The Flavoprotein Domain of P450BM-3: Expression, Purification, and Properties of the Flavin Adenine Dinucleotide- and Flavin Mononucleotide-Binding Subdomains†

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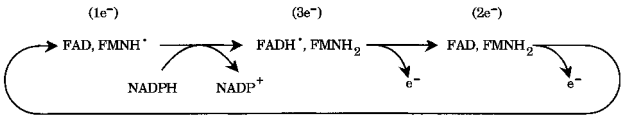
ABSTRACT: P450BM-3 is a self-sufficient fatty acid monooxygenase that can be expressed in *Escherichia coli* as either the holoenzyme or as the individual hemo- and flavoprotein domains. The flavoprotein domain (BMR) of P450BM-3 is soluble and contains an equimolar ratio of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and is functionally analogous to microsomal nicotinamide adenine dinucleotide phosphate (NADPH)−P450 reductases. These reductases have been proposed to have evolved through a fusion of genes encoding simple flavin-containing electron-transport proteins [Porter, T. D. (1991) *Trends Biochem. Sci. 16*, 154−158]. The gene encoding BMR has been divided into the coding regions for the FAD/NADPH- and FMN-binding domains. These proteins were overexpressed in *E. coli* and both domains were found to contain not less than 0.9 ± 0.05 mol of FAD or FMN/mol of protein. Compared to BMR, the electron-accepting properties of the recombinant flavin domains were mainly conserved. Titration of the FMN domain with sodium dithionite resulted in the conversion of the protein to the fully reduced $FMNH_2$ form without accumulation of intermediate semiquinone forms; however, a similar titration of the FAD domain gave clear evidence for the presence of a neutral, blue flavin semiquinone during the reduction. Titrations of the reduced forms of the domains with artificial electron acceptors indicated that the electron-transferring properties of both the FAD- and FMN domains were also conserved. The rate constants of reoxidation of the fully reduced FAD and FMN domains by molecular oxygen at 20 °C were found to be 2.5 and 0.1 min^{-1}, respectively. The cytochrome *c* reductase activity of BMR could be fully reconstituted with the individual domains. The data presented support the hypothesis that BMR has a discrete multidomain structure.

The flavoprotein NADPH−P450 reductase is an integral component of the microsomal P450-dependent monooxygenation system, transferring electrons from NADPH to P450 (Masters, 1980; Nebert et al., 1981; Williams & Kamin, 1962; Phillips & Langdon, 1962). Microsomal NADPH−P450 reductases contain 1 mol each of FAD and FMN/mol of enzyme (Iyanagi & Mason, 1973; Dignam & Strobel, 1975). During catalytic turnover with P450 as the electron acceptor, microsomal NADPH−P450 reductase was proposed to cycle between 1 − e^- and 3 − e^- (or 2 − e^- and 4 − e^-) (Backes & Reker-Backes, 1988) reduced levels of the flavins with only the two-electron reduced FMN being the electron donor to the heme of P450 (Iyanagi et al., 1981; Vermilion et al., 1981; Guengerich, 1983; Backes & Reker-Backes, 1988) as illustrated in Scheme 1. NADPH first transfers a hydride ion to the FAD moiety of the 1 − e^- reduced reductase and there is a rapid equilibration of the electrons between the two flavins in accordance with the redox potentials of the four one-electron redox couples involved (Iyanagi et al., 1974; Vermilion & Coon, 1978; Oprian & Coon, 1982). There follows the sequential 1 − e^- transfer from $FMNH_2$ to the heme of P450.

P450BM-3[1] (CYP102), a fatty acid monooxygenase, is the only prokaryotic P450 known to resemble the mammalian microsomal P450 monooxygenases that require an FAD- and FMN-containing reductase (Nelson et al., 1993). Fulco's group discovered and purified this catalytically self-sufficient enzyme from *Bacillus megaterium* and they cloned, sequenced, and expressed its gene in *Escherichia coli* (Miura & Fulco, 1974, 1975; Narhi & Fulco, 1986, 1987; Ruettinger et al., 1989). P450BM-3 contains heme, FAD, and FMN bound to a single polypeptide chain of 1048 residues (M_r 119 000) (Narhi & Fulco, 1986). The protein can be separated, by limited trypsinolysis, into two domains, one containing the heme (M_r 55 000) and the other containing

Scheme 1[a]



[a] In this scheme, the one-electron reduced form of the reductase ($1e^-$) is the air-stable "resting" state of the enzyme (Iyanagi et al., 1974, 1978; Yasukochi et al., 1979).

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[1] Abbreviations: P450BM-3, the soluble P450 isolated from *Bacillus megaterium* (the product of the CYP 102 gene); BMR, the recombinantly expressed flavoprotein domain (reductase) of P450BM-3; BMR-52, the 52-kDa proteolytic fragment of BMR; FAD domain, the recombinantly expressed nicotinamide adenine dinucleotide phosphate/flavin adenine dinucleotide-binding domain of BMR; FMN domain, the recombinantly expressed flavin mononucleotide-binding domain of BMR; DCIP, 2,6,-dichlorophenolindophenol; MOPS, morpholinopropanesulfonate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; rTEV protease, the recombinantly expressed site-specific protease purified from *Escherichia coli*.

