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*

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Comparison of the amino acid sequences of several microsomal cytochromes P-450 reductases to the flavoprotein domain (BMR) of cytochrome P-450BM-3 has revealed that this class of flavoproteins contains evolutionarily conserved regions that are important for their interaction with nucleotide substrates and cofactors. In order to understand the properties of BMR, the region encoding this protein, beginning at residue Lys-472 of cytochrome P-450BM-3, was subcloned and expressed in Escherichia coli. The recombinant protein (more than 50% of host-soluble proteins) was purified to homogeneity using conventional purification procedures. BMR (M, 66,000) showed typical flavo-enzyme absorbance spectra, contained FAD and FMN in a stoichiometry of 1:1, and catalyzed reduction of several artificial electron acceptors with rates comparable to those of the microsomal NADPH-cytochrome P-450 oxidoreductase.

Limited trypsinolysis of BMR, under non-denaturing conditions, revealed that the protease removed the NH2-terminal 122 residues. This region was postulated to contain amino acids that are important for FMN binding (Porter, T. D. (1991) Trends Biochem. Sci. 16, 154–158). Consistent with this hypothesis, the major tryptic product of BMR (BMR-52, M, 52,000) contained only FAD, in an equimolar ratio to the protein. Also, like the FMN-depleted microsomal NADPH-cytochrome P-450 oxidoreductase (Kurzban, G. P., Howard, J., Palmer, G., and Strobel, H. W. (1990) J. Biol. Chem. 265, 12272–12279), BMR-52 was active for only catalyzing ferricyanide reduction. These data provide strong experimental evidence for a discrete multidomain structure of BMR, as proposed for the membrane-bound reductases, with an amino-terminal FMN binding region and carboxyl-terminal FAD- and NADPH binding regions. Thus, BMR strongly resembles the microsomal cytochrome P-450 reductase and offers an opportunity to better understand the structure-function relationships of this class of flavoproteins.

Flavoprotein oxidoreductases catalyze various important redox reactions in the cellular milieu of eukaryotes and prokaryotes. These enzymes are involved in photosynthesis (1), assimilation of inorganic nitrogen (2) and sulfur (3), fatty acid oxidation (4) and methemoglobin biosynthesis (5, 6), and metabolism of many pesticides, drugs, and carcinogens (7). As an integral part of the microsomal redox system, NADPH-cytochrome P-450 reductase (EC 1.6.2.4) transfers electrons to cytochromes P-450 (8–11). Besides cytochromes P-450, the enzyme can also transfer reducing equivalents to other electron acceptor enzymes such as cytochrome b6 (12, 13), heme oxygenase (14), and squelene epoxidase (15). This flavoprotein (M, 78,225) contains 1 mol each of FAD and FMN (16, 17) and transfers reducing equivalents from NADPH to the heme of cytochromes P-450 via FAD and FMN (18, 19).

A bacterial cytochrome P-450 system, functionally analogous to the microsomal monoxygenase system, including the transfer of electrons to the heme from NADPH via FAD and FMN, was discovered in Fulco’s laboratory, viz. cytochrome P-450BM-3,1,2 the soluble fatty acid ω-hydroxylase of Bacillus megaterium (20–22). Cytochrome P-450BM-3 is a catalytically self-sufficient single polypeptide (M, 119,000) containing heme, FAD, and FMN in a stoichiometry of 1:1:1, respectively (23). This flavocytochrome is not only a fatty acid hydroxylase (23, 24), but also can function as an epoxidase (25). Narhi and Fulco (26) have shown that, upon limited trypsinolysis in the presence of the substrate, the protein is cleaved into two domains, one retaining the covalently linked heme and the other containing the non-covalently attached FAD and FMN. Even though these polypeptides, purified from the tryptic digest, appeared to retain their individual activities, such as the ability of the hemoprotein domain to bind substrate and that of the flavoprotein domain to catalyze NADPH-dependent cytochrome c reduction, reconstitution of fatty acid hydroxylation activity by combined trypic products could not be achieved (26). Similar results were obtained with our own recombinant preparations of cytochrome P-450BM-3 (24).

