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Probing the structure of the linker connecting the reductase and heme domains of cytochrome P450BM-3 using site-directed mutagenesis

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Abstract

Cytochrome P450BM-3 is a catalytically self-sufficient fatty acid hydroxylase containing one equivalent each of heme, FMN, and FAD. The heme and flavins reside in separate domains connected by a linker peptide. In an earlier study (Govindaraj S, Poulos T, 1995, *Biochemistry 34*:11221-11226), we found that the length but not the sequence of the linker connecting the heme and reductase domains is important for enzyme activity. In the present study, residues in the linker were replaced with Pro and Gly to probe the role that regular secondary structure plays in linker function. The rate of flavin-to-heme electron transfer and the fatty acid hydroxylase activities of the glycine and proline substitution mutants, including a six-proline substitution, did not change significantly relative to wild-type enzyme. These results indicate that the linker does not adopt any regular secondary structure essential for activity and that the length of the linker is the critical feature that controls flavin-to-heme electron transfer.

Keywords: cytochrome P450BM-3; electron transfer; linker region; secondary structure; site-directed mutagenesis

Cytochrome P450BM-3 is a bacterial enzyme that catalyzes the hydroxylation of fatty acid substrates (S in the scheme shown below) as follows:

$$S\text{-}H + NADPH + H^+ + O_2 \rightarrow S\text{-}OH + NADP^+ + OH^-.$$

Most bacterial P450s utilize a flavoprotein and an iron-sulfur protein to deliver reducing equivalents from NADPH/NADH to the P450, whereas microsomal P450s utilize a P450 reductase that contains both FAD and FMN. P450BM-3 is the bacterial homologue to microsomal P450s because P450BM-3 also utilizes a di-flavin reductase. However, in P450BM-3, the P450 and reductase are fused into a catalytically self-sufficient single polypeptide (Narhi & Fulco, 1986). The 119,000-Da protein contains one equivalent each of heme, FAD, and FMN. Narhi and Fulco (1987) have shown that, upon limited trypsinolysis in the presence of the substrate, the protein is cleaved into two domains, one retaining the heme and the other containing the noncovalently attached FMN and FAD. The individual domains retained their respective activities, such as substrate binding to the heme domain, and both cytochrome *c* and ferricyanide reductase activities for the reductase domain. However, reconstitution of fatty acid hydroxylase activity by combining the two domains has met with limited success (Narhi & Fulco, 1987). This shows that covalent attachment of the two domains is important for activity and suggests that the linker connecting the two domains may play an important role in controlling how the heme and reductase domains interact.

Recently, using site-directed and deletion mutagenesis, we found that the length of the linker and not the sequence are important in controlling the reductase-to-heme electron transfer reaction (Govindaraj & Poulos, 1995). However, this study did not address directly the issue of the type of secondary structure the linker might adopt. In the present study, an attempt has been made to identify whether the linker region adopts any regular secondary structure essential for function by substituting residues 471-473 with glycines and prolines and residues 468-473 with prolines. We also have made a three-alanine insertion into the linker to see what effect increasing the length of the linker has on activity. Quite unexpectedly, we find that these mutations

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Abbreviations: P450BM-3, cytochrome P450BM-3 isolated from *Bacillus megaterium*; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotine adenine dinucleotide phosphate reduced form; IPTG, isopropyl β-D-thio galacto pyranoside; 2′-AMP, 2′-adenosine monophosphate; Pro 3, Pro 6, mutant with residues 471-473 and 468-473, respectively, converted to proline; Gly 3, mutant with residues 471-473 converted to glycine; 3 Ala, mutant with three alanine residues inserted at 473.

have only a marginal effect on activity, indicating that the linker need not adopt a helical conformation and, very likely, no other regular secondary structure in order to maintain catalytic activity.

Results

Cytochrome c and potassium ferricyanide reductase activities

Purified wild-type P450BM-3 and the substitution mutants were analyzed for cytochrome *c* and ferricyanide reductase activities, characteristic activities associated with the flavin-containing reductase domain (Narhi & Fulco, 1987). As shown in Table 1, both cytochrome *c* and ferricyanide reductase activities of the substitution mutants are similar to the wild-type P450BM-3, except for the six-proline substitution mutant. With this mutant, the cytochrome *c* reductase activity increases to 150% of wild-type P450BM-3.

