Reconstitution of the Fatty Acid Hydroxylation Function of Cytochrome P-450BM-3 Utilizing Its Individual Recombinant Hemo- and Flavoprotein Domains*

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Cytochrome P-450BM-3 is a catalytically self-sufficient fatty acid ω-hydroxylase with two domains. Functional and primary structure analyses of the hemo- and flavoprotein domains of cytochrome P-450BM-3 and the corresponding microsomal cytochrome P-450 system have shown that these proteins are highly homologous. Prior attempts to reconstitute the fatty acid hydroxylating function of cytochrome P-450BM-3 utilizing the two domains, obtained either by trypsinolysis or by recombinant methods, were unsuccessful. In this paper, we describe the reconstitution of the fatty acid hydroxylation activity of cytochrome P-450BM-3 utilizing the recombinantly produced flavoprotein domain (Oster, T., Boddupalli, S.-S., and Peterson, J. A. (1991) J. Biol. Chem. 266, 22718–22725) and its hemoprotein counterpart. The rate of fatty acid-dependent oxygen consumption was shown to be linear when increasing concentrations of the hemoprotein domain are added to a fixed concentration of the flavoprotein domain and vice versa. The combination of the hemo- and flavoprotein domains in a ratio of 20:1 respectively, in the reaction mixture, results in the transfer of 80% of the reducing equivalents from NADPH for the hydroxylation of palmitate at 25°C. The ratio of the regioisomeric products obtained for lauric, myristic, and palmitic acids was similar to that obtained with the holoenzyme form of cytochrome P-450BM-3. The reconstitution of the fatty acid ω-hydroxylation activity, using the soluble domains of cytochrome P-450BM-3, without added factors such as lipids, may be useful for structure/function comparisons to their eukaryotic counterparts.

Microsomal cytochromes P-450 and their flavoprotein reductase counterpart have been shown to be involved in the oxidative metabolism of a wide spectrum of substrates including foreign chemicals, endogenous fatty acids, vitamins, steroids, eicosanoids, etc. (1, 2). The cytochrome P-450 gene (CYP) superfammily consists of an indeterminate number of members; approximately 150 genes have been sequenced to date from organisms ranging from bacteria to humans (3). This superfammy of enzymes can be divided into two classes based on the proximal electron donor for the cytochrome P-450. The first class (Class I) can be arbitrarily described as those enzymes which utilize an iron–sulfur protein as an electron shuttle between an FAD-containing flavoprotein reductase and the cytochrome P-450. The second class (Class II) of cytochromes P-450 are those which require a unique flavoprotein reductase, NADPH-cytochrome P-450 reductase, containing both FAD and FMN in an equimolar ratio.

Extensive biophysical as well as functional studies have been carried out on the reconstituted camphor monooxygenase system of Pseudomonas putida (4–7). This Class I cytochrome P-450 system is composed of a hemoprotein (cytochrome P-450cam), a flavoprotein (putidaredoxin reductase), and an iron–sulfur protein (putidaredoxin). The only high resolution structure of a cytochrome P-450, available to date, is that of cytochrome P-450cam (8). However, the differences between Class I and Class II monooxygenase systems indicate that the reconstituted cytochrome P-450cam system may not be the most appropriate model for understanding the enzymatic properties of the microsomal cytochrome P-450-dependent monooxygenase system (9).

The soluble fatty acid ω-hydroxylase of Bacillus megaterium, viz. cytochrome P-450BM-3 (M, 119,000), is a catalytically self-sufficient single polypeptide containing heme, FAD, and FMN in an equimolar ratio and requires NADPH as the source of electrons (10–14). Narhi and Fulco (15) demonstrated that upon limited trypsinolysis, the holoenzyme is cleaved into two portions, one (BMP) (M, 55,000) retains the heme and binds the substrate and the other (BMR) (M, 66,000) contains the flavins and carries out an NADPH-dependent reduction of cytochrome c. Analysis of the deduced amino acid sequence of cytochrome P-450BM-3 has revealed that this protein contains regions that strongly resemble microsomal ω-hydroxylases and NADPH-cytochrome P-450 oxidoreductase (16). Reconstitution of the fatty acid hydroxylation function of cytochrome P-450BM-3 utilizing either the purified tryptic products (15) or the recombinantly isolated domains (17) has been unsuccessful thus far. The present

