Cytochromes P450 utilize redox partners to deliver electrons from NADPH/NADH to the P450 heme center. Microsomal P450s utilize an FAD/FMN reductase. The bacterial fatty acid hydroxylase, P450BM-3, is similar except the P450 heme and FAD/FMN proteins are linked together in a single polypeptide chain arranged as heme-FMN-FAD. Sequence comparisons indicate that the P450BM-3 FMN and FAD domains are similar to flavodoxin and ferredoxin reductase, respectively. Previous work has shown that the heme and FMN/FAD domains can be separately expressed and purified. In this study we have expressed, purified, and characterized the following additional domains: heme-FMN, FMN, and FAD. Each domain retains their prosthetic groups although the FMN domain is more labile. The FAD domain retains both FAD and heme but not FMN. We also have investigated the dimerization pattern of the individual domains that lead to the following conclusions. Holo-P450BM-3 appears to dimerize via interactions that do not involve disulfide bond formation, whereas the reductase and FAD domains form intermolecular disulfides. This indicates that the Cys residues not available for dimerization in holo-P450BM-3 are unmasked in the individual domains.

Cytochrome P450s are heme containing monoxygenases that catalyze the hydroxylation of a vast array of hydrocarbons as shown in Reaction 1.

\[ R - H + O_2 + \text{NADPH} \text{ or NADH} + H^+ \rightarrow R - OH + H_2O + \text{NADP}^+ \text{ or NAD}^+ \]

**REACTION 1**

P450s can be classified according to the type of electron transfer system utilized to deliver electrons from NADPH or NADH to the P450 heme. Type I bacterial and mitochondrial P450s normally require two proteins to transport electrons from reduced pyridine nucleotide to the P450 heme as follows.

\[ \text{FAD protein} \rightarrow (\text{Fe} - S)_2 \text{ protein} \rightarrow \text{P450} \]

**REACTION 2**

In contrast, type II microsomal P450s utilize a single FAD/FMN reductase to deliver electrons to the P450.

\[ \text{FAD/FMN protein} \rightarrow \text{P450} \]

**REACTION 3**

The fatty acid monooxygenase, cytochrome P450BM-3 from *Bacillus megaterium*, is the first bacterial P450 found to belong to the microsomal class of P450s in that P450BM-3 uses a FAD/FMN reductase to deliver electrons to the P450. However, in P450BM-3 the P450 and FAD/FMN reductase are fused together in a single polypeptide chain of 119,000 daltons (1), whereas in microsomal P450s the reductase and P450 are separate polypeptides. Additionally, the sequence homology between the reductase domain of P450BM-3 and microsomal P450 reductase is high, whereas the heme domain more closely resembles in sequence certain classes of microsomal fatty acid P450 monooxygenases than other bacterial P450s (2, 3). Owing to these similarities, P450BM-3 has become an excellent model system for microsomal P450s with the distinct advantage that large amounts of P450BM-3 can be prepared from *Escherichia coli* recombinant expression systems (4) thus providing sufficient material for detailed biophysical studies.

Porter and Kasper (5) first noted that the N-terminal part of the diflavin P450 reductase exhibits good homology with FMN-containing bacterial flavodoxins, whereas the C-terminal half is homologous to the FAD-containing spinach ferredoxin reductase. This suggests that P450 reductase was constructed by the fusion of flavodoxin-like and ferredoxin reductase-like genes. This homology with flavodoxin and ferredoxin reductase extends to the P450BM-3 reductase domain. Such comparisons indicate that holo-P450BM-3 was pieced together by the fusion of at least three separate folding units: heme, FAD, and FMN. The architecture for nitric oxide synthase is remarkably similar (6). Apparently, once Nature discovers a useful functional unit, the various units are used in multiple ways by covalently tethering the domains via a gene fusion mechanism resulting in redox proteins with novel activities. If, as this view suggests, each of the P450BM-3 domains can fold as semi-autonomous units, it should be possible to recombinantly express the individual domains that retain both prosthetic groups and some functional activities. With P450BM-3 the heme and FAD/FMN reductase domains already have been separately expressed in recombinant systems, and with the heme domain, the crystal structures in the substrate-free (7) and -bound forms are known (8). The recombinantly expressed FMN and FAD domains of microsomal P450 reductase also have been characterized (9). In this report we show that the FMN and FAD reductase domains of P450BM-3 also behave as autonomous folding units. We have found that the FAD domain can be eliminated leaving behind the heme/FMN subdomain. In addition, we have probed the role of a large insertion in the reductase domain that is not present in either flavodoxin or ferredoxin reductase.
expression was induced by adding isopropyl-
b-100 were used to inoculate 1.5 liters of LB/ampicillin in 3-liter flasks and were grown at 37 °C in 1.5 liquid chromatography.

