Role of the Linker Region Connecting the Reductase and Heme Domains in Cytochrome $P450_{BM-3}$†

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ABSTRACT: Cytochrome $P450_{BM-3}$ is a fatty acid monooxygnease that contains the catalytic P450 heme domain covalently attached to a diflavin P450 reductase domain. The function of the linker region connecting the C-terminal end of the heme domain to the N-terminal end of the reductase domain has been studied by deleting parts of the linker and changing the sequence of the linker. Deleting three or six residues or changing an Arg-Lys-Lys stretch in the middle of the linker to Ala-Ala-Ala does not alter the functional properties of either domain. The mutants retain full cytochrome *c* and ferricyanide reductase activities characteristic of the P450 reductase domain. The heme domain in the mutants retains its ability to bind a fatty acid substrate giving the full low-to-high spin shift and exhibits the normal 450 nm absorption band characteristic of the reduced carbon monoxide complex. However, the six amino acid deletion mutant exhibits nearly undetectable levels of fatty acid hydroxylase activity, the three amino acid deletion mutant about 10% activity, and the three Ala substitution mutant about 50% activity. The mutants also exhibit slower rates of reductase-to-heme electron transfer rates that correlate with the loss in fatty acid hydroxylase activity. These results indicate that the length of the linker and, to a much less extent, the sequence are important for correctly orienting the reductase and heme domains, which apparently is necessary to achieve efficient reductase-to-heme electron transfer rates.

Cytochromes P450 catalyze the monooxygenation of a wide variety of hydrophobic substrates (Nebert & Gunzalez, 1987). The P450 microsomal monooxygenase system consists of a NADPH cytochrome P450 reductase which transfers reducing equivalents from NADPH to cytochrome P450, the substrate binding and catalytic component of the monooxygenase. The reductase component contains both FAD[1] and FMN and delivers electrons directly to P450 while in bacteria and mitochondria an iron−sulfur protein shuttles electrons between a FAD-containing reductase and P450. The first example of a bacterial P450 that uses the microsomal-like FAD/FMN reductase is the fatty acid monooxygenase from *Bacillus megaterium*, $P450_{BM-3}$ (Narhi & Fulco, 1986). The main difference is that the FAD/FMN reductase and heme-containing P450 are fused together as a single polypeptide chain (Narhi & Fulco, 1987). The DNA sequence and alignments show that the domain arrangement, from the N- to C-terminus, is heme−FMN−FAD (Ruettinger *et al.*, 1989). This architecture is very similar to that observed for nitric oxide synthase (Hevel *et al.*, 1991).

Narhi and Fulco (1987) have shown that the heme and FAD/FMN domains can be separated by limited tryptic digestion. Although the isolated domains retained their individual activities, the fatty acid hydroxylase activity could not be reconstituted by combining tryptic products. Li *et al.* (1991) found similar results using recombinantly expressed domains although Bodduppalli *et al.* (1992) found conditions under which some fatty acid hydroxylase activity could be reconstituted. These results indicate that it is necessary to have the flavin and heme domains covalently attached for full activity. These results, however, do not address how important the length and sequence of the linker are for activity. That the linker is so susceptible to tryptic hydrolysis shows that at least part of the linker is exposed and, perhaps, flexible. It then might be possible to remove part of the linker and still retain function. Moreover, engineering the linker might reveal important information regarding how precisely the reductase and heme domains must be oriented for electron transfer. To address the function of the linker region, we have generated both site-directed and deletion mutants in the linker region between the heme and flavin domains. The effect of these variants on the reductase-to-heme electron transfer reaction, substrate binding, and fatty acid hydroxylation activity are the topic of this paper.

MATERIALS AND METHOD

Materials. All chromatographic and electrophoretic materials were obtained from Pharmacia. NADPH, 2′-AMP, DTT, PMSF, EDTA, horse cytochrome *c* were purchased from Sigma. Sodium myristate was obtained from Fluka. [1-^{14}C]Myristate with a specific activity of 58 mCi/mmol was obtained from NEN-Research Products. CO was purchased from Liquid Carbonic Inc. T4 DNA ligase and DNA polymerase were purchased from New England Biolabs.

