A single mutation in P450 BM3 induces the conformational rearrangement seen upon substrate-binding in wild-type enzyme

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Running title: Crystal structure of P450 BM3 A264E
Summary

The multidomain fatty acid hydroxylase flavocytochrome P450 BM3 has been studied as a paradigm model for eukaryotic microsomal P450s, due to its homology with eukaryotic family 4 P450s 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 the 6th ligand water ligand to the heme iron. Concomitant changes in heme iron spin-state and heme iron reduction potential help to trigger electron transfer from the reductase and initiate catalysis. Surprisingly, the crystal structure of the substrate-free A264E heme domain mutant reveals the enzyme to be in the conformation observed for the 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 is not only caused indirectly by structural changes in the protein, but is a direct consequence of the presence of the substrate itself, similar to that observed for P450 cam. The crystal structure of the palmitoleate-bound A264E mutant reveals that substrate binding promotes heme ligation by Glu 264 with little other difference from the wild-type palmitoleate-bound structure observable. Despite having a protein-derived 6th 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.
Introduction

Cytochrome P450s (P450s) are among the most studied enzymes, in no small part due to the pivotal roles that hepatic P450s 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 a N-terminal fatty acid-binding P450 domain fused to its redox partner, an NADPH-dependent diflavin cytochrome P450 reductase (CPR, 3). It has been used as the paradigm model for studying the similar, but membrane-associated, eukaryotic microsomal P450 systems. This has been primarily due to the similarity in its heme domain to the eukaryotic fatty acid hydroxylases from P450 family 4, the fact that the enzyme is soluble, that it uses a eukaryotic-like CPR as the redox partner (as opposed to the two component ferredoxin reductase and ferredoxin systems found in many other bacterial systems) and since 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 due to the efficient electron transfer between the different redox modules afforded by their covalent linkage and hence close spatial organization (2). A sophisticated mechanism to avoid the unwanted generation of reactive oxygen species through futile cycling has been found in many P450s studied to date. The binding of oxygen only occurs to the reduced (ferrous) heme and the reduction of the ferric heme iron by electron transfer from the redox 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 P450 cam (the most intensively studied P450 enzymes) substrate-binding induces a heme iron spin-state...
shift and concomitant increase in reduction potential of the heme iron, favouring the one 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 P450s studied to date, although the substrate-binding induced displacement of water as the 6th ligand to the heme iron is a common feature (e.g. 8, 9). In P450 cam, the binding of substrate does not effect 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 hexa- to a penta-coordinated heme group. Indeed, there is no direct interaction observed between bound fatty acids and the water molecule in the 6th ligand position in the available crystal structures of the P450 BM3 heme domain. The conformational change in P450 BM3 involves a reorganisation of the I helix and it has been proposed that this creates a new water binding position (11). This position is mutually exclusive with the 6th 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 (e.g. 12). However, certain P450s do not contain any deformation of the I helix in the resting state (e.g. 13), while P450 cam has a bent I helix that does not significantly change conformation upon substrate binding (14).

We here present the crystal structures of both 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. The backbone of this residue hydrogen bonds to the 6th ligand water molecule in the unbound form and is substantially shifted upon substrate binding (8, 15). Interestingly, in several of the CYP 4 family of fatty acid oxygenase P450s, 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 manuscript, 17). In the fatty acid-free form, Glu 264 ligates the heme iron in a proportion of the molecules, creating a novel thiolate-carboxylate ligation that is pushed towards 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 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 on the mechanism of substrate-binding induced heme iron spin-state shift of P450-BM3 and cytochromes P450 in general are discussed, along with the ramifications for understanding conformational equilibria in cytochromes P450 and how binding of substrate impacts on these equilibria.

**Experimental procedures**

*Mutagenesis, expression and purification of mutant P450 BM3.*
The A264E mutant of P450 BM3 heme domain was created, expressed and purified as described in the accompanying manuscript (17). A final purification step by FPLC using Q-Sepharose resin (under the same conditions as those used for the low-pressure chromatographic purification described in the accompanying manuscript, 17) was used to produce homogeneous enzyme for crystallographic studies.

