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Published in:
ACS chemical biology

DOI:
[10.1021/acscchembio.8b00026](https://doi.org/10.1021/acscchembio.8b00026)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Khatri, Y., Jóźwik, I. K., Ringle, M., Ionescu, I. A., Litzenburger, M., Hutter, M. C., Thunnissen, A-M. W. H., & Bernhardt, R. (2018). Structure-based engineering of steroidogenic CYP260A1 for stereo- and regioselective hydroxylation of progesterone. *ACS chemical biology*, 13(4), 1021-1028.
<https://doi.org/10.1021/acscchembio.8b00026>

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Structure-Based Engineering of Steroidogenic CYP260A1 for Stereo- and Regioselective Hydroxylation of Progesterone

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S Supporting Information

ABSTRACT: The production of regio- and stereoselectively hydroxylated steroids is of high pharmaceutical interest and can be achieved by cytochrome P450-based biocatalysts. CYP260A1 from *Sorangium cellulosum* strain So ce56 catalyzes hydroxylation of C19 or C21 steroids at the very unique 1 α -position. However, the conversion of progesterone (PROG) by CYP260A1 is very unselective. In order to improve its selectivity we applied a semirational protein engineering approach, resulting in two different, highly regio- and stereoselective mutants by replacing a single serine residue (S276) of the substrate recognition site 5 with an asparagine or isoleucine. The S276N mutant converted PROG predominantly into 1 α -hydroxy-PROG, while the S276I mutant led to 17 α -hydroxy-PROG. We solved the high-resolution crystal structures of the PROG-bound S276N and S276I mutants, which revealed two different binding modes of PROG in the active site. The orientations were consistent with the exclusive 1 α - (pro-1 α binding mode) and 17 α -hydroxylation (pro-17 α -binding mode) of S276N and S276I, respectively. We observed that water-mediated hydrogen bonds contribute to the stabilization of the polar C3 and C17 substituents of PROG. Both binding modes of PROG may be stabilized in the wild-type enzyme. The change in regioselectivity is mainly driven by destabilizing the alternative binding mode due to steric hindrance and hydrogen bond disruption, caused by the mutations of Ser276. Thus, for the first time, the change in the selectivity of cytochrome P450-mediated steroid hydroxylation created by rational mutagenesis can be explained by the obtained 3D structures of the substrate-bound mutants, providing the basis for further experiments to engineer the biocatalyst toward novel steroid hydroxylation positions.

Cytochrome P450 monooxygenases (P450s) are heme-containing enzymes known for their remarkable ability to activate molecular oxygen and perform oxidative hydroxylation of various organic compounds, including complex molecules like steroids.¹ Biotechnological production of hydroxy-steroids in an environmentally friendly and economic way is a central aim of the pharmaceutical industry for the modern production of therapeutics or precursors to develop steroid-based drugs.² For this reason, soluble and easily expressible steroidogenic bacterial P450s are of high interest because of their great potential as industrial biocatalysts.³ So far, only few bacterial steroid hydroxylases have been described and characterized in more detail.⁴ As a result, bacterial genomes are being screened for novel steroid-converting P450s.⁵ However, the applicability of P450s for industrial purposes is often hindered because of low activity or poor regio- and stereoselectivities,^{3,6,7} suggesting the demand of P450-engineering either by rational design or directed evolution to create more selective and/or active P450 variants.^{8–10}

Two steroidogenic P450s, CYP260A1 and CYP260B1, were recently identified while investigating the CYPome of

Sorangium cellulosum So ce56 for novel myxobacterial P450s with potential pharmaceutical or biotechnological applications.¹¹ We have previously demonstrated that CYP260A1 catalyzes a highly selective 1 α -hydroxylation of C19- (testosterone and androstenedione)¹² and C21 (11-deoxycorticosterone (DOC)) steroids,¹³ whereas CYP260B1 selectively hydroxylates the C21 steroid cortodoxone at the 6 β position.¹⁴

In this study, based on an alternative predicted gene transcript from the NCBI gene database, we have cloned and expressed a “truncated” form of CYP260A1 (Δ CYP260A1), missing the first 50 amino acid residues at the N terminus compared with the previously described “full-length” CYP260A1 (FL-CYP260A1).¹² This Δ CYP260A1 enzyme was then engineered for a selective conversion of progesterone (PROG), a C21 steroid for which differently hydroxylated

Received: January 7, 2018

Accepted: March 6, 2018

Published: March 6, 2018

analogs have various medical applications.^{15–17} At first, we demonstrated that Δ CYP260A1 is fully functional and showed an identical product pattern compared with FL-CYP260A1, including the unselective hydroxylation of PROG. Engineering of Δ CYP260A1 resulted in the creation of two single-site mutants, S276N and S276I, which converted PROG into 1α -hydroxyprogesterone (1α -OH-PROG) and 17α -hydroxyprogesterone (17α -OH-PROG), respectively, with high regio- and stereoselectivity. The PROG-bound X-ray crystal structures of the two Δ CYP260A1 variants provided a rational explanation of the structural basis of the improved regioselectivities.

RESULTS AND DISCUSSION

Identification of a Functional “Truncated” Form of CYP260A1 (Δ CYP260A1). In our previous studies, FL-CYP260A1 from *Sorangium cellulosum* So ce56 containing a (His)₆ tag at the C terminus was expressed and purified^{12,13} based on the open reading frame (ORF) from the database of the Cytochrome P450 Homepage (<http://drnelson.uthsc.edu/cytochromeP450.html>; entry sce_040811_1461)¹⁸ leading to a protein of 444 amino acids. However, more recently the NCBI database (<https://www.ncbi.nlm.nih.gov/protein/CAN91746.1>) published a shorter version of the CYP260A1 sequence consisting of only 394 amino acid residues, missing the first 50 residues at the N terminus. Interestingly, in our earlier substrate-free crystal structure of FL-CYP260A1 (PDB entry 5LIV), the first 50 N-terminal residues were highly disordered and not included in the model.¹³ Here, we cloned and expressed this truncated variant of the protein (Δ CYP260A1). Spectral characterization of the purified Δ CYP260A1 form by UV/vis spectroscopy showed a Soret peak maximum at 417 nm and a characteristic peak at 448 nm in the dithionite reduced CO-bound enzyme (data not shown), identical with the FL-CYP260A1. Most importantly, both variants showed identical products in the *in vitro* conversion of androstenedione and testosterone, which were well characterized in our previous study as 1α -hydroxylated products¹² (Figure S1). Therefore, the Δ CYP260A1 was chosen here for detailed studies on the selectivity of steroid hydroxylation.