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[1] Abbreviations: $P450_{BM-3}$, cytochrome $P450_3$ isolated from *Bacillus megaterium*; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotine adenine dinucleotide phosphate reduced form; IPTG, isopropyl β-D- thiogalactopyranoside; 2′-AMP, 2′-adenosine monophosphate; PCR, polymerase chain reaction; CO, carbon monoxide; Ala_3, mutant with residues 471−473 converted to Ala; Δ_3, mutant with residues 471−473 deleted; Δ_6, mutant with residues 468−473 deleted.

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Mutagenesis. Oligonucleotide site-directed mutagenesis was carried out following the method of Kunkel *et al.* (1987) in the $P450_{BM-3}$ gene cloned into the pT7-7 vector (Darwish *et al.*, 1991). The PT7-7 (Darwish *et al.*, 1991) system was used to prepare wild type and the three variants: deletion of the residues 468 → 473 (Δ_6 mutant); deletion of residues 471 → 473 (Δ_3 deletion mutant) and substitution of residues 471 → 473 by alanines (Ala_3 substitution mutant). Single-stranded uracil-encoded plasmid was isolated by growing the pT7-7 vector in an appropriate host (RZ1032) and used as a template for annealing, extension, and ligation by the appropriate mutant oligonucleotide *in vitro*. The reaction mixture then was transformed into *Escherichia coli* JV30, which has a mechanism for deleting the uracil-encoded DNA. Mutants were selected by restriction enzyme digestion. Mutant plasmids were used to transform competent BL21 cells for expression of the mutant enzymes and for making plasmids for sequencing. Mutants were confirmed after screening by dideoxy sequencing (Sanger *et al.*, 1977) using the Sequenase kit (United States Biochemical) and also by Promega fmol™ DNA Sequencing System using PCR.

Purification of $P450_{BM-3}$ and Its Mutants. Transformant colonies picked from LB/agar plates with 100 μg/mL ampicillin were grown at 37 °C in 1.5× LB/ampicillin medium. Overnight cultures were used to inoculate 1.5 L of LB/ampicillin in 3 L flasks and incubated at 37 °C. When the optical density at 600 nm reached 1.0, another 100 μg/mL ampicillin was added and T7-RNA polymerase expression was induced by adding IPTG to 0.6 mM. Growth was allowed to continue for another 12−15 h at 30 °C. Five hundred milliliters of the culture was used for plasmid preparation, and the rest of the cells were harvested and stored at −70 °C as cell pellet until further use. All further handling of cells or enzyme preparations was performed in the cold. Frozen cell paste was suspended in 3−4 volumes of buffer A, 30 mM phosphate, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF, pH 7.4. The cells were lysed using a French press and collected by centrifugation for 60 min at 4 °C. The supernatant in buffer A was loaded on to a Q-Sepharose anion exchange column (2.6 × 55 cm) equilibrated with buffer A. Two to three bed volumes of buffer B (0.1 M phosphate with protease inhibitors) was used to wash the column at a flow rate of 80 mL/h. Finally, the protein was eluted using a gradient of 0.1−0.5 M phosphate buffer. The fractions with a high R_z value were concentrated using YM30 Amicon membrane, loaded onto 2′,5′-ADP-Sepharose affinity column (1.5 × 4 cm). Elution of the protein from the affinity column was performed as described by Black *et al.* (1994). The purified fractions from the affinity column loaded on to Sephadex G-25 column (1.5 × 22 cm) to remove the 2′-AMP. As shown in Figure 1, the proteins were homogeneous on SDS PAGE. All three mutants showed no difference in spectral properties compared to the wild type, which indicated that the prosthetic groups are intact in the mutants.

