The crystal structure of the complex between the heme and FMN-containing domains of Bacillus megaterium cytochrome P450BM-3 (Sevrioukova, I. F., Li, H., Zhang, H., Peterson, J. A., and Poulos, T. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1863–1868) indicates that the proximal side of the heme domain molecule is the docking site for the FMN domain and that the Pro382—Gln387 peptide may provide an electron transfer (ET) path from the FMN to the heme iron. In order to evaluate whether ET complexes formed in solution by the heme and FMN domains are structurally relevant to that seen in the crystal structure, we utilized site-directed mutagenesis to introduce Cys residues at positions 104 and 387, which are sites of close contact between the domains in the crystal structure and at position 372 as a control. Cys residues were modified with a bulky sulfhydryl reagent, 1-dimethylaminonaphthalene-5-sulfonate (dansyl)-L-cystine; DTT, di-thiothreitol; DRF, dRFH, the oxidized and semiquinone forms of 5-deazariboflavin; WT, wild type proteins; cyt c, cytochrome c; CcP, cytochrome c peroxidase.

Electron transfer (ET) processes between metallo-, hemo-, and flavoproteins require specific protein recognition for proper alignment of donor and acceptor molecules to achieve rapid electron flow. Despite the wealth of kinetic, thermodynamic, site-directed mutagenesis, and structural data on electron transferring enzymes, many questions in the area of biological ET remain unclear. For example, which chemical forces are most important for maintaining a functional protein-protein complex, how are electrons transferred from one redox center to another, and are the ET complexes observed in crystal structures functionally relevant?

Crystalization of redox partner complexes, such as cytochrome c peroxidase (CcP) and cytochrome c (cyt c) (1), methylene dehydrogenase and amicyanin (2), and the latter complex and cytochrome c551 (3), has provided valuable structural information about the interacting surfaces, orientation of cofactors, and possible pathways for the electron flow. The x-ray crystal structure of 1:1 complexes between yeast CcP and yeast and horse cyt c has revealed that the crystallographic CcP cyt c complex (1) differs significantly from the proposed model based on the complementarity of surface charges (4). With only a few hydrogen bonds at the interface, hydrophobic and van der Waals interactions are the predominant forces holding the two proteins together. The functional relevance of the binding orientation observed in the crystal structure is supported by mutagenesis and kinetic and chemical modification studies (5–7).

A single crystal polarized absorption study on binary and ternary complexes of methylene dehydrogenase with its redox partners demonstrated that both complexes are competent for substrate oxidation and ET (8). Also it was shown that the orientation of methylene dehydrogenase and amicyanin in the solution complex is the same as the one seen in the crystal structure (9). However, amicyanin binding to cytochrome c551 in solution appeared to occur at different sites when amicyanin is free or when it is in complex with methylene dehydrogenase (10).

Multidomain or multisubunit electron transferring proteins serve as examples of protein complexes where the interaction between partners is optimized by the covalent linkage of the domains. Several structures of multidomain electron transfer proteins are available (11–15). Among them, flavocytochrome b2 is the most intensively studied. Analysis of structural information on this enzyme has allowed the identification of structural elements and amino acid residues that are important in controlling ET from FMN to heme. The hinge region and one particular interface residue, Tyr143, were found to play an important role in modulation of interdomain ET (16–19). Flavocytochrome P450BM-3, a catalytically self-sufficient
fatty acid monoxygenases from *Bacillus megaterium* (20, 21), is an example of a multidomain protein in the superfamily of cytochrome P450 enzymes. Unlike other P450s, the heme domain of P450BM-3 is naturally fused with an FMN/FAD-containing reductase domain, which provides electrons required for P450 catalysis. Being a structural and functional analog to the microsomal P450s and P450 reductase, P450BM-3 is a very attractive model system for studying structure/function relationships and mechanism of ET in the P450-dependent monoxygenase system. Recombinant expression of functional domains of P450BM-3, including heme- (BMP), FMN/FAD-, FMN-, FAD-, and heme/FMN-binding fragments (22–27), has provided simplified systems and facilitated studies on domain-domain interaction in this complex enzyme. Since the FMN is the site donating electrons to the heme of P450, the heme/ FMN-binding domain (BMP/FMN) of P450BM-3 represents the simplest model for the electron transfer complex (28).

The heme domain of P450BM-3 has been structurally characterized. Both substrate-free and substrate-bound x-ray structures are available (29, 30). Recent crystallographic studies on BMP/FMN have provided information about the structure of the FMN domain and defined the interaction site between the flavin and heme domains (31). Although the linkage between the two domains was prototyped during crystallization, the manner of domain-domain interaction was consistent with previous studies on P450s. Moreover, the precise positioning of the FMN toward the heme-binding loop led to the conclusion that the complex between the FMN and heme domains is specific and that the Pro382—Cys400 peptide of BMP can provide an electron pathway from the flavin to the heme iron.

The present study is the first attempt to test the physiological relevance of the crystallographic complex. We have utilized laser flash photolysis techniques to examine the effect of chemical modification of specifically engineered cysteine residues of the heme domain of P450BM-3 on interdomain ET in both the reconstituted system consisting of the separate heme and FMN domains and intact BMP/FMN. The sterically bulky group of dansylalanine (DC) was covalently attached to the cysteines, which were introduced into the proximal side of the heme domain molecule to cause steric hindrance in the domain-domain interaction. The results indicate that the proximal side of the heme domain molecule in both separate BMP and intact BMP/FMN is the interaction site for the FMN domain and that the structure of the solution complex is consistent with that seen in the BMP/FMN crystal structure (31).