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the flavins (M_r 66 000) (Narhi & Fulco, 1987). During anaerobic titration of either P450BM-3 or its flavoprotein domain (BMR) with NADPH or sodium dithionite, the high-potential flavin (presumably FMN) was reduced first without the accumulation of semiquinone intermediates, and the reduction of FMN was complete before the production of FAD semiquinone was observed (Peterson & Boddupalli, 1992; Sevrioukova & Peterson, 1995). Stopped-flow spectrophotometric studies of the kinetics of reduction of the flavins in the reductase domain alone and of the heme iron in the holoenzyme of P450BM-3 indicated that only the FMN semiquinone is capable of reducing the heme iron and that two-electron reduced FMN is unreactive in electron transfer to the heme[2] (Sevrioukova & Peterson, 1995).

BMR exhibits about 33% identity with mammalian NADPH−P450 reductases with highly conserved segments involved in the binding of the flavins and pyridine nucleotide (Ruettinger et al., 1989). An analysis of the sequence similarity of NADPH−P450 reductases with other flavoproteins has shown (Porter & Kasper, 1985, 1986) that the N-terminal, FMN-binding region of the reductase shows significant sequence homology to the FMN-containing bacterial flavodoxins, while the NADPH- and FAD-binding domain is related to the FAD-containing ferredoxin−$NADP^+$ reductases (Karplus et al., 1991; Correll et al., 1993). It was proposed that, during the course of evolution, NADPH−P450 reductase arose through a fusion of genes encoding simple flavin-containing electron-transport proteins (Porter, 1991). Because of the strong sequence homology between eukaryotic NADPH−P450 reductases and BMR, BMR was also proposed to have discrete regions, resulting from the gene fusion described above, with an N-terminal FMN-binding region and C-terminal FAD- and NADPH-binding regions (Porter & Kasper, 1985; Oster et al., 1991). Tryptic proteolysis of BMR results in cleavage at K593 to produce a fragment (BMR-52) containing FAD (M_r 52 000) and traces of FMN (Oster et al., 1991). BMR-52 catalyzed NADPH-dependent ferricyanide reduction, which was expected for the FAD-binding region. This was the first direct experimental evidence of the multidomain structure of P450 reductases. Even stronger experimental evidence in support of the multidomain hypothesis was provided when the FAD/NADPH- and FMN-binding domains of human NADPH−P450 reductase (Smith et al., 1994) and the FAD/NADPH-binding domain of rat NADPH−P450 reductase (Hodgson & Strobel, 1996) were independently expressed in *E. coli*. The separate domains of the human NADPH−P450 reductase were capable of partial reconstitution of P450 monooxygenase activity (Smith et al., 1994).

Since eukaryotic microsomal NADPH−P450 reductase employs $FMNH_2$ as the reductant of P450 while BMR[2] employs $FMN^{\bullet -}$ (Sevrioukova & Peterson, 1995), we were interested in the mechanism of domain−domain interaction and interdomain electron transfer in P450BM-3. We have separately expressed in *E. coli* the flavin subdomains of P450BM-3 and obtained large quantities of these domains for mechanistic and structural studies. In this paper, we report the spectral and electron-accepting properties of the individual flavin domains of BMR and the reactivity toward molecular oxygen of their reduced forms.

[2] I. F. Sevrioukova, C. Shaffer, D. P. Ballou, and J. A. Peterson, unpublished experiments.

EXPERIMENTAL PROCEDURES

Materials. Bacto-tryptone, yeast extract, and Bacto-agar were purchased from Difco Laboratories, Detroit, MI. Agarose, acrylamide, bisacrylamide, and rTEV protease were purchased from Bethesda Research Laboratories, Inc., (BRL), Gaithersburg, MD. Protein molecular weight standards were obtained from Bio-Rad Laboratories, Hercules, CA. Protease inhibitors, NADPH, ampicillin, cytochrome *c*, DCIP, and potassium ferricyanide were purchased from Sigma Chemical Co., St. Louis, MO. Sodium dithionite was purchased from Hardman and Holden, Ltd., Manchester, England, and was stored in an inert atmosphere. The Ni-NTA resin, used in the purification of the proteins, was obtained from Qiagen, Inc., Chatsworth, CA. All other reagents were of the purest grades available.

E. coli Strains, Media, and Plasmids. *E. coli* strains DH5α and DH5αF′IQ were obtained as competent cells from BRL. Media were prepared as previously described (Sambrook et al., 1989) and contained ampicillin (50 μg/mL final concentration). 2×YT and TB media were used for performing small- and large-scale cell growth, respectively. The *E. coli* clone (JM109, pBM3-2A) containing the 9.2-kb plasmid encoding P450BM-3 was originally obtained from Dr. A. J. Fulco, Department of Biological Chemistry, University of California, Los Angeles, CA. The plasmid was subcloned into *E. coli* strain DH5α for overexpression of P450BM-3 (Boddupalli et al., 1990). The plasmids pProEX-1 and pTA were obtained from BRL and Stratagene Cloning Systems, La Jolla, CA, respectively. The pProEX-1 vector was designed for expression of proteins and incorporated a sequence of six histidine residues (6×His) fused to the amino terminus of the desired protein to facilitate affinity purification (Hoffmann & Roeder, 1991). The vector also encodes the recognition sequence for the TEV protease following the histidine tag so that the 6×His tag can be removed from the purified protein by proteolytic digestion.

DNA Methods. Recombinant DNA methods were carried out as previously described (Sambrook et al., 1989), using enzymes, oligonucleotide primers, and reagents from BRL.

