A Single Mutation in Cytochrome P450 BM3 Induces the Conformational Rearrangement Seen upon Substrate Binding in the Wild-type Enzyme*

Received for publication, February 17, 2004, and in revised form, March 12, 2004
Published, JBC Papers in Press, March 12, 2004, DOI 10.1074/jbc.M401717200

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The multidomain fatty-acid hydroxylase flavocytochrome P450 BM3 has been studied as a paradigm model for eukaryotic microsomal P450 enzymes because of its homology to eukaryotic family 4 P450 enzymes and its use of a eukaryotic-like diflavin reductase redox partner. High-resolution crystal structures have led to the proposal that substrate-induced conformational changes lead to removal of water as the sixth ligand to the heme iron. Concomitant changes in the heme iron spin state and heme iron reduction potential help to trigger electron transfer from the reductase and to initiate catalysis. Surprisingly, the crystal structure of the substrate-free A264E heme domain mutant reveals the enzyme to be in the conformation observed for substrate-bound wild-type P450, but with the iron in the low-spin state. This provides strong evidence that the spin-state shift observed upon substrate binding in wild-type P450 BM3 not only is caused indirectly by structural changes in the protein, but is a direct consequence of the presence of the substrate itself, similar to what has been observed for P450cam. The crystal structure of the palmitoleate-bound A264E mutant reveals that substrate binding promotes heme ligation by Glu284, with little other difference from the palmitoleate-bound wild-type structure observable. Despite having a protein-derived sixth heme ligand in the substrate-bound form, the A264E mutant is catalytically active, providing further indication for structural rearrangement of the active site upon reduction of the heme iron, including displacement of the glutamate ligand to allow binding of dioxygen.

Cytochrome P450 enzymes are among the most studied enzymes, in no small part because of the pivotal roles that hepatic P450 enzymes play in mammalian drug metabolism (1). Recent years have seen an explosion in the structural data available for these systems, a substantial proportion of which is on cytochrome P450 BM3 (2). This multidomain enzyme is isolated from Bacillus megaterium and contains an N-terminal fatty acid-binding P450 domain fused to its reductase partner, an NADPH-dependent diflavin-cytochrome P450 reductase (3). It has been used as the paradigm model for studying the similar, but membrane-associated eukaryotic microsomal P450 systems. This has been primarily because its heme domain is similar to the eukaryotic fatty-acid hydroxylases from P450 family 4, because the enzyme is soluble and uses a eukaryote-like cytochrome P450 reductase as the reductase (as opposed to the two-component ferredoxin reductase and ferredoxin systems found in many other bacterial systems), and because it is a convenient catalytically self-sufficient fusion protein enzyme (2–4). Cytochrome P450 BM3 is a fatty-acid hydroxylase that displays an unusually high rate of oxygenation of long-chain fatty acids (e.g., >15,000 turnovers/min with arachidonic acid) (5), likely because of the efficient electron transfer between the different redox modules afforded by their covalent linkage and hence close spatialization (2). A sophisticated mechanism to avoid the unwanted generation of reactive oxygen species through futile cycling has been found in many P450 enzymes studied to date. The binding of oxygen occurs only with the reduced (ferrous) heme, and the reduction of the ferric heme iron by electron transfer from the reductase partner is, in turn, dependent on the binding of substrate, effectively gating initiation of the reaction by substrate binding (6, 7). In P450 BM3 and the Pseudomonas putida camphor hydroxylase P450cam (the most intensively studied P450 enzyme), substrate binding induces a heme iron spin-state shift and a concomitant increase in the reduction potential of the heme iron, favoring the 1-electron reduction that commits the enzyme to the catalytic cycle (6, 7). The molecular mechanism whereby substrate binding induces this shift seems to be somewhat different in the P450 enzymes studied to date, although the substrate binding-induced displacement of water as the sixth ligand to the heme iron is a common feature (see Refs. 8 and 9). In P450cam, the binding of substrate does not affect any large-scale changes in the protein structure, and the displacement of water is due to direct steric hindrance with the camphor molecule (10). In contrast, P450 BM3 undergoes large-scale conformational changes upon binding of fatty acids, and these changes have been proposed to drive the conversion of a six- to a five-coordinate heme group. Indeed, there is no direct interaction observed between bound fatty acids and the water molecule in the sixth ligand position in the available crystal structures of the P450 BM3 heme domain. The conformational change in P450 BM3 involves a reorganization of the I helix, and it has been proposed that this creates a new water binding position (11).