Engineering of Δ CYP260A1 for the Regioselective Hydroxylation of Progesterone. Considering PROG as potential substrate, its conversion showed a very unspecific though identical hydroxylation pattern by both the FL-CYP260A1 and Δ CYP260A1 (Figure S2). Since PROG is the basic steroidal core to synthesize C21 steroid derivatives of pharmaceutical significance,^{15–17} in this study, we performed engineering of Δ CYP260A1 to promote regioselective hydroxylation of progesterone. The *in vitro* conversion of PROG by Δ CYP260A1 revealed seven products: two major (P3 and P5) and five minor (P1, P2, P4, P6, and P7; Figure 1, Table S1) products, which are identical with the products obtained after the conversion by the FL-CYP260A1 (Figure S2). In an effort to create mutants of Δ CYP260A1 with improved regioselectivity of progesterone hydroxylation, a combination of rational design and directed evolution (strategy according to Reetz¹⁹) was employed.

First, molecular docking of PROG into the crystal structure of FL-CYP260A1 (PDB entry 5LIV¹³) was carried out to identify potential selectivity-determining residues. Analysis of the docking poses with the lowest binding free energies suggested that PROG may adopt two alternative binding modes in the active site pocket. Both binding modes have the steroid rings oriented parallel to the heme plane, with the C17

Figure 1. HPLC chromatogram of the *in vitro* conversion of progesterone catalyzed by Δ CYP260A1 from *Sorangium cellulosum* So ce56. The major products (P3 and P5), minor products (P1, P2, P4, P6, and P7), and substrate peak for progesterone (PROG) are shown.

substituent of the D-ring either directed toward residues of substrate recognition site 5 (SRS5; pose I) or to residues of the I helix (pose II). Besides the predicted mainly hydrophobic PROG binding residues, there are three small polar residues that are located at opposite sides of the active site pocket, in the I helix (Ser275) or in the SRS5 region (Ser325, Ser326). The docking results suggest that these residues may interact with polar groups of PROG (the C3 and C20-keto groups) during catalysis (Figure 2). In particular, Ser326 is predicted to form a

Figure 2. Molecular models showing two predicted progesterone binding modes in the active site of CYP260A1, as obtained by docking. The binding poses with similar binding free energies place the progesterone (green sticks model) molecule parallel to the heme but differ by a rotation of $\sim 180^\circ$, inverting the positions of the C3 and C17 substituents. (a) Docking pose I. (b) Docking pose II. The heme is shown as a red stick model. Residue numbering in bold corresponds to the long form of CYP260A1 and in brackets, to Δ CYP260A1.

hydrogen bond with the C3-keto oxygen of PROG for pose II. Interestingly, mutation of this residue to an asparagine was previously shown to increase the regioselective conversion of DOC toward 1α -hydroxylated DOC.¹³

Given their predicted potential to affect the steroid binding mode in the active site pocket, the three serine residues in Δ CYP260A1 (Ser225, Ser275, and Ser276), corresponding to Ser275, Ser325, and Ser326 in the FL-CYP260A1, were

selected as “hot spots” for protein engineering. The three residues were randomized by NDC codon degeneracy (allowing mutations to 12 different amino acid residues: Leu, Ile, Arg, His, Asp, Phe, Asn, Cys, Gly, Tyr, Val, and Ser).¹⁹ The obtained clones were screened for PROG conversion in a 96-well plate using *E. coli* based whole-cell systems, and the resulting conversions were analyzed by HPLC. In total, 150 clones (50 per each of the three created mutation sites, S225x, S275x, and S276x) were randomly selected for the analysis of PROG conversion (Table S2). We observed that all the selected S225x and S275x clones were either inactive or displayed the same product profile of PROG conversion as Δ CYP260A1. However, out of the 50 selected S276x clones, five displayed an altered product profile, while the remaining clones were either inactive or showed an identical product profile as Δ CYP260A1. Sequencing of the plasmid DNA of the five positive colonies displaying an altered product profile identified the mutants as S276N, S276I, S276C, S276L, and S276V.

Biochemical Characterization of the Five S276 Variants. The five S276 variants (S276N, S276I, S276C, S276L, and S276V) of Δ CYP260A1 with an altered product profile of PROG were successfully expressed and purified (Figure S3). The yields of the purified proteins for S276N, S276I, S276C, S276L, and S276V were 1432, 404, 1300, 2192, and 778 nmol L⁻¹ of *E. coli* cell culture, respectively. The purified CYP260A1 variants exhibited characteristic CO-difference spectra with a peak maximum at ~448–450 nm, typical for the ferrous–CO heme adduct, indicating correct incorporation of the heme (Figure S4).