mant colonies picked from LB/agar plates with 100 and FAD/FMN-(471–1048) followed the following protocol. Transfor-
mated with the domain with the exception of the holo-P450BM-3 that is

mRNA plasmids for sequencing.

transform system using polymerase chain reaction. The plasmids were used to

mM phenylmethylsulfonyl fluoride, pH 7.4). The cells were lysed using

frozen cell paste was suspended in 3–4 until further use. All further handling of cells or enzyme preparations

Purification—In the following, each of the domains will be indicated by the residue number and the prosthetic group expected to be associ-

the method of Kunkel using the DEAE-Sephacel column (2.6

performed in the cold. Frozen cell paste was suspended in 3–4

with holo-P450BM3 gene in the pT7-7 system as the template. All the

FIG. 1. Subdomains of P450 reductase domain made for this study. The first and last amino acid of the different subdomains made are indicated. Calculated molecular mass of the different subdomains are FMM-(471–664), 23 kDa; FAD-(654–1048), 47 kDa; FAD-(483–1048), 64.8 kDa, Δ110 mutant, 107 kDa, heme/FMN-(1–664) 72 kDa, heme-(1–625), 89 kDa.

MATERIALS AND METHODS

Constructs—Fig. 1 is a schematic diagram showing the regions that were cloned, expressed, and purified. The various domains were cloned by polymerase chain reaction using the pT7 BM3 as the template (10). The 5'-oligonucleotides were synthesized with an overhanging BamHI restriction site, whereas the 3’-oligonucleotides contained an EcoRI site. The polymerase chain reaction product was digested with EcoRI and BamHI and ligated to the pT7-7 vector digested with BamHI and EcoRI. The 110-amino acid deletion mutant (Δ110) was constructed by the method of Kunkel et al. (11) using the single-stranded DNA from the wild type P-450BM3 gene in the pT7-7 system as the template. All the mutants generated were confirmed by Promega fmol DNA sequencing system using polymerase chain reaction. The plasmids were used to transform E. coli BL21 (DE3) for the expression of the mutant enzymes and for making plasmids for sequencing.

Purification—In the following, each of the domains will be indicated by the residue number and the prosthetic group expected to be associ-

the holoprotein, holo-P450BM-3 that is missing residues 711–821. This mutant will be referred to as Δ110 (Fig. 1). The purification of the FADD-(654–1048), FAD-(483–1048) reductase, and FAD/FMN-(471–1048) followed the following protocol. Transform-

FIG. 2. A, SDS-polyacrylamide (10–15% gradient) gel electrophoresis of the different subdomains purified. Lane 1, FMM-(471–664); lane 2, FAD-(654–1048); lane 3, FAD-(483–1048); lane 4, FAD/FMM-(471–1048); lane 5, 110 mutant; lane 6, heme/FMN-(1–664); lane 7, heme-(1–625); lane 8, Pharmacia low molecular mass markers (94, 67, 43, 30, 20.1, and 14.4 kDa). B, SDS-polyacrylamide (10–15% gradient) gel electrophoresis of the different subdomains purified under reducing and nonreducing conditions. Lane 1, FAD/FMN-(471–1048, nonreducing); lane 2, FAD/FMN-(471–1048 reducing); lane 3, FAD-(654–1048, nonreducing); lane 4, FAD-(654–1048, reducing); lane 5, FAD-FMN-(471–664, nonreducing); lane 6, heme/FAD/FMN-(1–1048, nonreducing); lane 7, heme/FAD/FMN-(1–1048, reducing); and lane 8, Pharmacia low molecular mass markers.

the column was further purified using Sephacryl S-100 (1.8 × 75 cm) gel filtration column. SDS-polyacrylamide gel electrophoresis of the various purified domains is shown in Fig. 2A.

