,3-DHB resulted in the consumption of 111 ± 3.4 nmol of O2 and the production of 109 ± 1.2 nmol of HOPDA. For DoxGSMA2, 111 ± 3.0 nmol of O2 were consumed, and 110 ± 0.3 nmol of HOPDA were formed. In these assays the complete depletion of the substrate was confirmed by HPLC. These results demonstrate that the consumption of O2 was tightly coupled to the cleavage of DHB in both enzymes.

Enzymatic assays were performed in air-saturated phosphate buffer, pH 7.0, (I = 0.1 M) at 25 °C using 1,2-DHN, 2,3-DHB, and 3,4-DHB as substrates (Table Three). The apparent $k_{cat}$ of the variant for 3,4-DHB was increased by a factor of 2.4 relative to the WT enzyme. However, the $K_m$ value of the variant for 3,4-DHB was greatly reduced, resulting in specificity 770 times above that of WT enzyme. The specificities of the variant for 1,2-DHN and 2,3-DHB were also changed with respect to the WT enzyme but to much lesser extents (6.7- and 0.5-fold, respectively). Notably, for each substrate, the $k_{cat}$ values of the two enzymes varied by less than a factor of three.

As discussed below, the L242S substitution occurs on one side of the substrate binding pocket, whereas the L190M and S191W substitutions occur on the other. To evaluate the effect of these substitutions on the substrate preference of DoxG, the L242S and L190M/S191W variants of DoxG were prepared by fragment ligation, as described under "Materials and Methods." The relative specific activity of the four variants...
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**TABLE TWO**

| Purification step | Total protein | Total activity | Specific activity | Yield |
|-------------------|--------------|---------------|------------------|-------|
| Raw extract       | 730          | 210           | 0.29             | 100   |
| Mono Q            | 100          | 130           | 1.30             | 59    |
| Superdex 200      | 80           | 150           | 1.90             | 70    |

**TABLE THREE**

| Substrate | Variant | Apparent steady-state kinetic parameters of DoxG variants |
|-----------|---------|-----------------------------------------------------------|
|           |         | $k_{cat}$/s$^{-1}$ | $K_m$/M | $k_{cat}/K_m$ |
| 1,2-DHN   | WT      | 9.6 (0.8)         | 120 (20) | 77 (7)   |
| 2,3-DHB   | WT      | 1.8 (0.04)        | 2.6 (0.3) | 680 (60) |
| 3,4-DHB   | WT      | 1.6 (0.07)        | 600 (100)| 2.6 (0.3) |
| DoxGSMA2  | 1,2-DHN | 6.1 (0.3)         | 11.8 (1.9)| 520 (70) |
| DoxGSMA2  | 2,3-DHB | 0.70 (0.03)       | 2.0 (0.4) | 350 (60) |
| DoxGSMA2  | 3,4-DHB | 3.8 (0.1)         | 1.9 (0.3) | 2000 (300)|

**DISCUSSION**

A directed evolution experiment combining a single round each of error-prone PCR, saturation mutagenesis, and DNA-shuffling yielded a DoxG variant, DoxGSMA2, that cleaved 3,4-DHB with an apparent specificity constant that was 770-fold higher than that of the wild-type enzyme. Interestingly, the increase in apparent $k_{cat}$ was relatively modest (4-fold) despite the fact that the concentrations of 3,4-DHB used to screen colonies ought to have saturated the enzyme. Extradiol dioxygenases catalyze the cleavage of preferred substrates with $k_{cat}$ and $k_{cat}/K_m$ values of up to 1350 s$^{-1}$ and 6.2 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$, respectively (9, 17). These values suggest that the 3,4-DHB-cleaving activity of DoxG could be further improved by as much as 2 orders of magnitude. Nevertheless, engineering an increase of 770-fold in any enzyme specificity constant is significant. Directed evolution studies typically report improvements of 10–50-fold (29). There are few reports of improvements of 2–3 orders of magnitude (30).

The success of the current study was due in part to the development of a relatively effective, inexpensive screening method utilizing digital image analysis. This method greatly simplified the visual inspection of colonies, enabling the screening of more than 10$^5$ clones/h. With respect to the experiments and procedures described herein, this represents a 10-fold increase in throughput compared with direct visual examination. Importantly, the imaging-based approach facilitated the initial rank-ordering of enzyme variants. Remarkably, this ranking correlated well with that based on the activities measured in microplates using the raw extracts (results not shown). With the availability of colored filters, the described screening strategy can be easily adapted to any biocatalyst whose reaction can be coupled to a colorimetric change. Indeed, colorimetric changes at particular wavelengths (e.g. red and blue) would not require any image filtering, and the color intensity of active clones could be quantified directly. For example, the engineering of indigo- and indirubin-producing cytochrome P450 monoxygenases (31) and the directed evolution of a $\beta$-furcosidase activity from the $\beta$-galactosidase (LacZ) of E. coli (32) would have been facilitated by the present screening method. Finally, the method could be extended to non-colorimetric reactions by coupling the desired enzymatic activity to the expression of a reporter such as LacZ, which produces a measurable indigo compound in the presence of 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-gal).