1 The nomenclature of cytochrome P-450 is adapted from Nebert et al. (3).
2 The abbreviations used are: cytochrome P-450BM-3, the soluble cytochrome P-450 isolated from B. megaterium (the product of CYP102 gene); BMP, the recombinantly expressed hemoprotein domain of cytochrome P-450BM-3; BMR, the recombinantly expressed flavoprotein domain (reductase) of cytochrome P-450BM-3; MOPS, 4-morpholinepropanesulfonate; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.
paper describes the conditions required to express and purify the hemoprotein domain and the reconstitution of the fatty acid hydroxylation activity using the recombinantly obtained hemo- and flavoprotein domains of cytochrome P-450BM.3.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled fatty acids were obtained from Nu Chek Prep, Inc. (Elysian, MN). 1-^1^C-Fatty acids (lauric, myristic, and palmitic acids with specific radioactivities of 26, 31, and 50 mCi/ mmol, respectively) were obtained from Amersham Corp. Diizalld (N- methyl-N'-nitroso-p-benzenediazonium) and chromium(VI) oxide were obtained from Aldrich. Bacto-tryptone, yeast extract, and Bactoagar were purchased from Difco Laboratories (Detroit, MI). Agarose, acrylamide, bisacrylamide, and protein molecular weight standards were purchased from Life Technologies, Inc. (Gaithersburg, MD). Ampicillin, lysozyme, protease inhibitors, NADPH, and catalase were obtained from Sigma. DE82 ion-exchange resin was obtained from Whatman, Ltd. (Hillsboro, OR). UstilagR A4 was purchased from Pharmacia LKB Biotechnology Inc., and sterile Millipore GV filter units were obtained from Millipore (Bedford, MA). All other reagents used were of the purest grades available.

**Bacterial Strains and Plasmids**—Escherichia coli strain DH5a (F, endA1, hsdR17, supE44, thi-1, lac, recA1, gyrA96, relA1, Δ(argF-lacZΔM15) was obtained as frozen competent cells from Life Technologies, Inc. Bacterial growth medium, 2xYT containing ampicillin (50 μg/ml, final concentration), was prepared as described by Sambrook et al. (18). The 12-kilobase pairs BM3.2, plasmid (16), containing the 9.2-kilobase pairs insert having the flanking and cytochrome P-450BM.3 encoding regions, was purified from the E. coli clone (JM109, BM3.2), which was provided by Dr. A. J. Fulco (Department of Biological Chemistry, University of California, Los Angeles, CA). The plasmid cloning vector, plpB124, was obtained from International Biotechnologies, Inc. (Hartford, CT).

**DNA Methods**—Recombinant DNA methods were carried out according to Sambrook et al. (18), using enzymes and reagents from Boehringer Mannheim and Life Technologies, Inc. Oligonucleotide primers were synthesized on an Applied Biosystems (Foster City, CA) 380A synthesizer and purified by Sep-Pak C8 (Waters Associates, Bedford, MA) column chromatography. Polymerase chain reaction was performed on a DNA thermal cycler apparatus using AmpliTaq DNA polymerase, both from Perkin-Elmer Cetus (Norwalk, CT). Nucleotide sequences were verified with the Bst sequencing kit from Boehr-Rad.

**Analytical Methods**—Spectrophotometric measurements and absorbance spectra were recorded on an IBM model 9420 UV-visible spectrophotometer (Danbury, CT). Protein concentrations, in fractions collected after column chromatography, were determined spectrophotometrically, as described by Warburg and Christian (19). Protein concentrations in pure enzyme samples were assayed according to Lowry et al. (20). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (21), with 4% stacking and 10% running gels, using a Bio-Rad Mini-Protean apparatus. Proteins were visualized after silver staining. The NH2-terminal amino acid sequence was determined by Edman degradation, utilizing a gas phase sequencing kit from Boehringer Mannheim and Life Technologies, Inc. Nucleotide sequences were verified with the Bst sequencing kit from Boehringer Mannheim and Life Technologies, Inc. Absorbance spectra were recorded on an IBM model 9420 UV-visible spectrophotometer (Danbury, CT). Protein concentrations, in fractions collected after column chromatography, were determined spectrophotometrically, as described by Warburg and Christian (19). Protein concentrations in pure enzyme samples were assayed according to Lowry et al. (20). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (21), with 4% stacking and 10% running gels, using a Bio-Rad Mini-Protean apparatus. Proteins were visualized after silver staining. The NH2-terminal amino acid sequence was determined by Edman degradation, utilizing a gas phase protein sequencer 470A attached to the analyzer (120A PTH, Applied Biosystems).