Enzyme Assays—All protein concentrations were estimated by the Bio-Rad protein estimation method. The amount of heme in wild type P450BM-3 and the Δ110 mutant were determined by reduced pyridine hemochromogen method (13). Purified FAD/FMN-(471–1048), FAD-(483–1048), FAD-(654–1048), and Δ110 were checked for their ability to carry out NADPH-dependent electron transfer to the artificial electron acceptors, ferricyanide and cytochrome c. All spectrophotometric assays were carried out using a Cary 3 spectrophotometer. Cytochrome c reductase, ferricyanide reductase, and NADPH oxidation activities were determined as described (14). Substrate binding was estimated using spectrophotometric titration by following the characteristic low to high spin transition as indicated by the shift of the main Soret absorp-

| Heme Domain | FMM Domain | FAD/NADPH Domain |
|-------------|------------|------------------|
| 1           |            |                  |
| 471         | 664        | 1048             |
| 654         |            |                  |
| 483         | 648        | 1048             |
| 625         | 664        | 1048             |
| Δ110 mutant |            |                  |

1 The abbreviations used are: DTT, dithiothreitol; FPLC, fast protein liquid chromatography.
residues 1–664, however, did give a soluble protein that contained 0.5 ± 0.05 mol of FMN per mol of protein (Table I) indicating that the additional 39 residues were essential for FMN incorporation. The decrease in absorbance near 500 nm in heme/FMN (1–664) is indicative of flavin reduction (Fig. 4). Based on this observation we cloned the FMN domain (471–664) and it contained 0.93 mol of FMN per mol of protein. The FMN domain showed absorption maxima at 277, 386, and 467 nm. The FMN domain could not be reduced by excess NADPH (Fig. 5).

The sequence comparisons indicated that the FMN domain of holo-P450BM3 contains an extra 12 residues at the N terminus not present in flavodoxin. To test the importance of these extra residues, a construct of the FAD/FMN domain was prepared consisting of residues 483–1048 rather than the usual 471–1048. During purification of FAD/FMN (483–1048), most of the protein bound to the affinity column very tightly, and only a small amount of protein was obtained using 50 mM 2-AMP in 0.5 M phosphate buffer. The purified protein also showed increased susceptibility to proteases when stored at 4 °C. Analysis of flavins showed 0.77 eq of FAD but no FMN (Table I), and the FMN domain was reduced to semiquinone form by NADPH as shown in Fig. 5. This indicates that the additional 12 residues, 471–482, are important for FMN binding.

**FAD Reductase Domain**—Spinach ferredoxin-NADP⁺ reductase is a FAD containing protein that catalyzes electron transfer from reduced ferredoxin to NADP⁺ during photosynthesis (17). Comparison of the P450BM3 reductase to ferredoxin-NADP⁺ reductase reveals striking similarity over stretches of amino acids in the C-terminal region of P450BM3 reductase especially the key residues involved in the binding of FAD and NADPH. Fig. 3 shows the alignment of the C-terminal segment of P450BM3 reductase residues 625–1048 with the complete sequence of ferredoxin-NADP⁺ reductase. Careful alignment of the two proteins indicates an overall shift of sequence toward the C terminus is due to an insertion of 110 residues in the C terminus of ferredoxin-NADP⁺ reductase. Careful alignment of the two proteins indicates an overall shift of sequence toward the C terminus is due to an insertion of 110 residues in the C terminus of ferredoxin-NADP⁺ reductase.