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents for bacterial growth were purchased from Difco, pProEX™ HTh vector, rTEV protease, and Fuso DNA polymerase were from Life Technologies, Inc. Restriction endonucleases and other modifying enzymes were purchased from Roche Molecular Biochemicals. Didansyl-l-cystine was from Molecular Probes. The Ni²⁺-nitrilotriacetic acid resin was obtained from Qiagen. Other chemicals were purchased from Sigma.

**Cloning and Site-directed Mutagenesis**—The 6-histidine tag fused cytochrome P450BM-3 holoenzyme and its heme-, FMN-, and heme/FMN-binding domains were cloned by PCR using the plT7 BM3 as the template (32). The 5'-oligonucleotide primers for the holoenzyme, the heme/FMN- and heme-binding domains were synthesized with an overhanging EcoRI restriction site, whereas the 5'-oligonucleotide for the FMN domain contained an overhanging EheI restriction site. The 3'-oligonucleotides for all fragments of P450BM-3 contained a stop codon followed by a KpnI restriction site. The PCR products were ligated to a pNEB-BMP, pNEB-FMN, and pNEB-FMN/BMP plasmid carrying the heme/FMN- and heme-binding domain, the 5'-oligonucleotide providing a polyA tail to the amino terminus of the heme domain were used in the first PCR amplification (underlines indicate mutated codons). Synthesized 0.2–0.5-kilobase pair fragments were ligated into pProEX/BMP digested with BglI. To build double mutants C156S/L104C, C156S/E572C, and C156S/E572C/L104C, the fragments for the C156S and L104C mutations were cloned into pProEX-BMP, respectively, and used as templates for amplification with the 5'-oligonucleotide for the amino terminus of the heme domain, and the 3'-oligonucleotide for the C156S/L104C or C156S/E572C/L104C mutation. To build a triple mutant C156S/L104C/E572C, the fragments for the C156S and L104C mutations were cloned into pProEX-BMP digested with BglI, and the 3'-oligonucleotide for the E572C mutation was used as template for amplification with the 3'-oligonucleotide for the heme-binding loop led to the conclusion that the proximal side of the crystallographic complex. We have utilized laser flash photolysis techniques to examine the effect of chemical modification of specifically engineered cysteine residues of the heme domain of P450BM-3 on interdomain ET in both the reconstituted system consisting of the separate heme and FMN domains and intact BMP/FMN. The sterically bulky group of dansylalanine (DC) was covalently attached to the cysteines, which were introduced into the proximal side of the heme domain molecule to cause steric hindrance in the domain-domain interaction. The results indicate that the proximal side of the heme domain molecule in both separate BMP and intact BMP/FMN is the interaction site for the FMN domain and that the structure of the solution complex is consistent with that seen in the BMP/FMN crystal structure (31).
Spectral and redox properties were similar to those of previous expression of the properly folded 479–635 FMN domain, whose amino acid carboxyl-terminal truncation did not affect either spectral or substrate binding properties of BMP/FMN. Similar amino acid carboxyl-terminal truncation did not affect either shorter 1–635 BMP/FMN domain of P450BM-3. Fourteen carboxyl-terminal residue to produce the 6-histidine tag fused construct was very low. A five amino acid extension of the carboxyl-terminal residue to produce the 6-histidine tag fused for the PCR reaction. Surprisingly, the expression of the 479–

The heme and flavin cofactors and residues that were inaccessible hydrophobic heme pocket (29). Cys62 is located close to the surface of the heme domain and has 8.62 Å² and 0.96 Å² solvent-accessible surfaces in BMP and BMP/FMN, respectively. However, Cys62 has never been found to interact with the heavy atoms during preparation of the heavy atom derivatives of the crystals of either the heme domain or BMP/FMN. In contrast, Cys156, whose solvent-accessible surfaces are equal to 2.3 Å² and 1.7 Å² in BMP and BMP/FMN, respectively, was found to be easily derivatized with mercury compounds during short time soaks of both BMP and BMP/FMN crystals. For this reason, the highly reactive cysteine 156 was replaced by serine in BMP, BMP/FMN, and the holoenzyme of P450BM-3. The C156S mutation did not affect the spectral and substrate binding properties and rates of NADPH oxidation and cyt c reduction by the holoenzyme (data are not shown). Therefore, C156S mutants can be considered as control proteins in the studies described below.

Three single cysteines were introduced in the heme-binding domains of different constructs at positions 104, 372, and 387 to produce the double mutants C156S/L104C, C156S/E372C, and C156S/Q387C. In the x-ray structure (Fig. 1), residues Leu104 and Gln387 were found to be at the interface between the heme and flavin domains of P450BM-3. Glu372, located on the side of the heme domain that does not contact the FMN domain, was replaced by cysteine as a control. The double mutants of BMP, BMP/FMN, and the holoenzyme of P450BM-3 incorporated heme and had UV-visible absorption spectra similar to those of the WT proteins. The addition of an excess of arachidonic acid to the mutated hemoproteins resulted in a near complete conversion of the heme iron to the high spin form. The NADPH oxidation and cyt c reduction activities of the double mutants of P450BM-3 were similar to those of the WT holoenzyme (data are not shown).