Enzymatic Activities. All the mutant and wild-type P450 enzyme concentrations were estimated from the reduced CO-bound spectra using an extinction coefficient of 91 mM^{-1} cm^{-1} at 448 nm (O'Keeffe *et al.*, 1978). The stock solution of [1-^{14}C]-myristate was prepared by mixing the radioactive substrate with the aqueous solutions of fatty acid in 50 mM K_2CO_3 to obtain a specific radioactivity of 1000 cpm/nmol. The reaction system for the monooxygenation of fatty acids

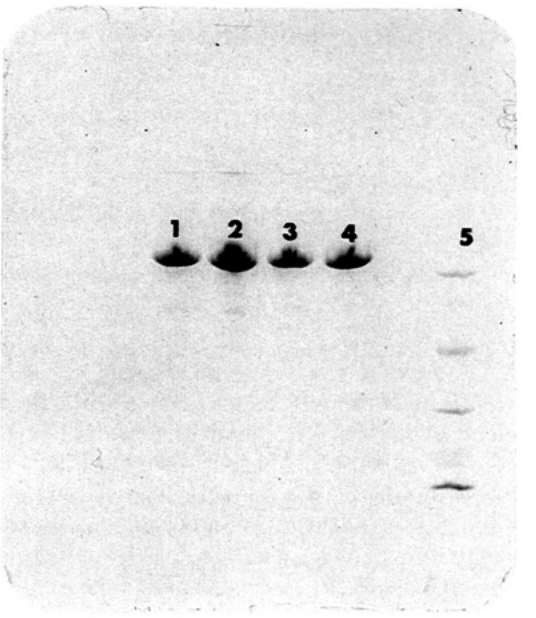

FIGURE 1: SDS−polyacrylamide gel electrophoresis of the wild-type $P450_{BM-3}$ and the mutants of the linker region. Lane 1, wild-type $P450_{BM-3}$; lane 2, Δ_6 mutant; lane 3, Δ_3 mutant; lane 4, Ala_3 mutant; lane 5, Phamacia molecular weight markers (phosphoryulase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; tyrpsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa).

contained 0.1 M potassium phosphate buffer, pH 7.4, 50 nM enzyme, 200 μM of fatty acid, and 600 μM NADPH in a total volume of 200 μL. The reaction was initiated by the addition of NADPH. At different time points the reaction was terminated by the addition of HCl to a final concentration of 2 N. The reaction mixture was extracted three times with 20 mL of ethyl acetate (HPLC grade). The pooled extracts were evaporated to dryness and redissolved in 0.3 mL of methanol and analyzed by reverse-phase HPLC according to Govindaraj *et al.* (1994).

All spectrophotometric assays were carried out using a Cary 3 spectrophotometer. NADPH oxidation was followed at 340 nm in a sample containing 1 mL of 9 pmol of enzyme, 100 nmol of myristate, and 200 nmol of NADPH in 0.1 M KP_i buffer, pH 7.4, by following the change in optical density at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Cytochrome *c* reductase activity was determined by measuring the increase in absorbance at 550 nm in a 1 mL sample containing 5 nmol of cytochrome *c*, 2 pmol of enzyme, and 200 nmol of NADPH in 0.1 M KP_i buffer pH 7.4, at room temperature against a blank of the same solution without NADPH. An extinction coefficient of 21.0 mM^{-1} cm^{-1} was used to calculate the number of moles of cytochrome *c* reduced per min per mole of enzyme. The NADPH oxidation in the presence of cytochrome *c* was measured under the identical conditions as above, but the decrease in the absorbance at 340 nm was measured using an extinction coefficient of 9.2 mM^{-1} cm^{-1}.

Ferricyanide reductase activity was measured in a 1.0 mL reaction volume consisting of 2 pmol of enzyme, 500 nmol of potassium ferricyanide, and 200 nmol of NADPH in 0.1 M phosphate buffer, pH 7.4. The extinction coefficient of 1.02 mM^{-1} cm^{-1} at 420 nm was used to calculate the rate of ferricyanide reduction.