Analytical Procedures. Protein concentration was determined by a published procedure using reagents purchased from Sigma Chemical Co. (Lowry et al., 1951). The reduction of 100 μM cytochrome *c*, 1.8 mM potassium ferricyanide, or 40 μM DCIP was carried out in 50 mM MOPS buffer, pH 7.4, at 30 °C. The reaction was initiated by the addition of NADPH (100 μM final concentration). Specific activities were calculated employing molar extinction coefficients of 21 mM^{-1} cm^{-1} at 550 nm, 1.02 mM^{-1} cm^{-1} at 420 nm, and 21 mM^{-1} cm^{-1} at 600 nm for cytochrome *c*, potassium ferricyanide, and DCIP, respectively. Extinction coefficients for the oxidized FAD- and FMN domains were determined after releasing the flavins from the proteins by heating samples in sealed tubes protected from light. The FAD and FMN concentrations were determined using extinction coefficients at 450 nm of 12.2 mM^{-1} cm^{-1} for FMN (Whitby, 1953) and 11.3 mM^{-1} cm^{-1} for FAD (Beinert, 1960).

Spectrophotometry. The optical absorbance spectra were recorded with an IBM Instruments Co. Model 9420 UV/visible spectrophotometer. The spectra were digitized by the instrument and transmitted to a microcomputer that was used to correct for baseline as well as dilution.

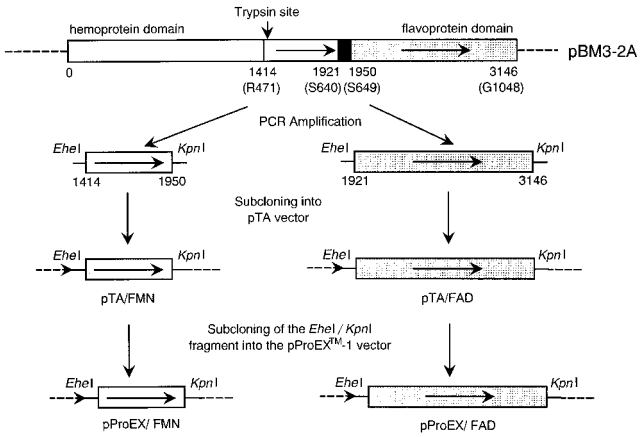

FIGURE 1: Strategy for subcloning the coding regions of the FAD- and FMN domains. The methods used are described in detail under Experimental Procedures. The shaded and black areas indicate the coding regions, and the arrows depict the direction of translation.

Anaerobic Titrations. The samples were made oxygen-free by repeated evacuation and flushing with prepurified argon as has been previously described (Peterson, 1971; Peterson et al., 1977). The titrants were prepared in anaerobic buffer and standardized. Sodium dithionite was standardized by anaerobically titrating a solution of cytochrome *c* while the NADPH concentration was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm. The reaction mixtures contained 50 mM MOPS buffer, pH 7.4, 35−38 μM FAD- or FMN domain, and an oxygen-scavenging system consisting of 0.02 unit of protocatechuate 3,4-dioxygenase and 100 μM protocatechuic acid (Bull & Ballou, 1981). All manipulations of the titrator assembly were performed in a glove bag that was continuously flushed with prepurified nitrogen to minimize contamination with oxygen. Following the addition of each aliquot of titrant, the reaction mixture was permitted to stand at room temperature until no further absorbance change occurred.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to published procedures (Laemmli, 1970), with 4% stacking and 12% running gels, using a Bio-Rad Mini-Protean apparatus. Proteins were visualized after Coomassie Blue staining.

RESULTS

Construction of Recombinant Vectors Encoding the FMN- and FAD Domains of BMR. The oligonucleotides for performing PCR amplification of the desired coding fragments of the pBM3-2A plasmid were designed using the sequence of P450BM-3 and the sequence alignment of homologous reductases reported previously (Ruettinger et al., 1989; Porter, 1991). R471 and S649 of P450BM-3 were chosen as the amino- and carboxy-terminal residues of the FMN domain, while the FAD domain included residues from S640 through G1048. The 5′-oligonucleotide primers were synthesized with an overhanging *Ehe*I restriction site, whereas the 3′-oligonucleotide primers contained a stop codon followed by a *Kpn*I site (Figure 1). After PCR amplification, the DNA fragments were cloned into the pTA vector resulting in the plasmids pTA/FMN and pTA/FAD. The nucleotide sequences of the desired coding regions of these plasmids were confirmed. After digestion of the pTA/FMN and pTA/FAD vectors by *Ehe*I and *Kpn*I, the 0.6- and 1.3-kb fragments containing the coding regions for the FMN- and FAD-binding domains were cloned into unique *Ehe*I/*Kpn*I sites of the expression plasmid pProEX-1, resulting in pProEX/FMN and pProEX/FAD. These plasmids were used to transform *E. coli* strain DH5αF′IQ.

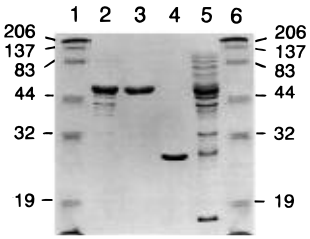

FIGURE 2: Level of expression of the FAD- and FMN domains of BMR in DH5αF′IQ. SDS−polyacrylamide gel electrophoresis was performed as described under Experimental Procedures. Lanes 1 and 6, molecular weight standards; lanes 2 and 5, the supernatant solution from the cell lysates with expressed FAD- and FMN domains, respectively; lanes 3 and 4, 2 μg of purified FAD- and FMN domains, respectively.