**Subcloning of the DNA Region Encoding the Hemoprotein Domain**—After isolation by conventional techniques (22), plasmid BM3.2 was digested with BglII and Sall. The resulting 3.2-kilobase pairs fragment, encoding the hemoprotein domain and a portion of the 5' flanking region of the cloned domain of cytochrome P-450BM.3, was purified and subcloned into the pBl24 vector digested with BamH1 and Sall as illustrated in Fig. 1. Using a 26-mer primer (5'-GGG TCG ATT CAG GTA AGA AGG ATA ACA TG-3'), the 3'-end of which corresponded to the region encoding the NH2-terminus of the hemoprotein domain and another primer matching the region around the PstI site, PCR amplification was performed to synthesize a DNA fragment containing a unique EcoRI site at its 5'-end, translation initiation signals, and the unchanged 3'-region encoding the hemoprotein domain of cytochrome P-450BM.3 (BMP). The plasmid pP450-1 was digested with EcoRI/NarI, and the fragment, corresponding to the vector and the 3'-end of the cytochrome P-450 coding region, was purified by agarose gel electrophoresis. The PCR-amplified fragment of DNA (153 base pairs), which had been EcoRI/ NarI-digested, was ligated into the purified vector containing the 3'-region coding for the hemoprotein domain. This step removed the flanking region 5' to the cytochrome P-450 coding region in BM3.2.

A second PCR amplification was performed using a primer, corresponding to the region around the PstI site, and a 27-mer primer (5'-GGG TCG ATT TAG COT ACT TT TTA GCA-3'), the 3'-end of which was complementary to the region encoding the COOH-terminus of the hemoprotein domain. This PCR amplification synthesized a DNA fragment of 851 base pairs containing a stop codon to terminate the translation of the hemoprotein domain and a unique XhoI site. The plasmid, pP450-2, was digested with PstI/XhoI, and the fragment containing the vector and the 5'-coding region of cytochrome P-450BM.3 was ligated with the DNA fragment for the 3'-end prepared by PCR amplification. Subsequently, clones were selected after small scale DNA purification (22) by restriction analysis. The nucleotide sequence of the DNA fragments obtained by PCR amplification was determined to verify the presence of the desired changes.

The plasmid pP450-3 was used to transform E. coli DH5a competent cells. The cells were grown for 24 h in 2xYT medium containing 50 μg/ml ampicillin at 37 °C which provided the optimal level of expression of BMP.

**Lysis of E. coli Cells Containing Expressed BMP**—Cells grown to late stationary phase, as described above, were harvested by centrifugation and washed in 20 mM MOPS buffer, pH 7.4, containing 20 mM KCl. The pellet was frozen at −70 °C and then thawed at 4 °C. Freeze-thaw cycles were performed four times, and the cell paste was resuspended as i/g/4 ml in 20 mM MOPS buffer, pH 7.4, containing 20 mM KCl, 0.1 mM Na3EDTA, and 0.1 mM DTT (buffer A). The cell suspension was treated with lysozyme as previously described (24). After centrifugation of the cell lysate at 40,000 × g for 30 min, the supernatant solution was transferred to a fresh tube. Under these conditions, approximately 75% of BMP in the whole cells was recovered in the supernatant solution. Subsequent purification steps were performed at 0–4 °C as described in detail under “Results.”

**Oxygen Uptake Determinations**—Oxygen consumption was measured with a Gilson 5/6 Oxygraph (Middleton, WI) with an enclosed 2-ml reaction chamber. Stock solutions of the fatty acids (25 mM) were prepared in 50 mM potassium carbonate. The reaction system for performing the reconstitution experiments typically contained 50
mm MOPS buffer (pH 7.4), the desired concentrations of the enzymes (BMP and BM-R), and the fatty acid substrate. After a preincubation of reactants for 2 min at room temperature, oxygen consumption was initiated by the addition of NADPH.