**Crystallization**

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 (approximately 6 x the Kᵅ value obtained from spectral binding titration data, and a well solution of 100 mM magnesium acetate, 20 % PEG 2000 MME and 100 mM cacodylic acid at pH 6.3. Substrate-free crystals were obtained with a well solution of 10mM manganese sulfate, 20% PEG 2000MME and 100 mM cacodylic acid at pH 6.3. Crystals were formed in both cases after approximately 7 days. Crystals were immersed in 10 % PEG 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**

Data used for refinement were collected at ESRF, Grenoble, France on ID14-EH1 using an ADSC Q4R CCD detector. Crystals were cooled at 100 K and diffraction data 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 P450 BM3 wild-type crystal structure (PDB code 2HPD) as a search model (19). The wild-type palmitoleate-bound crystal structure was used a starting model for the palmitoleate-bound mutant form. In both cases positional and B-factor refinement were 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 1. Structure factors and final coordinates for both crystal structures have been deposited in the PDB with codes 1SMI (substrate free A264E mutant) and 1SMJ (substrate bound A264E mutant).

**Results and Discussion**

*Crystal structure of the substrate-free form*

Initial crystallization 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 sulphate to substitute for magnesium acetate led to related conditions that generated large, diffraction quality crystals. The structure was solved to 2.0 Å and contained 2 molecules in the asymmetric unit. The overall conformation of both molecules is similar (r.m.s.d. 0.46 Å for all Cα atoms), with molecule B having a significant higher average B-factor (39.1 Å² compared to 47.6 Å²) due to less packing constraints. In this manuscript, molecule A will be used for discussion and calculations unless mentioned otherwise.
Due to 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 approximately 62% of the structure (11). Surprisingly, it was found that the A264E structure is structurally similar (r.m.s.d. 0.54 Å for all Cα) with the substrate-bound form of the wild-type enzyme (hereafter referred to as conformation SB) and shows a significant difference (r.m.s.d. 1.37 Å for all Cα) with the substrate-free wild-type structure (hereafter referred to as conformation SF) (8, 15) (Figure 1a,b). However, no substrate was added to the A264E mutant during either purification or crystallization, nor could any substrate 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 less well defined in A264E molecule B, indicating substantial plasticity of this region in absence of the 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 observed in the vicinity of the heme iron between the two molecules in the asymmetric unit. Molecule B has the side chain of Glu 264 pointing away from the heme, its carboxylate group stacking with the aromatic group of Phe 87 (Figure 2a). In contrast, molecule A has the carboxylate group coordinating the heme iron (Figure 2b). This heterogeneity of glutamate ligation was predicted from
solution spectrophotometric studies, and from EPR analysis (see accompanying paper, 17) and the thiolate-carboxylate ligation is novel to cytochromes P450, 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 Glu 264 between its two detected conformations on a) the heme iron, or b) stacking with Phe 87. 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 solution state the equilibrium is poised at ~ 3-4:1 in favour of the heme iron ligand “off” form (17). The interaction with Phe 87 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. Phe 87 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 behaviour 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 is mimicking 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 to available wild-type structures. We therefore tried to place the Glu 264 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. Phe 87, Thr260 and Ile263, in 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 conformation SB, but rather destabilises the SF conformation of the enzyme, to the extent that the SB conformation, even in absence of the 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 Ala 264. This suggests 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 Ala 264, a residue that acts as a sensitive trigger for the conformational conversion. In the A264E mutant, unfavourable 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 SF and SB forms, with the equilibrium being strongly favoured towards the SF form 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 towards this form as substrate concentration is increased. This model is also consistent with the behaviour observed for the A264E heme domain. For several substrates tested, the apparent binding constants (K_d values) determined are considerably lower that those for the wild-type heme domain, indicating much tighter binding (17). Given that the SB conformation is favoured in the fatty acid-free form of the A264E heme domain,
the “tight-binding” form of the protein is over-represented in solution with respect to that seen for wild-type P450 BM3 (Figure 3).