¶ Supported by the Biotechnology and Biological Sciences Research Council (BBSRC).
§ Supported by the Engineering and Physical Sciences Research Council (EPSRC).
† Supported by the BBSRC and the EPSRC.
‡ Supported by the European Community Access to Research Infrastructure Action of the Improving Human Potential Program to the European Molecular Biology Laboratory Hamburg Outstation under Contract HPRI-CT-1999-00017. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The atomic coordinates and structure factors (codes 1SMI and 1SMJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
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This paper is available on line at http://www.jbc.org
This position is mutually exclusive with the sixth ligand binding position and is proposed to have greater affinity, leading to an effective switch in heme coordination by transfer of the water to the new position. The majority of the P450 structures indeed show a conserved bend in the I helix, and this mechanism might therefore be of a general nature (see Ref. 12). However, certain P450 enzymes do not contain any deformation of the I helix in the resting state (see Ref. 13), whereas P450cam has a bent I helix that does not significantly change conformation upon substrate binding (14).

We present here the crystal structures of both the substrate-free and palmitoleic acid-bound forms of the A264E mutant in the P450 BM3 heme domain. The alanine occupies a key position in the I helix of the P450 enzyme. The backbone of this residue hydrogen bonds to the sixth ligand water molecule in the unbound form and is substantially shifted upon substrate binding (8, 15). Interestingly, in several of the CYP4 family of fatty-acid oxygenase P450 enzymes, a conserved glutamate residue at position 264 (BM3 numbering) is known to covalently ligate the heme macrocycle through autocatalytic turnover-dependent attachment to the 5-methyl group of the porphyrin (16). To investigate the possibility of creating a similar protein-heme link in the related P450 BM3 heme domain, the A264E mutant was created. Although no covalent modification of the heme was observed, this mutant has several unique features (see accompanying article (17)). In the fatty acid-free form, Glu264 ligates the heme iron in a proportion of the molecules, creating a novel thiolate-carboxylate ligation that is pushed toward full ligation by binding of the substrate. Surprisingly, the crystal structure of the substrate-free form of this mutant reveals the protein to be in the conformation previously considered to be induced by substrate binding, despite the fact that the P450 enzyme is still in a low-spin state and free of fatty acid. We show that binding of fatty acid does not then introduce any further gross conformational change in the protein structure, although a change in the proportion of molecules in which glutamate ligates the heme iron is effected. The implications of these observations for the mechanism of the substrate binding-induced heme iron spin-state shift of P450 BM3 and P450 enzymes in general are discussed, along with the ramifications for understanding conformational equilibria in P450 enzymes and how the binding of substrate impacts on these equilibria.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification of Mutant P450 BM3**—The A264E mutant of the P450 BM3 heme domain was created, expressed, and purified as described in the accompanying article (17). A final purification step by fast protein liquid chromatography using Q-Sepharose resin (under the same conditions as used for the low-pressure chromatographic purification described in the accompanying article (17)) was used to produce homogeneous enzyme for crystallographic studies.

**Crystal Structure of the Substrate-free Form**—The P450 BM3 A264E heme domain was crystallized in both the palmitoleic acid-bound and substrate-free forms by the sitting drop method at 4 °C. Sitting drops were prepared by adding 2 μl of mother liquor to 2 μl of 15 mg/ml enzyme. Palmitoleic acid-bound crystals were obtained by co-crystallizing the enzyme with 1.2 μM palmitoleic acid (~6 times the Kd value obtained from spectral binding titration data) and a well solution of 100 mM magnesium acetate, 20% polyethylene glycol 2000MME, and 100 mM cacodylic acid at pH 6.3. Substrate-free crystals were obtained with a well solution of 10 mM manganese sulfate, 20% polyethylene glycol 2000MME, and 100 mM cacodylic acid at pH 6.3. Crystals were formed in both cases after ~7 days. Crystals were immersed in 10% polyethylene glycol 200 in mother liquor as a cryoprotectant, before being mounted on a nylon loop and flash-cooled in liquid nitrogen.

