The full-length cytochrome P450 enzyme CYP102A1 dimerizes at its reductase domains and has flexible heme domains for efficient catalysis

Received for publication, October 25, 2017, and in revised form, March 28, 2018 Published, Papers in Press, April 4, 2018, DOI 10.1074/jbc.RA117.000600

© Haoming Zhang†, Adam L. Yokom‡, Shen Cheng§, Min Su†, Paul F. Hollenberg‡, Daniel R. Southworth†, and Yoichi Osawa†,‡,§,∥

From the Departments of †Pharmacology and §Biological Chemistry and the ¶Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109

Edited by F. Peter Guengerich

The cytochrome P450 enzyme CYP102A1 from Bacillus megaterium is a highly efficient hydroxylase of fatty acids, and there is a significant interest in using CYP102A1 for biotechnological applications. Here, we used size-exclusion chromatography–multitangle light scattering (SEC–MALS) analysis and negative-stain EM to investigate the molecular architecture of CYP102A1. The SEC–MALS analysis yielded a homogeneous peak with an average molecular mass of 235 ± 5 kDa, consistent with homodimeric CYP102A1. The negative-stain EM of dimeric CYP102A1 revealed four distinct lobes, representing the two heme and two reductase domains. Two of the lobes were in close contact, whereas the other two were often observed apart and at the ends of a U-shaped configuration. The overall dimension of the dimer was ~130 Å. To determine the identity of the lobes, we FLAG-tagged the N or C terminus of CYP102A1 to visualize additional densities in EM and found that anti-FLAG Fab could bind only the N-tagged P450. Single-particle analysis of this anti-Flag Fab–CYP102A1 complex revealed additional density in the N-terminally tagged heme domains, indicating that the heme domains appear flexible, whereas the reductase domains remain tightly associated. The effects of truncation on CYP102A1 dimerization, identification of cross-linked sites by peptide mapping, and molecular modeling results all were consistent with the dimerization of the reductase domain. We conclude that functional CYP102A1 is a compact globular protein dimerized at its reductase domains, with its heme domains exhibiting multiple conformations that likely contribute to the highly efficient catalysis of CYP102A1.

Bacterial CYP102A1, also known as cytochrome P450 BM3 or BM3, is a member of the cytochrome P450 (CYP) superfamily. Like other members of the superfamily, CYP102A1 shares the canonical feature of CYP enzymes that the catalytic center is a protoporphyrin IX ligated to a cysteinyl thiolate. The catalytic center accepts electrons from NADPH via cytochrome P450 oxidoreductase (POR) to activate inert hydrocarbon bonds. However, unlike other bacterial and mammalian CYP enzymes, the heme domain (BMP) and reductase domain (BMR) of CYP102A1 are fused in a single polypeptide chain with the N-terminal BMP domain being connected by a linker region to the C-terminal BMR domain. This structural arrangement is analogous to nitric-oxide synthase (NOS) and believed to grant CYP102A1 extraordinary catalytic prowess for hydroxylating fatty acids. Studies have shown that CYP102A1 hydroxylates long chain fatty acids at a turnover rate of >3,000 min⁻¹ (1). A turnover rate of 17,100 min⁻¹ was reported for oxidation of arachidonate by WT CYP102A1 (2). This is the highest turnover rate of any CYP enzymes and more than ~3 orders of magnitude higher than those of mammalian CYP enzymes. Furthermore, the oxidation of fatty acids by CYP102A1 is nearly 100% coupled to NADPH consumption (3).

The highly efficient catalysis of CYP102A1 has stimulated great interests in utilization of CYP102A1 as a biocatalyst for the biosynthesis of pharmaceuticals and detoxification of environmental pollutants. Through site-directed and random mutagenesis, a large collection of CYP102A1 variants have been found to oxidize a wide range of chemicals with enhanced activities and regioselectivity, including steroids (4), statins (5), numerous pharmaceutical drugs (6–8), small alkanes (6, 9), and polycyclic hydrocarbons (10). A single amino acid substitution of Ala82→Phe converts CYP102A1 from fatty acid hydroxylase to a human CYP2C19-like enzyme capable of oxidizing omeprazole at a rate of 1,460 min⁻¹ (11). These mutagenic studies have provided a wealth of information regarding the roles of specific residues, largely in the catalytic BMP domain.

This work was supported by National Institutes of Health Grants GM077430 and GM110001A, the University of Michigan Protein Folding Diseases Initiative, and the University of Michigan M cuales fund. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Fig. S1.

1 To whom correspondence may be addressed: Dept. of Pharmacology, University of Michigan Medical School, 1150 West Medical Center Dr., Ann Arbor, MI 48109. E-mail: haom@umich.edu.
2 To whom correspondence may be addressed: Dept. of Pharmacology, University of Michigan Medical School, 1150 West Medical Center Dr., Ann Arbor, MI 48109. E-mail: osawa@umich.edu.

The abbreviations used are: CYP, cytochrome P450; BMP, heme domain of CYP102A1; BMR, reductase domain of CYP102A1; DSS, disuccinimidyl suberate; Fab, fragment antigen-binding; NOS, nitric-oxide synthase; nNOS, neuronal NOS; POR, cytochrome P450 oxidoreductase; SEC–MALS, size-exclusion chromatography–multitangle light scattering; PDB, Protein Data Bank.

© 2018 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Architecture of dimeric CYP102A1

Early work by Black and Martin (12) in the 1980s showed that CYP102A1 was primarily a dimer in solution. It was later demonstrated by Munro’s lab (13) and Peterson’s lab (14) that the dimeric form of CYP102A1 is the functionally active form. Even though the crystal structures of the individual BMP and BMR domains of CYP102A1 are available, the structure of a full-length dimeric CYP102A1 is not. This greatly impedes our understanding of the holistic mechanisms of CYP102A1 and thus our ability to fully harness its catalytic power.