A substrate-binding induced spin-state shift has been observed in a large number of P450s studied to date (e.g. 25, 26). It is generally accepted that this behaviour 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, favouring electron transfer from the redox partner (6, 7). The molecular mechanism underlying this change in heme iron spin-state has been proposed to either be a direct displacement of the 6th ligand water molecule (as in P450 cam) or a more indirect displacement via substrate-induced changes in the protein structure (as for P450 BM3, 11). We have shown that the structure of fatty acid-free A264E does show all the structural hallmarks of a substrate-bound BM3 enzyme, but in the absence of substrate. An intriguing difference regarding A264E is the fact that, in contrast to substrate-bound wild type-enzyme, A264E does not show any significant high-spin character, in either 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 substrate-free A264E heme domain, and that this species does not lose the aqua ligand and convert to high-spin 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-palmitoyl-glycine-bound wild-type structure has lead to the suggestion 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 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 heme iron coordination state and hence conversion to the high-spin form (11). The A264E mutant heme domain structure shows all ligands to the H site to be in identical position as for the N-palmitoyl-glycine-bound wild-type P450 BM3 structure (Figure 4a). However, the water molecule is still occupying site L (the heme iron) for molecule B, while in molecule A Glu 264 ligates the heme iron. In both cases the H site remains unoccupied due to steric hindrance with either the water (in molecule B) or the Glu 264 side chain (in molecule A) at site L. It can therefore be concluded that site L (heme iron) remains the higher affinity site for water in the absence of substrate in conformation SB.

As has been pointed out previously, 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 structure of wild-type P450 BM3 indicates that secondary conformational changes of protein/substrate must occur following reduction of the heme iron, since 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, however, clear that upon binding of the fatty acid analogue N-palmitoyl glycine 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 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 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 spin-state equilibrium towards high-spin (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 towards the H site.

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 crystallise in the same space group as that reported previously for the substrate-bound wild-type P450 BM3 (8). As observed for the wild-type enzyme, the resolution and the quality of the data obtained for this particular crystal form is rather poor by comparison with 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 4 molecules in the asymmetric unit of the A264E structure, the Glu 264 side chain ligates the heme iron (Figure 4b). This finding indicates that the presence of substrate induces movement of the glutamate onto the iron to replace water as the 6th heme ligand, and is completely consistent with the spectroscopic
studies reported in the accompanying paper (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 towards 426 nm at apparent substrate saturation. This is in contrast to the blue shifts observed following substrate addition to wild-type P450 BM3 (towards 390 nm) and reflects the increasing proportion of the glutamate-ligated, low-spin form of the A264E mutant following substrate addition, as opposed to the pentacoordinated, high-spin form of the wild-type that accumulates through substrate-induced displacement of the water ligand from the heme iron (17, 28). From the substrate-free A264E structure, it is clear that two conformations are possible for the Glu 264 side chain and solution studies indicate the equilibrium ratio between both states to be strongly dependent on the solution conditions. Upon palmitoleate binding, the substrate effectively occupies the volume of the non heme iron-ligating conformation, driving the enzyme towards a completely ligated state, as observed in solution studies. Specifically, palmitoleate interacts with Phe 87, preventing Glu 264 from occupying the position observed in molecule A in 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 ligation state by minimising the degrees of freedom available to the Glu 264 sidechain. Strong heme ligands such as azoles function as potent inhibitors for P450s, and many are used as antifungal drugs to inactivate the sterol demethylase P450 CYP51 (30). It is therefore surprising to note that, despite the fact that the substrate-bound oxidised A264E structure shows fully hexa-coordinated heme iron, catalytic turnover for this mutant can still be observed, albeit at lower levels than those observed for wild-type P450 BM3 (17). This is a further indication that, upon reduction of ferric iron to ferrous, the position of the substrate with respect to the heme is critical for catalysis.
to the heme and perhaps the structure of the enzyme itself change dramatically, releasing the strong conformational lock on the glutamate side-chain and allowing oxygen to bind to the iron. Presumably this commits the enzyme to its “regular” catalytic cycle, and prevents coordination of the glutamate to the iron until it returns to a ferric form following product formation. A further interesting aspect of this study arises from the structural change and its effect on thermodynamic properties of the P450. In both the substrate-free and arachidonate-bound forms of the A264E mutant, the reduction potential of the heme iron is $\sim -315$ mV (see accompanying paper). By contrast, the reduction potential of the conformationally different substrate-free form of P450 BM3 is $-427$ mV, rising to $-289$ mV on binding of arachidonate and an extensive switch in spin-state equilibrium towards high spin (31). In A264E, the heme iron remains predominantly low-spin in both substrate-free and substrate-bound states. Thus, a possibility that arises is that the conformational change and its effects on the electronics of the heme system is of considerable importance in controlling the reduction potential of the heme iron. This is under further study, using A264 variants in which the sidechain of the introduced amino acid does not ligate the heme iron in substrate-free or substrate-bound forms.