**Data Collection, Structure Elucidation, and Refinement**—The data used for refinement were collected at the European Synchrotron Radiation Facility (Grenoble, France) on ID14-EH1 using an ADSC Q4R CCD detector. Crystals were cooled to 100 K, and diffraction data were collected in oscillations of 1°. Data were processed and scaled using the HKL package programs DENZO and SCALEPACK (18). The substrate-free crystal structures were solved via molecular replacement using the program AMoRE and the high-resolution wild-type P450 BM3 crystal structure (Protein Data Bank code 2HFD) (19) as a search model. The palmitoleate-bound wild-type crystal structure was used a starting model for the palmitoleate-bound mutant form. In both cases, positional and B-factor refinement was carried out using REFMAC5 with manual rebuilding of the model at regular intervals in TURBO-FRODO (20, 21). Only in the case of the low-resolution substrate-bound crystal form were strong NCS restraints imposed throughout refinement. Data collection and final refinement statistics are given in Table I. The atomic coordinates and structure factors for both crystal structures have been deposited in the Protein Data Bank with codes 1SMI (substrate-free A264E mutant) and 1SMJ (substrate-bound A264E mutant).

**RESULTS AND DISCUSSION**

**Crystal Structure of the Substrate-free Form**—Initial crystalization trials for the substrate-free form of the A264E heme domain of P450 BM3 using the published conditions for the wild-type heme domain proved unsuccessful. The use of manganese sulfate in place of magnesium acetate led to related conditions that generated large diffraction-quality crystals. The structure was solved to 2.0 Å and contained two molecules in the asymmetric unit. The overall conformation of both molecules is similar (root mean square deviation of 0.46 Å for all Cα atoms), with molecule B having a significantly higher...
average B-factor (39.1 Å² compared with 47.6 Å²) due to fewer packing constraints. In this study, molecule A will be used for discussion and calculations unless mentioned otherwise.

Because of the different space groups of the orthorhombic A264E mutant and the wild-type monoclinic crystal form, comparison of the structures was made after overlay using the structurally invariant residues, as described by Haines et al. (11), representing ~62% of the structure. Surprisingly, it was found that the A264E structure is structurally similar (root mean square deviation of 0.54 Å for all Cα atoms) to the substrate-bound form of the wild-type enzyme (hereafter referred to as the SB conformation) and shows a significant difference (root mean square deviation 1.37 Å for all Cα atoms) from the substrate-free wild-type structure (hereafter referred to as the SF conformation) (8, 15).

However, no substrate was added to the A264E mutant during either purification or crystallization, and no substrate could be observed in the electron density maps. Similar to the changes seen upon substrate binding in wild-type P450 BM3, the majority of residues that are in significantly different positions between the substrate-free structures of A264E and the wild-type heme domain are located in the "lid domain" of the substrate access channel, which consists of the F and G helices, the loop between them, and the B′ helix (8). Several of these residues are well defined in A264E molecule B, indicating substantial plasticity of this region in the absence of substrate. However, there is no significant large-scale difference between both A264E molecules in the asymmetric unit.

In contrast, upon closer inspection of the active site of the A264E enzyme, there is a marked difference in the vicinity of the heme iron between the two molecules in the asymmetric unit. Molecule B has the side chain of Glu264 pointing away from the heme, its carboxylate group stacking with the aromatic group of Phe87 (Fig. 2, left). In contrast, molecule A has the carboxylate group coordinating the heme iron (Fig. 2, right). This heterogeneity of glutamate ligation was predicted from solution spectrophotometric studies and from EPR analysis (see accompanying article (17)), and the thiolate-carboxylate ligation is novel to P450 enzymes and (as far as we are aware to date) to cytochromes in general. There do not seem to be any significant conformational changes to the overall P450 structure associated with the "switch" of Glu264 between its two detected conformations on (a) the heme iron or (b) stacking with Phe87. It is likely that, in solution, the carboxylate continually switches between the "on" and "off" heme iron states without major accompanying protein reorganization. According to spectroscopic data, in the solution state, the equilibrium is poised at ~3–4:1 in favor of the heme iron ligand off form (17).