Modification of C156S/L104C, C156S/E372C, and C156S/Q387C Mutants with Didansylcysteine—Optimal conditions for sulphydryl group modification were found by varying the molar ratio of DDC to protein and incubation time. Fluorescence spectra of free DC and DC-modified double mutants of BMP and BMP/FMN are shown in Fig. 2. Binding to the proteins was accompanied by an enhancement of the dansyl group fluorescence and a characteristic blue shift in the excitation from 535 to 495 nm. The 2-fold higher fluorescence yield of DC bound to BMP/FMN compared with that bound to BMP (Fig. 2B) is indicative of a more hydrophobic environment of the fluorescence probe in the intact heme/FMN domain, most likely because of the presence of the covalently linked flavin domain.

The specificity and stoichiometry of SH group modification were determined by addition of DTT to the labeled proteins. Loss of more than 85% of fluorescence within a few minutes due to bound DC upon DTT treatment (Fig. 2, spectra 5–7) indicates that fluorescent label binds specifically to the sulphydryl groups of the proteins. The estimation of the fluorescence intensity of free DC released from the proteins after DTT reduction enabled us to determine the molar ratio of the fluorescent probe to protein, which was near stoichiometric and varied from 0.8 to 1.2 for many experiments with various mutants. To check the possibility of interaction of DDC with cysteine 62, C156S mutants of BMP and BMP/FMN were incubated with the label under the same conditions as the double mutants. There was partial labeling of Cys62 in both proteins (Fig. 2, spectra 4). Calculation of the amount of the released label after addition of DTT to C156S mutants of BMP and BMP/FMN showed that less than 15% of the cysteines reacted with DDC. DC-labeled C156S mutants were used as controls in all experiments. The sulphydryl group modification did not affect the substrate bind-

Fig. 1. Complex between the heme and FMN domains of P450BM-3 (31). The heme and flavin cofactors and residues that were mutated to cysteines are shown in bold.
Electron Transfer in P450BM-3

The reaction solution contained 4 μM DC or 4 μM proteins in 20 mM phosphate buffer, pH 7.4. Cell path length was 1 cm. The spectra, recorded at room temperature with excitation at 330 nm, are as follows: I, C156S mutant in the absence of substrate (substrate-free and substrate-present); 2–5, C156S, C156S/L104C, C156S/E372C, and C156S/Q387C mutants, respectively, in the presence of 20 μM arachidonic acid.

![Fluorescence spectra of free DC and DC-modified heme (A) and heme/FMN (B) domains of cytochrome P450BM-3.](Image)

**Fig. 2.** Fluorescence spectra of free DC and DC-modified heme (A) and heme/FMN (B) domains of cytochrome P450BM-3. The reaction solution contained 4 μM DC or 4 μM proteins in 20 mM phosphate buffer, pH 7.4. Cell path length was 1 cm. The spectra, recorded at room temperature with excitation at 330 nm, are as follows: I, C156S mutant in the absence of substrate (substrate-free and substrate-present); 2–5, C156S, C156S/L104C, C156S/E372C, and C156S/Q387C mutants, respectively, in the presence of 20 μM arachidonic acid.

![Absorption spectra of DC-modified BMP/FMN mutants.](Image)

**Fig. 3.** Absorption spectra of DC-modified BMP/FMN mutants. The sample cuvette contained 1.2–1.4 μM proteins in 20 mM phosphate buffer, pH 7.4. The spectra, recorded at room temperature, are as follows: I, C156S mutant in the absence of substrate (substrate-free and substrate-present); 2–5, C156S, C156S/L104C, C156S/E372C, and C156S/Q387C mutants, respectively, in the presence of 20 μM arachidonic acid.

![Time-resolved laser-flash photolysis difference spectrum for the reduction of the FMN domain of P450BM-3.](Image)

**Fig. 4.** Time-resolved laser-flash photolysis difference spectrum for the reduction of the FMN domain of P450BM-3. The reaction solution contained 100 mM phosphate buffer, pH 7.0, 100 μM dRF, 2 mM semicarbazide, and 3 μM protein. The net absorbance change at 10 ms following a laser flash was measured at 10-nm intervals and plotted.

The wavelengths of 380 and 475 nm were chosen to monitor the formation and disproportion of the anionic FMN semiquinone, FMN\textsuperscript{-}, and the extent of reduction of the flavin of the FMN domain, respectively. The plots of $k_{\text{obs}}$ versus the FMN domain concentration for the FMN reduction by dRFH measured at pH 6.0, 7.0, and 8.0 versus the FMN domain concentration were similar and appeared to be hyperbolic suggesting that dRFH forms a pre-reaction complex with the FMN domain (Fig. 5A). A hyperbolic fit for all three data sets had a limiting value of $2.9 \times 10^{4}$ s\textsuperscript{-1} for $k_{\text{obs}}$ at infinite [FMN domain] and a dissociation constant, $K_d$, of 13.1 μM for the interaction of the free dRFH with the FMN domain.