The *in vitro* conversion of PROG by S276N and S276C demonstrated significantly increased selectivity for product P3 compared with Δ CYP260A1 (57% and 48% compared with 36%), while the mutants S276I, S276L, and S276V showed significantly increased selectivity for product P5 compared with Δ CYP260A1 (64%, 62%, and 58%, respectively, compared with 39%; Figure 3, Figure S5). Of the two variants displaying preference for product P3, S276N was more selective than S276C. In addition to product P3, the conversion of PROG by

S276N resulted in three other detectable side products (P1, P2, and P5), which is equivalent to less than 23% of the total product, whereas S276C showed significant formation of side products: P5 (~22%), P7 (~13%), P4 (~12%), and P2 (~7%). Notably, the formation of P1 by the S276N variant was increased from 3% to 16% compared to the conversion by Δ CYP260A1. Among the three variants that were able to shift the selectivity to product P5 (S276L, S276V, and S276I), the S276L and S276V variants showed more side products compared with S276I. Mutant S276I produced P1, P2, P3, and P4 as very minor side products (all together <7% of the total product), while the major product P5 was >64% compared with Δ CYP260A1. Therefore, the two very promising variants S276N and S276I, which not only showed a substantial increase in the regioselectivity of PROG conversion but also favored formation of two completely different oxidized products, were selected for further characterization.

Product Identification and Potential Biotechnological Application of Δ CYP260A1 Mutants. In order to determine the identity of the main hydroxylated products resulting from PROG conversion catalyzed by the S276N and S276I variants by nuclear magnetic resonance (NMR) spectroscopy, an *E. coli* based whole-cell bioconversion of PROG using these two mutants was established. Sufficient amounts of pure products P3 and P5 (~5 mg) were isolated and identified by ¹H and ¹³C and 2D NMR spectroscopy 1 α -hydroxyprogesterone (1 α -OH-PROG) and 17 α -hydroxyprogesterone (17 α -OH-PROG), respectively (Table S3). Unfortunately, the yield of product P1, with [M⁺ + H]⁺ = 347.2 indicating a dihydroxylated product ($\Delta m/z$ of +32; Table S1), was too low to be characterized further by NMR. Thus, while FL-CYP260A1 and Δ CYP260A1 produce a more or less equimolar mixture of 1 α -OH-PROG (P3) and 17 α -OH-PROG (P5) as main products, together with other side products, the S276N mutant produces mainly 1 α -OH-PROG, while the S276I mutant produces mainly 17 α -OH-PROG (Scheme 1, Figure 3).

Scheme 1. Reactions Catalyzed by the S276N and S276I Variants of Δ CYP260A1 from *Sorangium cellulosum* So ce56

Figure 3. Products formed in *in vitro* progesterone conversion experiments with Δ CYP260A1 or its five variants (S276N, S276I, S276C, S276L, and S276V). The products P1 (pink bar), P2 (gray bar), P3 (red bar), P4 (yellow bar), P5 (blue bar), P6 (cyan bar), and P7 (orange bar) are shown. The absence of any of the above-mentioned bars (P1–P7) represents the lack of relevant product formation with the corresponding mutant. The bar diagram was plotted neglecting the minor side products (<1%).

Both hydroxy-metabolites of PROG are potentially very interesting chemicals to be produced on a large scale by the chemical/pharmaceutical industries. Modifications on C1 and C17 allowing subsequent derivatizations like methylation *etc.* at these positions are highly important for the synthesis of anabolic steroids like mesterolone and methenolone.^{20,21} However, the synthetic route for a 1 α -hydroxylation of the A ring of a steroid is a very complicated process and requires either nine synthetic steps from dihydrotestosterone²² or a somewhat shorter route from 17 β -hydroxyandrost-1,4-diene-

3-one.²³ In addition, the yields of the 1 α -hydroxylated product in both chemical routes were very low. Attempts to produce 1 α -hydroxylated products using mutants of the P450 BM3 revealed 1 α -hydroxylated androstenedione with high regioselectivity. However, activity of these BM3 mutants toward progesterone was either missing or very poor, giving only trace amounts of three unidentified products.^{24,25} On the contrary, the engineered P450 BM3 mutant F87A from *Bacillus megaterium* previously showed good PROG conversion and high selectivity, yet the oxidized products showed an 18:82 mixture of 2 β - and 16 β -hydroxylated products.⁸ Therefore, the ability of CYP260A1 and its S276N mutant to perform C1-hydroxylation of PROG shows a so far unmet industrial potential.

Likewise, the derivatives of 17 α -OH-PROG, the parent compound of progestin drugs, carry immense pharmaceutical potential for the synthesis of steroidal drugs like chlormadinone acetate, cyproterone acetate, hydroxyprogesterone caproate, medroxyprogesterone acetate, and megestrol acetate.^{26,27} Those synthetic progestins are known to have distinct biological effects.²⁸ For example, the 17 α -hydroxyprogesterone caproate is used medically for prevention of the preterm delivery.¹⁵ The chemical synthesis of steroids including 17 α -OH-PROG is also a very tedious and demanding multistep process.²⁹ Although the membrane-associated human and bovine CYP17A1 can perform 17 α -hydroxylation of PROG,³⁰ due to a rather poor heterologous expression and fragile nature of this protein, broad biotechnological application is hindered. Thus, the bacterial soluble P450s, like CYP260A1 and its S276 variants (S276N and S276I) described herein, retain an exceptionally high demand to produce regio- and stereoselectively hydroxylated steroids.

Determination of Steady-State Kinetics. The determination of steady-state kinetic parameters of PROG conversion catalyzed by Δ CYP260A1 and the mutants S276N and S276I revealed typical Michaelis–Menten behavior. We obtained similar PROG conversion rates ($V_{\max}$) of 5 ± 0.4 , 5 ± 0.6 , and 6 ± 1.0 [nmol-product nmol-P450⁻¹ min⁻¹], with K_m values of 124 ± 15 , 127 ± 24 , and 146 ± 51 μ M for Δ CYP260A1, S276N, and S276I, respectively (Figure 4). Thus, the kinetic parameters of Δ CYP260A1 are not significantly affected by introduction of S276N and S276I mutations.

