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Cytochrome $P450_{cin}$ (CYP176A1) D241N: Investigating the role of the conserved acid in the active site of cytochrome P450s

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ABSTRACT

$P450_{cin}$ (CYP176A) is a rare bacterial P450 in that contains an asparagine (Asn242) instead of the conserved threonine that almost all other P450s possess that directs oxygen activation by the heme prosthetic group. However, $P450_{cin}$ does have the neighbouring, conserved acid (Asp241) that is thought to be involved indirectly in the protonation of the dioxygen and affect the lifetime of the ferric-peroxo species produced during oxygen activation. In this study, the $P450_{cin}$ D241N mutant has been produced and found to be analogous to the $P450_{cam}$ D251N mutant. $P450_{cin}$ catalyses the hydroxylation of cineole to give only (1*R*)-6β-hydroxycineole and is well coupled (NADPH consumed: product produced). The $P450_{cin}$ D241N mutant also hydroxylated cineole to produce only (1*R*)-6β-hydroxycineole, was moderately well coupled ($31 \pm 3\%$) but a significant reduction in the rate of the reaction (2% as compared to wild type) was observed. Catalytic oxidation of a variety of substrates by D241N $P450_{cin}$ were used to examine if typical reactions ascribed to the ferric-peroxo species increased as this intermediate is known to be more persistent in the $P450_{cam}$ D251N mutant. However, little change was observed in the product profiles of each of these substrates between wild type and mutant enzymes and no products consistent with chemistry of the ferric-peroxo species were observed to increase.

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1. Introduction

The cytochromes P450 (P450s) are a family of enzymes that commonly use electrons ultimately derived from a nicotinamide cofactor (NADH or NADPH) to activate molecular oxygen and produce a high-valent iron-oxo species, recently characterised by Rittle and Green [1]. They typically then utilise this iron-oxo species to catalyse the insertion of an oxygen atom into an unactivated carbon–hydrogen bond. Additionally, they are responsible for a number of other transformations, including heteroatom oxidations, epoxidations and C–C bond cleavages. Some of these reactions are believed to be catalysed by other molecular oxygen-derived oxidants that usually exist as intermediates in the normal P450 catalytic cycle. For example, a number of P450s, such as aromatase (CYP19) [2–5], CYP17A [6,7], CYP51 [8,9] and CYP2B4 [10] are all believed to employ the ferric-peroxo species as a nucleophile that adds to a reactive carbonyl to initiate the oxidation of their substrates. $P450_{cam}$ (CYP101) from *Pseudomonas putida* has been extensively used as a model system to establish the general mechanism of cytochrome P450s [11]. $P450_{cam}$ catalyses the oxidation of (1*R*)-camphor to 5-*exo*-hydroxycamphor, initiating its biodegradation and permitting *P. putida* to live on camphor as its sole source of carbon and energy. In the catalytic mechanism of P450s (Fig. 1), an almost universally conserved threonine/carboxylic acid pair (Asp251/Thr252 in $P450_{cam}$; Fig. 2), is proposed to assist and direct the protonation of the distal oxygen of iron bound dioxygen (Fig. 1E to F). This in turn initiates the scission of the O–O bond, release of a water molecule and generation of the high valent iron-oxo species (ferryl porphyrin cation radical) (Fig. 1G) [12]. This ferryl species is then responsible for the subsequent oxidation of the substrate. Replacement of the conserved threonine with an alanine (T252A in $P450_{cam}$) results in an enzyme that consumes NADH at approximately the same rate as the wild type enzyme but produces only a small amount of product (5% as compared to wild type for $P450_{cam}$) [13]. Most of the consumed reducing equivalents are diverted to the production of hydrogen peroxide [13,14], believed to arise from protonation of the bound dioxygen at the proximal oxygen. It has been reported that production of hydrogen peroxide in this case demonstrates the essential nature of the threonine in oxygen protonation in $P450_{cam}$ and is a specific example of uncoupling (NADH consumption without oxidised product formation) [13]. Subsequent studies with a range of eukaryotic and prokaryotic P450s have shown that mutation of the conserved threonine (or in some cases serine) often results in a significantly uncoupled enzyme [10,13–19]. Studies from several groups [7,15,20–26] also demonstrated that the conserved carboxylic acid (Asp251 in $P450_{cam}$) was important in maintaining the rate but not specificity of oxygen activation and substrate oxidation. Substitution of the Asp251 in $P450_{cam}$ with an asparagine reduced the rate to 1% of typical wild type oxidation [20,21,24]. The initial study [20] suggested that 14% of the reducing equivalents used by the $P450_{cam}$ D251N mutant are funneled directly into the production of hydroxycamphor whilst further reports suggested

