Obligatory Intermolecular Electron-Transfer from FAD to FMN in Dimeric P450BM-3[†]

Tatsuya Kitazume,[‡] Donovan C. Haines,[§] Ronald W. Estabrook,[‡] Baozhi Chen,[‡] and Julian A. Peterson[‡,*]

Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9038, and Department of Chemistry, The University of Texas at Dallas, 2601 N. Floyd Road, Dallas, Texas 75083-0688

Received May 26, 2007; Revised Manuscript Received July 24, 2007

ABSTRACT: Cytochromes P450 typically catalyze the monooxygenation of hydrophobic compounds resulting in the insertion of one atom of dioxygen into the organic substrate and the reduction of the other oxygen atom to water. The two electrons required for the reaction are normally provided by another redox active protein, for example cytochrome P450 reductase (CPR) in mammalian endoplasmic reticulum membranes. P450BM-3 from *Bacillus megaterium* is a widely studied P450 cytochrome in which the P450 is fused naturally to a diflavin reductase homologous to CPR. From the original characterization of the enzyme by Fulco's laboratory, the enzyme was shown to have a nonlinear dependence of reaction rate on enzyme concentration. In recent experiments we observed enzyme inactivation upon dilution, and the presence of substrate can diminish this inactivation. We therefore carried out enzyme kinetics, cross-linking experiments, and molecular weight determinations that establish that the enzyme is capable of dimerizing in solution. The dimer is the predominant form at higher concentrations under most conditions and is the only form with significant activity. Further experiments selectively knocking out the activity of individual domains with site-directed mutagenesis and measuring enzyme activity in heterologous dimers establish that the electron-transfer pathway in P450BM-3 passes through both protein molecules in the dimer during a single turnover, traversing from the FAD domain of one molecule into the FMN domain of the other molecule before passing to the heme domain. Analysis of our results combined with other analyses in the literature suggests that the heme domain of either monomer may accept electrons from the reduced FMN domain.

Cytochromes P450 typically catalyze the monooxygenation of hydrophobic compounds, as shown in eq 1, resulting in the insertion of one atom of dioxygen into the organic substrate and the reduction of the other oxygen atom to water.

$$AH + O_2 + NAD(P)H + H^+ \rightarrow AOH + H_2O + NAD(P)^+ \quad (1)$$

Monooxygenation requires two electrons and these are usually derived from NADPH in eukaryotic systems. In prokaryotic systems the source of electrons is most often NADH. The mode of delivery of these electrons from the reduced pyridine nucleotide to the heme iron of the P450 can be used to divide the vast majority of P450 systems into two different classes: (1) P450s which utilize an FAD/FMN containing P450 reductase (CPR[1]), and (2) P450s which utilize an FAD-containing reductase and a diffusible electron carrier, e.g., an iron sulfur protein.

Over 30 years ago, Fulco began the characterization of soluble fatty acid hydroxylation systems found in *Bacillus megaterium* (*1*, *2*). One of these, P450BM-3, has become a model for eukaryotic microsomal P450 systems (*3*). This enzyme is so useful because it has a CPR-like domain, as well as a P450-domain, all on a single polypeptide chain (*4*), and it is soluble, stable, and readily expressed in *Escherichia coli* either as the holoenzyme or various domain combinations (*5*−*8*). Sequence alignments very early showed that this enzyme was more like eukaryotic than prokaryotic P450 systems (*9*). Sequence alignments of the heme domain of P450BM-3 with both eukaryotic and prokaryotic P450s showed that there were insertions in the sequence that would result in changes in the putative redox partner binding surface of the P450 (*10*). P450BM-3 has one of the highest turnover numbers for a monooxygenation reaction of any P450 with a value of 3200 mol of arachidonic acid oxidized per min per mole of enzyme at 30 C (*11*). The physiological substrate of P450BM-3 is currently unknown; however, Wolf's group has speculated that the role of P450BM-3 is to protect *B. megaterium* by removing toxic fatty acids from the environment (*11*).

[†] This work was supported in part by NIH Grants (GM43479 and GM50858) and an American Heart Association Grant (0150611N) to J.A.P.

* Corresponding author. Tel: 214-648-2361, Fax: 214-645-9361. E-mail: Julian.Peterson@UTSouthwestern.edu.

[‡] The University of Texas Southwestern Medical Center at Dallas.

[§] The University of Texas at Dallas.

[1] Abbreviations: FNR, ferredoxin nucleotide reductase; BMR, the NADPH-P450 reductase domain of P450BM-3; P450BM-3, CYP102A1; BMP, the P450 heme domain of P450BM-3; DTT, dithiothreitol; NPG, *N*-palmitoylglycine; ETF, electron transfer flavoprotein; CPR, NADPH-cytochrome P450 reductase.

10.1021/bi701031r CCC: $37.00
Published on Web 09/29/2007

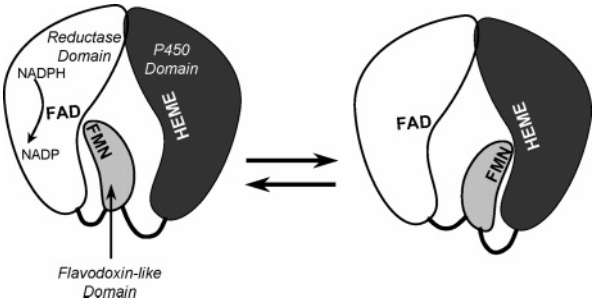

FIGURE 1: Cartoon showing the hypothetical interaction of the domains of P450BM-3.

For a number of years, we have been interested in the mechanism of transfer of electrons within this multidomain protein and found among other things that while the path of electrons from NADPH to FAD to FMN that had been elucidated for CPR was valid for P450BM-3, the FMN-binding domain did not utilize the same reduced intermediates to transfer the electrons from the FAD to the heme-domain. This FMN-binding domain cycles between fully oxidized and the anionic semiquinone (one electron reduced) form (*12*, *13*). The reduction potential of the fully oxidized/semiquinone form for the FMN-binding domain of BMR is about the same as that of the semiquinone/hydroquinone couple of CPR (*14*).

The commonly assumed interactions between the various domains of P450BM-3 are illustrated in the cartoon in Figure 1. On the left side of this figure, the various domains and their linkers are indicated with the flavodoxin-like domain docked to the FAD-containing reductase domain. This interaction is consistent with the domain interactions in CPR (*15*). In our studies of the kinetics of reduction of the reductase domain of P450BM-3 (BMR), NADPH delivers electrons to the FAD and presumably, because of the proximity of the FMN to the FAD, detection of intermediates in the transfer process through the FAD and on to the FMN is extremely difficult (*12*, *13*). Following reduction of the FMN, the delivery of the electron to the P450 heme occurs. We presume that there is rotation and translation of the flavodoxin domain . The distance between the FMN and the closest that the heme of BMP could approach without this rotation and translation is probably beyond 20 Å (*6*). The rotation and translation illustrated here was proposed for another very simple reason: the docked form of the flavodoxin domain to BMP in the X-ray crystal structure of this bidomain complex is similar to that shown in the right-hand portion of this figure (*6*) and is only compatible with rotation and translation of the flavodoxin-like domain. To complete the delivery of two electrons from NADPH to the P450, the flavodoxin-like domain would have to return to the FAD-binding domain for the second electron.

In 1994, Black and Martin reported that sedimentation equilibrium studies of P450BM-3 showed that the enzyme existed under some conditions as a higher order oligomer and that DTT could cause an interconversion of these oligomeric forms (*16*). More specifically, monomers, dimers, trimers, and higher order oligomers appeared to exist in their preparations of the enzyme, and preincubation in the presence of DTT resulted in conversion of the enzyme to predominately *the dimeric form*. The effect of DTT on the oligomeric state of P450BM-3 was interpreted as *not* being due to the reduction of disulfide bonds (*16*). Unfortunately, parallel enzymatic assays were not reported (*16*).