EM is a powerful tool for elucidating structures of macromolecules, particularly their complexes, which are dynamic in nature. Recent advancements in sorting algorithms and software available aid in studying complex protein conformations (15). We previously reported the architecture of neuronal NOS (nNOS) using negative stain EM. 2D analysis of reference free class averages showed flexible reductase domains around the oxygenase dimer interface (16). Because of the structural similarity between nNOS and CYP102A1, we applied this technique to elucidate the architecture of dimeric CYP102A1 in this work. Size-exclusion chromatography (SEC)–multiangle light scattering (MALS) analyses of our CYP102A1 preparations show four distinct densities representing the four domains of CYP102A1 homodimer. The four domains exhibit multiple orientations, indicative of the dynamic feature of CYP102A1. Studies from Fab labeling, progressive truncation, chemical cross-linking, and molecular modeling are consistent with dimerization of the BMR domains with flexibility in the BMP domains.

Results

Solution conformation, activity, and negative stain EM of full-length CYP102A1

Although the structures of the heme containing BMP and the flavin containing BMR domains are known, the architecture of the full-length CYP102A1 complex is unknown. To address this, we first examined the overall size and shape of the purified recombinant CYP102A1 by SEC–MALS. The CYP102A1 elutes predominantly as a single peak exhibiting an average molecular mass value of 235 ± 5 kDa (Fig. 1A). This indicates that CYP102A1 exists as a stable dimer in solution because the molecular mass is consistent with the theoretical mass of 238 kDa calculated from the amino acid sequence for a full-length CYP102A1 existing as a homodimer. To characterize the overall shape of CYP102A1 in solution, the hydrodynamic radius of 60 ± 1 Å was determined from the dynamic light scattering measurements. Based on comparison to the known globular protein tetramer of catalase (molecular mass = 243 kDa) with an $R_H$ of 51.2 Å (17), the CYP102A1 would be predicted to be globular with some flexibility. The NADPH oxidation activity of the dimeric CYP102A1 exhibited a turnover rate of 1,222 nmol/min/nmol P450, which is comparable with that previously reported (11). Clearly the active full-length CYP102A1 exists as a dimer in solution, consistent with previous reports (13, 14).

Distinct conformational states of CYP102A1 by 2D EM analysis

To explore the conformation and architecture of the CYP102A1, we employed single particle EM with high-contrast...
negative stain methods. The eluted material from the SEC–MALS study was collected and immediately prepared for EM analysis. In EM micrographs, single CYP102A1 particles were distinctly seen and selected for 2D classification. CYP102A was found to be generally globular with a dimension of ∼130 Å, in agreement with our SEC–MALS analysis (Fig. 1B). Additional conformations were prevalent, including a U-shaped structure with variable opening of the U, as well as closed structures. This suggested that CYP102A1 exists in different conformational states.

To further define the architecture and conformational states of CYP102A1, we performed 2D reference-free averaging and analysis using the iterative stable alignment and clustering method (18). The four lobes of density seen in single particles are well-defined in nearly all the 2D averages (Fig. 1C) consistent with the two BMP and two BMR domains of a full-length homodimer of CYP102A1. Because of the low resolution of these 2D images and the nearly equal size of the domains, we were unable to discern the identities of the four domains. However, this analysis clearly revealed a U-shaped image with two of the lobes always intimately associated, whereas the opposite lobes could be found together or apart. This suggests that two of the lobes could be in a more dynamic environment. To further discern the identity of the lobes, we utilized the tagging approach described below.

SEC analysis of the complex of FLAG-tagged CYP102A1 with anti-FLAG Fab

To help determine the identity of the four lobes of density in the 2D images, we constructed CYP102A1 tagged with FLAG either on the N terminus (N-FLAG) or C terminus (C-FLAG) so that after addition of anti-FLAG Fab, additional density could be visualized in EM studies. This procedure has been used successfully in EM studies of other small proteins (19). We first assessed the binding of Fab to CYP102A1 by SEC analysis. As shown in Fig. 2A, incubation of anti-FLAG Fab with untagged CYP102A1 had no effect on elution volume. In contrast, the same treatment decreased the elution volume of N-FLAG (green) compared with the control N-FLAG (blue), indicative of formation of the N-FLAG–Fab complex. Consistent with this interpretation is the concomitant decrease in the free Fab. The same study with C-FLAG gave no change in the elution volume of the tagged CYP102A1 or the amount of free Fab, indicating the lack of the C-FLAG–Fab complex (Fig. 2B).

To further validate the preparations of FLAG-tagged proteins, we examined the catalytic activity of the CYP102A1 by measuring the rate of NADPH oxidation. As shown in Fig. 2C, the untagged CYP102A1 gave a rate of 1.222 ± 62 min⁻¹, consistent with the activity of the dimeric form reported previously (11). The activities of the N-FLAG and C-FLAG CYP102A1 were determined to be 832 ± 75 and 1022 ± 29 min⁻¹, respectively. This clearly indicates that the dimeric form of each protein is active but with somewhat a diminished rate. More importantly, in each case, the addition of anti-FLAG Fab did not affect the activity of the CYP102A1. Thus, binding of Fab to N-FLAG CYP102A1 had no effect on the enzymatic activity.
plex was determined to be 320 kDa, corresponding to a net increase of 80 kDa. Because the molecular mass of the Fab is 50 kDa, we speculate that there is a mixture of partial and fully occupied N-FLAG CYP102A1 dimer bound to Fab fragments.