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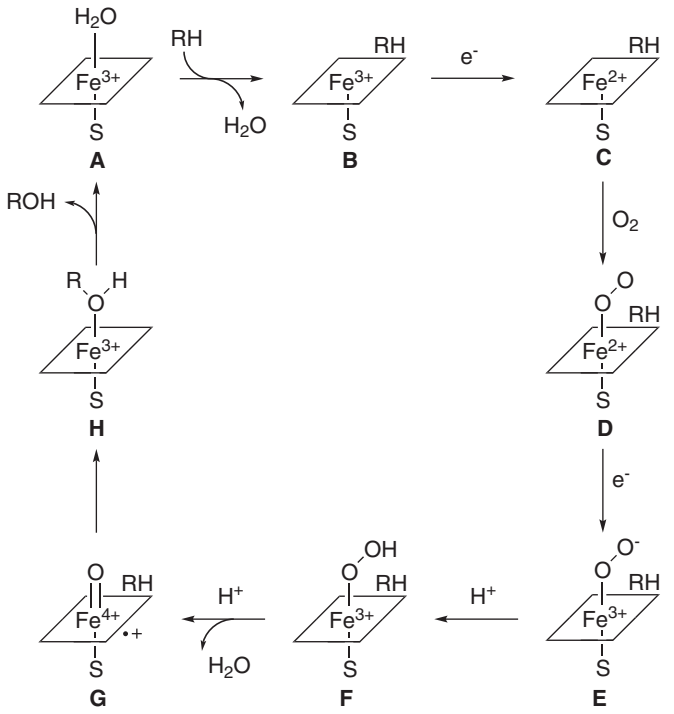

Fig. 1. Catalytic cycle for oxidation reactions catalysed by cytochromes P450.

this percentage is considerably higher (90%) [21,24]. This discrepancy presumably reflects the very low rate of NADH consumption, which in turn leads to difficulty in obtaining precise results and such problems are common to many studies of this type of mutant.

An analogous reduction in the rate of the oxidation has also been observed in a number of other conserved aspartic/glutamic acid mutants (Table 1) [7,15,22,23,25–28]. $P450_{BM3}$ (CYP102A1) is a bacterial P450 where the heme domain and the reductase domain are fused [29]. In this enzyme the conserved acid is a glutamate (E267) and the E267Q mutant has been characterised. Depending on the substrate, the rate of product formation is decreased to approximately 16% of wild type for laurate and to only 0.3% of wild type for palmitate [28]. Very little uncoupling to hydrogen peroxide was observed but it was proposed that uncoupling to water was significant in this case. In eukaryotic, xenobiotic metabolising P450s (CYP1A2 and CYP2A1) significant reductions in the rate of product formation (6–18% relative to wild type) were observed, dependent on both the substrate and the P450 [15,22,23,27]. Where coupling was measured in these systems it too was generally decreased relative to wild type (Table 1). The analogous mutants in two biosynthetic, eukaryotic P450s with specific substrate preferences have also been investigated [7,26]. The catalytic activity of the CYP19A1 (aromatase) D309A and D309N mutants was observed to drop to 1% and 5% of wild type, respectively [26]. CYP17A1 can catalyse both the hydroxylation of pregnenolone and the subsequent carbon-carbon bond cleavage of the product to

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                          **
CYP17A1   296  IGDIFGAGV**ET**TTSVVKWTLAFLL   319
CYP1A2    309  VNDIFGAGF**ET**VTTAIFWSILLLV  332
CYP19A1   300  ILEMLIAAP**DT**MSVSLFFMLFLIA   323
P450cam   242  CGLLLVGGL**DT**VVNFLSFSMEFLA   265
P450cin   232  FTILLLGGI**D**NTARFLSSVFWRLA   255
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NCBI Accession numbers: $P450_{cin}$ -AAL57614; $P450_{cam}$ -P00183; CYP19A1 -P11511; CYP1A2 -P04799; CYP17A1 -P05093.