The flavoprotein domain (BMR) of cytochrome P-450BM-3 is the only soluble FAD- and FMN-containing protein known to date that is functionally analogous to the microsomal NADPH-cytochrome P-450 oxidoreductases. The sequence homology of BMR to the eukaryotic cytochrome P-450 reductases (27, 28) indicates that BMR will be important in obtaining

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1 The abbreviations used are: cytochrome P450BM-3, the soluble cytochrome P-450 isolated from B. megaterium (the product of CYP102 gene); BMR, the individually expressed flavoprotein domain (reductase) of cytochrome P-450BM-3; BMR-52, the 52-kDa tryptic product of BMR; DCIP, 2,6-dichlorophenol indophenol; DTT, dithiothreitol; MOPS, morpholinopropanesulfonate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.
2 The nomenclature of cytochromes P-450 is adapted from Nebert et al. (70).
ing a better understanding of the mechanisms of these flavoproteins. In order to study the structure-function relationships of BMR and its resemblance to the mammalian enzymes, it will be necessary to obtain BMR in large amounts. Even though BMR can be possibly prepared by the trypsino-
ysis of cytochrome P-450BM-3 (26), in order to eliminate the possible side effects of proteolysis on the flavoprotein, recombinant DNA techniques were utilized in the current investigation.

In this paper, we report the catalytic properties and the experimental evidence for the importance of the amino-
terminal region in FMN binding of the recombinant BMR.

EXPERIMENTAL PROCEDURES

Materials—Bacto-tryptone, yeast extract, and Bacto-agar were purchased from Difco Laboratories. Agarose, acrylamide, bisacryl-
amide, and protein molecular weight standards were purchased from Bethesda Research Laboratories (BRL). Protease inhibitors, FAD, PMN, NADH, NADPH, ampicillin, cytochrome c, DCIP, and potassium ferricyanide were obtained from Sigma. Palmitic acid was obtained from Nu Chek Prep, Inc. All other reagents used were of the purest grades available.

Escherichia Coli Strain, Media, and Plasmids—E. coli strain DH5α (P'. endA1, hsdR17 (rK, mK), supE44, thi-1, recA1, gyrA96, relA1, Δ(lac-proAB)1567lacZΔM15), was obtained as component cells from BRL. Media were described as prepared by Sambrook et al. (29) and contained ampicillin (50 μg/ml final concentration). While 2 × YT was the medium used for preparing small scale plasmid purification and transformations, TB medium was used for large scale cultures. For long term storage of the cell lines, clones were individually picked from a plate, resuspended in 1 ml of 2 × YT containing ampicillin (50 μg/ml final concentration) and 15% gly-
cerol, frozen in a dry ice/ethanol bath, and stored at -70 °C until used. The 12-kilobase BM3-3α plasmid (27), containing the 9.2-kilobase DNA region encoding the flavoprotein domain, was purified and digested with EcoRV and BglII. The resulting 2.8-kilobase fragment, obtained after conventional techniques (37), was subcloned into the pIB124 vector digested with HincII and BamHI. Subsequently, clones were selected after small scale DNA purification (38) by restriction analysis and DNA sequencing.

Expression Studies—The plasmid pIB124 was used to transform E. coli DH5α competent cells. Recombinants were grown for 24 h, in TB medium containing 50 μg/ml ampicillin at 37 °C, as previously reported (24). Different conditions for inoculation and cell growth were studied to optimize the level of expression of pIB124.

Purification of BMR—Cells grown to late stationary phase 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. The cell paste was resuspended as 1 g/ml in 50 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM Na2EDTA, 0.1 mM DTT, and then concentrated to their original volume by centrifugation, using Centricon 1 concentrators (cut off 20,000; Sartorius), to ensure removal of the protease inhibitors added to the enzymes before storage at -70 °C. Then, the enzymes were subjected to limited trypsinolysis, essentially as described by Narhi and Pulco (28). The reaction system contained 100 mM potassium phosphate, 0.1 mM DTT, pH 7.4, buffer, 100 μg of cytochrome P-450BM-3, 0.33 μg of trypsin, 60 μg palmitic acid, in a final volume of 0.2 ml. For trypsin-
olysis of BMR, identical reaction conditions were employed except for the omission of the fatty acid. At selected intervals (0.5, 1, 1.5, 2, and 3 h), aliquots were taken and the digestion was inhibited by the addition of soybean trypsin inhibitor to a final concentration of 1 μg/ml. Samples were immediately assayed for NADPH-cytochrome c reductase activity and were subjected to SDS-PAGE. Products present in the BMR sample, obtained after 1 h of trypsinolysis, were separated by gel filtration on a fast protein liquid chromatography system, using a 10 × 300 mm Superose 12 HR 10/30 column (Phar-
macia LKB Biotechnology Inc.). Tryptic digests of cytochrome P-
450BM-3 were also assayed for palmitic acid hydroxylase as a function of oxygen consumption.