**RESULTS**

**FMN (471–664) Domain**—Sequence alignments of the FAD/FMN reductase domain of P450BM-3 with clostridial flavodoxin and spinach ferredoxin NADP⁺ reductase were carried out using the PASTA module in the BISOINSIGHT II package (Fig. 3). The FNM and FAD binding regions identified from the known X-ray crystal structures (15, 16) were manually aligned and held fixed prior to automated alignment for the rest of the sequence. These alignments then were used as guides for preparing the various constructs needed to express the domains. Initially we attempted to express the heme/FMN domain consisting of residues 1–625. A protein of the correct molecular weight was expressed but did not contain FMN. Expression of residues 1–664, however, did give a soluble protein that contained 0.5 ± 0.05 mol of FMN per mol of protein (Table I) indicating that the additional 39 residues were essential for FMN incorporation. The decrease in absorbance near 500 nm in heme/FMN (1–664) is indicative of flavin reduction (Fig. 4). Based on this observation we cloned the FMN domain (471–664) and it contained 0.93 mol of FMN per mol of protein. The FMN domain showed absorption maxima at 277, 386, and 467 nm. The FMN domain could not be reduced by excess NADPH (Fig. 5).

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The apparent dimerization of the FAD reductase domain was further analyzed by gel filtration chromatography using FPLC gel filtration chromatography (Superdex 200 HR 10/30). In 0.1 M phosphate buffer, pH 7.4, with 0.1 M NaCl, the protein was found to elute as two peaks (Fig. 6). In comparison to the calibration standards, these two peaks corresponded to molecular mass of 120 kDa (40%) and 42.9 kDa (60%). However, when the FAD reductase was incubated with 50 mM DTT for 5 min and run in the presence of 10 mM DTT in the same buffer, most of the protein eluted as a monomer. We also studied P450BM3 reductase, FMM/FAD (471–1048), under similar conditions. The FMN/FAD (471–1048) domain also eluted as two peaks in 0.1 M phosphate buffer, pH 7.4, with 0.1 M NaCl corresponding to molecular mass of 159.5 kDa (65%) and 90 kDa (35%, Fig. 6). FMM/FAD (471–1048) incubated with 50 mM...
The heme, FAD, and FMN contents are expressed in mol prosthetic group per mol of protein. Enzyme activities are in mol per min per mol of prosthetic group. Cyt c red., cytochrome c reductase.

| Domain                          | [Heme] | [FMN] | [FAD] | Cyt c red. activity | NADPH oxidation in the presence of cyt c | Ferricyanide reductase activity | NADPH oxidation in the presence of ferricyanide |
|--------------------------------|--------|-------|-------|--------------------|----------------------------------------|---------------------------------|-----------------------------------------------|
| FAD/FMN (471–1048)             | 0.94   | 0.97  | 1.0   | 997 ± 44           | 571 ± 45                               | 15220 ± 1351                   | 8106 ± 181                                   |
| FAD (482–1048)                 | 0.59 ± 0.08 | 0.77 ± 0.03 | 2656 ± 128 | 1442 ± 74         | 20593 ± 205                          | 8255 ± 221                       | 2418 ± 152                                   |
| FAD (654–1048)                 | 0      | 0.60 ± 0.10 | 3.3 ± 0.43a | 4.9 ± 0.26a       | 6045 ± 530                            | 2507 ± 196                     | 2372 ± 223                                   |
| FMN (471–664)                  | 0.93 ± 0.05 | 0     | 5.6 ± 0.3a | 3.8 ± 0.4a        | 4885 ± 368                            | 120 ± 7a                       | 101 ± 20a                                    |
| Heme/FMN (1–664)               | 0.53   | 0     | 0.52 ± 0.05 | 81.8 ± 4.5a      | 35.3 ± 3a                              | 8657 ± 731                     | 5192 ± 29                                    |
| Heme (1–625)                   | 0.7    |       |       | 0.09a             | 259 ± 15a                             | 16.02 ± 0.9                    | 763 ± 89                                     |
| Heme (1–625) + FMN + FAD (654–1048) 1:1:1 | 3.059 ± 0.2a | 0.99a | 731 ± 60                 | 422 ± 54                               | 106 ± 29                                  |
| Heme/FMN (1–664)               | 0.64   | 0.50 ± 0.05 | 0.758 ± 0.1a | 282 ± 16a       | 196 ± 221                              | 731 ± 60                      | 422 ± 54                                     |
| Heme/FMN (1–664) FMN + FAD (654–1048) 1:1:1 | 16.02 ± 0.9 | 0.758 ± 0.1a | 763 ± 89                 | 301 ± 30                                | 196 ± 221                               |