**Analysis of Fatty Acid Metabolites—**The reaction system for monooxygenation of fatty acids contained (unless otherwise mentioned) 50 mM MOPS, pH 7.4, 500 μM fatty acid, BMP, and BM-R, and 1 mM NADPH. The substrate was always added first followed by BMP and BM-R. After preincubation of the enzymes for about 5 min, the reaction was started by the addition of the reduced pyridine nucleotide. Aliquots of the reaction mixture (0.5 ml) were taken, and the reaction was terminated with 0.1 ml of 1 N HCl. The solutions were extracted twice with 5 ml of ethyl acetate. The combined extracts were washed with 2 ml of water and evaporated with nitrogen gas. The residue was taken up in methanol, and a portion was subjected to reverse phase HPLC on an analytical μBondapak C18 column (30 × 1 cm, Waters Associates). The radioactivity of the eluate was monitored with a radioactive flow detector (Flo-One, Model HP, Radiometrics, Meridian, CT). Metabolites were eluted using a gradient of 50-100% acetonitrile (prepared in 0.1% acetic acid).

Methyl esters of each of the compounds were prepared using an ethereal solution of diazomethane. Diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Diazald apparatus obtained from Aldrich. An aliquot of the ethereal solution of diazomethane was prepared using the Mini Dia...
Table I
Comparison of the fatty acid-dependent equilibrium association constants of cytochrome P-450BM3 and BMP

To obtain comparable results, the relative concentrations of cytochrome P-450BM3 and BMP in the reaction system were maintained constant (10–12 μM heme). Titrations were performed at 25 °C. Enzymes were diluted in 3 ml of 50 mM MOPS (pH 7.4) buffer to obtain the desired concentration, and the spectra were recorded after adding aliquots of fatty acids. Stock solutions of the fatty acids were prepared in 50 mM potassium carbonate. The equilibrium constants were calculated using a method similar to that described by Peterson (6). For each experiment, the reciprocal of the change in absorbance, for each addition of fatty acid, was plotted versus the reciprocal of the fatty acid concentration. The intercept on the y axis was taken as the inverse of the maximum absorbance change for each experiment. This value was used to calculate the molar absorbance change for the conversion of substrate-free to substrate-bound enzyme. Using this molar absorbance and the total concentration of enzyme and fatty acid, the concentration of E, ES, and S could be calculated. The equilibrium association constant for the reaction E + S ↔ ES could then be evaluated. Each experiment was repeated several times with more than five additions of fatty acids to each sample, and the values presented in the table represent the mean ± the S.D. of the equilibrium association (K_a) constant.

| Substrate       | K_a × 10^9 M^-1 |
|-----------------|-----------------|
| Laurate (12:0)  | 3.9 ± 0.7       |
| Myristate (14:0)| 12.8 ± 1.0      |
| Pentadecanoate (15:0)| 173.5 ± 53.2 |
| Palmitate (16:0)| 100.1 ± 7.9     |

Fig. 2. Effect of concentration of the domains on the rate of oxygen consumption. The rate of oxygen consumption is plotted against the product of the concentration of the two domains [BMR] [BMP] (μM)(μM). Although there are only 12 points indicated in this figure, each point is the mean of various experiments in which the concentrations of BMR varied from 0.25 to 5 μM and those of the BMP from 2.5 to 30 μM.

out effective electron transfer to its natural redox partner (BMP), oxygen consumption in the presence of an excess of BMP, reduced pyridine nucleotide, and palmitic acid was measured. The reaction was found to be linear with the concentration of BMP added in the reconstituted system. These experiments were repeated with various concentrations of BMR (0.25–5 μM) and BMP (2.5–30 μM) and the rate of oxygen consumption measured. Fig. 2 is a plot of the rate of oxygen consumption versus the product of the concentrations of BMR and BMP.