RESULTS

Limited Trypsinolysis of Cytochrome P-450BM-3—Narhi and Pulco (28) had shown that during the time course of trypsi-

nolysis of cytochrome P-450BM-3, while the myristic acid hy-
Flavoprotein Domain of Cytochrome P-450BM.3

Cytochrome P-450BM.3 was incubated, at 4 °C, with 1/300 ratio of trypsin (w/w). At indicated time intervals, the reaction was inhibited by the addition of trypsin inhibitor and the activities were determined. The activity of NADPH-cytochrome c reductase was determined at 556 nm in a 1-ml reaction system containing 20 mM MOPS, 0.1 M KCl, pH 7.4, as buffer, 0.5 μg of protein, and 0.1 mM NADPH. Palmitate hydroxylation was measured using a Clark-type oxygen electrode in a 1.6-ml reaction system containing 50 mM MOPS, pH 7.4, buffer, 1 mM potassium palmitate, 15 μg of protein, and 1 mM NADPH. Each value represents the mean of four independent determinations.

| Time (h) | NADPH-cytochrome c reductase activity | Palmitate hydroxylation |
|---------|---------------------------------------|-------------------------|
| 0       | 20                                   | 20                      |
| 0.5     | 29                                   | ND                      |
| 1.0     | 36                                   | 3.3                     |
| 1.5     | 42                                   | 2.0                     |
| 2.0     | 42                                   | 1.1                     |

*In the presence of 25 μM FMN in the reaction system.

hydroxylation was dependent on the amount of native undigested protein, NADPH-dependent cytochrome c reductase was unaltered. Using our recombinant enzyme preparations, we reexamined the above results under similar experimental conditions. Consistent with the earlier results, we observed that palmitate hydroxylation, as measured by the substrate-dependent consumption of oxygen, was related to the length of incubation of the enzyme with trypsin (Table I).

However, in contrast to the earlier results (26), enhanced NADPH-dependent cytochrome c reductase was observed during the time course of trypsinolysis of the enzyme (Table I). The NADPH-dependent cytochrome c reductase activity increased 2-fold after 2 h of digestion, probably due to easier accessibility of cytochrome c to the flavoprotein region. The ability of cytochrome c to compete for the electrons from the reductase portion of P-450BM.3 to the hemoprotein domain indicates that cytochrome c may interact specifically with the reductase domain. These results suggest that catalytic properties of the flavoprotein reductase domain are retained even after the protease digestion.

**Construction of a Recombinant Vector Encoding BMR and Its Expression in E. coli DH5α**—The amino terminus of the flavoprotein domain, resulting from the trypsinolysis of cytochrome P-450BM.3, had 2 lysines corresponding to positions 472 and 473 of the native undigested protein (27). To construct a recombinant vector which would produce this protein, a piece of DNA isolated from BM3.2A was subcloned, the 5'-end of which would encode these 2 lysine residues.

To achieve expression, a 43-mer oligonucleotide was designed (Fig. 1), a portion of which contained the translation initiation signals (ribosome-binding site, start codon) identical to that determined by Ruettiger et al. (27) for cytochrome P-450BM.3 gene. This oligonucleotide was used as one of the two primers for the PCR performed to synthesize a linker enclosing these signals. This linker was ligated 5' to the coding region of BMR. As shown in Fig. 1, the resulting DNA piece was inserted into pBlB24, downstream from the lacZ promoter. The recombinant plasmid (pRed2) was used to transform E. coli DH5α cells. The nucleotide sequence was determined to confirm successful gene engineering.