Purification of the Domains. Single colonies of pProEX/FMN and pProEX/FAD cells containing the genes for the desired proteins were used to inoculate 5 mL of 2×YT medium, which was grown at 37 °C for 8 h. An overnight culture (100 mL) of 2×YT medium was inoculated with 1 mL of the starter culture. TB medium, 500 mL in a 2.8-L Fernbach flask, was inoculated with 5 mL of the overnight culture and the cells were grown at 37 °C until an absorbance of 0.6−0.7 at 600 nm was reached. Isopropyl β-thiogalactoside (IPTG) was then added (0.6 mM final concentration) to induce the expression of the desired protein. Although maximal expression of both proteins per gram wet weight of cells was observed 4 h after addition of IPTG, approximately 8% and 50% of the soluble protein for the FMN- and FAD domains, respectively (Figure 2, *lanes* 2 and 5), extending the growth time of the cultures to 24−48 h before harvest gave the maximal yield of cells (10−12 g/L) and desired proteins.

The wet cell paste was resuspended as 1 g/4 mL of lysis buffer containing 50 mM sodium phosphate buffer, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride (PMSF) (buffer A). All subsequent steps were performed at 4 °C. Lysozyme (1 mg/mL) was added to the bacterial suspension and the cells were stirred for 1 h. DNase (1 μg/mL) and magnesium chloride (8 mM) were added to the suspension to hydrolyze the DNA and decrease the viscosity of the solution. The suspension was incubated for an additional hour. After centrifugation at 40000*g* for 30 min, the supernatant solution was added to 20 mL of a 50% slurry of Ni-NTA agarose resin, previously equilibrated in buffer A, and stirred on ice for 60 min. The resin was packed into a column (1.6 × 10 cm) and washed with 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl, 10 mM β-mercaptoethanol, 10 mM imidazole, and 10% glycerol (buffer B) until the absorbance of the effluent at 280 nm was less than 0.01. The protein was eluted with buffer B containing 100 mM imidazole. Fractions, 1 mL, were collected and analyzed by SDS−PAGE. The purest fractions were combined, diluted with 50 mM MOPS buffer, pH 7.4, and concentrated by ultrafiltration in a Centricon concentrator, 10 or 30 for the FMN- and FAD domains, respectively. At this stage, both proteins were observed to be homogeneous (Figure 2, *lanes* 3 and 4). Cleavage of the 6×His tag was performed by incubating 10

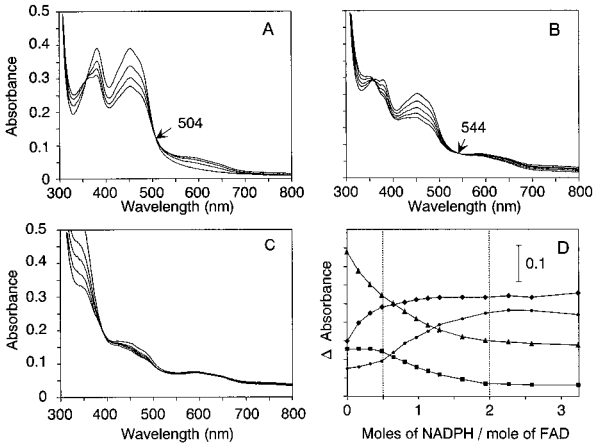

FIGURE 3: Anaerobic titration of the oxidized FAD domain with NADPH. The FAD domain (38 μM) in 50 mM MOPS buffer, pH 7.4, was titrated at room temperature with NADPH (4 mM). The absorbance spectra have been corrected for dilution. Panels A, B, and C represent a single titration of the protein. Sets of curves are separated in the figure to show isosbestic points occurring during the titration. Panel D is a plot of absorbance changes at 452 nm (▲), 504 nm × 2 (■), 585 nm × 3 (◆), and 750 nm × 5 (●) as a function of the moles of NADPH added per mole of FAD domain in the reaction mixture.

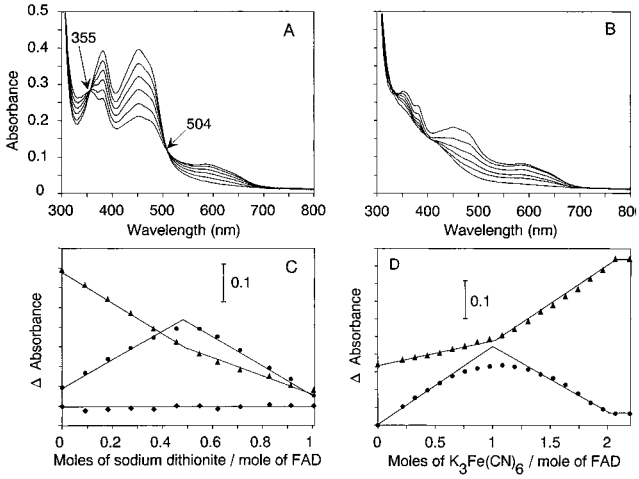

FIGURE 4: Anaerobic titration of the oxidized FAD domain with sodium dithionite. The FAD domain (38 μM) in 50 mM MOPS buffer, pH 7.4, was titrated at room temperature with sodium dithionite (4 mM). The absorbance spectra have been corrected for dilution. Panels A and B represent a single titration of the protein. Sets of curves are separated in the figure to show isosbestic points occurring during the titration. Panel C is a plot of absorbance changes at 452 nm (▲), 585 nm × 3 (●), and 750 nm × 5 (◆) as a function of sodium dithionite added. Panel D is a plot of absorbance changes at 452 nm (▲) and 585 nm × 3 (●) nm during the back titration of fully reduced FAD domain with potassium ferricyanide as a function of $K_3Fe(CN)_6$ added.