The peak corresponding to the N-FLAG CYP102A1 dimer in complex with Fab was collected and visualized via negative stain EM. Single particles were collected similar to those for unlabeled CYP102A1 (Fig. 1B). However, the 2D class average of the complex did not reveal Fab density that allowed unequivocal assignment of the BMP or BMR domains (data not shown). This is likely due to the dynamic nature of the CYP102A1 dimer and Fab labeling. Therefore, single particles of the N-FLAG CYP102A1 labeled with Fab were aligned to 2D averages of the unlabeled CYP102A1 dimer (Fig. 3B). Based upon these aligned single particles, we observed that the Fab densities exist on the ends of the U. Because Fab is attached to the N terminus of CYP102A1, we conclude that the BMP domains are flexible and comprise the ends of the U-shaped architecture, whereas the flavin-containing BMR domains remain tightly associated as a dimer and comprise the base of the U. Some of the particles only have one Fab visible, consistent with the SEC–MALS data shown above.

**SEC–MALS analysis of truncated forms of CYP102A1**

The results from SEC–MALS analysis of N-FLAG CYP102A1–Fab complex suggest that CYP102A1 is dimerized at the reductase domains (Fig. 3). To further confirm and identify the region responsible for dimerization, we progressively truncated full-length CYP102A1 from the C → N termini and examined the effects on dimerization. The results are presented in Fig. 4 and Table 1. As shown, the BMR construct showed a range of oligomers including dimer and trimer (data not shown). The determined molecular mass values for BMP and HFMN are significantly lower than the theoretic molecular mass expected for dimeric BMP (108 kDa) and HFMN (148 kDa) but higher than the molecular mass expected for corresponding monomers, suggesting that BMP and HFMN may exist as a mixture of monomer and dimer in rapid equilibrium. To confirm our hypothesis, we analyzed the molecular mass of the BMP fractions by MALDI. As shown in Fig. S1, we observed both monomeric BMP at 54 kDa and dimeric BMP at 108 kDa, confirming that BMP is a mixture of monomer and dimer. Based on this observation, we estimate that 48% BMP, 45% HFMN, 84% HFAD, and 98% full-length CYP102A1 are in dimeric forms. In contrast, the SEC profile of the BMR construct showed a range of oligomers including dimer and trimer (data not shown).

**Chemical cross-linking and peptide mapping of BMR**

To identify the site of dimerization, we examined the effect of a chemical cross-linking reagent disuccinimidyl suberate (DSS) on CYP102A1. Fig. 5 shows the Coomassie-stained cross-linked products of BMP, BMR, and full-length CYP102A1 at four DSS concentrations. As shown, a high molecular mass band was observed at ∼300 kDa after incubation of full-length CYP102A1 with DSS.
A homology model is fit relatively well in the density volume of CYP102A1 (PDB code 4DQL) with RMS values of 5 Å. The homology model was constructed with Modeler 9v8 as described previously (39). To fit the homology model into the density volume, we first built a BMR homology model (residue 478–1048) using the crystal structure of rat POR (PDB code 1AMO). The BMR homology model is highly homologous to the crystal structure of rat POR and the FAD domain as the template. The BMR homology model was fit to the 3D density volume using Chimera. The BMR homology model were fit relatively well in the density volume of CYP102A1 dimer clearly show four lobes of densities represented by green, yellow, and brown colors, respectively. The proximal side of the heme facing the FMN domain. Because of the conformational dynamics of the dimer, the linker region between BMP and BMR domains was not resolved.

**Table 1**

**Average molecular mass of full-length (FL) and truncated CYP102A1 as determined by SEC–MALS**

Details are provided under “Experimental procedures” for determination of molecular mass by SEC–MALS.

| Construct | Residue | Molecular mass kDa | Monomer molecular mass (Theor) kDa | Dimer |
|-----------|---------|--------------------|-----------------------------------|-------|
| FL        | 1–1048  | 235 ± 5            | 119                                | 98    |
| HFAD      | 1–883   | 184 ± 3            | 100                                | 84    |
| HFMN      | 1–650   | 110 ± 3            | 74                                 | 45    |
| BMP       | 1–470   | 80 ± 6             | 54                                 | 48    |
| BMR       | 480–1048| 72 ± 4             | 64                                 |       |

*Theoretic molecular mass of monomer.*

CYP102A1 with 5 μM DSS. The intensity of this band is increased at elevated DSS concentrations. In contrast, BMP does not exhibit any higher molecular mass bands even at 500 μM DSS, suggesting that it is not cross-linked. Cross-linking of BMP by DSS gives rise to multiple bands from 130 to 300 kDa. The band at ~130 kDa corresponds to cross-linked dimer of BMP. For peptide mapping this band was excised and subject to in-gel proteolysis with trypsin. Analysis of the tryptic digests by LC-MS/MS revealed three cross-linked peptides with <5% FDR. The three pairs of cross-linked lysines are Lys777–Lys790 identified from chemical cross-linking studies are far from the interface prior to fitting the density volume.

**3D modeling of dimeric CYP102A1**

To shed light on the nature of the dimerization interface, we constructed a 3D model of dimeric CYP102A1 using constraints of the EM density volume and our finding that the region 650–883 of the BMR domain is involved in dimerization as supported by SEC–MALS analysis of truncated CYP102A1. As shown in Fig. 6, the volumetric densities of the BMR domains are connected, and each is shaped like a bowl. This geometry is similar to that of rat POR as reported by Kim’s group (20). The modeling results reveal that residues Lys777 and Lys790 identified from chemical cross-linking studies are located in close proximity to the dimerization interface. The distance from Lys777 to Lys790 of the opposing BMR molecule is ~20 Å. This intermolecular distance should be considered an estimate because the 3D model does not have atomic resolution. To ensure objectivity of the model, we did not impose any specific constraints other than positioning the region 650–883 near the interface prior to fitting the density volume.