The interaction with Phe87 is particularly interesting, given the fact that this residue is absent from the CYP4 enzymes in which covalent ligation of the heme methyl group has been demonstrated. Phe87 interacts with the ω-methyl group of fatty acid substrate(s) in wild-type P450 BM3 and is considered to be a critical regulatory residue that controls regioselectivity of substrate oxygenation (8, 22, 23). A particular difference in the behavior of P450 BM3 with respect to eukaryotic CYP4 enzymes is the inability of the former to hydroxylate at the ω-position (24).

Close examination of the A264E structure reveals no clear direct structural explanation for the fact that this mutant mimics the conformation of the substrate-bound form of the wild-type enzyme. In particular, no extra stabilizing features involving the newly introduced glutamate side chain can be found in comparison with available wild-type structures. We therefore tried to place the Glu264 side chain in the corresponding substrate-free wild-type structure. All of the conformations available to the glutamate result in severe steric clashes with several other residues nearby (e.g. Phe87, Thr360, and Ile363, in

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The abbreviations used are: SB, substrate-bound; SF, substrate-free.
addition to the heme macrocycle itself), clearly resulting in the need for a protein conformational rearrangement to accommodate for the increased bulk of residue 264. It therefore seems likely that the A264E mutation does not particularly stabilize the SB conformation, but rather destabilizes the SF conformation of the enzyme to the extent that the SB conformation, even in the absence of substrate, is preferred.

In light of the above observations, it is interesting to note that, in the substrate-bound wild-type P450 BM3 structures, the substrates are in close contact with the side chain of Ala264. This suggests that the mechanism by which substrate binding switches P450 BM3 to a different conformational state does not involve simply the expulsion of water molecules from the substrate-binding cavity, but in addition exploits the force exerted by the substrate on Ala264, a residue that acts as a sensitive trigger for the conformational conversion. In the A264E mutant, unfavorable steric interactions of the glutamate side chain induce the switch to the SB conformation, without necessity for the substrate interaction. An alternative explanation that could be put forward is that the P450 BM3 heme domain is in rapid conformational equilibrium between the SF and SB conformations, with the equilibrium being strongly favored toward the SF conformation for wild-type P450 BM3 in the absence of substrate. In this model, the substrate binds preferentially to the SB conformation, effectively shifting the equilibrium toward this form as the substrate concentration is increased. This model is also consistent with the behavior observed for the A264E heme domain. For several substrates tested, the apparent binding constants ($K_a$) determined are considerably lower than those for the wild-type heme domain, indicating much tighter binding (17).

A substrate binding-induced spin-state shift has been observed in a large number of P450 enzymes studied to date (see Refs. 25 and 26). It is generally accepted that this behavior serves to avoid the potentially dangerous generation of active oxygen species that would occur through binding and subsequent reduction of molecular oxygen in the absence of substrate. Spin-state change induces a positive change in the heme iron reduction potential, favoring electron transfer from the redox partner (6, 7). The molecular mechanism underlying this change in the heme iron spin state has been proposed to be either a direct displacement of the sixth ligand water molecule (as in P450cam) or a more indirect displacement via substrate-induced changes in the protein structure (as in P450 BM3) (11). We have shown that the fatty acid-free A264E structure does show all the structural hallmarks of a substrate-bound P450 BM3 enzyme, but in the absence of substrate. An intriguing difference regarding A264E is the fact that, in contrast to the substrate-bound wild-type enzyme, A264E does not show any significant high-spin character in either the solution or crystalline state. A low-spin configuration would be expected for the glutamate-ligated species, but both spectroscopic and crystallographic studies show that there is a large population of non-glutamate-ligated protein in the substrate-free A264E heme domain and that this species does not lose the water ligand and convert to the high-spin form despite the change to the SB conformation. This suggests strongly that, even in wild-type P450 BM3, the spin-state shift is a direct (rather than an indirect) result of substrate binding. The high-resolution N-palmitoylglycine-bound wild-type structure has lead to the sug-
gestion that substrate binding-induced protein rearrangement creates a new water-binding site (designated site H) adjacent to the heme (11). The proposed higher water affinity of site H over the heme iron ligation site (designated site L) and the fact that these sites are mutually exclusive (so that only a single water molecule can bind at either site L or H at any given time) led to the proposal that site H effectively pulls the water molecule away from site L, leading to the observed shift in the heme iron coordination state and hence conversion to the high-spin form (11). The A264E heme domain structure shows all ligands to site H to be in identical positions compared with the N-palmitoylglycine-bound wild-type P450 BM3 structure (Fig. 4 upper).