**Table I**

| Time (h) | NADPH-cytochrome c reductase activity | FMN | +FMN |
|---------|---------------------------------------|-----|------|
| 0       | 20                                    | 20  | 8.1  |
| 0.5     | 29                                    | ND  | 5.3  |
| 1.0     | 36                                    | 3.3 | 2.0  |
| 1.5     | 42                                    | 1.1 | 1.1  |

**Fig. 1. Strategy for subcloning the BMR encoding region.** Methodology was described in detail under “Experimental Procedures.” Hatched areas indicate the coding regions and the arrows depict the direction of translation. The oligonucleotides utilized for performing the PCR on pRed1 are: an 18-mer primer, complementary to the ClaI region, and a 43-mer primer, pGGGAATTCTGAAAGGGAGAATACAGTGAAMAGAAGGGGATTAACTGAAAAGGCGAGAAACGGC, to create an EcoRI site, a stop codon, a Shine-Dalgalzo sequence, and a start codon optimally separated, from the 5' to the 3'-end.

showed that the linker was correctly inserted in the 5'-end of the coding region, and that the sequence of the DNA synthesized by PCR did not contain any undesired mutations.

The overexpression of the gene coding for cytochrome P-450BM.3 in E. coli DH5α was previously reported (24). The maximal expression occurred when cells were grown in TB medium containing ampicillin (50 μg/liter), after 24 h at 37 °C. The enzyme represented almost 20% of the soluble proteins of these cells without supplementing riboflavin or heme in the growth medium. The cell density of the inoculum (A600 between 0.6 and 0.8) was observed to be crucial for a high level expression of BMR. Under these conditions, even though the biomass of the cells was similar to that obtained for cytochrome P-450BM.3, the expression of BMR was much higher compared to that of the flavocytochrome. SDS gels revealed a polypeptide (Mr 45,000) only in the cell lysates of E. coli that was transformed with pRed2 (Fig. 2A), specifically detected in immunoblots probed with anti-cytochrome P-450BM.3 antisera (Fig. 2B), and estimated to correspond to more than 50% of the soluble proteins. Since apoenzyme would be expected to be unstable and susceptible to proteolytic degradation, the approximate quantitation of BMR in cell lysates would represent that of the holoprotein, substantiating the conclusion that endogenous flavin biosynthesis in strain DH5α can support the massive production of a flavoprotein. To our knowledge, BMR is the only foreign protein reported to be very highly produced as a holoenzyme in E. coli, with neither a selective pressure nor an inducer for its expression.

**Purification of BMR—Narhi and Fulco (26) purified the flavoprotein domain, from the trypsin digests of cytochrome P-450BM.3, that contained lower concentrations of flavin and residual specific activity for NADPH-cytochrome c reductase compared to the native enzyme. Also, it was reported that FMN was partially lost during anion-exchange chromatography (27). This probably was due to the ease of the nucleotide to be dissociated from the enzyme due to salt concentration,
pH, possible interactions with resins, etc. Although such a possibility was not observed during the purification of recombinant cytochrome P-450<sub>BM3</sub>, the protein was included in the wash buffer for the anion-exchange chromatography. The activity of the recombinant cytochrome P-450<sub>BM3</sub> was almost homogeneous (Fig. 2).

For the purpose of comparison to BMR, we determined the activities of BMR in the presence of excess of FMN in the reaction system. The turnover number of the microsomal reductase in the presence of excess of FMN in the reaction system increased the cytochrome c reduction. While the NADPH-cytochrome P-450<sub>BM3</sub> reductase activity of BMR to equal that of cytochrome P-450<sub>BM3</sub>. The reason for this decreased turnover of the flavoprotein domain is unknown at this time. We rule out the possibility that this was due to the loss of FMN from BMR by including excess FMN in the assay system and reexamining all the activities. While the presence of exogenous FMN in the reaction system increased the cytochrome c reductase activity of BMR to equal that of cytochrome P-450<sub>BM3</sub>, it had no significant influence on any other activity. Hence, the enhanced NADPH-cytochrome c reductase activity of BMR in the presence of excess of FMN in the reaction system may not be due to any apoenzyme contamination, but may be a nonspecific increase for cytochrome c reduction alone.

While the NADPH-cytochrome c reductase activity of BMR was similar to that of the microsomal enzyme, ferricyanide and DCIP reductase activities were twice compared to those of the membrane-bound enzyme, under identical experimental conditions. The turnover number of the microsomal reductase in various electron transfer reactions is known to be dependent on the ionic strength of the reaction buffer (39). For the purpose of comparison to BMR, we determined the turnover number of the recombinant enzyme in 20 mM MOPS, 0.1 M KCl, pH 7.4, buffer. It may be pertinent to
mention in this context that the apparent $K_m$ values for NADPH-dependent DCIP reduction for BMR and cytochrome P-450BM_{3.3}, 14 and 12 $\mu$M, respectively, were similar to those obtained for the pig liver microsomal cytochrome P-450 reductase (40).