The density volume of BMP is globular in general, similar to the crystal structure of BMP. However, the density volume of BMP is larger than the size of the crystal structure of BMP. As a result the BMP molecule can only be rationally fit into the density volume with the proximal side of the heme facing the FAD domain. Because of the conformational dynamics of the dimer, the linker region between BMP and BMR domains was not resolved.

**Discussion**

In this work we report for the first time a detailed study of the CYP102A1 dimerization interface using negative stain 2D EM combined with SEC–MALS analysis, chemical cross-linking, and molecular modeling. The 2D class averages of the CYP102A1 dimer clearly show four lobes of densities representative of the four domains of CYP102A1 homodimer. The overall dimension of the dimer is ~130 Å, consistent with the $R_g$ of 60 ± 1 Å determined by dynamic light scattering (see Fig. 1A). Compared with the 2D class average of nNOS that shows an elongated conformation, the overall conformation of CYP102A1 is globular. The overall geometry of CYP102A1 dimer is consistent with the findings by atomic force microscopy showing that the shapes of CYP102A1 molecules are globular in the form of

**Figure 6. 3D model of dimeric CYP102A1 showing the close view of the dimerization interface.** The 3D EM density shown in solid surface was constructed from the 2D class average images as described under “Experimental procedures.” The crystal structure of the BMP (PDB code 4KEW) and the BMR homology model were fit to the 3D density volume using Chimera. The BMR homology model was constructed with Modeler 9v8 as described previously (39). BMP molecule is shown in magenta, and the FMN, FAD, and nucleotide-binding domains of the BMR are shown in green, blue, and cyan, respectively. Co-factor FMN, FAD, and heme are shown in orange, yellow, and red, respectively. Residues Lys777 and Lys790 are shown in brown and gray, respectively.
Architecture of dimeric CYP102A1

Table 2

Cross-linked peptides in DSS-linked dimeric BMR

BMR was cross-linked with DSS-d25/d25, and the cross-linked dimeric BMR was subjected to in-gel tryptic digestion and LC-MS/MS analysis. The cross-linked peptides were identified by searching the MS/MS data set using StavroX software. The experimental details are provided under "Experimental procedures."

| m/z       | Charge | Δ ppm | Peptide a | Peptide b | Score |
|-----------|--------|-------|-----------|-----------|-------|
| 795.190063476563 | +4 | 4.92 | 7771TVBPPHKVELALLEK786 | 787EQYEOQVLK794 | 110 |
| 631.738952636719 | +5 | 0.56 | 7271LEAEEEKALHPLAK742 | 7573AmAATVBPMPK777 | 99 |
| 742.1416625976563 | +4 | 0.04 | 7951LTMLEELKYDABmK811 | 7851EQLVK797 | 88 |

a Cross-linked Lys residues are italicized and in bold. Letters B and m stand for S-carboxymethylated cysteine and oxygenated methionine residues, respectively.

compact objects with lateral dimensions of <500 Å (21). The 2D class averages also show that the conformation of CYP102A1 in solution is dynamic (see Fig. 1). We observed a range of 2D class averages with the four domains in multiple orientations. This likely indicates that CYP102A1 exhibits an ensemble of conformational states with significant domain mobility. Although the four domains are well-defined, individual BMP and BMR domains are not identified because of the low resolution of the 2D class averages. Nonetheless we were able to discern the BMP and BMR domains by obtaining single particles of an N-FLAG CYP102A1–Fab complex.

Fab is frequently used in cryo-EM to identify protein domains because the 50-kDa Fab bound to protein of interest is readily visible under EM (19, 22). We constructed both N-FLAG and C-FLAG CYP102A1 and incubated them with excess anti-Fab Fab to form the Fab complexes for negative stain EM analysis. As expected, Fab binds to the N-FLAG CYP102A1, leading to an increase in molecular mass by 80 kDa (see Fig. 3A). It is of note that Fab does not bind to the FLAG epitope in C-FLAG CYP102A1 under the same conditions used for N-FLAG CYP102A1–Fab complex, suggesting that the C-terminal FLAG epitope is shielded from the Fab. The inaccessibility of C-FLAG to Fab may arise from the fact that the C terminus of CYP102A1 is part of the FAD-binding pocket and less solvent-exposed. Alternatively, dimerization of the BMR domain may also play a role to limit the accessibility of the C terminus of CYP102A1.

The 2D class averages of the N-FLAG CYP102A1–Fab complex reveal an array of single particles bound with Fab fragments (see Fig. 3B). We examined the single particles in the U-shaped conformation and aligned them to unlabeled CYP102A1 2D classes. Of a large number of single particles in the U-shaped conformation, we observed additional density to CYP102A1. Without exception, the additional densities were observed in the two end domains of CYP102A1 dimer (see Fig. 3B). Because the epitope is located at the N terminus of the dimer, we conclude that the end domains are BMP domains, and therefore CYP102A1 is dimerized at the BMR domains.

To narrow the region for dimerization in the BMR domain, we progressively truncated full-length CYP102A1 from the C → N termini and examined the effect of truncation on dimerization. Deletion of 164 residues from the C terminus (Δ884–1048), which are part of the NADPH-binding domain, results in a loss of dimer by ~16% (see Table 1). Progressive deletion of another 233 residues (Δ651–883), which are part of the FAD-binding domain, results in a sharp decrease in dimerization by 55%. Deletion beyond residue 651 has no effect on dimerization because the percentages of dimer in BMP and HFMN remain at ~45%. These results suggest that the peptide segment (residues 651–883) is important for CYP102A1 dimerization. Based on the molecular mass calculation by SEC–MALS (see Table 1), BMP and HFMN would be mixtures of monomers and dimers. However BMP and HFMN elute as single broad peaks on SEC columns (see Fig. 1). We postulate that the BMP and HFMN dimers are loosely associated and in rapid equilibrium with monomers. This is consistent with the observation that BMP was not cross-linked by DSS, whereas cross-linking BMR and full-length CYP102A1 yielded dimeric products (see Fig. 5).