Substrate Binding. Substrate binding was estimated using spectrophotometric titrations by following the characteristic

Table 1: Various Activities of the Linker Mutants Compared to Wild-Type Enzyme[a]

	wild type	Ala_3	Δ_3	Δ_6
ferricyanide reductase	17598 ± 98	16102 ± 26	15584 ± 539	14240 ± 588
cytochrome *c* reductase	967 ± 43	1302 ± 26	1083 ± 13	1244 ± 36
NADPH oxidation in the presence of cyt *c*	554 ± 44	777 ± 49	659 ± 26	778 ± 54
myristate hydroxylation	1330.5	621.2 (46.7%)	140.8 (10.6%)	3.6 (0.27%)
NADPH oxidation in the presence of myristate	2291.0	878.5 (38.3%)	496.5 (21.7%)	15.4 (0.67%)
heme reduction (min^{-1})	19938 ± 222	12589 ± 209 (63%)	7797 ± 132 (39%)	1747 ± 28 (8.7%)

[a] The numbers in parentheses are % of wild-type activities. The various activities with the exception of the heme reduction rate are expressed as moles of product formed per mole of enzyme per minute. The heme reduction rate is a first-order process and is expressed as min^{-1}.

low- to high-spin transition as indicated by the shift of the main Soret absorption band from 418 to 390 nm. Myristate was dissolved in 50 mM K_2CO_3, and the final volume changes were less than 2%. The reaction mixture contained 2 μM enzyme in 0.5 M KP_i, pH 7.8, and difference spectra were recorded from 360 to 460 nm after each addition of myristate to both the blank and sample cuvettes. The spectral dissociation constant, K_D, was determined from the x intercept of double-reciprocal plot of the $A_{390}-A_{418}$ versus the free myristate concentration. The concentrations of free myristate were calculated using

$$[\text{myristate free}] = [\text{total myristate}] - [\text{P450}]\Delta\text{absorbance}/\Delta\text{absorbance}_{max}$$

Heme Reduction Measurement. A 0.1 M phosphate buffer was made anaerobic and saturated with CO after bubbling with deoxygenated nitrogen gas for 1 h followed by CO for 30 min. The kinetics of CO binding were analyzed using a Hi-Tech Ltd model SU-40 stopped flow system. Measurements were carried out at room temperature and initiated by rapid mixing of 100 μL of a CO-saturated solution containing 400 μM NADPH with equal volume of solution containing 2.0 μM enzyme and 100 μM of myristate in CO-saturated buffer. In all cases the absorbance change was monitored at 448 nm. Signal-to-noise ratios were improved by averaging three to five individual experiments. The time course of absorbance change was fit to a single-exponential equation

$$\Delta A = A_0 e^{-kt} + B$$

where k = the first-order rate constant, t = time, and B is a constant.

RESULTS

Functional Analysis of the Reductase Domain. Each of the domains exhibit unique activities that can be utilized to assess the functional properties of the mutants. The NADPH-dependent reduction of ferricyanide depends only on the FAD domain since the electron flow is NADPH-to-FAD-to-ferricyanide (Oster *et al.*, 1991). The NADPH-dependent cytochrome *c* reductase activity depends on both FAD and FMN with the electron flow going from NADPH-to-FAD-to-FMN-to cytochrome *c*. Klein *et al.* (1993) found that, in $P450_{BM-3}$, there is a good correlation between FMN content and cytochrome *c* reductase activity. Therefore, the ferricyanide and cytochrome *c* reductase activities are good measures of the functional integrity of the reductase domain. As shown in Table 1, both activities in all three sets of mutants are very similar to those found in the wild-type enzyme.

Another basis for comparison is the ratio of cytochrome *c* reduced to NADPH oxidized. Cytochrome *c* is a one-electron acceptor, so if the reaction is tightly coupled, the ratio of cytochrome *c* reduced to NADPH oxidized should be 2. This ratio is approximately 1.6−1.7 for the wild type and mutants. These close similarities between the mutants and wild type in both the ferricyanide and cytochrome *c* reductase activities indicate that the reductase domain has not been significantly altered by mutations in the linker region.