Because of the long time scale (>2 s) and the weak signal at 380 nm, the disproportional reaction of FMN\textsuperscript{-} to form the fully oxidized and fully reduced FMN was difficult to study. However, it should be pointed out that, under all studied conditions, there was no detectable protonation of the FMN\textsuperscript{-} and consequent formation of the blue, neutral FMN semiquinone, FMNH\textsuperscript{-}; in the reaction mixture. This is in agreement with the structural data (31) that indicate that the FMN-binding site of the FMN domain of P450BM-3 is different from that of flavodoxins and microsomal P450 reductase in that it does not have structural elements required for the formation and stabilization of FMNH\textsuperscript{-}. Taken together, we conclude that FMN\textsuperscript{-}, the only semiquinone form produced upon 1-electron reduction of the flavin in the FMN domain under physiological conditions, is the species that donates electrons to the heme iron.
of P450BM-3.

Reaction of the heme domain of P450BM-3 with dRFH was carried out in the presence of arachidonic acid and carbon monoxide. The CO complex formation was monitored at 460 nm. Co binding by reduced BMP was previously shown to be fast and proceed with a second order rate constant of 4 × 10^6 M^-1 s^-1 (40). In our reaction system with saturating concentrations of carbon monoxide (approximately 1 mM at room temperature), the rate of CO complex formation was limited by the slower BMP reduction and, thus, reflected the rates of the inter- or intramolecular ET between FMN^− and oxidized heme. dRFH was capable of reducing the separate heme domain of P450BM-3 with k_{obs} values ranging from 1,000 to 2,400 s^-1 within the studied concentration range. The non-linear plot of k_{obs} versus the BMP concentration was fit to a hyperbolic equation to obtain a limiting value of 2,660 s^-1 for k_{obs} and a K_d of 1.03 μM for the interaction of the free dRFH with the heme domain (Fig. 5B). It is important to point out that the K_d and k_{obs} limiting values for direct reduction of BMP by dRFH are an order of magnitude lower than those for the FMN domain.

Laser Flash-induced Reduction of the Mixture of the WT Heme and FMN Domains—In the system containing separate heme and FMN domains of P450BM-3, the flavin domain was present in excess and was reduced by dRFH first. The FMN reduction was monitored at 475 nm due to flavin reduction was two times larger in 10 mM phosphate buffer, heme reduction appeared to be biphasic with the rate constant for the fast phase an order of magnitude larger than that measured in 10 mM phosphate buffer (Table I) (Fig. 6). It should be noted that the rate constants for BMP reduction were comparable with those for FMN reoxidation. In addition, the reduction of BMP in the reconstituted system was 2 orders of magnitude slower than its direct reduction by dRFH. Taken together, these results demonstrate that in the reconstituted system BMP receives electrons from the reduced FMN domain and not directly from dRFH. The higher rates of the intermolecular ET between the FMN and heme domains at low ionic strength suggest that electrostatic interactions are important for complex formation and ET between the two proteins. For this reason, the study of the effect of DC modification on the interdomain ET was carried out in 10 mM phosphate buffer.

Effect of DC Modification on the Heme Domain Reduction in the Reconstituted System—Kinetic parameters of the reactions of FMN reoxidation and heme reduction in the reconstituted system consisting of the separate FMN domain and the WT and DC-labeled cysteine mutants of BMP are compared in Table II. Similar to WT and C156S BMP, up to 90% of DC-modified C156S/E372C and C156S/Q387C mutants of BMP were reduced in the fast phase. However, the rate constants for reduction of these proteins were two times smaller than WT. The attachment of a dansyl group to cysteine 104 had the most dramatic effect on the reduction of the hemoprotein by the FMN domain. Less than 60% of the mutant was reduced in the fast phase. Moreover, the rate constants for reduction of C156S/L104C BMP in both phases were an order of magnitude smaller than those for WT and C156S BMP. It is important that the unmodified C156S/L104C mutant was reduced by the flavin domain in a manner similar to that of WT, indicating that the mutation itself does not affect interdomain ET. The plots of k_{obs} for the fast phase of the heme domain reduction in the reconstituted system versus BMP concentration were linear for all heme domain mutants within the concentration range studied, indicating a bimolecular process (Fig. 7).

Effect of DC Modification on the Heme Reduction in the Intact Heme/FMN Domain—Similar to the nonlinear plots of k_{obs} versus the concentration of the separate FMN domain, the analogous plots for the flavin reduction in BMP/FMN in the absence and presence of substrate appeared to be hyperbolic (Fig. 5A). In the absence of substrate, the accessibility of the FMN for interaction with dRFH in BMP/FMN was decreased by the covalently tethered heme domain. At high protein concentrations, compared with the separate FMN domain, the FMN reduction rates in BMP/FMN were approximately 30% slower. A limiting rate constant of 1.4 × 10^4 s^-1 for reduction of the FMN prosthetic group and an apparent K_d of 4.8 μM were calculated from a hyperbolic plot. The presence of arachidonic acid in the reaction mixture not only completely restored the ability of the FMN in BMP/FMN to accept electrons from
Rate constants were determined by the laser flash photolysis technique at room temperature. The reaction mixture contained 100 μM dRF, 2 mM semicarbazide, 11 μM FMN domain, 100 μM arachidonic acid, CO-saturated 10 or 100 mM phosphate buffer, pH 7.0, and different concentrations of BMP. The FMN reduction and reoxidation and the CO-reduced BMP complex formation were followed by an absorbance change at 475 and 460 nm, respectively. Data were analyzed using KINFIT (OLIS Co., Jefferson, GA).