Limited Trypsinolysis of BMR—SDS-PAGE of the tryptic products followed by silver staining detection had revealed that after 4 h of trypsinolysis of cytochrome P-450BM_{3.3}, the intensity of the signals of each of the proteolytic products were different (Fig. 4). The signal of the polypeptide at apparent molecular weight 66,000 (corresponding to the flavoprotein domain) was observed to be less intense than that of the one at approximately 55,000 (corresponding to the hemoprotein domain). We utilized recombinant preparations of BMR to verify whether this was due to the cleavage of the flavoprotein domain by the protease. SDS-PAGE profiles (Fig. 4) of the tryptic digests of BMR have disclosed the ability of the protease to lyse BMR into a protein (apparent molecular mass 52,000 Da). These data explain the decrease in the intensity of the 66-kDa primary tryptic product of cytochrome P-450BM_{3.3} and the increased intensity of the signal for the apparent 55-kDa products. Better resolution and densitometric examination of these products on SDS-gels (Fig. 4) have indicated the presence of polypeptides corresponding to molecular weights 55,000 (hemoprotein domain) and 52,000 (the major tryptic product of BMR).

Consistent with the proteolytic profiles, the ability of the tryptic digests of BMR to carry out NADPH-dependent cytochrome c reduction depended on the length of incubation of the flavoprotein with trypsin (Fig. 5). The presence of excess FMN in the assay buffer could not restore the original activity, indicating that this was not due to the loss of FMN during the prolonged incubation of the enzyme. However, NADPH-ferricyanide reductase activity was unaltered for 4 h of trypsinolysis of BMR, and decreased at a slow rate thereafter (Fig. 5).

To confirm that the 52-kDa tryptic product (BMR-52) retained the ferricyanide reductase activity, we purified residual undigested BMR and BMR-52 from the BMR trypsinolysis reaction mixture obtained after 1 h (Fig. 6). Activities of purified BMR and BMR-52 toward NADPH-dependent reduction of cytochrome c, DCIP, and ferricyanide, are shown in Table III. Consistent with the data obtained for the tryptic digests, compared to native BMR, BMR-52 had residual activities for cytochrome c and DCIP reductions and had unaltered activity for ferricyanide reduction. These results suggested the possibility that BMR-52 lacked FMN-dependent catalytic properties, however, retaining those dependent on FAD. It may be mentioned here that studies on the microsomal cytochrome P-450 reductase have demonstrated that removal of FMN from the enzyme resulted in the loss of cytochrome c and DCIP reductions without altering the ferricyanide reductase activity (18, 41-45). In addition, preparations of FAD-depleted microsomal reductase failed to catalyze the transhydrogenase and ferricyanide reductase activi-
ties, indicating that electron entry occurs at the FAD site (46). Thus, BMR-52 may be equivalent to a FAD-reductase.

**Absorbance Spectra**—Fig. 7 shows the absorbance spectra of BMR and BMR-52. Both proteins, as isolated, exhibited typical oxidized flavin spectra without absorbance in the longer wavelength regions (where partially reduced flavins could be detectable). Maximal absorbance was observed at 456 and 382 nm for BMR and at 452 and 380 nm for BMR-52. The ratios of the peak absorbances for each of the proteins were identical (1.12). In contrast, FMN-depleted microsomal reductase could be detectable. Maximal absorbance was observed at 455 nm using ε_{max} of 10.8. While BMR had approximately 2 mol of flavin/mol of enzyme, the concentration of the flavins in BMR-52 was equimolar to that of the enzyme. These results were further substantiated by the FAD and FMN contents of these enzymes.

**Flavin Contents**—Earlier reports on the flavin contents of cytochrome P-450BM_{3} have indicated that 1 mol of enzyme contained approximately 0.5 mol each of FAD and FMN (23, 47). Determination of the flavin content in purified samples of cytochrome P-450BM_{3} and BMR have revealed that enzyme preparations contained 1 mol each of FAD and FMN/mol of protein (Table IV). Interestingly, BMR-52 contained only FAD as the nucleotide cofactor at an equimolar ratio to the protein. In relevance to the results reported for the microsomal reductase (41, 45), these data justify the lack of FMN-dependent activities observed for BMR-52. Also, excellent correlation between the theoretical and measured contents of the flavins in BMR and BMR-52 rules out any possibility for loss of FMN during the purification and confirms that these proteins were holoenzymes.

