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The role of Thr268 and Phe393 in cytochrome P450 BM3

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Abstract

In flavocytochrome P450 BM3 there are several active site residues that are highly conserved throughout the P450 superfamily. Of these, a phenylalanine (Phe393) has been shown to modulate heme reduction potential through interactions with the implicitly conserved heme-ligand cysteine. In addition, a distal threonine (Thr268) has been implicated in a variety of roles including proton donation, oxygen activation and substrate recognition. Substrate binding in P450 BM3 causes a shift in the spin state from low- to high-spin. This change in spin-state is accompanied by a positive shift in the reduction potential (ΔE_m [WT + arachidonate (120 μM)] = +138 mV). Substitution of Thr268 by an alanine or asparagine residue causes a significant decrease in the ability of the enzyme to generate the high-spin complex via substrate binding and consequently leads to a decrease in the substrate-induced potential shift (ΔE_m [T268A + arachidonate (120 μM)] = +73 mV, ΔE_m [T268N + arachidonate (120 μM)] = +9 mV). Rate constants for the first electron transfer and for oxy-ferrous decay were measured by pre-steady-state stopped-flow kinetics and found to be almost entirely dependant on the heme reduction potential. More positive reduction potentials lead to enhanced rate constants for heme reduction and more stable oxy-ferrous species. In addition, substitutions of the threonine lead to an increase in the production of hydrogen peroxide in preference to hydroxylated product. These results suggest an important role for this active site threonine in substrate recognition and in maintaining an efficiently functioning enzyme. However, the dependence of the rate constants for oxy-ferrous decay on reduction potential raises some questions as to the importance of Thr268 in iron-oxo stabilisation.

Keywords: Cytochrome P450; Oxygen activation; Electron transfer; Oxyferrous species; Iron-oxo stabilization

1. Introduction

The cytochromes P450 [1] are a large and extensively studied family of heme containing redox enzymes [2–4]. They are responsible for a variety of biologically important reactions on a huge range of substrates in almost every organism from bacteria to humans [5–7], primarily catalysing the insertion of an atom of oxygen into a CH bond [8]. There are currently around 4500 named P450 sequences identified on the cytochrome P450 homepage [9], a number which is steadily increasing as more genomic sequences become available.

There are many differences between individual P450s in terms of redox cofactors [10–14], substrates [5] and sequence [9], however there are several conserved features found throughout the P450 superfamily. The overall fold and tertiary structure are consistent [15] and it is apparent from sequence alignments that there are several highly conserved and hence possibly catalytically important residues [16]. Of these conserved residues, a threonine [17,18] located at the distal side of the heme has been associated with a variety of roles including oxygen activation

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[19,20], proton delivery [21–24], substrate recognition [25,26], electron transfer [27] and the positioning of the heme-ligand water [28]. This residue forms part of the I-helix which runs across the distal face of the heme [16,17,29]. Its location at the active site and role in forming the oxygen binding pocket has led to speculation that it can hydrogen bond to the ferrous-dioxygen complex [19,20], stabilising it and aiding the formation of the catalytically important oxy ferryl or ferric hydroperoxy species [30]. However, a recent study detailing the crystal structure of the dioxygen complex of P450cam has indicated that the threonine residue does not form a hydrogen bond with the dioxygen complex but instead suggests it may be a hydrogen bond acceptor from the hydroperoxy intermediate [31]. As the nature of oxygen activation and the precise mechanism of substrate hydroxylation are still a matter of some debate, a significant amount of research has been carried out to determine the exact role of this residue in a variety of P450 enzymes [19,27,32,33].