*Enzyme activities expressed in mol/mol of protein.

**Table I**

Flavin content and various activities of the sub-domains of P450BM-3 reductase domain

**Fig. 4.** Absorption spectra of the heme-(1–625) and heme/FMN-(1–664) before and after the addition of dithionite.

DTT at 4 °C and run in the presence of 10 mM DTT chromato-
graphed mostly as one peak at 90 kDa. This is higher than the expec-
ted size of 63 kDa possibly due to the shape of the mole-
cule. These results indicate that both the FAD-(654–1048) and
FAD/FMN-(471–1048) dimerize via formation of intermolecu-
lar disulfide bonds. Holo-P450BM-3 dimerizes via some other
mechanism. With or without the preincubation with 50 mM
FAD/FMN-(471–1048) dimerize via formation of intermolecu-
lar disulfide bonds. Holo-P450BM-3 dimerizes via some other
mechanism. With or without the preincubation with 50 mM
FAD/FMN-(471–1048) behaves as a monomer further indicating that disulfide
bridges are not responsible for the dimeric chromatographic behavior of holo-P450BM-3.

**Heme-(1–625) and Heme/FMN-(1–664)—** As evident from
Fig. 4, only the heme/FMN-(1–664) contained FMN indicating
that the additional 39 residues are essential for FMN incorpo-
ration. The FMN in the heme/FMN-(1–664) could not be re-
duced with NADPH, but dithionite was able to reduce the FMN
to the fully reduced hydroquinone form.

**Δ110 Deletion Mutant—** As noted earlier, the FAD-(654–1048)
domain of P540BM-3 and microsomal P450 reductase have a
≈110-residue insertion relative to spinah ferredoxin
reductase (Fig. 3). To investigate the significance of this inser-
tion, the 110 residues were deleted from holo-P450BM3 (Fig. 1), and the deletion mutant was designated Δ110. Despite such
a large internal deletion, the protein was overexpressed and
could be purified. However, the Δ110 bound much more tightly
to the 2’5’-ADP-Sepharose affinity column than wild type
P450BM-3 and could not be eluted using 50 mM AMP. There-
fore, we switched to ion exchange chromatography for purifi-
cation, and the resulting purified protein contained 0.5 eq of
heme, 0.5 eq of FAD but no FMN (Table I). Reconstitution of the
Δ110 mutant by incubating the enzyme with 5-fold excess of
free FMN on ice overnight did not improve the FMN content of
the enzyme. The KD value estimated using the 14-carbon fatty
acid, myristic acid, was 0.588 μM which is very close to the KD
value for the wild type (0.48 μM).

**Functional Activities of the Different Domains—** The purified
domains were tested for their ability to carry out NADPH-dep-
endent electron transfer to ferricyanide and cytochrome c. In
comparing % activities, the FAD/FMN-(471–1048) reductase
domain was taken as 100%. A comparison of such activities is
shown in Table I. FAD-(654–1048) retained 25% of the ferri-
cyanide reductase activity but very low cytochrome c reductase
activity. Similar studies carried out with FAD domain from
microsomal P450 reductase showed 50% ferricyanide reductase
activity and no cytochrome c reductase activity (9). Ferricya-
nide reduction by FAD-(654–1048) was ionic strength-depend-
tent electron transfer to ferricyanide and cytochrome c
reductase activity.
cytochrome c reductase activity increased slightly in comparison to FAD-(654–1048) alone.