Crystal Structures of the S276N and S276I. In order to rationalize the regioselectivity of PROG hydroxylation at 1 α and 17 α positions by S276N and S276I, we solved the crystal structures of the substrate-free (S276N, S276I) and PROG-bound (S276N-PROG, S276I-PROG) forms of both mutants at resolutions ranging from 2.05 to 1.35 Å (see Table S4 for the relevant crystallographic data statistics). In the final models, well-defined and continuous electron density is observed for two complete copies of the polypeptide chain, from amino acid residue Met1 to His394, including the heme group that is bound to Cys340. The C-terminal His₆ tags (residues 395 to 400) were not visible in the electron density and could not be modeled. In the PROG-bound structures (S276N-PROG and S276I-PROG), the electron density maps clearly defined the binding mode of the PROG molecule in the active site pocket. Surprisingly, well-defined electron density for a bound active site ligand is also observed in the substrate-free structures, which we interpreted as a histidine that coordinates the heme-iron as a sixth axial ligand (Figure S6). The origin of the bound histidine is unclear: since this amino acid was not present in any of the crystallization solutions, it must have entered the active

Figure 4. Determination of steady-state kinetic parameters of progesterone conversion catalyzed by Δ CYP260A1 and its S276N and S276I mutants. $V_{\max}$ and K_m values were determined by hyperbolic fitting (SigmaPlot software) done for the plotted velocities of product formation versus increasing substrate concentration, for the Δ CYP260A1 (black circles), S276N (green triangles), and S276I (blue squares). The error bars in the figures represent the standard deviation of three independent measurements.

site during protein purification. On the other hand, UV–vis spectra of the purified P450 proteins revealed a normal substrate-free Soret absorption band at ~ 417 nm, which shifted to ~ 393 nm upon titration with a saturated concentration of progesterone (~ 20 -fold excess, Figure S7), indicative of tight steroid binding. Most likely, the histidine-bound form represents only a small fraction of the purified proteins in solution, which, although favorable for crystallization, does not affect progesterone binding.

In all four crystal structures, the protein molecules adopt a closed conformation, with the FG helices together with BC-loop residues tightly covering the entrance to the active site pocket. This conformation is highly similar to the closed conformation observed previously for three of the four unique protein molecules in the crystal structure of FL-CYP260A1, which contain two bound glycerol molecules (PDB entry 5LIV;¹³ Figure 5). However, in the closed conformation of the Δ CYP260A1 variants, the active site pocket is not completely buried from the solvent; a narrow channel is running between the I helix and the BC loop and is filled with bound water

Figure 5. Superposition of the substrate-free CYP260A1 structure in its closed conformation (5LIV, chain C, light blue) with the S276I-PROG structure of Δ CYP260A1 (chain A, beige). The active site entrance is closed from the top by the FG helices and in the front by residues in BC loop. PROG is shown in blue sticks as bound in the S276I-PROG structure. The heme is shown as a red stick model.

molecules (Figure S8). This channel may serve as a water access/egress route that is required during catalysis. Careful inspection of the electron density maps further revealed that in three of the four Δ CYP260A1 mutant structures (S276N, S276I, and S276N-PROG), the heme group is bound in two alternate orientations (Figure S9). One orientation of the heme plane is identical to that in the previously reported crystal structure of FL-CYP260A1 (normal orientation, SLIV), while the other involves a 180° flip of the heme plane. Although similar reversed heme binding orientations have been observed in other P450s, i.e., CYP121 and CYP154A1,^{31,32} the cause and significance of this “heme-flipping” event are unclear. In the refined S276N, S276I, and S276N-PROG structures, the relative occupancies of heme bound in the reversed orientation varied between 40 and 70%.

Binding Mode of Progesterone in S276N and S276I.

The progesterone-bound crystal structures of S276N and S276I display two distinct binding modes (Figures 6 and 7), similar to

Figure 6. Quality of the electron density maps presented by substrate-bound complexes of the S276N/S276I variants of Δ CYP260A1. (a) Progesterone (PROG, blue sticks) bound above the heme in the S276N-PROG structure. (b) Progesterone bound in the S276I-PROG structure. Black mesh is the composite omit $2F_o - F_c$ electron density map calculated at 1.75 and 1.9 Å for S276N-PROG and S276I-PROG, respectively, and contoured at 1.5σ . The hydroxylation sites (C1 or C17) are shown as balls in magenta. The heme is shown as a red stick model.

the binding poses that have been observed in our docking studies. The PROG binding mode in S276N is similar to the docking pose I, while in S276I the PROG is bound similarly to docking pose II. Both binding modes position the 4-ring structure of PROG parallel to the heme plane with its α -side facing the heme group, but they differ by $\sim 180^\circ$ rotation of their ring skeleton, reversing the positions of the C3 and C17 substituents in the active site pocket (Figure 7). The binding modes of PROG are present at full occupancy in the final crystal structures, with no signs of any additional bound ligand

Figure 7. Progesterone binding interactions in the S276N/S276I variants of Δ CYP260A1. (a) Progesterone binding interactions in the S276N-PROG structure. (b) Progesterone recognition in the S276I-PROG structure. Superposition of the PROG binding modes versus the I helix is shown, as found in the two unique protein molecules in the asymmetric unit. Leu159 (F-helix) and Val382 (SRS6) are not shown for image clarity.

in the active site, clearly showing that the steroid fully replaced the bound histidine observed in the steroid-free structures.