Substrate Binding. $P450_{BM-3}$ shows a Soret peak maximum at 418 nm which indicates that the heme iron is predominantly in the hexacoordinate low-spin form. In the presence of substrate, the 418 nm peak shifts to 397 nm corresponding to a shift from low- to high-spin (Narhi & Fulco, 1986). This shift provides a convenient means of estimating substrate dissociation constants and is a good indicator of whether or not the active site has been perturbed. Results from spectrophotometric titrations using the 14-carbon long chain fatty acid substrate, myristic acid, are shown in Figure 2. The K_D values indicate a slightly tighter affinity for some of the mutants, but no more than a factor of 2−3. In all mutants, the spectral shift was very similar to that of the wild-type enzyme. Another good measure of the quality of the heme domain is the spectral shift that occurs upon reduction in the presence of carbon monoxide. If the ligation environment involving coordination between the Cys sulfur atom and the iron is normal, a 450 nm band in the reduced CO complex is observed. Some as yet unknown perturbation in the S−Fe bond leads to a band at 420 nm. All the mutants gave the reduced-CO band at 448 nm characteristic of the wild-type enzyme (data not shown). Therefore, as with the reductase domain, mutations in the linker do not significantly alter the heme domain.

Myristic Acid Hydroxylation. While the linker mutations do not alter the individual functional properties of the reductase and heme domains, the overall enzyme activity is dramatically altered. As shown in Figure 3 and Table 1, the rate of myristic acid hydroxylation is significantly lower in some of the mutants, especially the Δ_6 mutant where this activity is nearly undetectable. Also shown in Table 1 is a similar assay, except here the rate of NADPH oxidation was measured. This activity also is much lower in the mutants. In a perfectly coupled system, the ratio of NADPH oxidized to fatty hydroxylated should be 1.0. As shown in Table 1, this ratio is about 1.7 for the wild-type enzyme, indicating that even in the wild-type enzyme not all reducing equivalents can be accounted for as hydroxylated product. The NADPH oxidized/fatty acid hydroxylated ratio increases to >3 for the two deletion mutants, indicating that deletions in the linker significantly increase uncoupling.