During the course of our studies of fatty acid amide derivatives of amino acids as alternative substrates for P450BM-3, the results obtained indicated that P450BM-3 was only catalytically active as a dimeric species. This manuscript describes those experiments and the structural interpretation of the results obtained, which clearly indicate that intermolecular electron transfer between the FAD domain of one monomer to the FMN domain of the other monomer is obligatory during turnover of P450BM-3.

MATERIALS AND METHODS

Materials. The heme-, FAD-, and FMN-binding domains of P450BM-3 were purified as previously described (*7*, *12*). The reductase domain of P450BM-3 (BMR) was purified as previously described (*17*). The concentration of P450 in any preparation was determined by the method of Omura and Sato (*18*). The concentration of flavin in those samples containing only flavin prosthetic groups was determined spectrophotometrically at 450 nm (*7*). UV−visible spectroscopy was performed on either a Hewlett-Packard model 8452A Diode Array spectrophotometer or a Varian Cary 100 double beam instrument. *N*-Palmitoylglycine was synthesized and purified as previously described (*19*, *20*). NADPH, sodium dithionite, horse heart cytochrome c, Sepharose CL-6B, glutaraldehyde, fatty acids, and buffer components were obtained from Sigma Aldrich.

Assay for Oxygen Consumption. Oxygen consumption was measured with an Instech Laboratories, Inc. (Plymouth Meeting, PA) Model 110 Fiber Optic Oxygen Monitor. The standard probe was used either with the reaction chamber supplied with the apparatus or with a larger volume reaction chamber. The oxygen monitor was used at 25 °C, and the oxygen concentration in the buffers was calibrated enzymatically with P450BM-3, 500 μM fatty acid substrate, and known amounts of NADPH.

Cytochrome c Reductase Assay. To measure the cytochrome c reductase activity (*21*), a stock solution of about 10 μM wild-type P450BM-3 in 5 mM KPi pH 7.4 was diluted in the same buffer to concentrations of 25 nM to 3 μM. The solutions were incubated for 1 h at room temperature to ensure full equilibration. The cytochrome c reductase activity was then assayed by diluting these samples into fresh 5 mM KPi pH 7.4 containing 35 μM horse heart cytochrome c and 100 μM NADPH. The solution was rapidly mixed and the change in absorbance at 550 nm was monitored. The final P450BM-3 concentrations in the cuvettes was normally 10 nM, but increased to 25 nM for the samples with lowest cytochrome c activity. The cytochrome c reduction rate was calculated from the absorbance change at 550 nm using an extinction coefficient of 21 100 M^{-1} cm^{-1} for reduced−oxidized cytochrome c. Initial rates were calculated from the first 20 s of reaction using the HP 8453 software's initial rate fitting routines and were corrected for a low level of background (nonenzymatic) activity. The specific activity was calculated based on the final P450BM-3 concentration in the cuvette.

Glutaraldehyde Cross-Linking. Purified P450BM-3 was cross-linked with glutaraldehyde using a published procedure (*22*). Solutions of P450BM-3, with the preincubation condi-

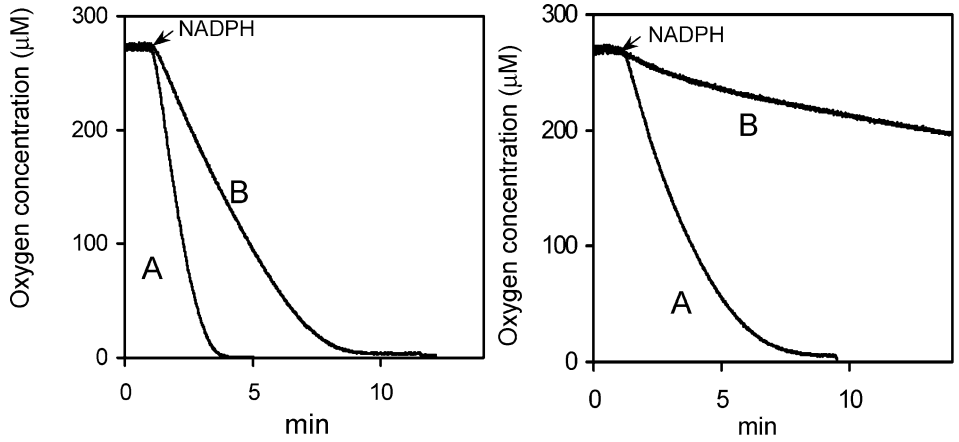

FIGURE 2: Effect of preincubation conditions on the activity of P450BM-3. P450BM-3 was added to 50 mM potassium phosphate buffer pH 7.4 with (left panel) or without (right panel) 300 μM sodium palmitate. After either no preincubation (A) or a 10 min preincubation (B), the reaction was initiated by the addition of 300 μM NADPH. In the instances that the enzyme was preincubated in the absence of sodium palmitate, the fatty acid was added to the reaction mixture approximately 15 s prior to the addition of NADPH.

tions described in each figure (50 μL), were incubated with an aqueous solution of glutaraldehyde (50 μL) for 5 min at room temperature. The reaction mixture was treated with $NaBH_4$ (20 μL of 2 M solution) at 30 °C for 30 min. The cross-linked protein was precipitated by adding 50 μL of 80% trichloroacetic acid, washed, and separated on SDS/PAGE gels with an 8−18% linear acrylamide gradient with a 2-amino-2-methyl-1,3-propanediol/glycine/HCl buffer system (*23*). Samples for SDS/PAGE were boiled in SDS sample buffer containing 50 mM dithiothreitol for 5 min and then loaded onto the stacking gel. The dependency of cross-linking on the concentration of glutaraldehyde was checked over the range from 0.05 to 500 mM (data not shown). Based on these results, the final concentration of glutaraldehyde in the cross-linking experiments was 100 mM.

Site-Directed Mutagenesis. The QuikChange site-directed mutagenesis kit (Stratagene) was used for introducing mutations into P450BM-3, by following the manufacturer's manual with oligonucleotide primers as follows: G570D-F 5′-CCGTATTTGGATGCGATGATAAAAACTGGGCTACTACG-3′, G570D-R 5′-CGTAGTAGCCCAGTTTTTATCATCGCATCCAAATACGG-3′(*24*),W1046A-F5′-GCAAAAGACGTGGCGGCTGGGTAAATTAAAAAGAGG-3′,W1046A-R 5′-CCTCTTTTTAATTTACCCAGCCGCCACGTCTTTTGC-3′. The F87Y mutant has been made by our laboratory previously (*25*).

Size Exclusion Chromatography and Activity Measurement after the Chromatography. A 1.5 × 90.0 cm Sepharose CL-6B Gel (Sigma-Aldrich) column was prepared for size exclusion chromatography. Approximately 10 μM P450BM-3 was applied to the column, which had been pre-equilibrated with 50 mM Tris chloride buffer, pH 7.5, containing 100 mM KCl. The elution of P450BM-3 was monitored at both 280 nm to follow protein elution and 418 nm to follow elution of the low-spin form of P450BM-3. The activity measurements of the fractions from chromatography were performed by diluting the column fractions to a final concentration of 50 nM P450BM-3 without reconcentrating to avoid changes in the dimerization of the enzyme.