In the S276N-PROG structure, the C3-keto group of the A ring is bound close to Ser225 of the I helix, while the C17 substituent is bound close to residues 275–277 of the SRS5 site (Figure 7A). The two polar substituents of PROG are anchored to the protein by water-mediated hydrogen bonds. Additionally, the C17 substituent is in van der Waals contact with the side chain of Asn276. The C3-keto group may form a direct hydrogen bond with the side chain of Ser225, but this interaction is not very strong considering the observed conformational disorder observed for Ser225. The remaining protein–ligand interactions in S276N-PROG are hydrophobic contacts between the steroidal rings and residues Phe64, Leu69 (SRS1), Thr233 (SRS4), Asn276, Phe277, Gly278 (SRS5), and Val382 (SRS6). Interestingly, the S276N-PROG bound orientation in the active site is such that the C1 carbon is placed optimally for a hydrogen abstraction and formation of the 1α -hydroxylated product: e.g., in monomer A, the distance between the C1 carbon and the heme iron is 4.4 Å and the $C1-H_{\text{pro}}-\alpha-Fe$ angle is 165° . For successful hydroxylation, the distance between reactive carbon and the heme-Fe should be up to 4.9 Å, and the angle between the carbon, hydrogen, and the heme-Fe should be $135-180^\circ$.^{33,34} On the basis of these distance and angle criteria, the other carbons of PROG close to the heme iron are excluded as possible hydroxylation sites (Table S5).

In the S276I-PROG crystal structure, the C3-keto group of PROG is bound near the residues 275–277, while the D ring of the steroid with the C17 substituent is located near Ser225 of

the I helix (Figure 7B). In this binding mode, only one polar substituent is stabilized by a water-mediated hydrogen bond (C20-keto group of PROG with the side chain of Gln221 (SRS4)). Other residues interacting with PROG are Ala74 (SRS1), Leu159 (SRS2), Ser225, Leu228, Gly229, Thr233 (SRS4), Asn276, Phe277, and Gly278 (SRS5). The C17 carbon of PROG is at an optimal position for the formation of 17 α -OH-PROG, e.g., in monomer A, the C17 is at 4.7 Å distance from the heme iron and the C₁₇–H_{pro- α} –Fe angle is 140°. Heme-iron distances and angle cutoffs calculated for the other possible steroid hydroxylation sites in PROG (the C16, C15, C6, C11, or C12 carbons) are unfavorable for oxidation (Table S5).

Taken together, this study demonstrates that mutagenesis at sequence position 276 of Δ CYP260A1 displayed a significant effect on the regioselectivity of PROG hydroxylation. A dramatic influence on the regioselectivity of substrate hydroxylation has also been observed for the residue(s) at an equivalent sequence position in various other P450 enzymes.^{35–38} The position has been commonly referred to as “position 5 after the ExxR motif”³⁹ or “standard position 328”⁴⁰ and is considered to be a crucial selectivity-determining residue in most P450s. Mutations at this position were believed to influence the orientation of the substrate during catalysis, but the structural basis accounting for the observed changes in regioselectivity have to the best of our knowledge not been explained yet using experimentally determined protein structures.

In this work, characterization of product profiles combined with the analysis of crystal structures of the two optimized S276N and S276I variants allowed us to rationalize how the Ser276 mutations in Δ CYP260A1 affect the regioselectivity of PROG conversion. The active site pocket in CYP260A1 (FL-CYP260A1 and Δ CYP260A1) provides similar stabilization for both binding modes of PROG, consistent with 1 α -hydroxylation (pro-1 α binding mode) and 17 α -hydroxylation (pro-17 α binding mode). Mutation of Ser276 to an asparagine does not significantly affect the interactions in the pro-1 α binding mode, as observed in the S276N-PROG structure, in which the C3- and C20-keto groups of PROG are stabilized by water-mediated hydrogen bonds. However, the S276N mutation disrupts the predicted hydrogen bond interaction of this residue with the C3-keto group of PROG, thereby destabilizing the pro-17 α binding mode. Thus, the pro-1 α binding mode of PROG is strongly favored in the S276N variant, resulting in predominant conversion toward 1 α -OH-PROG. Similarly, the mutation of Ser276 to an isoleucine does not result in a large net change of stabilizing/destabilizing interactions with the pro-17 α binding mode as observed in the S276I-PROG structure. The side-chain of Ile276 forms a hydrophobic contact with the A-ring of PROG, as observed in the S276I-PROG structure, which cannot be formed by S276 in Δ CYP260A1. This new interaction probably compensates for the loss of the hydrogen bond with the C3-substituent of PROG. However, the S276I mutation will destabilize the pro-1 α steroid binding mode due to steric hindrance of the C17 substituent by the bulky hydrophobic side chain of the isoleucine residue. The isoleucine side chain additionally may preclude formation of the water-mediated hydrogen bond with the C20-keto group of PROG. Thus, by destabilizing the pro-1 α binding mode the pro-17 α binding mode of PROG is strongly favored in the S276I variant, resulting in predominant conversion toward 17 α -OH-PROG. Most likely, a similar structural explanation applies to the

S276V and S276L mutants characterized in this study, which also favor PROG oxidation at the 17 α position. Among the five studied S276 variants of Δ CYP260A1, the S276C variant shows the lowest regioselectivity toward PROG. We hypothesize that this mutation has a destabilizing effect on both the pro-1 α and pro-17 α binding modes, so there is no large net change in the observed regioselectivity.

In conclusion, Δ CYP260A1 from *Sorangium cellulosum* So ce56 was successfully engineered, and two active and regioselective mutants were obtained, able to produce two different hydroxylated PROG derivatives that are of high impact for biotechnological applications. The structural bases are provided for the role of residues at sequence position 276 in determining the regiospecificity of PROG hydroxylation catalyzed by the Δ CYP260A1 variants.

METHODS

Plasmids, Strains, and Chemicals. All chemicals were purchased from standard sources and were of the highest purity available, as described in Supporting Information S1.1.

Cloning, Expression, and Purification. The FL-CYP260A1 was cloned as described previously.¹² The expression construct for the “truncated” CYP260A1 was designed in pET22b with *Nde* I and *Kpn* I restriction sites (pET22b- Δ CYP260A1) and using primers as described in Table S6. The nucleotide sequences were confirmed by automated sequencing (MWG-Biotech AG). Expression and purification of P450s investigated in this work were performed as described previously.⁴¹ The detailed methods are illustrated in Supporting Information S1.2. The truncated bovine adrenodoxin (Adx_{4–108}) and bovine adrenodoxin reductase (AdR) were expressed and purified as described elsewhere.^{42,43}

In Vitro Substrate Conversion and HPLC Analysis. The *in vitro* conversions of tested steroids (testosterone, androstenedione, or progesterone) were performed using the same batch of purified P450 variant (0.5 μ M), Adx_{4–108} (10 μ M), and AdR (1.5 μ M) [CYP260A1/Adx/AdR ratio of 1:20:3⁴⁴]. The conversion was analyzed by reversed phase HPLC as described previously.¹² Further experimental details are illustrated in Supporting Information S1.3. and S1.4.