As has been pointed out previously (11), there are no direct steric clashes between the bound fatty acid substrates of P450 BM3 and the water molecule at site L. The palmitoleic acid-bound wild-type P450 BM3 indicates that secondary conformational changes in the protein/substrate must occur following reduction of the heme iron because the substrate is too distant from the heme iron for oxidative attack at catalytically relevant positions on the fatty acid chain (8). NMR studies of the substrate-bound form of ferrous, fatty acid-bound wild-type P450 BM3 are consistent with a significant reorientation of the substrate in this enzyme form (27). It is clear, however, that, upon binding of the fatty acid analog N-palmitoylglycine, the surroundings of site L become more hydrophobic, decreasing the water affinity and ultimately shifting the water molecule to predominantly occupy site H. Spectroscopic studies of wild-type P450 BM3 at catalytically relevant temperatures (by both electronic absorption and resonance Raman) have shown that, even in the presence of apparently saturating concentrations of substrate, an equilibrium exists between the high-spin five-coordinate and the low-spin six-coordinate states of the heme iron. Depending on the nature of the fatty acid substrate used, varying amounts of low-spin heme iron are detected, with shorter chain saturated fatty acids (e.g., lauric acid) being less effective than longer chain ones (e.g., palmitic acid) at effecting the shift in the spin-state equilibrium toward the high-spin state (28, 29). This clearly indicates how, even in the presence of substrate, the possibility exists for water remaining bound at the heme iron (site L), and the water affinity of this site is strongly dependent on the nature of the substrate, although all drive the L-H equilibrium toward site H.

Crystal Structure of the Substrate-bound Form—In contrast to the requirement for screening novel conditions to obtain...
suitable crystals of the substrate-free A264E heme domain, the palmitoleate-bound form of A264E was found to crystallize in the same space group as that reported previously for substrate-bound wild-type P450 BM3 (8). As observed for the wild-type enzyme, the resolution and quality of the data obtained for this particular crystal form are rather poor by comparison with those for the substrate-free enzyme. Nevertheless, electron density clearly indicates no major changes between the palmitoleate-bound A264E and palmitoleate-bound wild-type P450 BM3 structures. The single exception is the fact that, in all four molecules in the asymmetric unit of the A264E structure, the Glu264 side chain ligation the heme iron (Fig. 4, lower). This finding indicates that the presence of substrate induces movement of the glutamate onto the iron to replace water as the sixth heme ligand and is completely consistent with the spectroscopic studies reported in the accompanying article (17).

Addition of long-chain fatty acids perturbs the UV-visible absorption spectrum of the A264E heme domain, inducing red shifts of the heme Soret band toward 426 nm at apparent substrate saturation. This is in contrast to the blue shifts observed following substrate addition to wild-type P450 BM3 (toward 390 nm) and reflects the increasing proportion of the observed following substrate addition to wild-type P450 BM3 shifts of the heme Soret band toward 426 nm at apparent state, as observed in solution studies. Specifically, palmitoleate formation, driving the enzyme toward a completely ligated form, renders the substrate-free A264E structure. Despite any significant further structural rearrangement induced following palmitoleic acid binding to the SB conformation of A264E, the substrate does influence directly the heme iron. This is under further study using Ala264 variants in which the side chain of the introduced amino acid does not ligate the heme iron in the substrate-free and substrate-bound forms.