μg of either protein with 1 unit of TEV protease at 20 °C for 2 h. Under these conditions, cleavage of the tag from the FAD domain was complete, while only 50% of the tag could be removed from the FMN domain. The 6×His tag and the protease, which also has a 6×His tag, were separated from the cut domains by passing the samples through a Ni-NTA agarose column. It should be noted that neither of the proteins discussed in this paper had the 6×His tag remaining on the purified proteins.

Absorbance Spectra. The absorbance spectrum of the FAD domain has maxima at 276, 380, and 452 nm and a pronounced shoulder at 476 nm, with $A_{276/452}$ and $A_{452/380}$ equal to 7.76 and 1.04, respectively. The absorbance spectrum of the FMN domain has maxima at 276, 388, and 466 nm and a shoulder at 500 nm, with $A_{276/468}$ and $A_{468/388}$ equal to 5.17 and 1.16, respectively. The extinction coefficients for the oxidized flavoproteins were found to be 10.0 $\pm$ 0.3 mM^{-1} cm^{-1} at 452 nm and 9.8 $\pm$ 0.2 mM^{-1} cm^{-1} at 466 nm for the FAD- and FMN domains, respectively. Analysis of flavin and protein content showed that both domains contained 0.9 $\pm$ 0.05 mol of flavin/mol of protein.

Anaerobic Titrations with NADPH. The addition of a 4-fold molar excess of NADPH to an anaerobic solution of the FMN domain resulted in no change in the absorbance spectrum (data not shown). The addition of aliquots of NADPH to the oxidized FAD domain (Figure 3) resulted in the reduction of the flavin moiety of the protein. Reduction of the FAD proceeded in two phases (Figure 3D). The first phase, defined by the appearance of an isosbestic point at 504 nm, was observed during addition of 0.5 mol of NADPH/mol of FAD. The absorbance decrease at 452 nm concomitant with the absorbance increase at 585 nm indicates that a neutral, blue FAD semiquinone is formed during this phase of the titration (Figure 3A,D). Absorbance changes at wavelengths >700 nm were insignificant during this phase. The second phase, defined by the appearance of an isosbestic point at 544 nm, was observed when up to 2 mol of NADPH/mol of FAD was added. The equilibrium between added NADPH and the reduced forms of FAD was achieved more slowly in this titration when compared to the sodium dithionite titrations (see below), and 5−10 min was required to reach equilibrium following each addition. Characteristic features of this phase were continuous bleaching of absorbance at 452 nm and a significant absorbance increase in the long-wavelength region, indicative of the formation of a charge-transfer complex between reduced flavin and oxidized pyridine nucleotide (Figure 3B,D). Addition of a third mole of NADPH per mole of FAD resulted in small absorbance changes at 452 nm, while the absorbance increase at 340 nm indicated accumulation of unreacted NADPH (Figure 3C,D).

Anaerobic Titrations with Sodium Dithionite. When the FAD domain was titrated with sodium dithionite, two phases of the reaction were observed. The first reducing equivalent added resulted in a decrease in the flavin absorbance at 452 nm and a spectral species, which has an absorbance spectrum characteristic of a neutral, blue flavin semiquinone (Massey & Palmer, 1966), was formed (Figure 4A,C). This reduction proceeds with isosbestic points at 355 and 504 nm and the maximum absorbance increase at 585 nm occurs at a stoichiometry of 1 electron equiv added/mol of FAD (0.5 mol of sodium dithionite/mol of flavin). During the second phase of the titration, when a second electron equivalent of reductant per mole of flavin was added, there was a smaller decrease in absorbance at 452 nm, and loss of the 585-nm absorbance was observed (Figure 4B,C). Addition of more sodium dithionite caused no further changes in the visible spectrum. The stoichiometry between sodium dithionite added and the FAD domain reduced showed that the flavoprotein accepted 2 electron equivalents during the reduction process. In order to ascertain the electron transfer properties of the FAD domain, the fully reduced protein was back-titrated with potassium ferricyanide. The changes in absorbance at 585 and 452 nm are shown in Figure 4D. The results indicate that 1 and 2 mol of potassium ferricyanide/

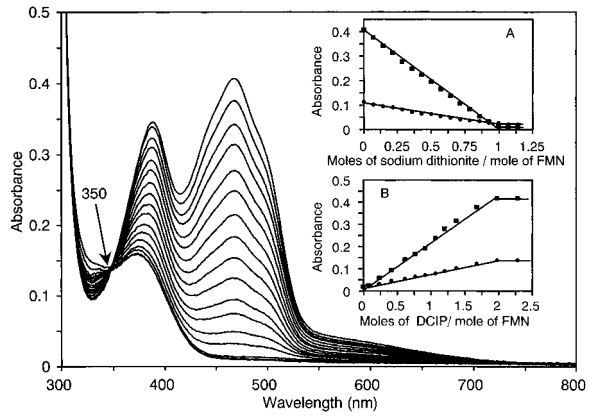

FIGURE 5: Anaerobic titration of the oxidized FMN domain with sodium dithionite. The FMN domain (38 μM) in 50 mM MOPS buffer, pH 7.4, was titrated at room temperature with sodium dithionite (4 mM). The absorbance spectra have been corrected for dilution. Inset A is a plot of absorbance changes at 466 nm (■) and 585 nm × 3 (●) as a function of sodium dithionite added. Inset B is a plot of absorbance changes at 466 nm (■) and 585 nm × 3 (●) nm during the back titration of fully reduced FMN domain with DCIP as a function of DCIP added.

mol of reduced flavin are required to reoxidize the flavoprotein to its semiquinone and fully oxidized states, respectively, and demonstrate the reversibility of reduction.