Another basis for comparison is the ratio of cytochrome *c* and ferricyanide reduced to NADPH oxidized. Cytochrome *c* and ferricyanide are one-electron acceptors, so, if the reaction is tightly coupled, the ratio of cytochrome *c* or ferricyanide reduced to NADPH oxidized should be 2.0. The ratio is approximately 1.7–2.0 for the wild type and the mutants, demonstrating that nearly all reducing equivalents derived from NADPH are used for substrate reduction. These close similarities between the substitution mutants and the wild type in both the ferricyanide and cytochrome *c* reductase activities indicate that the reductase domain has not been altered significantly by substitution of glycines and prolines in the linker region.

Enzyme activity

Two methods were used to determine enzyme activity. The simplest is to follow the rate of NADPH oxidation in the presence of substrate. The second is to follow the rate of substrate hydroxylation. In this case, the hydroxylation of ^{14}C-labeled myristic acid is determined as a function of time using an HPLC assay (see the Materials and methods). As shown in Figure 1 and Table 1, both the rate of NADPH oxidation and myristate hydroxylation are not altered significantly in the mutants. Moreover, the ratio of NADPH oxidized to myristate hydroxylated is close to 1.0 for all enzymes. Because 1 NADPH is required to oxidize 1 substrate molecule, a ratio of 1 indicates that electron transfer is tightly coupled to substrate hydroxylation and that little of the NADPH-derived reducing equivalents are funneled toward water or peroxide formation.

The rate of heme reduction was measured by mixing a CO-saturated buffer containing NADPH with a CO-saturated solution containing enzyme and myristate. Formation of the reduced P450-CO complex was followed in the stopped flow at 448 nm. Because the rate of flavin reduction by NADPH is fast compared to heme reduction (Sevrioukova & Peterson, 1995), this experiment measures the FMN → heme electron transfer rate. As shown in Table 1, the rate of heme reduction is very similar in the wild-type and mutant P450s.

Discussion

There were two reasons for suspecting that the linker region connecting the P450BM-3 heme and reductase domains might be helical. First, a comparison of the P450BM-3 linker sequence to sequences of other proteins where the three-dimensional structure is known matches well with a helical stretch in aconitase (Robbins & Stout, 1989). Second, P450BM-3 shares the same heme-linker-reductase domain architecture as nitric oxide synthase (Hevel et al., 1991). The linker in NOS binds calmodulin (Bredt & Snyder, 1990) and there now is structural evidence indicating that the NOS linker segment binds to calmodulin in a helical conformation (Zhang & Vogel, 1994). Our previous work, where deletion mutations blocked electron transfer from FMN → heme (Govindaraj & Poulos, 1995), and the relative insensitivity of replacing three residues in the linker with alanine, a helical "promoting" residue, are consistent with this idea.

The experiments described in this report were designed to more stringently test if the linker must adopt some regular structure to maintain a high level of activity. The outcome of these experiments provides strong evidence that the linker need not be helical. Replacing three or six residues with proline or insert-

Table 1. *Summary of various activities*[a]

Enzyme	Cyt.*c* reductase	NADPH ox. + cyt.*c*	Ferricyanide reductase	NADPH ox. + ferricyanide	NADPH ox. + myrstate	Myristate hydrox.[1]	Heme reduction
Wild type	1,015.3 ± 35.3 (4)	486.6 ± 19.7 (4)	16,159 ± 1,887 (4)	8,045 ± 175 (4)	1,550.4 ± 101 (4)	1,161	1,910 ± 219 (7)
Gly 3	1,068 ± 119 (3)	484 ± 36 (3)	15,866 ± 76.4 (5)	7,555.6 ± 188 (3)	1,869.8 ± 76.5 (5)	1,624	1,381 ± 72 (9)
Pro 3	1,053.3 ± 62.1 (5)	520.9 ± 50.2 (4)	12,565 ± 662 (3)	6,654 ± 473 (3)	1,920 ± 84.6 (3)	1,658	1,730 ± 168 (4)
Pro 6	1,680 ± 63.3 (4)	680.4 ± 142 (4)	15,066 ± 1,352 (3)	8,058 ± 450 (3)	1,469 ± 99 (4)	1,139	1,915 ± 205 (5)
3 Ala insertion	1,286.3 ± 23.8 (3)	648.3 ± 70.6 (3)	17,777 ± 910 (3)	8,742 ± 239 (3)	2,027 ± 109 (4)	1,253	2,487 ± 211 (6)

[a] All rates are in units of min^{-1}. Values in parentheses are the number of replicates used to calculate standard deviations.