Molecular Docking. The AutoDock Vina 1.1.2 program⁴⁵ was used to dock the substrate (PROG) into the crystal structure of CYP260A1 (PDB entry 5LIV, molecule C) and visualized in UCSF Chimera⁴⁶ as described in Supporting Information S1.5.

Creation of PCR-Based Saturation Mutagenesis Library. The megaprimers used for the creation of a library at three selected positions (225, 275, and 276) contained an NDC codon (N represents all bases; D represents either adenine thymine or guanine, and C represents cytosine) and were ordered from MWG-Biotech AG. Sequences of the designed primers are described in Table S6, and the PCR was performed in a DNA Engine PCR cycler (Bio-Rad Laboratories). Five cycles were used to create the megaprimer followed by 20 cycles to create the mutation. After all PCR cycles, the parental methylated DNA was digested by adding 1 μ L of *Dpn* I to the PCR mixture and followed by an incubation time of 1 h at 37 °C and 200 rpm. The digestion step was then repeated to obtain only plasmids containing a mutation.

Screening for Progesterone Conversion by the Whole Cells Expressing Δ CYP260A1 or Its Variants. In order to screen the mutant library, *E. coli* based whole cell conversion of PROG was performed either using Δ CYP260A1 or one of the created variants as described further in the Supporting Information S1.6.

Spectroscopic Characterization of the Purified P450 Enzymes. The UV–visible spectra of purified P450 and its concentration were measured as illustrated in Supporting Information S1.7.

Whole-Cell Conversion of Progesterone with S276N/I Variants, Product Isolation, and NMR Characterization. The whole-cell conversion was performed in *E. coli* BL21 (DE3) as described in Supporting Information S1.6. The product purification

was performed by a preparative reversed phase HPLC column (ec MN Nucleodur C₁₈ VP, 5 μ M, 8.0 $\times$ 250 mm (Macherey-Nagel)) using a gradient elution of 10–100% acetonitrile, with a flow rate of 3.3 mL min⁻¹. The fractions containing the desired product were pooled and evaporated to dryness. In our *in vivo* conversion system (500 mL culture volume), S276N and S276I produced $\sim$ 55 mg and $\sim$ 58 mg of products P3 (1 α -OH-PROG) and P5 (17 α -OH-PROG), respectively. A total of 5 mg of each product was dissolved in deuterated chloroform (CDCl₃) for subsequent NMR analysis.

The NMR data were recorded on a Bruker DRX 500 NMR spectrometer at 300 K. The chemical shifts were relative to CHCl₃ (δ 7.24 for ¹H NMR) or CDCl₃ (δ 77.00 for ¹³C NMR) using the standard δ notation in parts per million (ppm). The 1D NMR (¹H, ¹³C NMR and DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence.

Determination of Kinetic Parameters. To determine PROG hydroxylation kinetics, increasing substrate concentrations of 0–200 μ M were applied in *in vitro* reactions with fixed concentrations of CYP260A1 (0.5 μ M), Adx_{4–108} (5 μ M), and AdR (1.5 μ M). Thus, the ratio of CYP260A1/Adx/AdR of 1:10:3 was used.⁴⁴ The conversion reactions, extraction, and HPLC analysis of PROG and its products were carried out as described above. The $V_{\max}$ and K_m values were determined by plotting the product formation rate versus the corresponding substrate concentration using a hyperbolic fit (Michaelis–Menten kinetics) applying the SigmaPlot software (Systat Software).

Crystallization and Determination of Crystal Structures. Four samples of CYP260A1 S276N (substrate-free, 33 mg mL⁻¹), S276N-PROG (complex with PROG, 30 mg mL⁻¹), S276I (substrate-free, 32 mg mL⁻¹), and S276I-PROG (complex with PROG, 22 mg mL⁻¹) were subjected to crystallization screening. The screening and extraction of grown crystals were performed as described in Supporting Information S1.8. Diffraction data were collected at the ID23-1 and ID29 beamlines of the European Synchrotron Radiation Facility (ESRF), Grenoble. The details on structure determination by the molecular replacement method and validation are presented in Supporting Information S1.8.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscchembio.8b00026](https://doi.org/10.1021/acscchembio.8b00026).

Detailed experimental procedures S1.1–S1.8, Figures S1–S9, and Tables S1–S6 (PDF)

Accession Codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 6F85 (histidine-bound Δ CYP260A1-S276N), 6F88 (progesterone-bound Δ CYP260A1-S276N), 6F8A (histidine-bound Δ CYP260A1-S276I), and 6F8C (progesterone-bound Δ CYP260A1-S276I).

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Author Contributions

Y.K. and R.B. conceived and coordinated the study. Y.K., M.R., I.A.I., and M.L. performed all biochemical experiments and collected data (prepared expression constructs, purified enzymes, performed protein engineering, collected HPLC data from steroid oxidation experiments, determined reaction kinetic parameters). Y.K., M.R., I.A.I., M.L., and R.B. analyzed and interpreted the biochemical data. I.K.J. performed molecular docking simulations, crystallized P450 mutants, collected crystallographic data, and solved the crystal structures. I.K.J. and A.-M.W.H.T. analyzed and interpreted the structural data. I.K.J., Y.K., M.L., M.H., A.-M.W.H.T., and R.B. wrote the paper. All authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to R.B. (BE 1343/23-1/23-2) and the People Programme (Marie Curie Actions) of the European Union's seventh Framework Programme (FP7/2007-2013) under REA Grant Agreement 289217 (P4FIFTY) to I.K.J., M.R., A.-M.W.H.T., and R.B. The authors thank B. Heider-Lips for the purification of Adx_{4–108} and AdR and J. Zapp for measuring the NMR samples. The authors also acknowledge the European Synchrotron Radiation Facility (ESRF) for provision of synchrotron radiation facilities and thank the beamline staff of ID29 and ID23-1 for their assistance.