| BMP concentration | FMN reduction | FMN reoxidation | CO complex formation |
|-------------------|---------------|-----------------|----------------------|
| 100 mM phosphate  | 4.0 ± 0.5a    | 0.8 ± 0.1       | 1.1 ± 0.1            |
| 0.8               | 9.1 ± 0.5     | 0.7 ± 0.1       |                      |
| 1.8               | 9.4 ± 0.6     | 0.5 ± 0.1       | 1.3 ± 0.4            |
| 5.5               | 9.2 ± 0.8     | 0.6 ± 0.1       | 1.3 ± 0.2            |

Fast phase % | Slow phase % | Fast phase % | Slow phase %
0.2 | 50 1.0 | — | —
0.9 | 43 1.3 | — | —
1.0 | 55 1.3 | — | —
1.6 | 48 1.1 | — | —
5.0 | 41 1.3 | 23.7 ± 3.8 | 60 1.3 ± 0.1 40

* The results are the means of two to three experiments.

** Not present.

The percentage of the protein reduced in the fast or slow phase of the reaction.

**Fig. 6. Transient kinetics observed upon laser flash excitation of the solution containing both the FMN- and heme-binding domains of P450BM-3. A and B, transients obtained at 475 nm to follow the FMN domain reduction (A) and reoxidation (B). C, transient obtained at 460 nm to monitor reduced BMP-CO complex formation. The smooth lines represent single and double exponential fits giving rate constants of 13,000 s⁻¹ (A), 18.0 and 3.0 s⁻¹ (B), and 16.0 and 3.4 s⁻¹ (C). The reaction mixture consisted of 19 μM FMN domain, 2.6 μM WT BMP, 100 μM dRF, 2 mM semicarbazide, and 100 μM arachidonic acid in CO-saturated 10 mM phosphate buffer, pH 7.0.

dRFH but also promoted ET. The limiting rate constant was increased almost 2-fold to 2.3 × 10⁴ s⁻¹ whereas Kₐ, 5.1 μM, was not significantly different from that in the absence of arachidonate. The data indicate that the conformational change caused by substrate binding to the heme domain alters the solvent exposure of the FMN in BMP/FMN and the mode of interaction between the domains. Thus, the solution complex between the flavin and heme domains is likely to be flexible and may undergo conformational changes during catalysis. Furthermore, the possibility should not be ruled out that binding of arachidonic acid to BMP/FMN also could promote dRFH to FMN ET by affecting the FMN redox potential.

The effect of DC modification on the interdomain ET in BMP/FMN mutants was studied in the presence of arachidonic acid. It is important to note that the reduction of the FMN by dRFH was not significantly affected by DC modification in any of the BMP/FMN mutants (data are not shown). The kinetics of the CO complex formation with the reduced heme iron in the intact heme/FMN domain were similar for WT, the DC-modified C156S, C156S/E372C, and C156S/Q387C mutants, and the unmodified C156S/L104C mutant of BMP/FMN (Fig. 8). Furthermore, the ET reactions from the FMN to the heme iron were found to be monophasic. Although an intramolecular ET reaction is expected to be independent of protein concentration, the plot of kobs versus [BMP/FMN] for the heme reduction in BMP/FMN is hyperbolic. Considering the hyperbolic character of the kinetics of the FMN reduction by dRFH in either the separate FMN domain or in the intact BMP/FMN (Fig. 5A), we assume that the hyperbolic dependence of kobs of the intramolecular FMN to heme ET is a consequence of the preceding reaction, i.e. FMN reduction. It should be mentioned that there was a direct correlation between the rate constants of FMN⁺ oxidation and the heme reduction/CO binding which demonstrates the occurrence of an intramolecular ET.

As seen from the Fig. 8, the rate of interdomain ET was dramatically diminished in the DC-modified C156S/L104C mutant of BMP/FMN. Both the reactions of FMN reoxidation and heme reduction of this protein appeared to be biphasic (Table II) and were 15 to 20 times slower than those observed for the WT and the other modified cysteine mutants of BMP/FMN. These results are in accord with the kinetic data on the reduction of the separate DC-labeled C156S/L104C BMP by the FMN domain in the reconstituted system and demonstrate that residue 104 is a part of the interface in the ET complex between the heme and flavin domains of P450BM-3 formed in solution.

**DISCUSSION**

A recent determination of the structure of the complex between the heme and FMN domains of P450BM-3 (31) was
the present study, we have defined the minimum length of the domains of P450BM-3, produced properly folded proteins. In the putative linker between the FMN- and FAD-binding heme/FMN domain, 1–649 and 1–664 (25, 26), containing part of the FMN domain, 471–649 and 471–664, and the N-terminal residue, whereas the choice of the carboxyl-terminal residue was different and appeared to affect the ability of the FMN domain of P450BM-3 with flavodoxins and homologous reductases, and what could be the electron pathway from the FMN to the heme iron. In the complex, the flavin domain interacts with the proximal side of the BMP molecule and forms contacts with the methyl groups of the FMN toward the heme-binding loop, with previous studies on P450s, and the precise positioning of the Pro382–Gln387 peptide that precedes the heme-binding loop, led to the conclusion that the crystallographic complex between the heme and FMN domains of P450BM-3 (Fig. 1) provided detailed information about the arrangement of cofactors and the domain-domain interaction. It also provides the first structural insights into the main interface. It also provides the first structural insights into the main interface. It also provides the first structural insights into the main interface. It also provides the first structural insights into the main interface.