Peptide mapping of the cross-linked BMR dimer allows us to identify three pairs of Lys residues cross-linked by DSS (see Table 2). According to the crystal structure of the FAD domain (PDB code 4DQL), all of the three pairs of cross-linked peptides are located in the connecting domains that link the FAD-binding domain to the FMN-binding domain (23). The intramolecular distances between Lys806 and Lys796 and between Lys777 and Lys790 are 8.1 and 24.7 Å, respectively. Because Lys734 is missing from the crystal structure, the intramolecular distance between Lys734 and Lys777 is unknown. The intramolecular distance between Lys777 and Lys790 is greater than the spacer arm distance of 11.4 Å of DSS. Therefore, peptide 7771TVCPPHKVLEALEK786 and peptide 7871QAYKEQVLK794 are likely involved in dimerization of BMR. CYP102A1 is a homodimer, and thus we were unable to discern whether the Lys806–Lys796 pair arises from intra- or interprotein linkage. Involvement of peptide 771TVCPPHKVLEALEK786 in dimerization seems to be consistent with the notion that Cys773 is involved in dimerization of the FAD domain (residue 660–1048) via an intermolecular disulfide bond, as reported by Joyce et al. (23).

These authors showed that treatment of the WT FAD domain with DTT led to partial formation of the monomeric FAD domain. Mutation of Cys773 → Ala resulted in formation of monomer only. The intermolecular disulfide bond may also play a partial role in the homodimer of CYP102A1 based on the observation that DTT treatment of the mixture of variant A264H and G570D significantly increased the activity of the heterodimer A264H/G570D, presumably because of enhanced exchange to form the active heterodimer after cleavage of the disulfide bond (13). Caution should be exercised on the role of intermolecular disulfide bond in CYP102A1 dimer because DTT treatment has no effect on dimerization of homodimer of CYP102A1. It is unclear whether the disulfide bond in dimeric CYP102A1 is completely shielded from DTT. The precise interface for dimerization of CYP102A1 remains to be elucidated.
Results from the 3D modeling shed light on the nature of the dimerization interface. As shown in Fig. 6, the two BMR domains exhibit a C2 symmetry where the FAD domain interacts with the FMN domain of the opposing BMR molecule. The nucleotide-binding domain is pointing away from the interface. Furthermore, both Lys777 and Lys790 identified from the chemical cross-linking studies are located in proximity to the interface. This arrangement is consistent with the observations that truncation of residue 650–883 results in dissociation of the homodimer, whereas truncation of residue 883–1048 in the nucleotide-binding domain has little effect (see Fig. 4). Because of the dynamic nature of the dimer and lack of a dimeric model of a full-length CYP102A1, the linker region between BMP and BMP was not identified, and the precise conformation of BMP remains to be elucidated. In one of the likely conformations, the proximal side of BMP is positioned toward the FMN co-factor. This would allow the shortest distance for electron transfer from FMN to the heme by swinging out the FMN domain with the dimeric interface as a hinge.

Collectively we have shown that CYP102A1 is dimerized at the BMR domain and the BMP domains are in multiple conformations. In the U-shaped conformation, the two BMP domains are split in an open conformation, whereas in the closed conformation, the two BMP domains are in proximity. Like the architecture of nNOS, the CYP102A1 dimer has a central dimerization point with flexible flanking BMP domains. However, the conformational range in CYP102A1 is reduced compared with nNOS because the flanking BMP domains remain closer to the dimerization point. This reduced conformational range is likely due to a much shorter linker (by ~70 residues) between the BMP and BMR domains because CYP102A1 lacks the regulatory domains like the calmodulin-binding domain and autoinhibitory helix domain present in nNOS. This shorter linker renders CYP102A1 a compact and globular architecture, in contrast to the elongated architecture of nNOS dimer. This compact architecture may have important implications for efficient electron transfer for catalysis.

The importance of the CYP102A1 architecture for efficient catalysis was recognized three decades ago because various attempts to reconstitute the catalytic activity of CYP102A1 with functional individual domains failed (24, 25). BMP and BMR domains separated by proteolysis were no longer associated with each other (24). These observations indicate that the architecture of dimeric CYP102A1 plays a critical role in modulating domain-domain interactions for efficient catalysis. Our findings on the architecture of dimeric CYP102A1 suggest a dynamic model for electron transfer as depicted in Scheme 1. In this model the two BMR domains are dimerized, whereas the two BMP domains may exhibit different orientations. For simplicity, we describe the mobility of the BMP domain as closed and open conformations. In reality the conformational state of the BMP would be more diverse, especially in 3D space.