Selection of suitable clones was further facilitated by verifying ring-cleaving activities in raw extracts using a microplate reader and two different substrates, 3,4- and 2,3-DHB. Nevertheless, to prevent the elimination of potentially interesting variants, those demonstrating a >4-fold increase in activity were selected regardless of their relative ability to catalyze the cleavage of the two substrates.
ant IP33 showed an unchanged 3,4-DHB/2,3-DHB activity ratio yet demonstrated a 5-fold increase of activity toward 3,4-DHB. Significantly, the rates observed in the high throughput enzymatic assays performed on the raw extract correlated well with the kinetics performed using pure enzyme preparations. Considering the concentrations of substrate used in the assays, the relative activities determined using the microplate were within a factor of 2 of those calculated from the steady-state kinetic parameters obtained using purified enzyme preparations. Furthermore, the relative activities determined spectrophotometrically using the microplate assays and raw extracts prepared from a 1-ml cell culture (TABLE ONE) correlated well with the relative activities measured using the Clark-type electrodes and raw extracts prepared from 100-ml cultures (TABLE FOUR). For DoxG<sub>SMA2</sub>, for example, the differences in relative activity toward 3,4-DHB was 11%, and the difference between the 3,4-DHB/2,3-DHB activity ratio was 4%.

Based on the crystal structure of the DoxG-3,4-DHB complex, the residues that are substituted in the DoxG<sub>SMA2</sub> variant are less than 9 Å from the bound substrate (Fig. 5B). The side chains of two of the mutated residues (L190M and L242S) face into the substrate binding site and may interact with the distal ring of the substrate or influence contacts between 3,4-DHB and other residues. Interestingly, the two sets of substitutions (L190M/S191W and L242S) influenced the enzyme apparent specific activity toward 3,4-DHB in an anti-cooperative fashion but influenced that toward 1,2-DHN and DHB in an antagonistic fashion (25). Although Míldvan’s analysis has not been applied to multiple substrates in a single enzyme, the substrate-dependent nature of the cooperativity between the residues is not surprising in light of the unique way in which each substrate interacts with the enzyme. Although the role of each substituted residue in substrate binding and catalytic turnover remains unclear, the nature of the substitutions (two bulkier residues on one side of the substrate and a smaller residue on the other, see Fig. 5B) are consistent with a reshaped binding pocket that binds 3,4-DHB in a different conformation. Overall, the dramatic changes in specificity were produced by relatively conservative changes in the substrate pocket of the enzyme.

The specificity of DoxG<sub>SMA2</sub> differs from that of DoxG in two other interesting respects. First, DoxG<sub>SMA2</sub> preferentially catalyzes the distal cleavage of 3,4-DHB (2.8-fold over DoxG). Hence, the presence of Ser at position 242 could stabilize a reaction intermediate arising during the distal cleavage of 3,4-DHB. Second, the specificity constant of DoxG<sub>SMA2</sub> for 1,2-DHN is only 7-fold higher than that of WT DoxG, reflecting a much smaller change in <i>K<sub>m</sub></i>. By way of explanation, Met-190 of DoxG<sub>SMA2</sub> may interact with the distal ring of 1,2-DHN to a lesser extent than that of 3,4-DHB because it is a smaller substrate. Although DoxG has been implicated in naphthalene catabolism, its specificity constant for 1,2-DHN is quite low, suggesting that this is not the optimal substrate of the enzyme. Moreover, the relatively large substrate binding pocket of DoxG (26) suggests that the natural substrate of the enzyme could be a polyaromatic catechol.

The effect of the substitution of Arg for Gln at position 101 is noteworthy because this residue is located at the surface of the DoxG monomer, more than 30 Å away from the active site iron atom. Despite this remote location, in the background of the L242S and P267L variant, this residue had a significant effect on the enzyme specificity (12-fold, complementing recent studies involving such variants.

The successful engineering of DoxG may be due to the relatively large substrate binding pocket of the enzyme, a feature that could translate into increased substrate pocket plasticity, more permissive to changes in proximity of its active site. A similar engineering strategy was applied to several 2,3-dihydroxybenzylphenyl dioxygenases with less success.7 Compared with DoxG, these enzymes have specificity constants for DHB that are close to 2 orders of magnitude higher. Thus, the present experiment constitutes an additional example supporting the idea that enzyme engineering could be facilitated by starting with enzymes characterized by low, broad specificities (28).

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