Second, the crystallographic complex between the heme and FMN domains of P450BM-3 (Fig. 1) provided detailed information about the arrangement of cofactors and the domain-domain interaction. It also provides the first structural insights into how P450s may interact with their reox partner, P450 reductase, and what could be the electron pathway from the FMN to the heme iron. In the complex, the flavin domain interacts with the proximal side of the BMP molecule and forms contacts with the residues from the C- and L-helices, His100 and Asn101, and with previous studies on P450s, and the precise positioning of the Pro382–Gln387 peptide that precedes the heme-binding loop, led to the conclusion that the crystallographic complex between the heme and FMN domains of P450BM-3 is specific (31).

**Designing of Cysteine Mutants**—To test this model, we attempted to modify the complex interface by introducing a sterically bulky group on the surface of the heme domain molecule to hinder sterically complex formation. For this purpose, we
designed a series of C156S/35 mutants of BMP, with previous studies on P450s, and the precise positioning of the methyl groups of the FMN toward the heme-binding loop, led to the conclusion that the crystallographic complex between the heme and FMN domains of P450BM-3 is specific (31).

**Comparison of the kinetic parameters of the FMN reoxidation and the CO-reduced heme domain complex formation in the reconstituted system consisting of the separate FMN domain and the WT or DC-modified cysteine mutants of BMP**

| Heme domain concentration | FMN reoxidation Rates | CO complex formation Rates |
|----------------------------|-----------------------|----------------------------|
|                            | Fast phase  | Slow phase |                            | Fast phase  | Slow phase |
|                            | %          | %          |                            | %          | %          |
|                            | s⁻¹        | s⁻¹        |                            | s⁻¹        | s⁻¹        |
| 0.8                        | 13.0 ± 1.0a | 2.1 ± 0.2 | 66                        | 15.6 ± 1.2 | 3.6 ± 0.7 | 60 |
| 2.6                        | 20.0 ± 1.8 | 3.3 ± 0.5 | 45                        | 17.6 ± 1.7 | 3.4 ± 0.6 | 37 |
| 5.7                        | 28.3 ± 2.0 | 4.5 ± 0.8 | 22                        | 30.7 ± 0.7 | 5.0 ± 1.9 | 15 |
| 9.1                        | 56.0 ± 14.0 | 5.8 ± 1.2 | 16                        | 65.5 ± 9.0 | 10.0 ± 0.3 | 17 |
| 14.8                       | 74.8 ± 5.1 | 100       |                            | 85.3 ± 3.1 | 23.0 ± 2.0 | 12 |

a Percentage of the heme domain reduced in the fast or slow phase of the reaction.

b The results are the means of two to four experiments.

Not present.
However, studies of the redox properties of the FMN
was found to be pH-independent within the
range to be measured. In the present paper, for the first time, we have obtained the redox difference spectrum of the FMN domain (25, 26). The reduction potentials of the FMN in the separate FMN domain and the holoenzyme of P450BM-3 were estimated, and the positive reduction potential difference in the FMN semiquinone/hydroquinone couple was detected (49). This confirmed that the FMN hydroquinone is more thermodynamically stable than the semiquinone form. The thermodynamic instability of the semiquinone form and the lack of 1-electron reductants for the FMN precluded studies on the characterization of the intermediate species produced during reduction of the FMN domain. Laser flash photolysis studies on the 1–664-truncated heme/FMN-containing domain (BM$_{z}$P450BM-3 (28) have demonstrated that dRFH can efficiently reduce FMN to the semiquinone state. Spectral changes observed upon reduction were consistent with the formation of FMN$^*$ but not FMNH$^-$. However, the strong absorbance of the heme in the 400 nm region did not allow the flavin-reduced minus oxidized difference spectrum for BM$_{z}$ in the 350–450 nm range to be measured. In the present paper, for the first time, we have obtained the redox difference spectrum of the FMN domain (Fig. 4) which clearly shows that upon 1-electron reduction this protein forms a red, anionic FMN semiquinone. The formation of FMN$^*$ was found to be pH-independent within the pH range from 6 to 8. The inability of the FMN domain of P450BM-3 to produce and stabilize the FMNH$^-$; a unique feature among the flavodoxin-like proteins, has been found to be defined by the structure of the FMN-binding site of the protein (31).

**Effect of DC Modification on the Interdomain ET**—The effect of DC modifications on the ET of the holoenzyme of P450BM-3 was monitored at 460 nm. Solid lines represent hyperbolic fits to the data.}

![Image](64x570 to 282x729)

**FIG. 7. Plots of $k_{obs}$ versus [BMP] for the reduction of the WT and DC-modified BMP/FMN mutants by the FMN domain in the fast phase of the reaction.** ○, WT; □, C156S; ▼, C156S/E372C; ▼, C156S/Q387C; ■, C156S/L104C; □, unmodified C156S/L104C. The reaction buffer was CO-saturated 10 mM phosphate buffer, pH 7.0, containing 100 $\mu$M dRF, 2 mM semicarbazide, 100 $\mu$M arachidonic acid, and 16–19 $\mu$M FMN domain. Kinetics were monitored at 460 nm. Solid lines are linear fits to the data.