RESULTS

Inactivation of P450BM-3. As seen in Figure 2, the ability of P450BM-3 to oxidize sodium palmitate is significantly decreased during a 10 min preincubation of the enzyme in dilute solution in the presence of 300 μM sodium palmitate (but in the absence of NADPH, which is known to inactivate P450BM-3 when a hydroxylation substrate is unavailable (*26*, *27*)). The time course of the inactivation and the effect of buffer components were determined (Supporting Information). In these experiments, the alternative substrate *N*-palmitoylglycine (NPG) was employed, but similar results were obtained when sodium palmitate was used as the substrate (data not shown). The activity of P450BM-3 is most rapidly lost when the enzyme is preincubated in a buffer of low ionic strength and in the absence of a fatty acid substrate or substrate analogue. Preincubation of the enzyme in a buffer of approximately physiological ionic strength delayed but did not prevent the inactivation of P450BM-3. Thus, the ionic strength of the buffer, in addition to the presence/absence of a substrate, has a significant affect on the rate of inactivation.

To test whether the loss of activity on incubation in dilute solution is in fact reversible, we designed a dilution/reconcentration protocol (Supporting Information). This protocol tests whether the loss of activity was due to dissociation of one of the enzyme cofactors, e.g., the FMN (*5*), as it would be irreversibly lost during reconcentration using an ultrafiltration membrane. Previous work in our lab has shown that the FMN cofactor can be removed from the FMN-binding domain of P450BM-3 (*5*). Using the same incubation conditions as those for the experiment in Figure 2, a loss of approximately 85% of the hydroxylation activity was determined in those samples that were preincubated in low ionic strength potassium phosphate buffer (5 mM, pH 7.4) (Supporting Information). A separate sample of the dilute enzyme was reconcentrated to 20 μM, after the 10 min incubation in dilute solution, using a membrane concentration system, and then assayed. The diluted and preincubated enzyme recovered approximately 75% of its original activity when concentrated. A similar experiment performed in the presence of 0.1 μM FMN to reconstitute any lost FMN gave essentially the same results demonstrating that loss of FMN from the flavodoxin domain was not the cause of enzyme inactivation.

Size Exclusion Chromatography. Remembering the report of Black's group that P450BM-3 was oligomeric (*16*) and

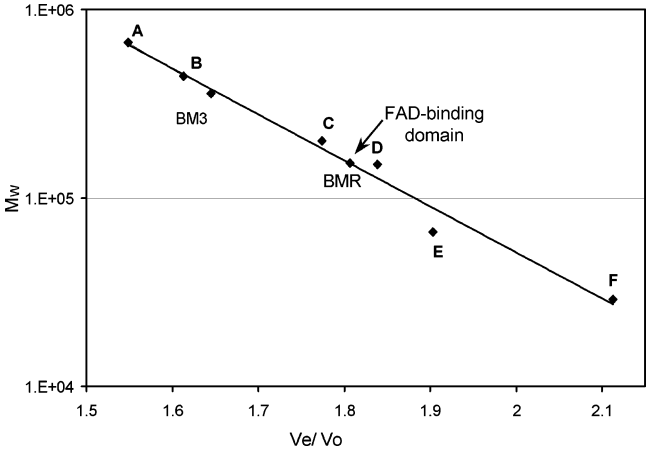

FIGURE 3: Elution of P450BM-3 and BMR compared to molecular weight standards. P450BM-3, BMR, and FAD-binding domain were separately chromatographed on Sepharose CL-6B as described in Figure 6 and their relative elution volumes (V_e/V_o) compared to that of the following globular proteins of known molecular weight: (A) thyroglobulin, 669 000; (B) apoferritin, 443 000; (C) β-amylase, 200 000; (D) alcohol dehydrogenase, 150 000; (E) bovine albumin, 66 000; (F) carbonic anhydrase, 29 000.

Fulco's early observations that the activity of crude P450BM-3 was highly concentration dependent (*28*), the molecular size of our preparations of P450BM-3, BMR, the FAD-binding domain of BMR and BMP was examined using size exclusion chromatography on Sepharose CL-6B. The column was equilibrated with 50 mM Tris-chloride buffer, pH 7.5, containing 100 mM KCl which has an ionic strength of about 150 mM. The elution profile of these proteins was compared to that of globular proteins. P450BM-3 elutes as a single molecular species with essentially a Gaussian elution pattern (Supporting Information). The R_f of P450BM-3, when compared to the standard proteins, indicates that P450BM-3 is oligomeric (Figure 3) with an estimated molecular weight of about 360 000 Da. The predicted dimer molecular weight would be 240 000 Da. This analysis presumes that the protein is essentially globular, but if it is not and instead is more ellipsoidal, the apparent molecular size will be larger. Thus, the species could be a dimer or a trimer. In a separate series of experiments, the elution properties of BMP were examined and this protein behaved as if it was a globular protein of approximately 50 000 Da (data not shown). In contrast, BMR eluted with a molecular weight of approximately 154 000 Da as if it was at least dimeric (Figure 3). The predicted molecular weight of the dimer would be 140 000 Da. The FAD-binding reductase domain has essentially the same elution profile as BMR, which contains both the FAD-binding reductase and the FMN-binding flavodoxin domain. Most of the mass of the combined reductase domain is in the FAD-binding domain (55 000 Da of 70 000 Da). The similarity between the elution of the FAD-binding domain and BMR therefore suggests the same degree of oligomerization. It should be noted that the buffers do not contain DTT or other reducing agents that might be expected to disrupt disulfide bonds or to exert other effects such as those described in Black's work (*16*).

It seemed plausible that oligomerization was occurring and affected catalytic activity, but the lack of complete recovery of the activity of P450BM-3 after it had been preincubated in dilute solution and then concentrated was difficult to understand. A sample of the concentrated enzyme solution,

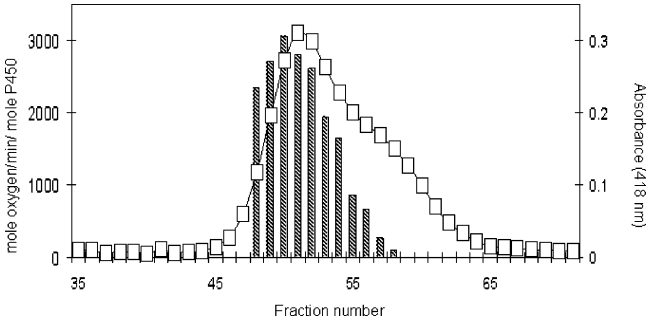

FIGURE 4: Elution of P450BM-3 after dilution and reconcentration. The open squares are of the absorbance at 418 nm and the bars of the specific activity of the enzyme in the oxygen consumption assay as described in Materials and Methods.

that had undergone the dilution reconcentration protocol, was chromatographed on Sepharose CL-6B as described in Figure 3 and the activity of each of the fractions measured as shown in Figure 4. The absorbance profile of the fractions that contain heme (418 nm) is no longer symmetric but is rather distorted with a shoulder on the low molecular weight side. The plot of turnover number versus fraction number clearly shows that the higher molecular weight fractions (dimers) had the same activity as the enzyme had prior to dilution and concentration. It is not clear at the present time why a fraction of P450BM-3 did not reform the higher molecular weight species, but this plot indicates that the lower molecular weight form of P450BM-3 is catalytically inactive in the oxidation of fatty acids. The relative proportions of the two peaks are consistent with the recovery of about 70−75% of the activity when P450BM-3 is diluted and reconstituted.