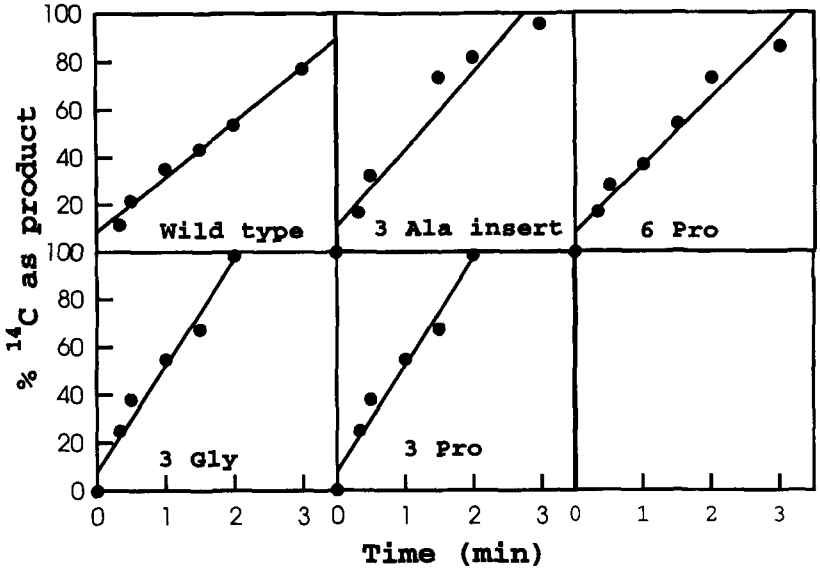

Fig. 1. Comparison of the rate of the hydroxylation of ^{14}C-myristate by the wild-type P450BM-3 and mutants in the linker region. The reaction was stopped at different time points and the product separated from the substrate by HPLC. The percent total counts emerging at the position of hydroxylated product is plotted as a function of time.

ing three extra residues has no effect on enzyme activity. Such results are incompatible with a helical linker being critical for activity. It also appears very unlikely that this region of the linker is involved in any regular ordered element of secondary structure, because a six-proline stretch would be incompatible with both helices and sheets. Therefore, the linker structure in P450BM-3 is unlikely to be the same as in NOS.

In looking for other homologous proteins, P450BM-3 does appear to resemble another multidomain, multiprosthetic group electron transfer enzyme, flavocytochrome *b*2. The linker in flavocytochrome *b*2 also has been subjected to deletion mutagenesis and here, too, activity is lowered (Sharp et al., 1994). The advantage with flavocytochrome *b*2 is that the crystal structure is known (Xia & Matthews, 1990). This structure shows that the region of the linker altered in the mutagenesis work is an elongated stretch on the surface of the protein not involved in any regular secondary structure. Deleting residues, however, forces a reorientation of the flavin and heme domains and hence, alters interdomain electron transfer. It appears that P450BM-3 is very similar. As shown in Figure 2, we envision the linker as an irregular stretch of polypeptide on the surface that can accommodate nearly any sequence, compatible with solubility and aggregation concerns, including a six-proline substitution. Insertion of residues has little effect because these extra residues simply extend into the surrounding medium. Deletions, however, tighten the connection between the two domains and thus force a reorientation of the reductase and heme domains, which dramatically lowers the FMN → heme electron transfer rate. The analogy with flavocytochrome *b*2 is striking and, as Sharp et al. (1994) have argued, correct orientation of the domains is critical for maintaining efficient rates of interdomain electron transfer.

Materials and methods

Chemicals

NADPH, 2′-AMP, DTT, PMSF, EDTA, and horse cytochrome *c* were purchased from Sigma Chemical Co. Sodium myristate was obtained from Fluka. [1-^{14}C]-myristate with specific activities