Sodium dithionite titration of the FMN domain resulted in a decrease in absorbance between 350 and 800 nm without the appearance of absorbance bands in the 580-nm region. These results indicate that there was no accumulation of flavin semiquinone in the equilibrium mixture during this titration (Figure 5). This result indicates that the hydroquinone form of FMN is thermodynamically preferable to either of the flavin semiquinone forms (red or blue), which must be transiently formed but which have been converted to the hydroquinone during the titration (5−10 min/point). The addition of 1 mol of the reductant/mol of flavin caused essentially complete bleaching of absorbance at 466 nm (Figure 5, inset A), while a new peak near 380 nm was formed. Addition of more sodium dithionite caused no further change in the visible spectrum. The absorbance spectra were isosbestic at 350 nm until there was an excess of sodium dithionite, which absorbs light at wavelengths <400 nm. The stoichiometry between sodium dithionite added and the FMN domain reduced showed that this protein also accepted 2 electron equivalents during reduction. When the fully reduced FMN domain was back-titrated with DCIP, the spectra (inset B) were similar to those shown in Figure 5. No formation of the semiquinone form of the FMN was observed.

Air Stability of the Reduced Domains. The reactivity of the two domains of BMR toward oxygen in 50 mM MOPS buffer, pH 7.4, was different (Figure 6). When the FAD domain, reduced with a 4-fold molar excess of NADPH, was mixed with oxygen-saturated buffer, there was an initial lag period prior to an increase in absorbance at 452 nm (Figure 6A). This lag period was a function of the amount of NADPH added to the reaction mixture and corresponded to the oxidation of the excess NADPH. Following this lag period there was a rapid loss of absorbance at 580 nm (not shown) and an increase at 452 nm. The reoxidation was complete within 2 min at room temperature (Figure 6A). The reoxidation reaction was found to be monophasic with a pseudo-first-order rate constant of 2.5 min^{-1}. We presume

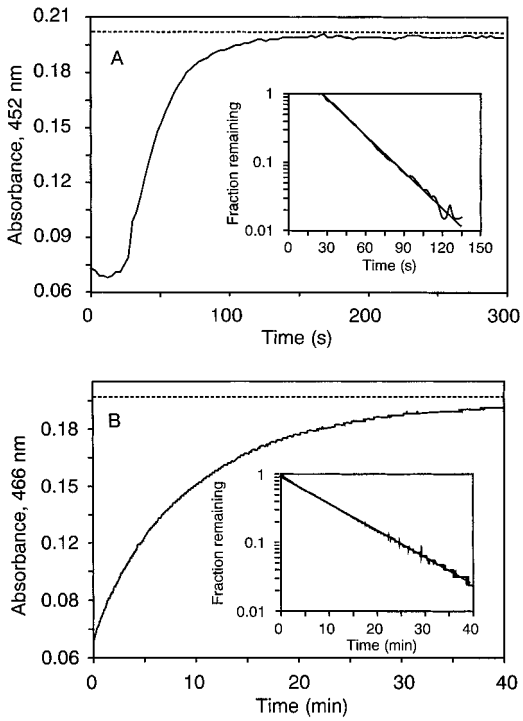

FIGURE 6: Reoxidation of fully reduced FAD- and FMN domains with molecular oxygen. The reaction was carried out in 50 mM MOPS buffer, pH 7.4, at room temperature. (A) The FAD domain (1.5 mL, 38 μM) was reduced with a 4-fold molar excess of NADPH and then mixed with 1.5 mL of oxygen-saturated buffer. The inset in this panel is a semilogarithmic plot of the "fraction remaining" of the absorbance change at 452 nm, where "fraction remaining" is $(\Delta A_{max} - \Delta A_t)/\Delta A_{max}$. (B) The FMN domain (1.5 mL, 38 μM) was reduced with a 2-fold molar excess of sodium dithionite and then mixed with 1.5 mL of oxygen-saturated buffer. The inset in this panel is a semilogarithmic plot of the "fraction remaining" of the absorbance change at 466 nm.

that the rate of this reaction is dependent on the oxygen concentration. Attempts to measure the rate constant for reoxidation of the FAD domain, which had been reduced with sodium dithionite, were difficult because of the reactivity of both sodium dithionite and the enzyme with molecular oxygen.

The FMN domain, which had been fully reduced by a 2-fold molar excess of sodium dithionite, was reoxidized with molecular oxygen relatively slowly (Figure 6B). The initial absorbance of the oxidized enzyme at 466 nm was recovered 1 h after mixing the reagents. No absorbance changes, consistent with the formation of a flavin semiquinone, were observed during the reoxidation. The increase in absorbance at 466 nm was monophasic with a pseudo-first-order rate constant of 0.1 min^{-1}. As with the reoxidation of the FAD domain, the rate of reoxidation of the FMN domain should be a function of oxygen concentration.