Glutaraldehyde Cross-Linking. To examine whether P450BM-3 existed as a monomer or a dimer under conditions that are closer to those of the enzyme activity experiments, glutaraldehyde, a cross-linking agent, was used to explore the oligomeric nature of this complex enzyme. As seen in Figure 5, if P450BM-3 is freshly diluted into buffer of low ionic strength and glutaraldehyde added immediately, the enzyme is predominately a monomer at 1 μM and essentially dimeric at 5 μM. At 1 μM P450BM-3, the dimer is stabilized in the presence of either or both 100 mM KCl and a slight molar excess of *N*-palmitoylglycine over enzyme. This result indicates that under the conditions identified that preserve activity, the enzyme is found predominantly in the dimeric form. Under the conditions that gave decreased activity, the enzyme is significantly monomeric. Figure 6 shows the effect of a 10 min preincubation before cross-linking and varying the ionic strength on the dimeric nature of the enzyme. As with the previous experiment, the addition of stock enzyme to low ionic strength buffer results in most of the enzyme being monomeric within the time frame of glutaraldehyde cross-linking (*29*). Increasing the ionic strength using KCl or a higher concentration of phosphate resulted in a stabilization of the dimer. The effect of preincubating the samples for 10 min at room-temperature prior to the addition of glutaraldehyde is shown in the right panel of this figure. Even with 100 mM KCl in the buffer, the enzyme is only a 50/50 mixture of monomer and dimer. It should be noted that the monomer/dimer interconversions described above are independent of the addition of DTT to the reaction mixture and thus

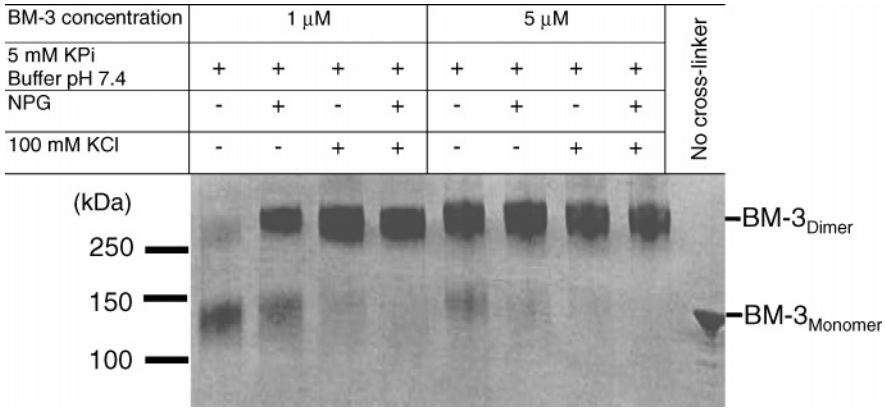

FIGURE 5: Chemical cross-linking of P450BM-3 as a function of protein concentration and buffer conditions. The conditions for each lane are shown in the table above the photograph of the PAGE gel. The same amount of protein was loaded in each lane.

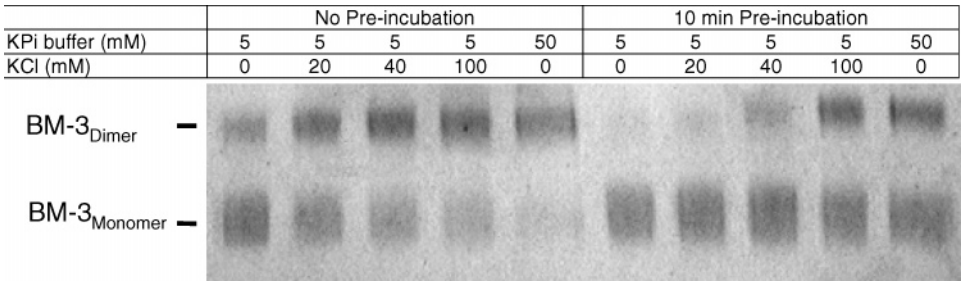

FIGURE 6: Chemical cross-linking of P450BM-3 with and without preincubation. The conditions of this experiment are given in the table above the PAGE gel.

preclude disulfide bridges as being important in these interconversions.

To get a more accurate correlation of activity to dimerization, activity measurements were taken under conditions similar to those used to study dimerization in Figure 6. These results, showing the increase in activity of P450BM-3 with increasing ionic strength of the buffer (Supporting Information), parallel the results shown in Figure 6 in which the fraction of enzyme that is dimeric increases with increasing ionic strength. Once again the percent maximal activity correlates well with the (visually estimated) fraction of P450BM-3 that exists as a dimer under identical conditions. At the present time, we do not know the reason that the activity of P450BM-3 decreases at ionic strengths greater than 0.5 M.

Sequence Alignment of BMR and CPR. A significant difference between the reductase domain of P450BM-3 and the NADPH-P450 reductase, whose structure has been determined, is the length of the linker between the FAD-binding domain and the FMN-binding domain. Using the structure of NADPH-P450 reductase (*15*) as a framework, the structure of the FMN-binding domain of P450BM-3 was overlaid and it became apparent that it would be very difficult to properly position the FMN to accept the electron from the FAD. The linker was simply not long enough. This suggested that the FMN domain from one molecule of P450BM-3 may be required to accept electrons from the FAD domain of the other molecule of P450BM-3 in the dimeric structure and would explain why the monomer lacks activity.

The reason the linker was not long enough is apparent when one compares P450BM-3 and related fusion P450s with cytochrome P450 reductase sequences (Figure 7). Overall the two groups of proteins align well, although they clearly segregate into two groups based on shared amino acids. More important than the amino acid similarities and differences, there is a significant gap (~16 amino acids distributed between two specific gaps in the region, 665−675 and 691−697) in the sequence of the fusions in the region joining the FMN binding domain of P450BM-3 and the other fusions to the hinge region of the reductase (which in turn is attached to the FAD binding domain). In the structure of rat CPR this region is extended, and it is hard to imagine how it can be shortened by 16 amino acids without disrupting the structure observed by displacing the FMN domain. In the rat CPR structure, the FMN domain nestles into the FAD domain, with the methyl groups of the isoalloxazine rings essentially in contact (*15*). Although solution studies suggest the FMN- and FAD-binding domains may not be as closely associated as in the crystal structure, the structure still appears to be a good picture of a conformation important during electron transfer (*13*, *30*). The structure seems ideal for FAD to FMN electron transfer, with the two cofactors in edge to edge contact. Shortening the linking tether (without other compensating changes in the sequence) would not allow the FNM binding domain to fit into the concave face of the FAD binding domain and place the FMN cofactor into contact with the FAD cofactor. This would seem to preclude efficient intramolecular electron transfer from FAD to FMN in the same monomer. The shorter linker would not interfere with intermolecular electron transfer in a dimer and would support active electron-transfer if the structure is similar to that shown in the model in Figure 11.