Since the FMN-(471–664) lacks the NADPH domain, little NADPH-dependent reductase activity was expected. As shown in Table I, this domain does not have significant levels of either ferricyanide or cytochrome c reductase activities.

The FAD/FMN-(483–1048), which has FAD but no FMN, retained about 25–30% ferricyanide reductase activity but very little cytochrome c reductase activity. This is in keeping with the expectation that FMN is required for cytochrome c reduction but not ferricyanide reduction (19). Preincubation of this domain with excess FMN did not restore any cytochrome c reductase activity. This indicates that the presence of residues 471–483 at the N terminus of the FAD/FMN reductase domain is important for FMN binding. P450 reductase domain of P450BM-3 where the N-terminal 120 amino acids cleaved by trypsin showed similar kinetics (20).

\[\text{FIG. 5. Absorption spectra of purified subdomains of P450BM-3 reductase.}\]

\[A, \text{FAD domain, residues 654–1048;}\]

\[B, \Delta_{110}, \text{residues deleted;}\]

\[C, \text{FAD domain, residues 483–1048;}\]

\[D, \text{FMN domain, residues 471–664.}\]

\[\text{DISCUSSION}\]

Autonomous Folding Units—Our present work together with recent work on the domain structure of P450 reductase (21) clearly show that cytochrome P450BM-3 and FAD/FMN P450 reductase are constructed of autonomous folding units. Of these, the heme and FAD domains are the most robust, whereas the FMN domain is most prone to losing the prosthetic group. For example, FAD/FMN-(471–1048) has both FMN and
The simplest criterion demonstrating that the crystal structure determination (22) has been crystallized in a form suitable for high resolution will be forthcoming shortly since a microsomal P450 reductase deletion of residues 711–821 is less clear. However, the answer to the protein (16). Why FMN binding should be so sensitive to the protein and forms a more extensive array of interactions with the spinach ferredoxin reductase is more deeply embedded in the larger FAD with two rings, isoalloxazine and adenine, in between the FMN and bulk solvent in flavodoxin. In contrast, the FMN domain is due to the loss of some key residues required for binding FMN or a more drastic unfolding or loosening of the FMN domain. An examination of the Clostridium Mp. flavodoxin x-ray structure (3FXN, see Ref. 15) shows that the N-terminal region is involved in a β-sheet structure and that the C-terminal region is helical and close to the N terminus. Disruption of either end might well lead to folding problems or a loosening of the FMN binding. This could explain the sensitivity of FMN binding to manipulation of the FMN domain’s N and C termini in P450BM-3. It also should be noted that it is relatively easy to remove FMN from P450 reductase (19), and C termini in P450BM-3. It also should be noted that it is relatively easy to remove FMN from P450 reductase (19), whereas the FAD is more rigidly held in place. This, too, is evident from the x-ray structures since a single Trp residue sits between the FMN and bulk solvent in flavodoxin. In contrast, the larger FAD with two rings, isoalloxazine and adenine, in spinach ferredoxin reductase is more deeply embedded in the protein and forms a more extensive array of interactions with the protein (16). Why FMN binding should be so sensitive to deletion of residues 711–821 is less clear. However, the answer will be forthcoming shortly since a microsomal P450 reductase has been crystallized in a form suitable for high resolution crystal structure determination (22).

We can be more definitive about the integrity of the FAD and heme domains. The simplest criterion demonstrating that the FAD domain remains intact is the ability to adhere to the affinity column used for purification. This property requires a correctly folded NADP site. Moreover, the ability to carry out NADPH-dependent ferricyanide reduction is a good indication that the FAD domain is correctly folded. The robustness of the heme domain is evidenced by determination of the heme domain crystal structure with (8) and without substrate (7) and by retention of the many spectral properties expected of a functional P450. Therefore, P450BM-3 consists of at least three domains (heme, FAD, and FMN) that can independently fold. This is consistent with a similar study on P450BM-3 recently published (21) and previous work on microsomal P450 reductase (9).