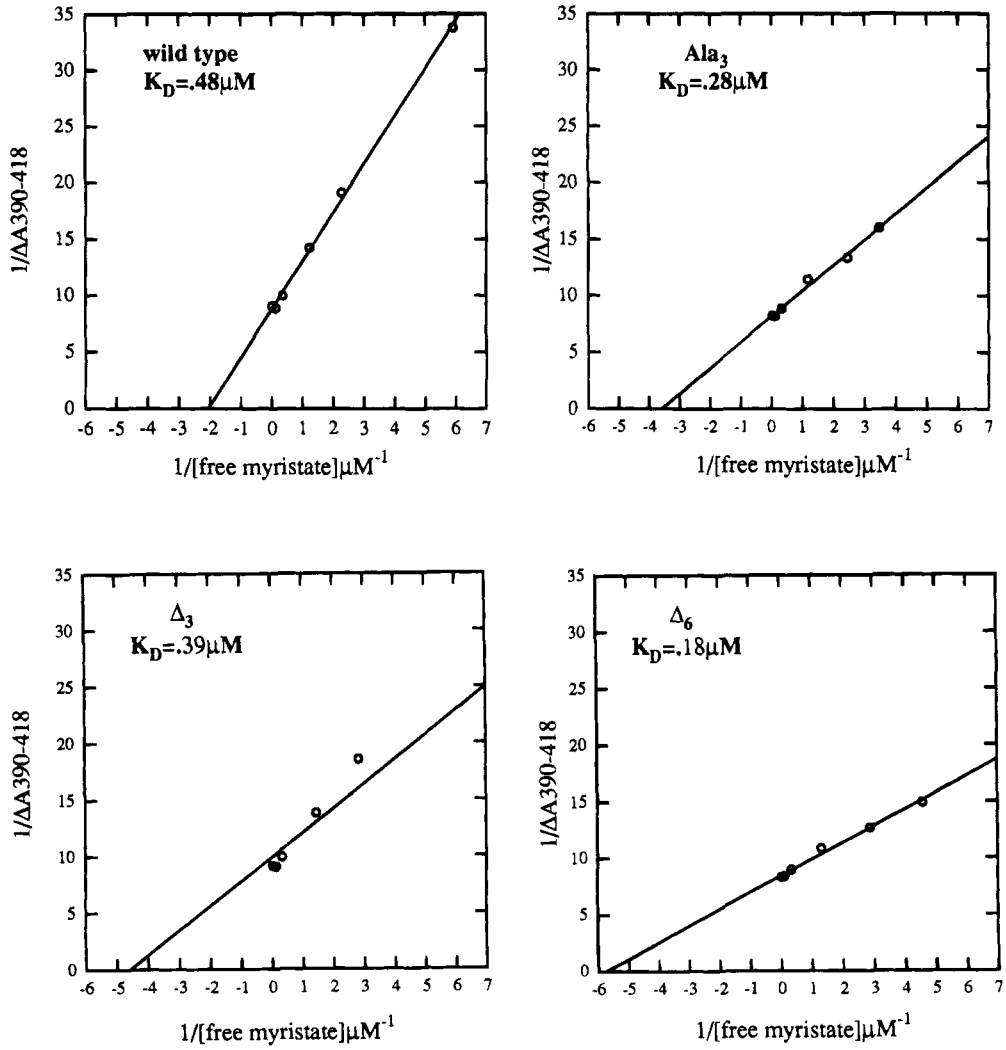

FIGURE 2: Determination of the spectral dissociation constant (K_D) for myristate for the wild-type P450$_{BM-3}$ and the mutants of the linker region. Difference spectra of P450 myristate were recorded using 0.15−100 μM myristate. The K_D value was derived from the x intercept.

Heme Reduction Rate. The results so far indicate that the mutants have a lowered fatty acid hydroxylase activity yet have normal properties expected for the individual domains. This further suggests that the linker mutations are selectively altering electron transfer from the reductase domain to the heme domain. If this is true, then a direct measure of the reductase-to-heme electron transfer reaction should also be impaired. This can be estimated by following the rate of heme reduction in the presence of CO at 448 nm using stopped flow methods. As shown in Figure 4 and Table 1, heme reduction in all the mutants and especially the Δ$_6$ mutant is slow. While not evident in Figure 4 owing to the requirement of placing the mutant data on the same scale as the wild type, the initial phase of the reaction in the mutants followed good first-order kinetics and could be readily estimated using the stopped flow system. For the wild-type enzyme, about 60% of the total heme was reduced. This is a lower estimate and is in error by 10−20% due to flavin absorbance near 448 nm so the actual amount of heme reduced is closer to 70−80%. For the mutants, not only is the rate of heme reduction slower but the total heme reduced is much less, and, for the Δ6-deletion mutant, total heme reduction was about 30% of wild type.

The correlation between the decrease in electron transfer and fatty acid hydroxylation strongly suggests that the reason the linker mutants exhibit decreased levels of fatty acid hydroxylase activity is due to impaired electron transfer from FMN to heme. It is of interest to note that the rate of heme reduction in the wild-type enzyme is much faster than fatty acid hydroxylation. Since the rate of heme reduction we have determined is the first of two electron transfer steps required to complete the catalytic cycle, it appears that the second electron transfer step or some subsequent step is rate limiting under steady-state turnover conditions.