Site Specific Mutations to Analyze Electron Flow. Prior experiments had shown that the substrate binding site mutant, F87Y, of P450BM-3 was inactive (*25*). The W1046A mutant was constructed because Wolf's group (*31*) had shown that CPR could be converted from an NADPH to an NADH

```
                          650       660       670       680
                    ....|....|....|....|....|....|....|....|
P450BM-3     613  TYEEWREHMWSDVAAYFNLDIENSE..........DNKST  642
CYP102A3     616  HRESWENRFWKETMDAFDINEIAQK..........EDRPS  645
CYP102A4     626  QLEQWKQRMWSDAMKVFGLELNKNME.........KERST  656
P450foxy     631  SDFEAWEDIVLWPGLKEKYKISDEE.............SG  657
CPR, Pig     215  DFITWREQFWPAVCEHFGVEATGEESSIRQYELVVHTDMD  254
CPR, Mouse   215  DFITWREQFWPAVCEFFGVEATGEESSIRQYELVVHEDMD  254
CPR, Rat     215  DFITWREQFWPAVCEFFGVEATGEESSIRQYELVVHEDMD  254
CPR, Rabit   216  DFITWREQFWPAVCEHFGVEATGEESSIRQYELVLHTDID  255
CPR, Human   215  DFITWREQFWPAVCEHFGVEATGEESSIRQYELVVHTDID  254

                          690       700       710       720
                    ....|....|....|....|....|....|....|....|
P450BM-3     643  LSLQFVDSAA.......DMPLAKMHGAFSTNVVASKELQQ  675
CYP102A3     646  LSITFLSEAT.......ETPVAKAYGAFEGIVLENRELQT  678
CYP102A4     657  LSLQFVSRLG.......GSPLARTYEAVYASILENRELQS  689
P450foxy     658  GQKGLLVEVS......TPRKTSLRQDVEEALVVAEKTLT.  690
CPR, Pig     255  TAVVYTGEMGRLKSYENQKPPFDAKNPFLAVVTTNRKLN.  293
CPR, Mouse   255  TAKVYTGEMGRLKSYENQKPPFDAKNPFLAAVTTNRKLN.  293
CPR, Rat     255  VAKVYTGEMGRLKSYENQKPPFDAKNPFLAAVTANRKLN.  293
CPR, Rabit   256  VAKVYQGEMGRLKSYENQKPPFDAKNPFLATVTTNRKLN.  294
CPR, Human   255  AAKVYMGEMGRLKSYENQKPPFDAKNPFLAAVTTNRKLN.  293
```

FIGURE 7: Amino acid sequence alignment of selected CPR and natural P450-CPR fusion sequences. The portion of the alignment shown represents the transition from the FMN-binding domain to the 'hinge' domain connecting to the FAD-binding domain. Highly conserved residues are shaded.

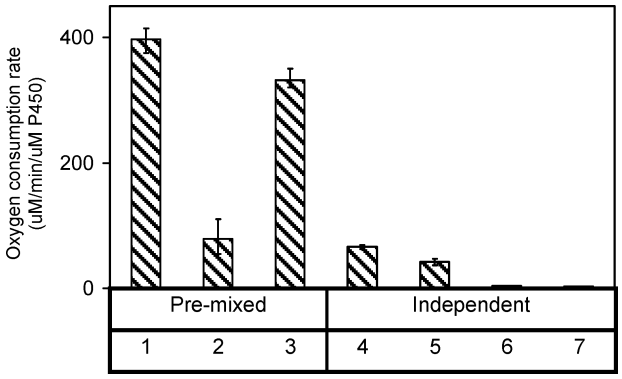

FIGURE 8: Complementary recovery of oxygen consumption activity by mixing two different mutants. Oxygen consumption activity was measured with following the reaction system, 50 mM potassium phosphate, monobasic (KH_2PO_4) (pH 7.4), 125 mM NPG, 6.6 mM glucose 6-phosphate, 2 U/mL glucose 6-phosphate dehydrogenase (sigma), 50 mM NADPH, 400 nM (premixed sample) or 200 nM (independent sample) enzyme, respectively. Premixed sample was prepared by mixing an equal amount of two different mutants in 50 mM KPi (pH 7.4) at room temperature and concentrated this till 120 μM. 1: Mixture of F87Y and W1046A, 2: Mixture of F87Y and G570D:W1046A (double mutant), 3: premixed W1046A and F87Y:G570D (double mutant), 4: W1046A, 5:F87Y, 6: G570D:W1046A (double mutant), 7: F87Y:G570D (double mutant).

requiring reductase with the analogous mutation. This tryptophan residue sits very close to the FAD in the FAD-binding domain of CPR (*15*). If electron transfer in the active dimer occurred from the FAD of one molecule of P450BM-3 to either FMN cofactor and into the P450 domain of the second molecule of P450BM-3, mixtures of the two mutant proteins may be active even though pure samples of each mutant should lack NADPH-dependent activity (*31*).

Seen in Figure 8 is the effect of W1046A and F87Y mutations on the ability of P450BM-3 to oxidize NPG (bars 4 and 5). Both of these mutations result in a very slow rate of oxygen consumption. To test whether the activity of P450BM-3 could be reconstituted with these inactive proteins, 30 μM stock solutions were mixed in a 1:1 molar ratio and incubated either in ice (4 C) or on the bench top at 23 C for 5 min and then diluted each into the normal assay mixture. As can be seen in column 1 of Figure 8, a 1:1 mixture of the inactive mutants gave an enzyme that is much more catalytically active in the oxidation of NPG than either mutant was alone. The mixture that was incubated for 5 min in ice was no more active than either of the mutants alone (not shown here). The initial velocity of the mixture was about 400 mol/min/mol while wild-type enzyme would have had an initial velocity of about 2500 mol/min/mol. Assuming that the reaction mixture had reached equilibrium, there should be a mixture of complexes: F87Y dimer; W1046A dimer; the heterodimer. These complexes should be present in the ratio of 1:1:2. We have shown in Figure 8 that the two homodimers are essentially inactive and the heterodimer should have 50% of the activity of the wild-type enzyme. Thus, we should expect that the maximal activity that could be observed in this mixture is $0.5 \times 0.5 \times 2500$ or 600 mol/min/mol. Thus, after equilibration, ~70% of the activity was recovered; this is essentially identical to the activity recovered when wild-type enzyme was diluted and reconcentrated.

To further probe the path of electron flow between the modules of P450BM-3, we created a set of double mutants of the enzyme. Fulco has shown that the G570D mutation in the FMN-binding site results in an enzyme that does not bind FMN (*24*). We created the F87Y, G570D double mutant, and as can be seen in column 7 in Figure 8, this enzyme as expected was inactive. Our second double mutant G570D, W1046A, shown in column 6 in Figure 8, also was inactive. Mixing these double mutants as described for the single mutants was done as shown in columns 2 and 3 of the figure. The mixture of W1046A and the double mutant F87Y, G570D had almost as much activity as the mixture of the F87Y and W1046A mutants. The cartoon shown in

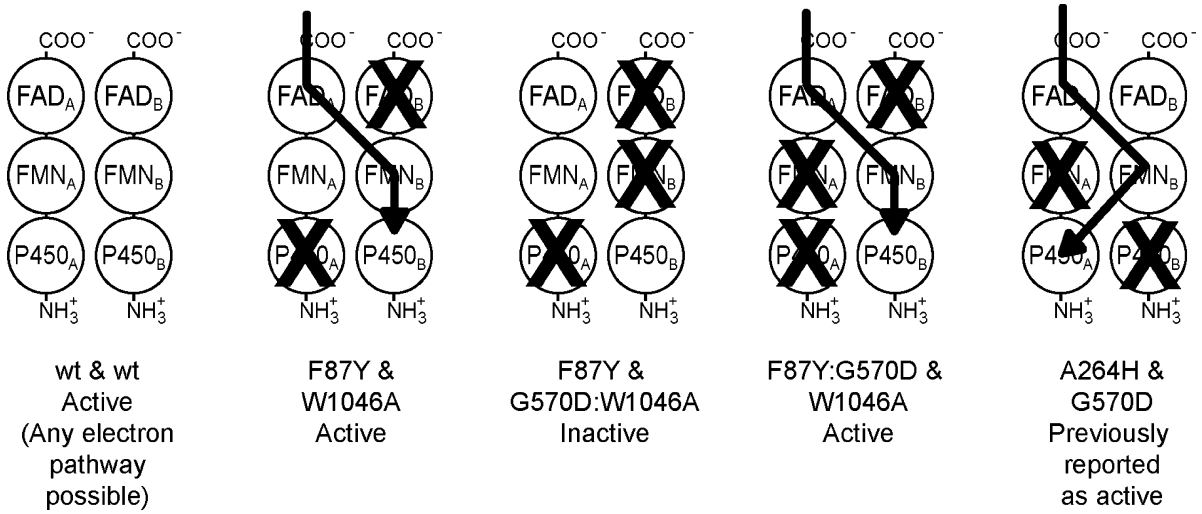

FIGURE 9: Schematic analysis of P450BM-3 electron-transfer pathways. Domains are arranged in order of reduction potential, with least positive at the top (FAD) and most positive at the bottom (P450). Alignment of two monomers is not intended to imply structural relationships but is consistent with the data obtained. Structural relationships are discussed elsewhere in the text. Domains marked with an 'X' are inactivated by mutations. Arrows show possible electron-transfer pathways in each case.