Further scrutiny of both the substrate-free and substrate-bound A264E structures reveal other important features of the P450 BM3 structure that relate to attempts to engineer covalent ligation of the heme macrocycle via the interaction of Glu 264 with the heme 5-methyl group (Figure 5). The active site organization in P450 BM3 is such that Phe 87, and likely the I helix residue Thr 260, obstruct access of Glu 264 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 in work to enable covalent heme ligation by Glu 264 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 Glu 264 to the relevant methyl group and could thus allow autocatalytic linkage to occur.

Conclusions

Crystallographic studies of the A264E variant of P450 BM3 confirm the proposals based on spectroscopic studies that the glutamate is able to ligate to the ferric heme iron of the mutant in the substrate-free form, and that substrate addition “forces” on the ligand – producing a completely hexa-coordinated low-spin species, as opposed to the extensively high-spin penta-coordinated form seen for the wild-type P450 BM3 (17). Structural studies explain clearly why substrate has this effect in the A264E enzyme, since palmitoleic acid occupies one of the two favoured positions for the Glu 264 sidechain. Glu 264 can no longer form an interaction with the key regiospecificity-determining residue Phe 87 in the palmitoleate-bound form, and is thus induced to move towards 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 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
Crystal structure of P450 BM3 A264E

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 Glu 264 ligates the heme iron or is positioned against Phe 87, and the enzyme is low-spin in both forms and aqua-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 Ala 264, and that the A264E mutation favours this conformational rearrangement independent of substrate due to steric restrictions to movement of the glutamate side chain in the SF conformation, and/or 2) that P450 BM3 is in a continual dynamic equilibrium between SF and SB conformations, and that the A264E mutation forces this equilibrium toward the SB form. 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 on substrate association (and the concomitant change in 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 accessible 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 favourable binding of the substrate to this conformer. This conclusion is supported by the fact that much tighter 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 other variants at position 264 – specifically
investigating A264 variants that induce the conformational switch to the SB conformation, but which do not in addition give rise to coordination to the heme iron.

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References


-19-


Figure legends

Figure 1: Stereoview of an overlay of substrate-free A264E heme domain (coloured in green ribbons). Panel a) with the substrate-free (in yellow) form of wild-type P450 BM3 heme domain Panel b) with the substrate-bound (in blue) form of wild-type P450 BM3 heme domain.

Figure 2: Conformations of Glu 264 in molecules A and B of the substrate-free A264 heme domain of flavocytochrome P450 BM3. The heme macrocycle along with residues Cys 400 and Glu 264 is displayed for molecule B (panel a) and molecule A (panel b) with the 2Fo-Fc-map contoured at 1σ superposed.

Figure 3: A scheme representing a model for the conformational equilibrium of P450-BM3 in solution and the influence of substrate binding. The left panel displays the wild-type equilibrium; the right panel the A264E equilibrium. The equilibrium in absence of substrate is drastically changed by introduction of the A264E mutation. In both cases, large grey arrows indicate the apparent shift on substrate binding.

Figure 4: Stereo view of the active site of A264E. Panel a) An overlay of the active site of A264E (in blue) and the N-palmitoylglyine bound wild-type BM3 (in green) (PDB-code 1JPZ). For clarity, the heme macrocycle is only displayed for the A264E mutant. Hydrogen bonding pattern to the H-site is indicated by dotted black lines. The ligating water molecule occupying the L-site in the A264E mutant is coloured in red. Panel b) Active site structure of the palmitoleic acid-bound form of the A264E heme
Crystal structure of P450 BM3 A264E

domain. Residues are coloured according to residue type, the substrate is depicted in purple.

Tables

Table 1: Crystallographic statistics

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Joyce et al.
Figure 2a and b, 3

Wild Type

A264E

Joyce et al.
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M. Gordon Joyce, Hazel M. Girvan, Andrew W. Munro and David Leys

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