Catalytic Activities. The ability of the flavin domains of P450BM-3 to carry out electron transfer from NADPH to various artificial electron acceptors was examined. The specific activities of BMR and the separate FAD- and FMN domains in these electron transfer reactions are shown in Table 1. The reduction of potassium ferricyanide by the FAD domain was found to have a specific activity 83% of that observed with BMR. The FAD domain also had residual activity for NADPH-dependent cytochrome *c* and DCIP reduction. In contrast, the NADPH-dependent reduction of electron acceptors by the FMN domain was very low,

Table 1: Activities of BMR and Its Fragments toward Various Electron Acceptors[a]

enzyme	turnover [mol min^{-1} (mol of flavin)$^{-1}$]		
	$K_3Fe(CN)_6$	cytochrome *c*	DCIP
BMR	7200 ± 300 (100%)	3600 ± 300 (100%)	2700 ± 150 (100%)
FAD domain	6000 ± 200 (83%)	20 ± 1 (0.6%)	240 ± 16 (9%)
FMN domain	600 ± 40 (8.3%)	10 ± 1 (0.3%)	90 ± 7 (3%)

[a] The reactions were carried out in 50 mM MOPS buffer, pH 7.4. The final concentrations of $K_3Fe(CN)_6$, cytochrome *c*, and DCIP in the reaction system, were 1.8 mM, 100 μM, and 40 μM, respectively. Each value is an average of at least three independent determinations

indicating again that this component cannot accept electrons from NADPH in the absence of the FAD domain. To evaluate the ability of the FMN domain to interact with and accept electrons from the FAD domain, the effect of varying concentrations of the FMN domain on cytochrome *c* reduction was measured. In this assay, the variable "substrate" of the FAD domain was the FMN domain and the rate of transfer of electrons from the FAD domain to the FMN domain was measured by the reduction of cytochrome *c*, which was present in excess. In this coupled assay, a Lineweaver−Burk plot of the inverse of the rate of reduction versus the inverse of the concentration of the FMN domain was a straight line (Dixon & Webb, 1964). The K_m of the FAD domain for the FMN domain in this assay was 1.9 μM and the V_{max} was 3800 ± 300 mol min^{-1} (mol of FAD domain)$^{-1}$. As can be seen in Table 1, the turnover number of BMR in the reduction of cytochrome *c* is 3600 ± 300 mol min^{-1} (mol of protein)$^{-1}$, which is within experimental error of the value found for the reconstituted cytochrome *c* reductase system described above. Since the FMN semiquinone species of the FMN domain was not observed during anaerobic titrations with sodium dithionite, and the FMN hydroquinone is incapable of reducing the heme iron of P450BM-3[2] (Sevrioukova & Peterson, 1995), we did not try to reconstitute the P450 reductase activity using the separate domains.

DISCUSSION

The mechanism of action of NADPH−P450 reductase is of interest for at least two major reasons: (1) the role of the enzyme as an electron carrier in P450-dependent microsomal monooxygenation reactions and (2) the unique nature of the catalytic cycle of the enzyme, containing FAD and FMN prosthetic groups. As a consequence of the central role which NADPH−P450 reductase has in microsomal electron transfer, many research groups have contributed to the development of the knowledge about this enzyme. Although the preparation of X-ray diffraction-quality crystals of a truncated form of microsomal NADPH−P450 reductase has been reported, the structure of this protein has not yet been determined (Djordjevic et al., 1995). Recently the cDNA for human NADPH−P450 reductase has been divided into regions that would correspond to the NADPH/FAD-binding reductase domain and the FMN-binding, flavodoxin-like domain and inserted into expression vectors (Smith et al., 1994). Interestingly, each of these domains could be expressed at high level (10−15% of the membrane-bound protein of *E. coli*) and contained between 0.63−0.67 and 0.7 mol of FMN and FAD/mol of FMN- and FAD/NADPH-binding domain, respectively. In a separate study, the FAD/NADPH-binding domain of rat NADPH−P450 reductase was also expressed in *E. coli* and its properties were reported (Hodgson & Strobel, 1996). Such an approach will permit an investigation of how the flavin domains interact with each other and with redox partners to form functional electron-transfer complexes. The availability of each of these subdomains may prove to be useful in establishing their structure using either X-ray diffraction techniques or NMR spectroscopy.

The flavoprotein domain of P450BM-3, which is functionally analogous to the microsomal NADPH-dependent P450 oxidoreductases, serves as a good model for structure−function relationships of these flavoproteins. For this reason, we utilized recombinant DNA techniques to construct vectors that could be used to produce the FMN- and FAD/NADPH-binding domains of the protein. The boundaries of the domains, for construction of the expression vectors, were decided using the sequence alignment of homologous reductases reported previously (Porter, 1991). We were assisted in the design of the fragments for expression by the data regarding which proteolytic fragments of P450BM-3 were stable (Narhi & Fulco, 1987; Oster et al., 1991; Black, 1994). Porter (1991) had proposed that A625 and A650 were likely to be the end and the beginning of the FMN- and FAD-binding regions of the reductase domain of P450BM-3, respectively. We decided to include the 10 residues S640−S649 from the putative domain linker region in both fragments to increase the possibility of correct folding of the domains. Indeed, the fact that both recombinant proteins contained a nearly stoichiometric amount of the flavin cofactor indicates that the domains are capable of folding independently. As an interesting aside, this strain of *E. coli* is capable of synthesizing massive quantities of either FAD or FMN in response to the synthesis of these recombinant proteins which bind these cofactors.