**NH_{2}-terminal Sequence Analyses**—As expected, the amino-terminal sequence of BMR (Fig. 8) was identical to residues Lys-471 to Pro-481 of cytochrome P-450BM_{3} (27), except for the first residue, a methionine encoded by the inserted translation start codon of the linker (Fig. 1). The molecular mass of BMR-52, estimated on SDS-gels, suggested that BMR may be cleaved by trypsin resulting in the loss of approximately 120 amino acid residues. Amino-terminal sequence of BMR-52 (Fig. 8) has confirmed that trypsinolysis occurred after Lys-122 in BMR.

**DISCUSSION**

The microsomal NADPH-cytochrome P-450 oxidoreductase is significantly different from the flavoprotein counterparts of the mitochondrial cytochrome P-450 monoxygenase system in that it contains both FAD and FMN and does not have an obligatory requirement for iron-sulfur proteins to transfer the electrons to cytochromes P-450. The genes encoding this unique microsomal flavoprotein have been cloned from several organisms such as yeasts (48, 49) and some vertebrates (50-54), including the human (55). Mammalian

**FIG. 8. Primary structure analysis of BMR.** The amino acid sequence of the flavoprotein domain of cytochrome P-450BM_{3} was deduced by Ruettinger et al. (27). Arrows start at the NH_{2}-terminus of BMR and BMR-52. Bold residues in the figure indicate the amino-terminal sequences of each of these two purified enzymes, and determined as described under "Experimental Procedures." Regions 1 and 2 were shown to be homologous to those comparable in rabbit hepatic microsomal cytochrome P-450 reductase (27). Regions 3-8 were identified by Karplus et al. (67) to be conserved with respect to spinach NADP*-ferredoxin reductase, the three-dimensional structure of which was determined. Sequences highlighted using bars contain conserved residues important for FMN-, FAD-PPI-, and NADPH-FAD binding among various NADPH-cytochrome P-450 reductases and BMR, as described by Porter (62).
reductases exhibit 90% amino acid sequence identity among themselves (56), 78% to trout (53), and 33% to yeast enzymes (48). Sequence homology studies between the microsomal reductases have indicated five functional domains in these proteins (49, 57): the amino-terminal domain, which is required for membrane anchorage, and interaction with cytochrome P-450 (58, 59), and the domains involved in the binding of FAD, FMN, NADPH, and that of cytochrome P-450 (60, 61). Based on the homology of the NH2-terminal domain of the microsomal NADPH-cytochrome P-450 reductase with flavodoxin and that of its COOH-terminal domain with ferredoxin-NADP+ reductase, Porter suggested that the ancestral genes encoding these FMN- and FAD-containing flavoproteins fused to give rise to a gene encoding a fusion protein, viz. the cytochrome P-450 reductase (62).

The flavoprotein domain of cytochrome P-450BM3, BMR, is the only known prokaryotic protein that contains both FAD and FMN on a single polypeptide, in a stoichiometry of 1:1, and is functionally analogous to the microsomal oxidoreductase. Moreover, Ruettinger et al. (27) reported that this bacterial protein shares 33 and 40% amino acid sequence identity to the rabbit and rat hepatic microsomal NADPH-cytochrome P-450 reductases, respectively, with highly conserved segments likely to be involved in the binding of the flavins. Yet another bacterial flavoprotein that resembles the microsomal oxidoreductases is the NADPH-sulfite reductase (3). This enzyme, involved in the biosynthesis of cysteine from sulfate, is a multimeric protein (αβ2), with the α-subunits containing four molecules of FAD and FMN and the β-subunits containing a FeS4 cluster and a siroheme group. Even though the stoichiometry of the FAD and FMN is not 1:1 for all the molecules in the multimer, the amino acid sequence of the monomer is 30% identical to the microsomal reductase. In view of the structural and functional homology of these bacterial forms to the relevant eukaryotic enzymes, it is interesting to compare the salient features of the amino acid sequence of BMR to those of the mammalian forms.