Our dynamic model is consistent with the notion that electron transfer from FAD to FMN occurs via a cis fashion within the same polypeptide chain (chain A or B), i.e. FAD_A → FMN_A or FAD_B → FMN_B, whereas electron transfer from FMN to the heme occurs in a trans fashion, i.e. FMN_A → HEM_A or FMN_B → HEM_B (13, 26). This notion is supported by the observations that mixing two nonfunctional CYP102A1 variants, i.e. A264H (defective heme) and G570D (lack of FMN), resulted in a significant increase in fatty acid hydroxylase activity. As depicted in Scheme 1, FAD_A → FMN_A could occur in either open or closed conformation because studies have shown that the FMN domains are flexible, and this flexibility plays a vital role in electron transfer between the FMN, FAD, and heme. For example, restricting the freedom of the motion of the FMN domain greatly impairs the electron transfer of FMN as demonstrated elegantly in rat POR and the reductase domain of nNOS (27, 28). As we have shown by 2D EM, the architecture of the BMR is a compact globular protein where the FMN and FAD domains are in proximity. This proximity would facilitate the cis electron transfer from FAD → FMN. However the trans electron transfer from FMN to the heme is unlikely to occur in the open conformation because of the long distance from the heme to FMN located on the opposing domain. Therefore the open conformation likely represents an inactive state for catalysis. Because the BMP domains are flexible as revealed by 2D EM (see Fig. 1), the trans electron transfer could occur via the motion of the BMP domain bringing itself closer to the FMN on the opposing domain. This may occur in the closed conforma-
Construction, overexpression, and purification of various forms of CYP102A1

The cDNA sequence encoding a full-length CYP102A1 A82F mutant gene was synthesized by Blue Heron Biotechnology (Bothell, WA). The coding region was then cloned to a pCWori vector using a pair of Ndel/NotI restriction sites. To facilitate purification, a His$_6$ tag sequence was introduced to the N terminus after the start codon ATG to construct the plasmid of pCW-CYP102A1A82F-His$_6$. Truncated and FLAG-tagged CYP102A1 were constructed by PCR using pCW-CYP102A1A82F-His$_6$ as template DNA and a pair of primers as shown in Table 3.

All forms of the CYP102A1 constructs were overexpressed in C41(DE3) cells in the presence of 0.1 mg/ml ampicillin. In brief, a single colony from the C41(DE3) cells transformed with the pCWori plasmid containing the desired gene was inoculated to 50 ml of LB medium, and the culture was grown at 30 °C/180 rpm overnight. An aliquot of 10 ml of the LB culture was used to inoculate 1 liter of Terrific broth medium. The Terrific broth medium was grown for 6 h, and overexpression of CYP102A1 was induced by the addition of 0.6 mM isopropyl β-D-1-thiogalactopyranoside and 0.5 mM δ-aminolevulinic acid. The induced cells were continued to grow for 16 h at 30 °C and then harvested by centrifugation at 2,500 x g for 25 min. All proteins except for BMR were purified with a Histrap HP column (5 ml; GE Health Sciences) as reported previously (29). BMR was purified with a 2’,5’-ADP affinity column (GE Health Sciences) as reported previously (30). The purified proteins were desalted to 0.1 M KPi, 15% glycerol buffer (pH 7.4) using PD-10 columns (GE Health Sciences) and stored in aliquots at −80 °C until use. The concentrations of CYP102A1 constructs were determined by the method of Omura and Sato (31), whereas the concentrations of BMR were determined using the extinction coefficient of 21.4 mm$^{-1}$ cm$^{-1}$ at 450 nm as reported previously (32).

Preparation of antigen-binding (Fab) fragment and CYP102A1–Fab complex

Fab was prepared from monoclonal anti-FLAG M2 IgG2 antibody using a Pierce Fab preparation kit according to the manufacturer’s protocol. To form the CYP102A1–Fab complex, FLAG-tagged CYP102A1 (8 μM) was incubated overnight at 4 °C in PBS solution with a 2-fold excess of the prepared Fab. Formation of the complex was confirmed by SEC–MALS analysis as described below.

SEC–MALS analysis of full-length and truncated forms of CYP102A1

SEC–MALS analysis was performed on a Bio-Rad NGC Quest chromatography system equipped with a miniDAWN TREOS MALS detector and an Optilab TrEX differential refractive index detector. The SEC–MALS system was calibrated with bovine serum album. An aliquot of 50 μl CYP102A1 (~10 μM) was loaded onto a WTC-050S5 column (7.8 × 300 mm, 5 μm, Wyatt Technology) and eluted in PBS buffer. The molecular mass was determined from the Raleigh ratio calculated by measuring the static light scattering and corresponding protein concentration of a selected peak using ASTRA VI software (Wyatt Technology). Sample injection of 20 μM CYP102A1 was centrifuge-filtered prior to being loaded onto a WTC-050S5 column for hydrodynamic radius determination. An ÄKTAmicro chromatography system equipped with a WyattQELS light scattering detector was used as a final purification step and taken directly to be stained for negative stain EM experiments.
EM sample preparation, data collection, and 2D processing

The samples were stained using ~0.75% uranyl formate (pH 5.5–6.0) on carbon-coated copper grids (Pelco). EM data were collected using a G2 Spirit transmission electron microscope (FEI) operated at 120 keV. For CYP102A1 alone ~150 micrograph images were taken at 52,000X nominal magnification using a 4k by 4k CCD camera (Gatan). Original pixel spacing of 2.16 Å was binned by 2 (4.32 Å per pixel) for image particle picking and processing. e2boxer was used to manual select 7809 single particles, which were extracted and classified using iterative stable alignment and clustering 2D classification (33, 34).

CYP102A1–Fab data were collected and processed the same as the CYP102A1 alone data. Specifically, 140 micrographs were taken resulting in 14,507 single particles selected within e2boxer manual particle selection. SPIDER was used to align the single particles of CYP102A1–Fab with the 2D class averages from CYP102A1 alone (35).

Chemical cross-linking and peptide mapping of the BMR domain

Chemical cross-linking of BMR was performed in 20 mM HEPES, 0.1 mM NaCl buffer (pH 7.8). Fresh DSS-d_10/d_13 stock solution was prepared by dissolving solid DSS- in N,N-dimethylacetamide. BMR (~8 μM) was incubated with varying concentrations of DSS-d_10/d_13 at 37 °C for 30 min. The reaction was then quenched with 50 mM ammonium bicarbonate. Cross-linked products were SDS–PAGE. For peptide mapping the dimer band of BMR was excised for in-gel tryptic digest as previously reported (36). The tryptic digest of BMR was dried and resuspended in 50% acetonitrile, 49.9% water, 0.1% TFA. Peptide mapping of the cross-linked BMR was performed on a Thermo Scientific Orbitrap fusion mass spectrometer at a resolution of 160,000. The cross-linked peptides were identified using StavroX software as described (37).