![Image](67x333 to 279x487)

**FIG. 8. Plots of $k_{obs}$ versus [BMP/FMN] for the reduction of the WT and DC-modified BMP/FMN mutants.** The heme reduction-CO complex formation was monitored at 460 nm. ○, WT; □, C156S; ▼, C156S/E372C; ▼, C156S/Q387C; ■, C156S/L104C; □, unmodified C156S/L104C. The reaction buffer was CO-saturated 10 mM phosphate buffer, pH 7.0, containing 100 $\mu$M dRF, 2 mM semicarbazide, and 100 $\mu$M arachidonic acid. Solid lines represent hyperbolic fits to the data.

replaced the natural Cys$^{156}$ with Ser and introduced novel cysteines on the surface of the heme domain in BMP and BMP/FMN at positions 104, 372, and 387, and we chemically modified them by the covalent attachment of the fluorescent dansyl group on the protein surface. DCC modification and ease of monitoring and evaluating the extent of DC labeling make dansylation an attractive tool for introducing a sterically bulky fluorescent group at desired sites in the protein surface.

**Redox Active Moiety of FMN**—Although the kinetics and thermodynamics of the transient intermediates in the catalytic cycle of P450BM-3 have been reported, there is no general agreement on the precise catalytic mechanism. Recent studies of the P450BM3 by stopped-flow spectrophotometry, it was proposed that the anionic FMN semiquinone is the species that donates electrons to the heme iron (46). Later, the red, anionic flavin semiquinone formed during the catalytic cycle of the holoenzyme of P450BM-3 was concluded to be FAD$^-$. However, studies of the redox properties of the individually expressed FMN- and FAD-binding domains of P450BM-3 (25, 26) disproves the latter conclusion by showing that the FAD domain was producing exclusively the blue, neutral semiquinone upon reduction by either NADPH or sodium dithionite. Titration of the FMN domain with sodium dithionite resulted in the conversion of the protein to the fully reduced FMN without accumulation of the intermediate semiquinone forms (25, 26). The reduction potentials of the FMN in the separate FMN domain and the holoenzyme of P450BM-3 were estimated, and the positive reduction potential difference in the FMN semiquinone/hydroquinone couple was detected (49). This confirmed that the FMN hydroquinone is more thermodynamically stable than the semiquinone form. The thermodynamic instability of the semiquinone form and the lack of 1-electron reductants for the FMN precluded studies on the characterization of the intermediate species produced during reduction of the FMN domain. Laser flash photolysis studies on the 1–664-truncated heme/FMN-containing domain (BM$_{z}$) of P450BM-3 (28) have demonstrated that dRFH can efficiently reduce FMN to the semiquinone state. Spectral changes observed upon reduction were consistent with the formation of FMN$^*$ but not FMNH$^-$. However, the strong absorbance of the heme in the 400 nm region did not allow the flavin-reduced minus oxidized difference spectrum for BM$_{z}$ in the 350–450 nm range to be measured. In the present paper, for the first time, we have obtained the redox difference spectrum of the FMN domain (Fig. 4) which clearly shows that upon 1-electron reduction this protein forms a red, anionic FMN semiquinone. The formation of FMN$^*$ was found to be pH-independent within the pH range from 6 to 8. The inability of the FMN domain of P450BM-3 to produce and stabilize the FMNH$^-$; a unique feature among the flavodoxin-like proteins, has been found to be defined by the structure of the FMN-binding site of the protein (31).

**Effect of DC Modification on the Interdomain ET**—The effect...
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| Table III | Kinetic parameters of the reduction of the DC-modified C156S/L104C mutant of BMP/FMN by dRFH |

Rate constants were determined by the laser flash photolysis technique at room temperature. The reaction mixture contained 100 μM dRF, 2 mM semicarbazide, 100 μM arachidonic acid, CO-saturated 10 mM phosphate buffer, pH 7.0, and different concentrations of DC-modified C156S/L104C mutant of BMP. The FMN reoxidation and the CO-reduced BMP complex formation were followed by an absorbance change at 475 and 460 nm, respectively. Data were analyzed using KINFIT (OLIS Co., Jefferson, GA).

| BMP/FMN concentration | FMN reoxidation | CO complex formation |
|-----------------------|-----------------|---------------------|
| μM  | Fast phase | Slow phase | % | Fast phase | Slow phase | % |
| 2.3 | 14.8 ± 5.0 | 58 | 3.2 ± 0.9 | 42 | 20.7 ± 0.3 | 56 | 3.6 ± 0.1 | 44 |
| 3.8 | 23.5 ± 6.0 | 55 | 2.2 ± 0.2 | 45 | 25.7 ± 9.0 | 55 | 4.2 ± 1.6 | 45 |
| 8.9 | 33.0 ± 10.0 | 56 | 2.7 ± 1.2 | 44 | 25.0 ± 8.0 | 62 | 3.9 ± 1.9 | 38 |
| 13.6 | 41.0 ± 11.0 | 50 | 4.2 ± 1.4 | 50 | 28.0 ± 7.0 | 60 | 4.7 ± 1.0 | 40 |

a Percentage of the heme domain reduced in the fast or slow phase of the reaction.

b The results are the means of two to four experiments.

d of DC modification of specific cysteine residues of the heme domain of P450BM-3 on the domain-domain interaction was evaluated by measuring the kinetics of ET from the FMN to the heme iron in the system consisting of either separate heme and FMN domains or intact BMP/FMN, utilizing the laser flash photolysis technique. This methodology has proven to be an excellent tool for studying both intermolecular ET between different redox partners and intramolecular ET in multicenter redox proteins (50–57).