The absorbance maxima of the oxidized form of the FAD domain (276, 380, and 452 nm) were similar to those of the proteolytically prepared NADPH/FAD-binding fragments of BMR and P450BM-3 (Oster et al., 1991; Black, 1994). However, the ratios $A_{276/452}$ and $A_{452/380}$ and the extinction coefficient at 452 nm were different from the previously reported values (Oster et al., 1991; Black, 1994), most likely because of different lengths of the proteins. There are two interesting features of the absorbance spectrum of the FMN domain: the characteristic flavin absorbance bands are red-shifted slightly to 388 and 466 nm, and there is long-wavelength absorbance extending from 550 to 700 nm. Because of these spectral properties of the protein-bound FMN, the color of the enzyme solution is yellow-green. The 14-nm difference between the absorbance maxima in the 450-nm region for the two flavins in BMR explains why its absorbance spectrum is broad with the maximum around 456 nm.

Anaerobic titrations of the FAD- and FMN domains indicate that the electron-accepting properties of the flavins appear to be essentially unaltered by their separation. As we have shown previously[2] (Sevrioukova & Peterson, 1995), reductive titrations of BMR with 1 mol equiv of either NADPH or sodium dithionite proceed without formation of flavin semiquinone. This behavior of BMR during the titrations was suggested to be because of the electron-accepting properties of the FMN moiety[2] (Sevrioukova &

Peterson, 1995). Reductive titrations of the FMN domain with sodium dithionite have reinforced this hypothesis. The spectra of the oxidized FMN domain at various intermediate stages of reduction are shown in Figure 5. Reduction of the flavin, with a single isosbestic point at 350 nm, was accompanied by bleaching of the long-wavelength absorbance and a progressive decrease in absorbance at 388 nm. At the end of reduction, the spectrum of the FMN domain has a maximum in the 380-nm region. The thermodynamic instability of the enzyme-bound FMN semiquinone, with respect to the two-electron reduced flavin, has been observed with two enzymes—old yellow enzyme and rat microsomal NADPH−P450 reductase—in which the native cofactor had been replaced by FMN derivatives (Vermilion et al., 1981; Stewart & Massey, 1985). To account for the titration of the enzyme-bound FMN derivatives, the redox potentials for the half-reactions for the addition of the second electron must be at least 65−120 mV more positive than for the addition of the first electron (Vermilion et al., 1981; Stewart & Massey, 1985). The same proposal was made for the redox potentials of the FMN of BMR[2] (Sevrioukova & Peterson, 1995) and are consistent with our titrations of the FMN domain.

During titrations with NADPH, the absorbance changes observed with the FAD domain were somewhat different from those with BMR[2] (Sevrioukova & Peterson, 1995). One of the differences was in the second phase of the titration of BMR. After the FMN moiety had been reduced, the absorbance increases at 585 and 750 nm were proportional, indicating that both neutral, blue flavin semiquinone and a charge-transfer complex between NADP(H) and $FADH_2$ were being formed. Titration of the FAD domain proceeded with two distinct phases for the spectral changes at 585 and 750 nm. In the first phase, the neutral, blue FAD semiquinone was formed without a significant increase in absorbance at 750 nm. After maximal formation of the 585-nm absorbance, the absorbance at 750 nm increased, indicating that the charge-transfer complex between reduced flavin and oxidized pyridine nucleotide was being formed. These differences can be explained if the presence of the FMN domain raised the redox potential for the addition of the second electron to the protein-bound FAD. This would permit the concomitant formation of blue semiquinone and fully reduced enzyme that could then form a charge-transfer complex with $NADP^+$. The FAD domain, like BMR (Mimura et al., 1994), could not be fully reduced with NADPH, but it could be fully reduced when the protein was titrated with sodium dithionite. Comparison of the spectra of one- and two-electron reduced forms obtained during titrations with sodium dithionite with those obtained with NADPH (Figures 3 and 4) indicates that at the end of the titration of either BMR or the FAD-binding domain with NADPH, the reaction mixture consists of one- and two-electron reduced forms of FAD.

Analysis of the reactivity of the two flavin domains toward oxygen has shown that the flavins of the separate domains react with oxygen at different rates. Although the reoxidation of BMR is biphasic, it is not clear at the present time whether the separate rates represent the different rates of reoxidation of the individual domains.

The fact that both reduced FAD- and FMN domains were capable of reducing artificial electron acceptors indicates that at least some of the electron transfer properties of the proteins were preserved after separation. Moreover, the turnover number of the FAD domain in potassium ferricyanide reduction approached that of BMR (Table 1). Anaerobic titrations with NADPH and very low NADPH-dependent cytochrome *c* and DCIP reductase activities of the FMN domain show that the FMN of BMR, like FAD-depleted microsomal reductase (Kurzban & Strobel, 1986) and its FMN domain (Smith et al., 1994), is incapable of accepting electrons from NADPH. The ability to fully reconstitute the cytochrome *c* reductase activity of BMR, employing the individual domains, demonstrates that the basic structure of these domains and their functions, at least in the case of cytochrome *c* reduction, have been maintained in the separate domains.

In summary, by dissection of BMR into the FAD/NADPH- and FMN domains and their expression in *E. coli*, we have demonstrated that this flavoprotein has a discrete multidomain structure. The two domains were found to have the redox and electron-accepting properties of the BMR-bound flavins. We propose that in the holoenzyme the presence of the FMN domain might slightly raise the redox potential of the protein-bound FAD and affect the ability of the enzyme to form a charge-transfer complex with $NADP^+$.

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