■ REFERENCES

- (1) Bernhardt, R. (2006) Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* 124, 128–145.
- (2) Donova, M. V., and Egorova, O. V. (2012) Microbial steroid transformations: Current state and prospects. *Appl. Microbiol. Biotechnol.* 94, 1423–1447.
- (3) Bernhardt, R., and Urlacher, V. B. (2014) Cytochromes P450 as promising catalysts for biotechnological application: Chances and limitations. *Appl. Microbiol. Biotechnol.* 98, 6185–6203.
- (4) Kiss, F. M., Khatri, Y., Zapp, J., and Bernhardt, R. (2015) Identification of new substrates for the CYP106A1-mediated 11-oxidation and investigation of the reaction mechanism. *FEBS Lett.* 589, 2320–2326.
- (5) Agematu, H., Matsumoto, N., Fujii, Y., Kabumoto, H., Doi, S., Machida, K., Ishikawa, J., and Arisawa, A. (2006) Hydroxylation of testosterone by bacterial cytochromes P450 using the *Escherichia coli* expression system. *Biosci., Biotechnol., Biochem.* 70, 307–311.
- (6) Urlacher, V. B., and Girhard, M. (2012) Cytochrome P450 monooxygenases: An update on perspectives for synthetic application. *Trends Biotechnol.* 30, 26–36.
- (7) Lundemo, M. T., and Woodley, J. M. (2015) Guidelines for development and implementation of biocatalytic P450 processes. *Appl. Microbiol. Biotechnol.* 99, 2465–2483.
- (8) Kille, S., Zilly, F. E., Acevedo, J. P., and Reetz, M. T. (2011) Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. *Nat. Chem.* 3, 738–743.