Studies on the separate FMN- and heme-binding domains of P450BM-3 have shown that both proteins can be easily reduced by dRFH. Direct second order reduction of the FMN or heme by dRFH was expected to yield linear plots of k_{obs} versus [protein]. However, this was not the case for either the FMN domain or BMP (Fig. 5). The approach of k_{obs} to limiting values suggests that, at higher protein concentrations, dRFH was capable of forming complexes with both domains of P450BM-3. It has been shown previously that FMN could directly interact with the heme domain and, in the presence of NADPH, transferred electrons and supported fatty acid hydroxylation (58). It is likely that dRFH interacts with the same site of BMP. In the flavin domain, the FMN-binding site could be a potential site for interaction with dRFH whose isoalloxazine ring could form a complex with the coplanar and partly exposed aromatic rings of the FMN and Trp574 (31). Indeed, in the absence of substrate in BMP/FMN, where the FMN-binding site is buried in the interface between the two domains, both the interaction with the protein and the ET from dRFH to FMN were perturbed.

There was no apparent complex formation between dRFH and the heme domain in the system reconstituted with the FMN domain. In these experiments, the flavin domain was present in excess and was reduced first. The biphasic kinetics of BMP reduction by the FMN domain in the reconstituted system at low ionic strength might be due to the existence of multiple FMN domain-binding sites on the surface of the heme domain. Appearance of the fast phase in the reaction of the heme reduction at lower ionic strength indicates that electrostatic forces are important for complex formation between the heme and FMN domains, and that the electrostatically stabilized complex is more effective in the FMN to heme ET. Although only one salt bridge was found in the 967-Å² area of interface between the two domains in the x-ray structure, the surfaces that interact were electrostatically complementary with the overall molecular dipoles oriented for maximum stability (31). Even at saturating concentrations of the FMN domain, reduction of the heme domain in the reconstituted system was an order of magnitude slower than in the intact heme/FMN domain. This indicates that the interaction between separate domains is not strong and is greatly facilitated by the covalent linkage in BMP/FMN. A similar conclusion was made during fluorometric studies on domain-domain interactions in P450BM-3 (59) and during analysis of the interaction site in the crystal structure of the complex (31).

The rate constants for the heme reduction in BMP/FMN measured in the present study were 2-fold higher than those determined previously for the 1–664-heme/FMN-containing fragment of P450BM-3 (28). The reasons for this could be the shorter (Δ29) length of the present heme/FMN-binding domain, which excludes the interference of the carboxyl-terminal peptide with the domain-domain interaction, and the lower ionic strength of the reaction mixture, which appeared to facilitate the interaction of the two domains. Also, using arachidonic, rather than myristic (28) acid as a substrate results in a larger conversion of the low to high spin state of the heme iron.

According to the analysis of intraprotein ET developed from ET measurements both in biological and in chemical systems, the interdomain ET rate constant of 100–500 s⁻¹ observed in BMP/FMN in the present study corresponds to an approximately 20-Å edge-to-edge distance between the FMN and heme (60). This is in a good agreement with the crystallographic complex, where the distance between the flavin and the heme iron was shown to be 18.4 Å. Therefore, the conformational changes in BMP/FMN that occur upon substrate binding probably are not large and do not result in an appreciable change in the separation of the cofactors.

The covalent attachment of the hydrophobic, sterically bulky dansyl group to cysteines of the BMP mutants altered to different extents the ability of the heme domain to interact with and accept electrons from the FMN domain (Fig. 7). Dansylation of Cys387 on the proximal side of BMP had the same effect as modification of the control Cys372. The reduction of both these mutants by the FMN domain was 2-fold slower than the WT. In contrast, DC modification of analogous cysteines in BMP/FMN did not perturb the interdomain ET rate. Modification of cysteine 104 had the most dramatic effect on the rates of the FMN to heme ET in both the reconstituted system and BMP/FMN. Heme reduction of the dansylated C156S/L104C mutant was 10–20 times slower compared with the WT. In accord with the structural data, these results demonstrate that the proximal side of the BMP molecule is the interaction site with the FMN domain. Although the backbone of Glu587 is the closest approach for the flavin in the crystals, the side chain of this residue is solvent-accessible. It is, therefore, possible that the dansyl group on Cys587 is not oriented toward the FMN domain surface and does not cause steric hindrance for the FMN domain binding. In contrast, residue 104 is buried at the interface, and its dansyl group cannot be accommodated at the interprotein interface without causing a significant conformational change.

In summary, a structural model of the ET complex between

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Data were analyzed using KINFIT (OLIS Co., Jefferson, GA).

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In summary, a structural model of the ET complex between
the heme and FMN domains of P450BM-3 has been tested. Three residues on the heme domain surface were mutated to cysteines and chemically modified. DC modification of Cys

was the most critical for the interdomain ET in both the reconstituted system and the intact heme/FMN domain. Dansylation of residues 372 and 387 had only moderate effects on the BMP reduction and did not affect ET in BMP/FMN. Although the results do not prove that the orientation of domains in the crystal structure is exactly the same as in ET complex in solution, the present study does demonstrate that the proximal side of the heme domain molecule in both separate BMP and intact BMP/FMN is the interaction site for the FMN domain and that the structure of the ET complex in solution is consistent with that seen in the crystalline complex.

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