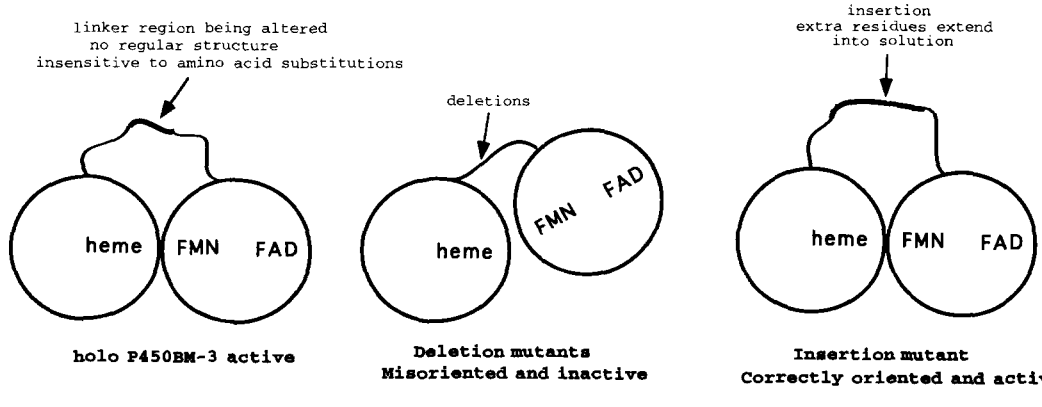

Fig. 2. Schematic representation of the domain movement about the flexible linker region in the wild-type and deletion and insertion mutants of P450BM-3. The heme and reductase domains of P450BM-3 are depicted as circles.

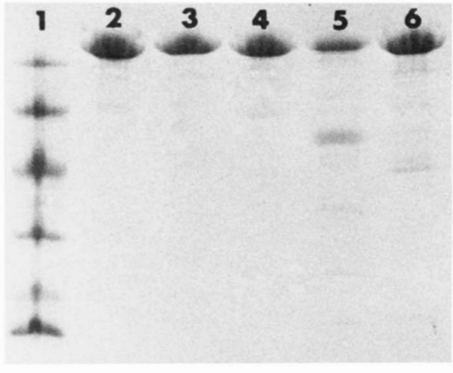

Fig. 3. SDS-PAGE of the wild-type P450BM-3 and the mutants of the linker region. Lane 1, Bio-Rad low molecular weight markers; lane 2, 3 Ala mutant; lane 3, Pro 6 mutant; lane 4, Pro 3 mutant; lane 5, Gly 3 mutant; lane 6, wild-type P450BM-3.

of 58 mCi/mmol was obtained from NEN-research products. CO was purchased from Liquid Carbonic, Inc. T4 DNA ligase, DNA polymerase, and other molecular biology reagents were purchased from New England Biolabs.

Construction of the Pro 3, Gly 3, Pro 6, and 3 Ala enzymes by site-specific mutagenesis

Oligonucleotide site-directed mutagenesis was conducted following the method of Kunkel et al. (1987) in the P450BM-3 gene with the six-amino acid deletion in the linker region (468–473) cloned into the pT7-7 vector (Govindaraj & Poulos, 1995). The six-amino acid deletion mutant DNA was used as the template for making the four variants: substitution of residues 468–473 by prolines (Pro 6 mutant); substitution of residues 471–473 by prolines and glycines (Pro 3 and Gly 3 mutants, respectively), and insertion of three residues at residue number 473 (3 Ala). Selection of the mutations was performed initially by *Pst*1 restriction enzyme analysis because introducing the prolines and glycines in the linker region removes the *pst1* site created by the six-amino acid deleted template DNA. 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 P450BM-3 and its mutants

Wild-type P450BM-3 and mutants were purified from the transformed BL21(DE3) strain by methods described previously (Govindaraj & Poulos, 1995). Enzyme purity was judged by SDS-PAGE (Fig. 3). All four 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 1,000 cpm/nmol. The reaction system for the monooxygenation of fatty acids contained 0.1 M potassium phosphate buffer, pH 7.4, 50 nM enzyme, 200 μM of fatty acid, and 600 μM of 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 conducted using a Cary 3 spectrophotometer. NADPH oxidation in the presence of myristate was performed in a 1-mL reaction mixture containing 9 pmol of enzyme, 100 nmol of myristate, 200 nmol of NADPH in 0.1 M potassium phosphate buffer, pH 7.4. Cytochrome *c* reductase activity was determined by measuring the increase in absorbance at 550 nm in a 1.0-mL sample containing 5 nmol of cytochrome *c*, 2 pmol of enzyme, and 200 nmol of NADPH in 0.1 M potassium phosphate 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 minute 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 potassium 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.

Rate of heme reduction

A 0.1-M potassium 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 made 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 of enzyme and 100 μM of myristate in CO-saturated buffer. In all cases, the absorbance change was monitored at 448 nm. Signal to noise ratio was 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 is the first-order rate constant, t is time, and B is a constant. An accurate estimation of total heme reduced cannot be determined directly at 448 nm using the stopped flow because the flavins also absorb in this region and are not completely reduced under these conditions. However, the increase at 448 nm is due to heme reduction because flavin reduction leads to a decrease in absorbance near 448 nm.

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