Figure 9 illustrates how electrons could be delivered from NADPH through the FMN-binding domain to the P450 heme for oxidation of the fatty acid. Shown in this figure, the FAD-binding domain of one of the dimer pairs accepts electrons from NADPH and transfers them to the FMN-binding domain of the other member of the dimer. This FMN-binding domain then transfers electrons on to the P450.

Cytochrome c Reductase Assay. After the completion of most of the work described in this manuscript and reported at an international conference in 2005, another group reported that P450BM-3 exists as an obligatory dimer (*32*). They arrived at a very different model of electron transfer, based largely on their observation that the cytochrome c reductase activity of P450BM-3, which occurs via electron transfer from NADPH to FAD to FMN to cytochrome c, does not change appreciably upon dilution of P450BM-3. They report a constant activity of 4200 μmol cytochrome c min^{-1} μmol P450BM-3 in the concentration range 2−50 nM with a slight drop to 3420 μmol cytochrome c min^{-1} μmol P450BM-3 at 0.5 nM (*32*). Because we could not reconcile that experiment with the results of our own studies, we set out to measure the cytochrome c reductase activity of both monomeric and dimeric P450BM-3.

To ensure both forms would be relevant under similar conditions, we carried out our assay in the low ionic strength buffer 5 mM KPi pH 7.4, a buffer in which our cross-linking studies clearly showed P450BM-3 was monomeric at low (sub-micromolar) concentrations and dimeric at higher concentrations. In preliminary experiments, if samples were not allowed to equilibrate for more than 30 s, the cytochrome c reductase specific activity was constant relative to the cuvette concentration of P450BM-3 but changed significantly with changes in the concentration of P450BM-3 stock solutions used to dilute into the assays. Further studies suggested that there was a strong time dependence of cytochrome c reductase activity upon dilution of P450BM-3 very similar to that observed for NPG hydroxylation activity.

We took advantage of the slowness of equilibration of the monomer−dimer equilibrium by making a range of stock solutions of P450BM-3 in 5 mM KPi pH 7.4 from a 10 μM

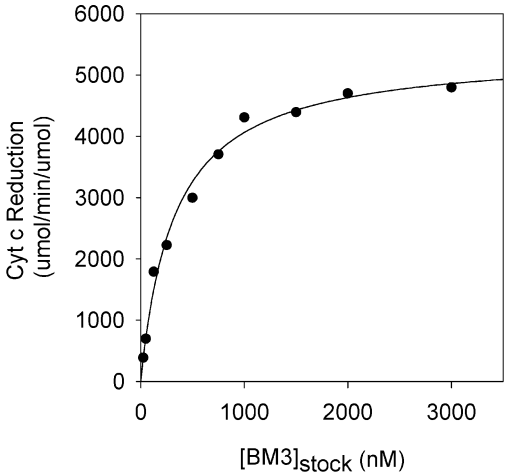

FIGURE 10: Cytochrome c reductase activity of P450BM-3. Stock solutions of P450BM-3 in 5 mM KPi pH 7.4 were equilibrated for 1 h at room temperature and diluted into cytochrome c assay solution composed of 35 μM cytochrome c and 100 μM NADPH in 5 mM KPi pH 7.4. The initial rate of cytochrome c reduction was measured. Activity is reported relative to the concentration of P450BM-3 in the cuvette (10−25 nM) and plotted against concentration of P450BM-3 in the equilibrated stock solution.

P450BM-3 stock solution. The initial (parent) 10 μM P450BM-3 stock solution was generated by desalting P450BM-3 into 5 mM KPi pH 7.4 using a PD-10 column to ensure that salt in the enzyme storage solution would not affect the assays. The samples were allowed to equilibrate for 1 h at room temperature and assayed immediately upon dilution into the cytochrome c reductase assay solution. By carrying out the experiment in this way, we could calculate the specific activity for cytochrome c reduction before the monomer−dimer equilibrium shifted significantly from its position in the stock solution. The results shown in Figure 10 clearly demonstrate a strong dependence of the specific cytochrome c reductase activity on the concentration of P450BM-3 in the stock solution. The activity drops from ~4800 μmol cytochrome c min^{-1} μmol P450BM-3 at 3 μM P450BM-3 to 690 μmol cytochrome c min^{-1} μmol P450BM-3 at 50 nM P450BM-3. Below 50 nM, reaction rates were even lower (data not shown), but we were unable to reliably

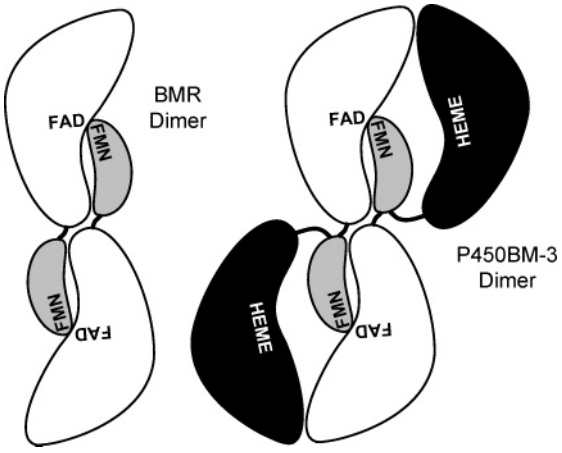

FIGURE 11: Model for domain interactions in the dimer of P450BM-3.

distinguish them from the low background (nonenzymatic) activity. The data were fit to a simple rectangular hyperbola, resulting in a maximal velocity of 5390 ± 170 μmol/min/μmol and a K_D for dissociation of dimer of 327 ± 36 nM. The K_D is lower than one would predict from our cross-linking studies (a K_D estimated from Figures 5 and 6 would be around 2−3 μM), but the cross-linking agent itself may interfere with dimer formation slightly. Clearly the activity of monomeric P450BM-3 is indistinguishable from zero and cannot be more than 5% of the activity of dimeric enzyme, consistent with an absolute restriction on electron transfer from FAD to FMN within a single P450BM-3 monomer.

DISCUSSION

As we began the present set of experiments, we believed that P450BM-3 functioned as a self-sufficient monomer and that even if oligomers had been previously observed (*16*), they had no relevance to the catalytic activity of this complex enzyme. Our observation that the ability of P450BM-3 to catalyze the oxidation of fatty acids was lost over time in dilute buffer of low ionic strength was troubling and complicating to our understanding of how this enzyme functioned. The data presented in this paper correlating the dimeric form with the active enzyme ultimately forced us to conclude that P450BM-3 was functional only in its dimeric form, but then we were left with devising a model that would *require* dimerization for activity. The observation that fatty acid hydroxylation required dimer formation implied that there was intermolecular electron-transfer, otherwise the monomer should be active. Since the FAD-binding FNR-like domain was dimeric even in the absence of the flavodoxin-like FMN-binding domain, as shown by both gel exclusion analysis and analytical ultracentrifugation (data not shown), we started the construction of the model as shown in the cartoon in Figure 11. In this cartoon, the FAD-binding FNR-like domain was assembled tail to tail. If this domain had been assembled head to tail, the protein could have formed higher order oligomers. The addition of the FMN-binding domain to this dimeric model did not increase the axial ratio and hence would not be expected to change the elution volume of the BMR from Sepharose. This BMR dimer can be seen in the cartoon in the left-hand panel of Figure 11 with the FMN-binding domain of one molecule bound to and in position to accept electrons from the FAD of the FNR-like domain. In the right panel of Figure 11, we have included the heme domain and have positioned it so that it would accept electrons from the FMN-binding domain on the same polypeptide. It should be obvious that there are a variety of other geometrical arrangements for intermolecular electron-transfer that would account for the data in this manuscript but for the present discussion we have chosen this model.