DISCUSSION

P450$_{BM-3}$ is the first P450 isolated which has all the components necessary for electron transfer on a single polypeptide, requiring only substrate, NADPH, and O_2 (Narhi & Fulco, 1986). P450$_{BM-3}$ has the largest turnover number of any P450 probably due to the faster electron transfer rate from the reductase domain to the heme domain which are covalently tethered to one another. The ease with which the two domains can be separated by limited tryptic digestion indicates that at least part of the linker is readily accessible and, possibly, part of a flexible region of the protein. In this case it might be possible to remove or alter parts of the linker without grossly effecting activity. What we found is that the electron transfer reaction between the reductase and

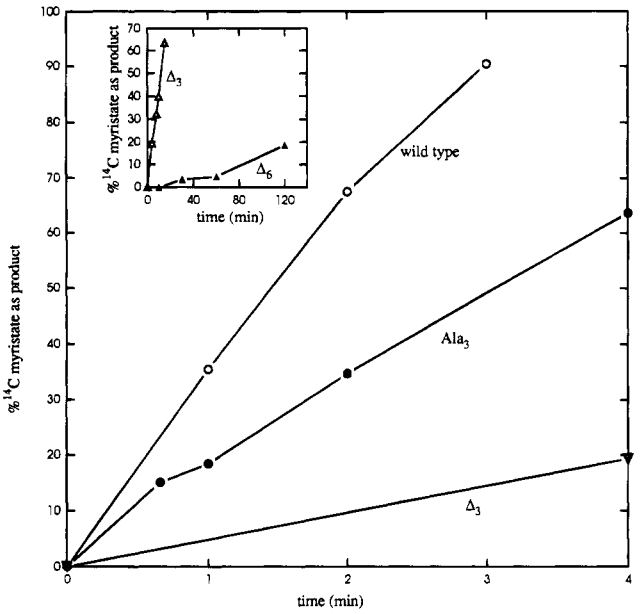

FIGURE 3: Comparison of the rate of the hydroxylation of [^{14}C]-myristate by the wild-type $P450_{BM-3}$ and the mutants of the linker region. The reaction was stopped at different time points and the product separated from the substrate by HPLC. The percent total counts emerging at the position of hydroxylated product is plotted as a function of time. The inset shows the slow hydroxylation rate of the Δ_6 mutant.

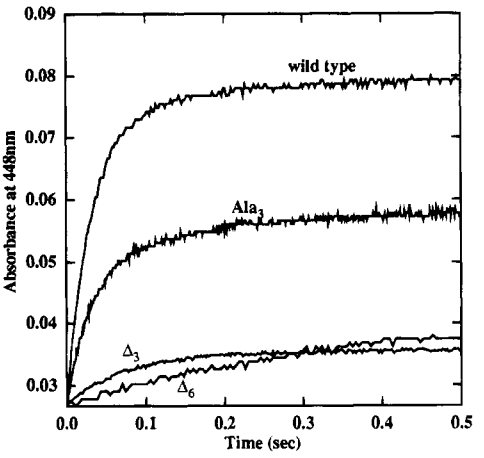

FIGURE 4: Comparison of the kinetics of the heme reduction of the wild type and the mutants of the linker region under CO atmosphere. A solution containing the enzyme and substrate was mixed with NADPH in a stopped flow apparatus under the conditions outlined in Materials and Methods.

heme domains is very sensitive to alterations in the length of the linker.

The individual activity of the domains, such as ferricyanide and cytochrome *c* reductase activities for the reductase domain and substrate binding for the heme domain, are relatively insensitive to linker mutations. This indicates that, in the mutants, the reactions of NADPH with FAD and the subsequent electron transfer from FAD to FMN is unaltered and the respective prosthetic group binding sites are unaltered. However, in the mutant where six residues are deleted, the fatty acid hydroxylase activity is <1% of wild-type activity, and the rate of FMN-to-heme electron transfer is less than 10% of wild type. In addition, much less heme is reduced in the mutants than in wild-type enzyme probably because in the mutants electron transfer from FMN to heme is uncoupled leading to the leak of reducing equivalents to oxygen.