Three-dimensional structure analysis of the Desulfovibrio vulgaris flavodoxin has shown that the amino-terminal residues form a loop surrounding the terminal phosphate group of FMN and that Trp-60 and Tyr-98 are involved in interactions with the isoalloxazine ring of the nucleotide (63). Sequence comparisons among several flavodoxins have confirmed the importance of these regions (64). Using a fluorescent cysteine probe and tryptic peptide mapping, it was shown that the region following the membrane-anchoring portion of the microsomal cytochrome P-450 reductase may be involved in the FMN binding of this protein (65). Porter and Kasper (60) aligned the rat liver microsomal reductase sequence to those of flavodoxins and hypothesized that Tyr-140 and Tyr-178 of the microsomal enzyme may be functionally equivalent to Trp-60 and Tyr-98 of the bacterial proteins. Site-directed mutagenesis of these aromatic residues in the mammalian enzyme altered FMN binding as well as NADPH-dependent cytochrome c reductase activities, confirming their functional importance (57). Ruettinger et al. (27) found two highly conserved regions, from Leu-12 to Glu-24 and Tyr-66 to Trp-104, among the bacterial and mammalian enzymes (Fig. 8, regions 1 and 2). Even though the three-dimensional structure of BMR is ultimately required to elucidate the importance of these regions for FMN binding, it may be predicted that region 1 could be involved in interactions with the terminal phosphate and region 2 may be essential for shielding the isoalloxazine ring. It is interesting to note that region 2 consists of 39 residues flanked by two aromatic amino acids, as it was the case for bacterial flavodoxin (64). Considering that tryptophan is a common replacement for tyrosine in the flavodoxins (66), Tyr-66 and Trp-104 may functionally correspond to Tyr-140 and Tyr-178 of the rabbit enzyme. Also, 18 of the 39 residues in region 2 of BMR are identical to the rabbit enzyme sequence flanked by these tyrosines.

In view of the above discussion, removal of the NH2-terminal 122-residue portion, containing these two regions in BMR, resulted in the loss of FMN (Table IV) and the activities dependent on its presence (Table III). Comparing the amino acid sequences of rat and yeast NADPH-cytochrome P-450 reductases with that of BMR, conserved residues were found to be mainly located into three domains. These domains have been identified by Porter (62) as the FMN-, the FAD-, and the NADPH-FAD-binding domains and are shown by bars in Fig. 8. Thus, BMR-52 unambiguously lacks a major part of the FMN-binding domain, containing amino acid residues important for interactions with FMN, especially Tyr-66 and Trp-104. The unaltered ferricyanide reductase activity BMR-52 (Table III) and the presence of 1 mol of FAD/mol of protein (Table IV) suggest that the amino-terminal portion of BMR is not obligatory for either FAD or NADPH binding. Karplus et al. (67) have determined the three-dimensional structure of spinach ferredoxin-NADP+ reductase to define the FAD- and NADP+-binding domains. While the FAD-binding domain has an antiparallel β-barrel core and a single α-helix, the NADPH-binding domain is shown to consist of a central five-strand parallel β-sheet surrounded by six helices. Regions important for these structural features (Fig. 8, regions 3-8) were observed to be conserved in several FAD-containing reductases, including eukaryotic cytochrome P-450 reductases and BMR (67). These, however, do not provide strong experimental evidence for a discrete multidomain structure of BMR, with an amino-terminal FMN-binding domain and carboxyl-terminal FAD- and NADPH-binding domains, in agreement with the fusion protein structure suggested by Porter (62).

In vitro studies on the interactions of the microsomal reductase with the cytochromes P-450 are complicated by their hydrophobicities and inherent tendencies to form oligomeric forms. Lipid-protein interactions occurring in the reconstructed system may potentially augment these complications (68, 69). Furthermore, unavailability of these proteins in large quantities limits extensive mechanistic investigations. Therefore, BMR, a soluble enzyme with structural and functional homologies to the microsomal flavoprotein, appears to be a promising model for better understanding the properties of the microsomal cytochrome P-450 reductase as well as the interactions of the flavoprotein with cytochrome P-450 for electron transfer. In this direction, we are currently characterizing the redox properties of flavins in BMR as well as its ability to transfer reducing equivalents to the cytochrome P-450 domain for fatty acid ω-hydroxylation.

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