Catalytic activities of CYP102A1 enzymes

The catalytic activities of CYP102A1 enzymes were determined by measuring the rates of NADPH consumption. In brief, aliquots of 0.5 ml of PBS buffer containing 0.1 μM CYP102A1 and 0.1 mM substrate omeprazole were incubated at 25 °C for 5 min. Aliquots of NADPH were then added to 0.2 mM to initiate the reaction. The absorbance at 340 nm was recorded for 2 min on a Shimadzu UV-2501 (PC) spectrophotometer. The rate of NADPH consumption was calculated based on the initial slope at 340 nm and the extinction coefficient of 6.2 mM⁻¹ cm⁻¹ as previously reported (38).

3D modeling of the dimeric structure of CYP102A1

Well-defined class average images showing the dimer were selected to generate the initial model using the program e2initialmodel.py (33). Then 3571 particles were extracted from those selected classes for 3D reconstruction using Relion (34). The initial model was filtered to 60 Å resolution and subjected to 3D auto-refinement with C2 symmetry enforced. The final step of the model building is to fit the structure of CYP102A1 to the 3D EM density using Chimera. Because the crystal structure of a full-length CYP102A1 is not available, we fit the crystal structure of the BMP domain (PDB code 4KEW, residues 3–455) and a BMR homology model (reside 478–1048) instead. The BMR homology model was constructed on the template of rat POR (PDB code 1AMO) using Modeler 9v8 as described previously (39).

Author contributions—H. Z. and Y. O. conceptualization; H. Z., A. L. Y., S. C., and M. S. data curation; H. Z., A. L. Y., S. C., M. S., and Y. O. formal analysis; H. Z. supervision; H. Z., D. R. S., and Y. O. funding acquisition; H. Z., A. L. Y., S. C., and M. S. investigation; H. Z., A. L. Y., and D. R. S. methodology; H. Z. and A. L. Y. writing-original draft; H. Z. and D. R. S. project administration; H. Z., P. F. H., D. R. S., and Y. O. writing-review and editing; P. F. H. and Y. O. resources.

Acknowledgment—We thank Dr. Brian Shay for analysis of the molecular mass of BMP by MALDI.

References

1. Narhi, L. O., and Fulco, A. J. (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in Bacillus megaterium. J. Biol. Chem. 261, 7160–7169 Medline
2. Noble, M. A., Miles, C. S., Chapman, S. K., Lysek, D. A., Mackay, A. C., Reid, G. A., Hanzlik, R. P., and Munro, A. W. (1999) Roles of key active-site residues in flavocytochrome P450 BM3. Biochem. J. 339, 371–379 CrossRef Medline
3. Truan, G., and Peterson, J. A. (1998) Th268 in substrate binding and catalysis in P450BM-3. Arch. Biochem. Biophys. 349, 53–64 CrossRef Medline
4. Venkataraman, H., Te Poede, E. M., Rosloniec, K. Z., Vermeulen, N., Commandeur, J. N., van der Geize, R., and Dijkhuizen, L. (2015) Biosynthesis of a steroid metabolite by an engineered Rhodococcus erythropolis strain expressing a mutant cytochrome P450 BM3 enzyme. Appl. Microbiol. Biotechnol. 99, 4713–4721 Medline
5. Kim, K. H., Kang, J. Y., Kim, D. H., Park, S. H., Park, S. H., Kim, D., Park, K. D., Lee, Y. J., Jung, H. C., Pan, J. G., Ahn, T., and Yun, C. H. (2011) Generation of human chiral metabolites of simvastatin and lovastatin by bacterial CYP102A1 mutants. Drug Metab. Dispos. 39, 140–150 CrossRef Medline
6. Chen, M. M., Snow, C. D., Vizcarra, C. L., Mayo, S. L., and Arnold, F. H. (2012) Comparison of random mutagenesis and semi-rational designed libraries for improved cytochrome P450 BM3-catalyzed hydroxylation of small alkanes. Protein Eng. Des. Sel. 25, 171–178 CrossRef Medline
7. Damsten, M. C., van Vugt-Lussenburg, B. M., Zeldenrust, T., de Vlieger, J. S., Commandeur, J. N., and Vermeulen, N. P. (2008) Application of drug metabolising mutants of cytochrome P450 BM3 (CYP102A1) as biocatalysts for the generation of reactive metabolites. Chem. Biol. Interact. 171, 96–107 CrossRef Medline
8. Otey, C. R., Bandara, G., Lalonde, J., Takahashi, K., and Arnold, F. H. (2006) Preparation of human metabolites of propranolol using laboratory-evolved bacterial cytochromes P450. Biotechnol. Bioeng. 93, 494–499 CrossRef Medline
9. Meinhold, P., Peters, M. W., Chen, M. M., Takahashi, K., and Arnold, F. H. (2005) Direct conversion of ethene to ethanol by engineered cytochrome P450 BM3. Chembiochem. 6, 1765–1766 CrossRef Medline
10. Carmichael, A. B., and Wong, L. L. (2001) Protein engineering of Bacillus megaterium CYP102. The oxidation of polycyclic aromatic hydrocarbons. Eur. J. Biochem. 268, 3117–3125 CrossRef Medline
11. Butler, C. F., Peet, C., Mason, A. E., Voice, M. W., Leys, D., and Munro, A. W. (2013) Key mutations alter the cytochrome P450 BM3 conformational landscape and remove inherent substrate bias. J. Biol. Chem. 288, 25387–25399 CrossRef Medline
Architecture of dimeric CYP102A1