Replacing the three charged residues, Arg-Lys-Lys (471–473), with Ala has a much less dramatic effect since this mutant still retains about 50% wild-type activity. Even deleting these three residues leaves about 10% activity. Taken together, these results show that the length of the linker and, to a far less extent, the sequence, is very important for FMN-to-heme electron transfer.

The relevant question centers on the structure of the linker region. It seems unlikely that this region consists of a flexible loop since one might expect that removing such a structure would not affect activity to such an extent. To obtain some idea of what structure the linker might adopt, the sequence of the linker was used to search the Protein Data Base for all available protein structures to see which element of structure the linker sequence best matches using the FASTA routine in the Biosym INSIGHT II package. The best match was with a helical region in aconitase (Robbins & Stout, 1989).

P450BM-3	S$_{460}$ P S T E Q S A K K V R K K A E$_{475}$
Aconitase	Y$_{18}$ D L L E K N I D I V R K R L N$_{33}$

Deleting six residues in a helix clearly would lead to some significant changes in domain orientations. Replacing three residues with Ala would be expected to have a much less dramatic effect since Ala is normally considered a helix-forming residue. This analysis, of course, does not prove the linker is helical but does suggest that the linker exhibits some ordered structure required for correctly orienting the heme and reductase domains.

Linkers have been implicated in the orientation of domains in other proteins. For example, in endoglucanase A (CenA) from *Cellulomonas fimi* the catalytic and cellulose binding domains are connected by a 23-residue linker called the Pro-Thr box (Wong *et al.*, 1986). Deletion of the Pro-Thr box alters the conformation of CenA by changing the relative orientation of the catalytic and cellulose binding domains. The change in the conformation reduces the catalytic efficiency of the enzyme and masks one of the protease sites between the domains implicating a role for the Pro-Thr box in maintaining the separation and relative orientation of the two domains necessary for normal function (Shen *et al.*, 1991).

While such sensitivity might be expected in enzymes where there are precise stereochemical requirements for bond making and breaking, the sensitivity of the FMN-to-heme electron transfer to linker mutations is somewhat surprising. It suggests that the electron transfer process requires a fairly precise orientation of the two redox partners. One view of electron transfer holds that it is primarily the distance between redox centers that controls electron transfer rates (Moser *et al.*, 1992). In contrast, others maintain that it is the intervening medium which controls rates and that the protein actively participates by providing a conduit from donor to acceptor (Karpishin *et al.*, 1994). Our results would favor this later view since electron transfer involving protein groups should be much more sensitive to subtle alterations in orientation. Although this is appealing, some caution should be exercised. While a 3–6 residue deletion in a protein containing >1000 residues is not much, such a disruption in a regular element of secondary structure

designed to hold two domains together could force a rather radical reorientation of the two domains. This in turn might significantly change the distance between donor and acceptor. It is of interest to note that the reductase-to-heme electron transfer could provide a possible regulatory control in an enzyme with a domain arrangement very similar to that of $P450_{BM-3}$, nitric oxide synthase. In nitric oxide synthase, a P450-like catalytic domain is followed by a diflavin P450 reductase domain. Nitric oxide synthase is activated by calmodulin which binds to the heme-reductase linker region (Abu-Soud *et al.*, 1994). It could be that calmodulin adjusts the domain interactions which favors reductase-to-heme electron transfer indicating that, in both nitric oxide synthase and $P450_{BM-3}$, the intramolecular electron transfer reaction is quite sensitive to domain interactions.

In summary, these results show that the linker connecting the heme and reductase domains in $P450_{BM-3}$ is not important in controlling functions unique to the individual domains. This should not be surprising since the individual functional domains can be prepared by tryptic digestion (Narhi & Fulco, 1987) or recombinantly expressed (Li *et al.*, 1991) and still retain their respective functional properties. However, the linker is quite important in controlling communication between the reductase and heme domains, especially along the FMN-to-heme electron transfer pathway. This further suggests that correct orientation of the two domains is critical for FMN-to-heme electron transfer.

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