Similar reconstitution experiments with the two domains were performed to monitor the oxidation of NADPH spectrophotometrically at 340 nm. A plot of the initial rate of oxidation of the reduced pyridine nucleotide was linear with the concentration of BMP in the reaction system. Also, the initial rate of NADPH oxidation was similar to the rate of oxygen consumption seen with equivalent concentrations of BMR and BMP. Similar reconstitution experiments, performed with the purified tryptic product of BMR lacking the amino-terminal 122 residues (27), elicited neither uptake of oxygen nor oxidation of NADPH, confirming that the FMN-binding domain of BMR is essential for successful electron transfer to the cytochrome P-450 domain.

The rate of substrate-dependent oxygen uptake catalyzed by cytochrome P-450BM3 is dependent upon the chain length of the fatty acid metabolized (13, 24). The rate of oxygen uptake in the reconstitution system was found to be maximal for palmitic and pentadecanoic acids and minimal for lauric acid and were directly dependent upon the concentration of BMP in the reaction system. With a ratio of BMP to BMP of 1:7.5, the turnover number for palmitic acid-dependent oxygen uptake was 180 mol of oxygen/min/mol of BMP. However, the rate of the reaction increased linearly with the concentration of BMP present in the reaction mixture. In view of this, a constant turnover number for the substrate-dependent oxygen uptake per mol of BMP could not be obtained.

Metabolism of Palmitic Acid—Analysis of the amount of oxygen and NADPH consumed and product formed during the oxidation of palmitic acid to monohydroxy palmitates by the reconstituted system showed that the ratio of the concentration of hydroxylated products formed to that of oxygen consumed was less than 1 (Table II). The extent of leakage of electrons was found to be independent of the concentration of BMP in the reconstituted system (Table II). Other experiments, not shown here, have indicated that the excess oxygen consumed, which is not incorporated into the hydroxylated fatty acid forms hydrogen peroxide. Whether this hydrogen peroxide is formed via the direct two-electron reduction of molecular oxygen or by the intermediate formation of superoxide anion has not been determined. The extent of coupling between the oxygen consumed and substrate oxidized varied with the ratio of BMP to BMR, but under optimal conditions the percentage incorporation into hydroxylated product was observed to be as large as 80% (Table II). In contrast, the ratio of oxygen consumed to hydroxylated product formed during the metabolism of palmitic acid (500 μM) by cytochrome P-450BM3 was always 1 with no detectable hydrogen peroxide formed during the reaction (24).

During the metabolic studies performed with cytochrome P-450BM3, we determined the relative abundance of the isomers of the monohydroxylated fatty acids by subjecting the isomeric mixture of the methyl esters to GC followed by chemical ionization mass spectrometry (25). In the reconstituted system, the stoichiometry between the oxygen consumed and product formed during palmitate hydroxylation by the reconstituted enzyme system is described under "Experimental Procedures." The ratio is the amount of monohydroxypalmitic acid produced to the amount of oxygen consumed during the reaction.

| Concentration       | Product amount | Difference | Ratio |
|---------------------|----------------|------------|-------|
| BMR/μM cmol/ml      | Oxygen/nmol/ml |            |       |
| 0.5                  | 5.0            | 80         | 29    | 0.36 |
| 0.5                  | 10.0           | 158        | 106   | 0.67 |
| 0.5                  | 15.0           | 230        | 185   | 0.80 |
| 0.5                  | 20.0           | 250        | 225   | 0.77 |
Reconstitution of Cytochrome P-450BM-3

The object of these studies was to demonstrate the functionality of the recombinant domains of cytochrome P-450BM-3 by reconstituting the monooxygenation activity seen with the holoenzyme. The intrinsic membrane binding properties of the eukaryotic enzymes, their tendencies to form oligomeric forms, and the requirement for the presence of exogenous lipid in the reaction system have hindered detailed studies on the structure and function of the individual enzymes and mechanism of electron transfer between the microsomal cytochromes P-450 and their reductase counterpart.

While cytochrome P-450BM-3 has proven to be an extremely useful model for the Class I cytochromes P-450, it is not clear yet whether extrapolations from cytochrome P-450BM-3 to microsomal cytochromes P-450 are equally valid in all instances.