Initial studies by Imai et al. showed that the hydroxyl group of threonine was essential for coupled enzymatic activity and that its removal resulted in a less stable oxy-ferrous complex [34]. They suggested that the threonine might play a role in both stabilising the iron oxo complex and in the proton donation pathway. However, a subsequent study by Kimata et al. [35] demonstrated that the substitution of the threonine in P450cam with the unnatural amino acid methoxythreonine (removing proton donating ability) resulted in a fully coupled enzyme. Hence the hydroxyl group of the threonine was not a pre-requisite for oxygen bond cleavage. Concominantly, similar work was being carried out on the extensively characterised enzyme of this type, P450cam [20,22], a camphor hydroxylase from *Pseudomonas putida*. The crystal structure of the P450cam active site mutant Thr252Ala shows how this substitution leads to structural changes in the active site and suggests that greater access for solvent water is the source of uncoupling [36]. Hence another role for the threonine is suggested in preventing active site access to solvent water molecules through the proton channel.

The second most commonly studied enzyme of this type is P450 BM3 from *Bacillus megaterium*, a convenient model for studying P450s due to its combination of redox cofactors [10,37]. The substitution of Threonine 268 with an alanine (analogous to Thr252Ala in P450cam) results in a decrease in the rate constant for NADPH (β-nicotinamide adenine dinucleotide phosphate) consumption and an increase in the conversion of electron equivalents into hydrogen peroxide instead of hydroxylated product [19,25]. However, while in P450cam, the threonine residue is necessary for any NADPH consumption, the Thr268Ala mutant in P450 BM3 exhibits a small, but significant amount of coupled activity [19,25]. It is suggested that this may indicate that while the distal threonine may fulfil a similar role in all P450s, it may do so with a differing degree of importance. Several papers by Peterson et al. [25,28] confirm the importance of Thr268 in maintaining coupled substrate hydroxylation.

There are numerous other examples of similar substitutions cited in the literature [21,38–41] highlighting this residues importance in electron delivery, proton transfer and the high/low-spin character of the ferric resting state. In P450 1A2 it is even reported that the T319A substitution leads to a 9-fold increase in $V_{\max}$, clearly a result which contradicts those discussed earlier.

These previous studies indicate several possible roles for the active-site threonine, without conclusively identifying its function within the P450 super-family. It has been suggested that this residue may fulfil a different role or have varying importance within the P450 family depending on the nature of the substrate and flexibility of the substrate-binding pocket.

Our earlier studies have shown that substituting the proximal residue Phe393 of P450 BM3 to a histidine results in alterations in the electronic nature of the heme environment which in turn influence the heme reduction potential [42–44]. Substitution of Phe393 with a histidine led to a positive shift in the E_m which was concomitant with an increase in the rate of first-electron transfer and importantly, an increase in the stability of the oxy-ferrous complex.

In order to attempt to increase the stability of the oxy-ferrous complex even further we decided to combine the F393H mutation with additional substitutions at Thr268. Such a stabilised species would provide a platform for future work on subsequent steps in the catalytic cycle. In addition it was hoped that these substitutions would help to further elucidate the function of the Thr268 residue and assess its importance in hydrogen bonding, proton donation and substrate recognition in P450 BM3.

2. Materials and methods

2.1. Mutagenesis

The P450 BM3 mutants characterised here were generated by site-directed mutagenesis using the Kunkel method as described previously [42,53]. The wild type (WT) plasmids pBM23 (holo P450 BM3) and pBM20 (P450 BM3 heme domain) were used as templates for making the T268A and T268N single mutants. The P450 BM3 F393H plasmids pCM37 (holo protein) and pCM81 (heme domain) were used for construction of the F393H/T268A and F393H/T268N double mutants [42].

Oligonucleotides used were T268A : 5′ GACCACTTGT$\underline{\text{TGC}}$TTCGTGTCCCGC and T268N: 5′ GACCACTTGT$\underline{\text{GTT}}$TTCGTGTCCCGC. Mismatches are underlined. The resulting expression plasmids were pCM174 and pCM166 (T268A holo protein and heme domain, respectively), pCM175 and pCM167 (T268N holo protein and heme domain), pCM172 and pCM164 (F393H/T268A holo protein and heme domain) and pCM173 and pCM165 (F393H/T268N holo protein and heme domain).