- (9) McIntosh, J. A., Farwell, C. C., and Arnold, F. H. (2014) Expanding P450 catalytic reaction space through evolution and engineering. *Curr. Opin. Chem. Biol.* 19, 126–134.
- (10) Nguyen, K. T., Virus, C., Gunnewich, N., Hannemann, F., and Bernhardt, R. (2012) Changing the regioselectivity of a P450 from C15 to C11 hydroxylation of progesterone. *ChemBioChem* 13, 1161–1166.
- (11) Khatri, Y., Hannemann, F., Perlova, O., Muller, R., and Bernhardt, R. (2011) Investigation of cytochromes P450 in myxobacteria: Excavation of cytochromes P450 from the genome of *Sorangium cellulosum* So ce56. *FEBS Lett.* 585, 1506–1513.
- (12) Khatri, Y., Ringle, M., Lisurek, M., von Kries, J. P., Zapp, J., and Bernhardt, R. (2016) Substrate hunting for the myxobacterial CYP260A1 revealed new 1 α -hydroxylated products from C-19 steroids. *ChemBioChem* 17, 90–101.
- (13) Khatri, Y., Carius, Y., Ringle, M., Lancaster, C. R., and Bernhardt, R. (2016) Structural characterization of CYP260A1 from *Sorangium cellulosum* to investigate the 1 α -hydroxylation of a mineralocorticoid. *FEBS Lett.* 590, 4638–4648.
- (14) Salamanca-Pinzon, S. G., Khatri, Y., Carius, Y., Keller, L., Muller, R., Lancaster, C. R., and Bernhardt, R. (2016) Structure-function analysis for the hydroxylation of Delta4 C21-steroids by the myxobacterial CYP260B1. *FEBS Lett.* 590, 1838–1851.
- (15) Romero, R., and Stanczyk, F. Z. (2013) Progesterone is not the same as 17 α -hydroxyprogesterone caproate: Implications for obstetrical practice. *Am. J. Obstet. Gynecol.* 208, 421–426.
- (16) Meis, P. J., Klebanoff, M., Thom, E., Dombrowski, M. P., Sibai, B., Moawad, A. H., Spong, C. Y., Hauth, J. C., Miodovnik, M., Varner, M. W., Leveno, K. J., Caritis, S. N., Iams, J. D., Wapner, R. J., Conway, D., O'Sullivan, M. J., Carpenter, M., Mercer, B., Ramin, S. M., Thorp, J. M., and Peaceman, A. M. (2003) Prevention of recurrent preterm delivery by 17 α -hydroxyprogesterone caproate. *N. Engl. J. Med.* 348, 2379–2385.
- (17) Tamm, J., Seckelmann, M., Volkwein, U., and Ludwig, E. (1982) The effect of the antiandrogen II α -hydroxyprogesterone on sebum production and cholesterol concentration of sebum. *Br. J. Dermatol.* 107, 63–70.
- (18) Nelson, D. R. (2009) The cytochrome P450 homepage. *Hum. Genomics* 4, 59–65.
- (19) Reetz, M. T. (2011) Laboratory evolution of stereoselective enzymes: A prolific source of catalysts for asymmetric reactions. *Angew. Chem., Int. Ed.* 50, 138–174.
- (20) Schänzer, W., and Donike, M. (1993) Metabolism of anabolic steroids in man: Synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta* 275, 23–48.
- (21) Camerino, B., and Sciacry, R. (1975) Structure and effects of anabolic steroids. *Pharmacol. Ther., Part B* 1, 233–275.
- (22) Mann, J., and Pietrzak, B. (1989) Synthesis of 1- α -hydroxytestosterone. *Tetrahedron* 45, 1549–1552.
- (23) Garside, D., Kirk, D. N., and Waldron, N. M. (1994) Synthesis of hydroxylated steroid hormones via conjugate addition of a silyl-cuprate reagent. *Steroids* 59, 702–711.
- (24) Liu, X., Wang, Z. B., Wang, Y. N., and Kong, J. Q. (2016) Probing steroidal substrate specificity of cytochrome P450 BM3 variants. *Molecules* 21, E760.
- (25) Liu, X., and Kong, J. Q. (2017) Steroids hydroxylation catalyzed by the monooxygenase mutant 139–3 from *Bacillus megaterium* BM3. *Acta Pharm. Sin. B* 7, 510–516.
- (26) Smith, H. J., and Williams, H. (1983) *Introduction to the Principles of Drug Design*, 1st ed., pp 187, Elsevier, Bristol, U.K.
- (27) Aronson, J. K. (2009) *Meyler's Side Effects of Endocrine and Metabolic Drugs*, 1st ed., pp 289, Elsevier, Oxford, U.K.
- (28) Schindler, A. E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J. R., Schweppe, K. W., and Thijssen, J. H. (2008) Classification and pharmacology of progestins. *Maturitas* 61, 171–180.
- (29) Blickenstaff, R. T., Ghosh, A. C., and Wolf, G. C. (1974) Total Synthesis of Steroids, in *Organic Chemistry: A Series of Monographs*, 1st ed., Vol. 30, Elsevier, New York.
- (30) Khatri, Y., Gregory, M. C., Grinkova, Y. V., Denisov, I. G., and Sligar, S. G. (2014) Active site proton delivery and the lyase activity of human CYP17A1. *Biochem. Biophys. Res. Commun.* 443, 179–184.
- (31) Leys, D., Mowat, C. G., McLean, K. J., Richmond, A., Chapman, S. K., Walkinshaw, M. D., and Munro, A. W. (2003) Atomic structure of *Mycobacterium tuberculosis* CYP121 to 1.06 Å reveals novel features of cytochrome P450. *J. Biol. Chem.* 278, 5141–5147.
- (32) Podust, L. M., Bach, H., Kim, Y., Lamb, D. C., Arase, M., Sherman, D. H., Kelly, S. L., and Waterman, M. R. (2004) Comparison of the 1.85 Å structure of CYP154A1 from *Streptomyces coelicolor* A3(2) with the closely related CYP154C1 and CYPs from antibiotic biosynthetic pathways. *Protein Sci.* 13, 255–268.
- (33) Szklarz, G. D., Ornstein, R. L., and Halpert, J. R. (1994) Application of 3-dimensional homology modeling of cytochrome P450 2B1 for interpretation of site-directed mutagenesis results. *J. Biomol. Struct. Dyn.* 12, 61–78.
- (34) Szklarz, G. D., and Halpert, J. R. (1997) Use of homology modeling in conjunction with site-directed mutagenesis for analysis of structure-function relationships of mammalian cytochromes P450. *Life Sci.* 61, 2507–2520.
- (35) Sawada, Y., and Ayabe, S. (2005) Multiple mutagenesis of P450 isoflavonoid synthase reveals a key active-site residue. *Biochem. Biophys. Res. Commun.* 330, 907–913.
- (36) Honda Malca, S., Scheps, D., Kuhnelt, L., Venegas-Venegas, E., Seifert, A., Nestl, B. M., and Hauer, B. (2012) Bacterial CYP153A monooxygenases for the synthesis of omega-hydroxylated fatty acids. *Chem. Commun. (Cambridge, U. K.)* 48, S115–S117.
- (37) Cools, H. J., Parker, J. E., Kelly, D. E., Lucas, J. A., Fraaije, B. A., and Kelly, S. L. (2010) Heterologous expression of mutated eburicol 14 α -demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. *Appl. Environ. Microbiol.* 76, 2866–2872.
- (38) Born, S. L., John, G. H., Harlow, G. R., and Halpert, J. R. (1995) Characterization of the progesterone 21-hydroxylase activity of canine cytochrome P450 PBD-2/P450 2B11 through reconstitution, heterologous expression, and site-directed mutagenesis. *Drug Metab. Dispos.* 23, 702–707.
- (39) Seifert, A., and Pleiss, J. (2009) Identification of selectivity-determining residues in cytochrome P450 monooxygenases: A systematic analysis of the substrate recognition site 5. *Proteins: Struct., Funct., Genet.* 74, 1028–1035.
- (40) Gricman, L., Vogel, C., and Pleiss, J. (2015) Identification of universal selectivity-determining positions in cytochrome P450 monooxygenases by systematic sequence-based literature mining. *Proteins: Struct., Funct., Genet.* 83, 1593–1603.
- (41) Khatri, Y., Hannemann, F., Ewen, K. M., Pistorius, D., Perlova, O., Kagawa, N., Brachmann, A. O., Muller, R., and Bernhardt, R. (2010) The CYPome of *Sorangium cellulosum* So ce56 and identification of CYP109D1 as a new fatty acid hydroxylase. *Chem. Biol.* 17, 1295–1305.
- (42) Sagara, Y., Wada, A., Takata, Y., Waterman, M. R., Sekimizu, K., and Horiuchi, T. (1993) Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization. *Biol. Pharm. Bull.* 16, 627–630.
- (43) Uhlmann, H., Kraft, R., and Bernhardt, R. (1994) C-terminal region of adrenodoxin affects its structural integrity and determines differences in its electron transfer function to cytochrome P-450. *J. Biol. Chem.* 269, 22557–22564.
- (44) Khatri, Y., Schiffrin, A., and Bernhardt, R. (2017) Investigating the effect of available redox protein ratios for the conversion of a steroid by a myxobacterial CYP260A1. *FEBS Lett.* 591, 1126–1140.
- (45) Trott, O., and Olson, A. J. (2010) AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461.
- (46) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612.