This model is consistent with the results of our experiments on the activity of heterodimers of mutants of P450BM-3. The pathway of electrons through the cofactors can reasonably be expected to flow from NADPH to FAD to FMN to heme, based on the reduction potentials of the various cofactors. The simplest models involve the transfer of electrons linearly, stepping through the FAD domain of one monomer or the other before transfer to the FMN domain of either monomer. Figure 9 shows a schematic summary of these experiments where the qualitative indications of activities are normalized for the concentration of active heme domain and 50% population of potentially active heterodimers (see Results). For the active F87Y and W1046A heterodimer, two such electron-transfer pathways are possible. First, electrons can enter $FAD_A \rightarrow FMN_B \rightarrow P450_B$ as illustrated by the arrow in this figure. Second, electrons can enter monomer A through the FAD domain (FAD_A) and then pass to the FMN domain of monomer A (FMN_A) and then (because the heme domain of monomer A is inactive due to mutation) into the heme domain of monomer B ($P450_B$) (not illustrated with an arrow). The second pathway can be ruled out, as the F87Y and G570D:W1046A heterodimer is inactive. This indicates that either electrons cannot be transferred intramolecularly from FAD to FMN or that electrons cannot be transferred intermolecularly from FMN to heme, so the second pathway described above does not function in P450BM-3. The F87Y:G570D and W1046A heterodimer is active, confirming the first pathway as a valid pathway in P450BM-3. Thus, electrons can travel from the FAD domain of monomer A to the FMN domain of monomer B and then pass into the heme domain of monomer B.

After the completion of most of the work described in this manuscript, another group reported that P450BM-3 exists as an obligatory dimer (*32*). However, this other study arrives at a very different picture of the electron-transfer pathway. Using a heme domain knockout A264H and the same FMN domain knockout G570D used here, they show that a heterodimer is active (when normalized) (*32*). This would require intermolecular electron transfer from the FMN domain of one monomer to the heme domain of the other monomer ($FMN_A \rightarrow P450_B$). Although the present results do not rule out this as a possible electron-transfer step (if $FAD_A \rightarrow FMN_A$ electron transfer does not occur), there is no single linear pathway that can satisfy the constraints of both their data (*32*) and the present results. We clearly see intramolecular transfer from FMN_A to $P450_A$ as effective and they clearly see intermolecular transfer from FMN_A to $P450_B$ as effective (*32*). Thus, we must conclude that a simple linear pathway of electron flow is not at work in P450BM-3.

Interestingly, a more complex model where either FMN domain could donate electrons to either heme domain would reconcile the two studies. In fact, this appears to be required

to support the data from the mutants used in both studies. A pathway (as shown in Figure 9) in which FAD_A transfers electrons to FMN_B (but not FMN_A), and FMN_B in turn can transfer electrons to either $P450_A$ or $P450_B$, would explain all the data on mutant heterodimer activity. The arrows in Figure 9 show this pathway as it would occur in each heterodimer. The inactive F87Y combined with G570D: W1046A combination fails to support substrate hydroxylation activity because the $FAD_A \rightarrow FMN_A$ intramolecular electron transfer cannot occur.

The other study carries out cytochrome c reductase activity measurements (*32*). Cytochrome c reduction occurs by electron-transfer specifically from the FMN domain of P450BM-3 (*32*). A lack of significant dependence of this reaction on the concentration of P450BM-3 was interpreted to indicate that $FAD_A \rightarrow FMN_A$ electron transfer does occur. Unfortunately, no direct measurements of oligomerization were carried out. The other study reported that at concentrations from 2 to 50 nM, the rate, ~4200 μmol cytochrome c min^{-1} μmol P450BM-3, was independent of the P450BM-3 concentration, but at 0.5 nM enzyme it was found to be reduced to 80% of this value. On the basis of this, the authors of that study (*32*) concluded that the limit of cytochrome c reductase activity at low enzyme concentrations must be close to these values. Our results reported here on cytochrome c reduction clearly provide a much different result and indicate that monomeric P450BM-3 present at low concentrations is inactive for cytochrome c reduction.

More generally, our data establish an important caveat for future studies on P450BM-3. The dimerization equilibrium was found to be very sensitive to both the ionic strength and to the presence or absence of substrate. Under most of the conditions tested, both monomer and dimer were clearly present in varying amounts (see for example Figure 6). Further, upon changes in the solution conditions, there was a slow time dependent shift in the amount of monomer and dimer (upon dilution for example). Any kinetic rate or biophysical characterization of the enzyme would also be expected to represent a combination of the two forms and would therefore be very sensitive to the enzyme concentration, ionic strength, presence of substrates and other ligands, and incubation time. Great care is required to control these variables in the design and performance of experiments with P450BM-3 to obtain reliable and reproducible data.

It is interesting to compare the final model with the current domain interaction model best describing the similar mammalian fusion enzyme nitric oxide synthase (NOS) (a review of the domain structure of NOS can be found in (*33*)). In NOS the catalytically active form of the enzyme is a homodimer with obligatory intermolecular electron transfer from the reductase domain of one monomer to the oxygenase domain of the other (*34*, *35*). Although numerous reports emerged that NOS dimerizes solely via the heme-binding oxygenase domain, recent evidence suggests that for the eNOS and nNOS isoforms, reductase−reductase dimerization, and reductase-oxygenase dimerization also play an important role in the dimeric structure (*33*, *36*−*38*). P450BM-3, as reported here, dimerizes via the reductase domain and shows no sign of dimerization via the heme-binding domain. Dimerization in NOS appears to assist in isoform-dependent regulation of enzyme activity (by calmodulin binding for example) (*37*, *38*). The benefit of dimerization in P450BM-3 is not yet clear .