Initially studies on cytochrome P-450BM-3 were important because this enzyme represented a different type of soluble cytochrome P-450, which had been isolated from a Bacillus rather than a catabolic Pseudomonas (28–30). With the recognition that cytochrome P-450BM-3 was a catalytically self-sufficient enzyme, which was structurally and functionally analogous to the eukaryotic microsomal cytochromes P-450 and the NADPH-cytochrome P-450 oxidoreductase (16, 17, 25, 27), avenues for utilizing cytochrome P-450BM-3 as a soluble model system for closely examining the structure/function relationship of the Class II type of cytochrome P-450 systems were opened.

Early studies of cytochrome P-450BM-3 established that this enzyme would hydroxylate fatty acids and that this reaction had one of the highest turnover numbers of any cytochrome P-450 (14, 24). Narhi and Fulco (13) demonstrated that the holoenzyme was sensitive to trypsin cleavage and that two domains and was comparable with that observed with cytochrome P-450BM-3. The preferred carbon for hydroxylation, like the holoenzyme (25), was dependent on the chain length of the fatty acid substrate. While lauric and myristic acids were hydroxylated at the ω-1 position, palmitic acid was hydroxylated predominantly on the ω-2-methylene group.

We have previously reported the heterologous expression of the reductase domain of cytochrome P-450BM-3 at a level of over 50% of E. coli soluble proteins and characterized this recombinant flavoenzyme (27). Consistent with the similarity in the primary structures (16), the properties of the flavoprotein domain were remarkably comparable with those of liver microsomal cytochrome P-450 reductase. Also, in agreement with the hypothesis that the NH2-terminal region of the flavoprotein participates in the binding of FMN (31), removal of the NH2-terminal 120 residues by limited tryptic digestion of the reductase domain abolished binding of the mononucleotide to the protein as well as activities associated with it (27). Therefore, we felt that the use of trypsin to prepare the individual domains of cytochrome P-450BM-3 may lead to undesirable side effects (as described above) and would hinder the progress of our studies on the mechanism of protein/protein interaction between the two domains.

In our current studies, we have constructed a plasmid which contains the coding sequence for the hemoprotein domain of cytochrome P-450BM-3 and have used this plasmid to transform E. coli strain DH5α. Like cytochrome P-450BM-3 and the recombinant reductase domain, this protein is expressed at a high level without the addition of either inducers like isopropylthio-β-D-galactose or precursors for the biosynthesis of the cofactors to the cells, and the recombinant enzyme can be readily purified to homogeneity in large amounts. The spectral properties of the purified hemoprotein domain are similar to those of the holoenzyme with the exception of contributions to the spectra attributable to the presence of both FAD and FMN in the holoenzyme. The equilibrium association constants for fatty acid binding to either cytochrome P-450BM-3 or the hemoprotein domain were changed only slightly (Table I).

Although we and others were unable to obtain reconstitution of the activity of cytochrome P-450BM-3 with the individual domains which had been cleaved by trypsin, our preparations of the recombinant forms of the individual domains are fully active in fatty acid monooxygenation. A plot of the rate of oxygen consumption versus the product of the concentrations of the hemoprotein and flavoprotein domains was linear (Fig. 2). We have interpreted this result to indicate that in the reconstituted enzyme system, the rate-limiting step is the productive collision of the individual domains to form an enzymatically active heterodimer. Since the reaction was performed in the presence of potassium palmitate, the cytochrome P-450 domain is initially in the high spin substrate-bound form. The solution also contains NADPH so the flavoprotein domain is reduced at least to the three-electron-reduced form. Thus, when these domains collide in the proper orientation for the transfer of the first electron required for the monooxygenation reaction, the remainder of the reaction cycle of cytochrome P-450, that is oxygen binding, transfer of the second electron and product formation, will occur prior to separation of these reaction partners.

In summary, we have described in this communication the construction of a plasmid encoding the hemoprotein domain of cytochrome P-450BM-3 and the expression of this domain in E. coli. The purification of this domain in large quantity has enabled us to study the reconstitution of these domains in the monooxygenation of fatty acids. The properties of the domain in binding fatty acid substrates and the products formed by the reaction are similar to those of the holoenzyme. Thus, the availability of this enzyme system and its domains in highly purified form, which is catalytically active in the oxidation of fatty acids, will provide us with an additional tool for comparison with eukaryotic microsomal cytochromes P-450. In this context, we have recently crystallized BMP, and x-ray diffraction data to a resolution better than 2.0 Å were also obtained.

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