12. Black, S. D., and Martin, S. T. (1994) Evidence for conformational dynamics and molecular aggregation in cytochrome P450 102 (BM-3). Biochemistry 33, 12056–12062 CrossRef Medline

13. Neeli, R., Girvan, H. M., Lawrence, A., Warren, M. I., Leys, D., Scorttun, N. S., and Munro, A. W. (2005) The dimeric form of flavocytochrome P450 BM3 is catalytically functional as a fatty acid hydroxylase. FEBS Lett. 579, 5582–5588 CrossRef Medline

14. Kitazume, T., Haines, D. C., Estbrook, R. W., Chen, B., and Peterson, J. A. (2007) Obligatory intermolecular electron transfer from FAD to FMN in dimeric P450BM-3. Biochemistry 46, 11892–11901 CrossRef Medline

15. Kimanian, D., Forsberg, B. O., Scheres, S. H., and Lindahl, E. (2016) Accelerated cryo-EM structure determination with parallelisation using GPUs in RELION-2. Elife 5

16. Yang, Z., Fang, J., Chittuluru, J., Asturias, F. J., and Penczek, P. A. (2012) Iterative stable alignment and clustering of 2D transmission electron micrographs. Structure 20, 237–247 CrossRef Medline

17. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., and Kim, J. J. (1997) Three-dimensional structure of NADPH-cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes. Proc Natl. Acad. Sci. U.S.A. 94, 8411–8416 CrossRef Medline

18. Bukharina, N. S., Ivanov, I. D., Pleshakova, T. O., Frantsuzov, P. A., Ivanova, N. D., Krokhin, N. V., Petushkova, N. A., and Archakov, A. I. (2015) Atomic force microscopy monitoring of temperature dependence of cytochrome BM3 oligomeric state. Biofizika 60, 80–87 Medline

19. Joyce, M. G., Ekanem, I. S., Roitell, O., Dunford, A. J., Neeli, R., Girvan, H. M., Baker, G. J., Curtis, R. A., Munro, A. W., and Leys, D. (2012) The crystal structure of the FAD/NADPH-binding domain of flavocytochrome P450 BM3. FEBS J. 279, 1694–1706 CrossRef Medline

20. Narhi, L. O., and Fulco, A. J. (1987) Identification and characterization of two functional domains in cytochrome P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in Bacillus megaterium. J. Biol. Chem. 262, 6683–6690 Medline

21. Sevioukova, I., Truan, G., and Peterson, J. A. (1997) Reconstitution of the fatty acid hydroxylase activity of cytochrome P450BM-3 utilizing its functional domains. Arch. Biochem. Biophys. 340, 231–238 CrossRef Medline

22. Girvan, H. M., Dunford, A. J., Neeli, R., Ekanem, I. S., Waltham, T. N., Joyce, M. G., Leys, D., Curtis, R. A., Williams, P., Fisher, K., Voice, M. W., and Munro, A. W. (2011) Flavocytochrome P450 BM3 mutant W1046A is a NADH-dependent fatty acid hydroxylase: implications for the mechanism of electron transfer in the P450 BM3 dimer. Arch. Biochem. Biophys. 507, 75–85 CrossRef Medline

23. Hamdane, D., Xia, C., Im, S. C., Zhang, H., Kim, J. I., and Waskell, L. (2009) Structure and function of an NADPH-cytochrome P450 oxidoreductase in an open conformation capable of reducing cytochrome P450. J. Biol. Chem. 284, 11374–11384 CrossRef Medline

24. Dai, Y., Haque, M. M., and Stuehr, D. J. (2017) Restricting the conformational freedom of the neuronal nitric-oxide synthase flavoprotein domain reveals impact on electron transfer and catalysis. J. Biol. Chem. 292, 6753–6764 CrossRef Medline

25. Zhang, H., Gruenke, L., Saribas, A. S., Im, S. C., Shen, A. L., Kasper, C. B., and Waskell, L. (2003) Preparation and characterization of a 5′-deazaFAD T491V NADPH-cytochrome P450 reductase. Biochemistry 42, 6804–6813 CrossRef Medline

26. Omura, T., and Sato, R. (1964) The Carbon monoxide-binding pigment of horse liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. 239, 2379–2385 Medline

27. Scheres, S. H. (2012) RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 CrossRef Medline

28. Frank, J., Radermacher, M., Penczek, P., Zhu, J., Le, Y., Ludtke, S. J. (2007) EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46 CrossRef Medline

29. Leith, A. (1996) SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. J. Struct. Biol. 116, 190–199 CrossRef Medline

30. depicted-to-kinetics of protein complexes and identification of cross-linking sites using LC-MS/MS and the xQuest/xProphet software pipeline. Nat. Protoc. 9, 120–137 Medline

31. Förster, M., Peltokunn, S., Schals, S., Bosse, K., Ihling, C. H., Krauth, F., Fritzschke, R., Kühn, U., and Sinz, A. (2012) StavroX-A software for analyzing crosslinked products in protein interaction studies. J. Am. Soc. Mass Spectrom. 23, 76–87 CrossRef Medline

32. Zhang, H., Hamdane, D., Im, S. C., and Waskell, L. (2008) Cytochrome b5 inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. J. Biol. Chem. 283, 5217–5225 CrossRef Medline

33. Lin, H. L., Zhang, H., Medower, C., Hollenberg, P. F., and Johnson, W. W. (2011) Inactivation of cytochrome P450 (P450) 3A4 but not P450 3A5 by OSI-930, a thiophene-containing anticancer drug. Drug Metab. Dispos. 39, 345–350 CrossRef Medline