Further scrutiny of both the substrate-free and substrate-bound A264E structures reveals other important features of the P450 BM3 structure that relate to attempts to engineer covalent ligation of the heme macrocycle via the interaction of Glu264 with the heme 5-methyl group. The active-site organization in P450 BM3 is such that Phe87 and likely the I helix residue Thr260 obstruct access of Glu264 to the relevant position on the porphyrin ring. The failure to obtain any significant degree of covalent ligation in the A264E mutant may thus be explicable through steric restrictions in the active site. To address these restrictions and to enable covalent heme ligation by Glu264 and produce a more robust and biotechnologically exploitable form of P450 BM3, we are currently generating secondary mutations at these locations that might facilitate access of Glu264 to the relevant methyl group and could thus allow autocatalytic linkage to occur.

Conclusion—Crystallographic studies of the A264E variant of P450 BM3 confirm the proposals based on spectroscopic studies that the glutamate is able to ligate the ferric heme iron of the mutant in the substrate-free form and that substrate addition “forces” on the ligand, producing a completely low-spin six-coordinate species, as opposed to the extensively high-spin five-coordinate form seen for the wild-type P450 BM3 (17). Structural studies explain clearly why substrate has this effect on the A264E enzyme since palmitoleic acid occupies one of the two favored positions for the Glu264 side chain. Glu264 can no longer form an interaction with the key regiospecificity-determining residue Phe87 in the palmitoleate-bound form and is thus induced to move toward its only other acceptable position, coordinating to the heme iron.

An unexpected finding, but one with enormous ramifications for understanding the conformational changes that occur in P450 BM3 (and P450 systems in general) and their consequences, is the fact that both the substrate-free and palmitoleic acid-bound forms of the A264E heme domain have overall structural conformations that are virtually identical to those found for the substrate-bound forms of wild-type P450 BM3, but are distinct from that of the substrate-free wild-type heme domain (8, 11, 15). This SB conformation is not dependent on whether Glu264 ligation the heme iron or is positioned against Phe87, and the enzyme is low-spin in both forms and water-coordinated in the latter form for substrate-free A264E. The most obvious explanations are 1) that the SB conformation in wild-type P450 BM3 is a consequence of substrate-induced deformation of the I helix in the region of Ala264 and that the A264E mutation favors this conformational rearrangement independent of the substrate due to steric restrictions to movement of the glutamate side chain in the SB conformation, and/or 2) that P450 BM3 is in a continual dynamic equilibrium between the SB and SF conformations and that the A264E mutation forces this equilibrium toward the SB conformation. For both cases, the fact that the mutant remains in a low-spin form in the SB conformation in the fatty acid-free structure suggests that the spin-state conversion observed in wild-type P450 BM3 upon substrate association (and the concomitant change in the reduction potential) is a consequence of the physical presence of the lipid in the environment of the heme, and not a result of the adoption of the SB conformation per se. Moreover, the fact that the SB conformation is clearly accessi-
Crystal Structure of Cytochrome P450 BM3 A264E

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ble in the substrate-free A264E mutant also suggests that the binding of fatty acid might not be essential for inducing this conformational rearrangement in the wild-type enzyme and that the adoption of the SB conformation in the palmitoleate-bound wild-type structure could merely be a consequence of favorable binding of the substrate to this conformer. This conclusion is supported by the fact that much lower $K_d$ values are observed for binding of several long-chain fatty acids to the A264E variant than to the wild-type P450 BM3 (17). The SB conformation predominates in the mutant. In ongoing work, we aim to validate further the hypotheses that arise from these findings through creation of other variants at position 264, specifically investigating Ala$^{264}$ variants that induce the conformational switch to the SB conformation, but that do not, in addition, give rise to coordination to the heme iron.

Acknowledgments—We are grateful for access to beamlines X11 and BW7A at the European Molecular Biology Laboratory Hamburg Out-station (Deutsches Elektronen-Synchrotron), which were essential to crystal improvement, and for access to ID14.1 at the European Synchrotron Radiation Facility, where the final data were collected.

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J. Biol. Chem. 2004, 279:23287-23293.
doi: 10.1074/jbc.M401717200 originally published online March 12, 2004

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