SUPPORTING INFORMATION AVAILABLE

Figures showing (1) oxygen consumption activity as a function of preincubation time, (2) the protocol for dilution and reconcentration of P450BM-3, (3) the effect of dilution, preincubation, and reconcentration on the activity of P450BM-3, and (4) the effect of KCl on the activity of P450BM-3 are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Miura, Y., and Fulco, A. J. (1974) (Omega-2) hydroxylation of fatty acids by a soluble system from bacillus megaterium, *J. Biol. Chem. 249*, 1880−1888.
2. Fulco, A. J. (1983) Fatty acid metabolism in bacteria, *Prog. Lipid Res. 22*, 133−160.
3. Munro, A. W., Leys, D. G., McLean, K. J., Marshall, K. R., Ost, T. W., Daff, S., Miles, C. S., Chapman, S. K., Lysek, D. A., Moser, C. C., Page, C. C., and Dutton, P. L. (2002) P450 BM3: the very model of a modern flavocytochrome, *Trends Biochem. Sci. 27*, 250−257.
4. Wen, L. P., and Fulco, A. J. (1987) Cloning of the gene encoding a catalytically self-sufficient cytochrome P-450 fatty acid monooxygenase induced by barbiturates in Bacillus megaterium and its functional expression and regulation in heterologous (Escherichia coli) and homologous (Bacillus megaterium) hosts, *J. Biol. Chem. 262*, 6676−6682.
5. Haines, D. C., Sevrioukova, I. F., and Peterson, J. A. (2000) The FMN-binding domain of cytochrome P450BM-3: resolution, reconstitution, and flavin analogue substitution, *Biochemistry 39*, 9419−9429.
6. Sevrioukova, I. F., Li, H., Zhang, H., Peterson, J. A., and Poulos, T. L. (1999) Structure of a cytochrome P450-redox partner electron-transfer complex, *Proc. Natl. Acad. Sci. U.S.A 96*, 1863−1868.
7. Sevrioukova, I., Truan, G., and Peterson, J. A. (1996) The flavoprotein domain of P450BM-3: expression, purification, and properties of the flavin adenine dinucleotide- and flavin mononucleotide-binding subdomains, *Biochemistry 35*, 7528−7535.
8. Oster, T., Boddupalli, S. S., and Peterson, J. A. (1991) Expression, purification, and properties of the flavoprotein domain of cytochrome P-450BM-3. Evidence for the importance of the amino-terminal region for FMN binding, *J. Biol. Chem. 266*, 22718−22725.
9. Graham-Lorence, S., and Peterson, J. A. (1996) P450s: structural similarities and functional differences, *FASEB J. 10*, 206−214.
10. Capdevila, J. H., Wei, S., Helvig, C., Falck, J. R., Belosludtsev, Y., Truan, G., Graham-Lorence, S. E., and Peterson, J. A. (1996) The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3, *J. Biol. Chem. 271*, 22663−22671.
11. Gustafsson, M. C., Palmer, C. N., Wolf, C. R., and von Wachenfeldt, C. (2001) Fatty-acid-displaced transcriptional repressor, a conserved regulator of cytochrome P450 102 transcription in Bacillus species, *Arch. Microbiol. 176*, 459−464.
12. Sevrioukova, I., Shaffer, C., Ballou, D. P., and Peterson, J. A. (1996) Equilibrium and transient state spectrophotometric studies of the mechanism of reduction of the flavoprotein domain of P450BM-3, *Biochemistry 35*, 7058−7068.
13. Gutierrez, A., Munro, A. W., Grunau, A., Wolf, C. R., Scrutton, N. S., and Roberts, G. C. (2003) Interflavin electron transfer in human cytochrome P450 reductase is enhanced by coenzyme binding. Relaxation kinetic studies with coenzyme analogues, *Eur. J. Biochem. 270*, 2612−2621.
14. Iyanagi, T., Makino, N., and Mason, H. S. (1974) Redox properties of the reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 and reduced nicotinamide adenine dinucleotide-cytochrome b5 reductases, *Biochemistry 13*, 1701−1710.
15. 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.

16. 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.
17. Boddupalli, S. S., Oster, T., Estabrook, R. W., and Peterson, J. A. (1992) Reconstitution of the fatty acid hydroxylation function of cytochrome P-450BM-3 utilizing its individual recombinant hemo- and flavoprotein domains, *J. Biol. Chem. 267*, 10375−10380.
18. Omura, T., and Sato, R. (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature, *J. Biol. Chem. 239*, 2370−2378.
19. Haines, D. C., Tomchick, D. R., Machius, M., and Peterson, J. A. (2001) Pivotal role of water in the mechanism of P450BM-3, *Biochemistry 40*, 13456−13465.
20. Lapidot, Y., Rappoport, S., and Wolman, Y. (1967) Use of esters of N-hydroxysuccinimide in the synthesis of N-acylamino acids, *J. Lipid Res. 8*, 142−145.
21. Vermilion, J. L., and Coon, M. J. (1974) Highly purified detergent-solubilized NADPH-cytochrome P-450 reductase from phenobarbital-induced rat liver microsomes, *Biochem. Biophys. Res. Commun. 60*, 1315−1322.
22. Renatus, M., Stennicke, H. R., Scott, F. L., Liddington, R. C., and Salvesen, G. S. (2001) Dimer formation drives the activation of the cell death protease caspase 9, *Proc. Natl. Acad. Sci. U.S.A 98*, 14250−14255.
23. Bury, A. F. (1981) Analysis of Protein and Peptide Mixtures - Evaluation of 3 Sodium Dodecyl Sulfate-Polyadrylamide Gel-Electrophoresis Buffer Systems, *J. Chromatogr. 213*, 491−500.
24. Klein, M. L., and Fulco, A. J. (1993) Critical residues involved in FMN binding and catalytic activity in cytochrome P450BM-3, *J. Biol. Chem. 268*, 7553−7561.
25. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S., Helvig, C., and Capdevila, J. H. (1997) An active site substitution, F87V, converts cytochrome P450 BM-3 into a regio- and stereoselective (14S,15R)-arachidonic acid epoxygenase, *J. Biol. Chem. 272*, 1127−1135.
26. Narhi, L. O., Kim, B. H., Stevenson, P. M., and Fulco, A. J. (1983) Partial characterization of a barbiturate-induced cytochrome P-450-dependent fatty acid monooxygenase from Bacillus megaterium, *Biochem. Biophys Res. Commun. 116*, 851−858.
27. Ruettinger, R. T., and Fulco, A. J. (1981) Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from Bacillus megaterium, *J. Biol. Chem. 256*, 5728−5734.
28. Matson, R. S., Hare, R. S., and Fulco, A. J. (1977) Characteristics of a cytochrome P-450-dependent fatty acid omega-2 hydroxylase from bacillus megaterium, *Biochim. Biophys. Acta 487*, 487−494.
29. Migneault, I., Dartiguenave, C., Bertrand, M. J., and Waldron, K. C. (2004) Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, *Biotechniques 37*, 790−802.
30. Bastiaens, P. I., Bonants, P. J., Muller, F., and Visser, A. J. (1989) Time-resolved fluorescence spectroscopy of NADPH-cytochrome P- 450 reductase: demonstration of energy transfer between the two prosthetic groups, *Biochemistry 28*, 8416−8425.
31. Dohr, O., Paine, M. J., Friedberg, T., Roberts, G. C., and Wolf, C. R. (2001) Engineering of a functional human NADH-dependent cytochrome P450 system, *Proc. Natl. Acad. Sci. U.S.A 98*, 81−86.
32. Neeli, R., Girvan, H. M., Lawrence, A., Warren, M. J., Leys, D., Scrutton, 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.
33. Ghosh, D. K., and Salerno, J. C. (2003) Nitric oxide synthases: domain structure and alignment in enzyme function and control, *Front Biosci 8*, d193−209.
34. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998) Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer, *J. Biol. Chem. 273*, 18950−18958.
35. Sagami, I., Daff, S., and Shimizu, T. (2001) Intra-subunit and inter-subunit electron transfer in neuronal nitric-oxide synthase: effect of calmodulin on heterodimer catalysis, *J. Biol. Chem. 276*, 30036−30042.
36. Stuehr, D. J. (1997) Structure-function aspects in the nitric oxide synthases, *Annu. Rev. Pharmacol. Toxicol. 37*, 339−359.
37. Garcin, E. D., Bruns, C. M., Lloyd, S. J., Hosfield, D. J., Tiso, M., Gachhui, R., Stuehr, D. J., Tainer, J. A., and Getzoff, E. D. (2004) Structural basis for isozyme-specific regulation of electron transfer in nitric-oxide synthase, *J. Biol. Chem. 279*, 37918−37927.
38. Venema, R. C., Ju, H., Zou, R., Ryan, J. W., and Venema, V. J. (1997) Subunit interactions of endothelial nitric-oxide synthase. Comparisons to the neuronal and inducible nitric-oxide synthase isoforms, *J. Biol. Chem. 272*, 1276−1282.

BI701031R