**Enzymatic Activities**—Each of the domains retains the expected catalytic activities. Nevertheless, intact holo-P450BM-3 is required for full fatty acid hydroxylation activity. Reconstitution of some activity has been achieved by mixing the heme and FAD/FMN domains (23), but no combination of domains gives the very high levels of fatty acid hydroxylation activity ($k_{cat} > 1,000$ min$^{-1}$) found with holo-P450BM-3. Some of our previous work indicates that this has less to do with the structure of the individual domains but rather the way in which the FMN and/or FAD domains interacts with the heme domain. The flow of electrons is NADPH-to-FAD-to-FMN-to-heme. The C terminus of the heme domain is attached to the N terminus of the FMN domain by a linker consisting of residues that may include residues 456–470. We say “may include” because it is not certain where the FMN domain begins. It does appear that the important part of the heme domain may end at residue 455 where the last β-sheet structure in the heme domain terminates. Residues immediately following 455 may belong to the linker between the heme and reductase domains. This might be why these residues are disordered in the crystal structure of the 1–471 heme domain (7). We have analyzed the role of this linker by making both amino acid substitution, deletion, and insertion mutants. Enzyme activity is insensitive to replacement of residues in the linker including a 6 Pro or Gly substitution (24) but is very sensitive to shortening the linker (14). The main conclusion drawn from this previous work is that the linker is critical for correctly orienting the reductase domain relative to the heme domain for efficient electron transfer. Although somewhat speculative, it is interesting to consider that Nature spent most of her evolutionary energy on how to link the various domains together rather than modifying the domains themselves. It appears that the correct length of linker had to be discovered in order to allow the reductase to sample the correct orientation for electron transfer. The importance of the linker is further evidenced by the example of nitric oxide synthase which has the same heme-FMN-FAD architecture as P450BM-3. One major difference is that the nitric oxide synthase linker binds calmodulin which switches on the FMN-to-heme electron transfer reaction. In this case, it is very likely that the linker must adopt a helical structure, the expected conformation for calmodulin binding (25).

![FPLC gel filtration chromatography](image)

**Fig. 6.** FPLC gel filtration chromatography of the FMN/FAD-(471–1048) and FAD-(654–1048) domains in the absence or presence of 10 mM DTT. The column used was a Pharmacia FPLC Superdex 200 HR 10/30 column in the presence and absence of 10 mM DTT operating at a flow rate of 250 ml/min. The thicker lines are the chromatograms run in the presence of DTT.
heme electron transfer reaction.

**Oligomeric Structure**—The FPLC and electrophoresis data indicate that the FAD/FMN-(471–1048) and FAD-(654–1048) dimerize through disulfide bonds. This suggests that removal of the heme domain makes available Cys residues that cannot readily form disulfides in holo-P450BM-3. This could be due to a physical unmasking of Cys residues when the heme domain is removed or be due to steric restrictions that are absent in the FAD/FMN and FAD domains. In contrast, holo-P450BM-3 appears not to dimerize via disulfides (27). Some hint as to what causes holo-P450BM-3 to dimerize stems from the crystal structure of the substrate-free (26) and -bound (8) heme domain. Each form of the heme domain, with and without substrate, crystallizes in different space groups with a different number of molecules in the asymmetric unit. Nevertheless, in each crystal form, the heme domain forms dimers at the same interface via an array of noncovalent contacts near Tyr-166. Dimerization of holo-P450BM-3 at the same interface could explain the differences in dimerization patterns observed in the various domains.

In addition to gaining some insights into the domain architecture of P450BM-3, an additional reason for undertaking this study was to develop simpler functional units for further detailed biophysical studies. One of the more interesting and important is intramolecular electron transfer between prosthetic groups which becomes a complex problem in holo-P450BM-3. The heme/FMN-(1–664) should be particularly useful for studying the FMN-to-heme electron transfer reaction which is the topic of the accompanying manuscript (28).

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