Fig. 2. Multiple sequence alignment of selected P450s highlighting conserved threonine and aspartic/glumatic acid pair.

Table 1
Comparison of aspartic and glutamic acid mutants from a variety of P450s.

P450	Mutant	Substrate	Rate[a] %	Coupling %	Ref.
$P450_{cam}$	D251N	(1*R*)-camphor	1[b]	14; 90	[20,21,24]
$P450_{BM3}$	E267Q	Laurate	16[b]	ND[c,d]	[28]
		Myristate	1.6[b]	ND[d]	[28]
		Palmitate	0.3[b]	ND[d]	[28]
CYP17A1	E305Q	Pregnenolone	35	ND	[7]
		17α-hydroxypregnenolone	21[e]	ND	[7]
CYP19A1	D309N	Androstenedione	5.4	ND	[26]
		Testosterone	7	ND	[26]
	D309A	Androstenedione	1	ND	[26]
CYP1A2	E318A	7-ethoxycoumarin	6–18	3.7[f]	[22,23]
		Methanol	25	16.2[f]	[15]
CYP2A1	E302G	Testosterone	7.8	ND	[27]

[a] Rate given as percentage of wild type.
[b] Rate of NAD(P)H consumption;
[c] Not Determined;
[d] Less than 3% hydrogen peroxide produced but "extra" water formation was reported, no traditional coupling calculated
[e] Lyase activity;
[f] Amount of oxygen consumed to that incorporated into the substrate.

dehydroepiandrosterone (DHEA); the latter process (called lyase activity) is thought to require the ferric-peroxo species [7]. Although reduction in the rates of catalysis of both hydroxylation and lyase activity were observed with the CYP17A1 E305Q mutant, it was not as substantial as that observed with $P450_{cam}$ (Table 1; 35% and 21% for hydroxylation and lyase respectively). In all of these cases (CYP1A2, 2A1, 17A1 and 19A1) significant effects on the K_m were also reported, and this was suggested, in part, to be responsible for the loss of activity.

Based on the above evidence it was proposed that the acid-threonine pairing ensured that the distal oxygen of the bound dioxygen is efficiently protonated (Fig. 1E to F) [20,21]. The threonine was essential for guaranteeing the regiospecificity of the protonation, with a number of slightly different roles suggested: anchoring a water molecule responsible for the protonation [30]; directly transferring the proton itself [31]; or activating the distal oxygen via hydrogen bonding [32]. The exact role of the conserved acid is much less clear. It was found that the rate-limiting step in the $P450_{cam}$ mutant was likely to be the first proton transfer that produces the ferric-hydroperoxo species (Fig. 1F) [33,34]. This was confirmed by EPR/ENDOR studies where the D251N mutant was trapped at the ferric-peroxo stage during 77 K cryoreduction and required a considerable amount of thermal activation to convert it to the ferric-hydroperoxo species, as compared to the wild type [34,35]. Mechanistically, it was initially suggested that the Asp251 sidechain might provide a "carboxylate switch", anchoring and activating a chain of water molecules to deliver the required protons to the active site. Recent computational studies have again promulgated this idea [31]. However, crystal studies of both $P450_{cam}$ and its D251N mutants have revealed no role for the acidic side chain but instead have been consistent with a "carbonyl switch" in which the backbone carbonyl indirectly stabilises water that delivers a proton to the bound dioxygen species. All proposals explain the experimentally observed increased lifetime for the ferric-peroxo species.

$P450_{cin}$ (CYP176A) is a bacterial P450 involved in the hydroxylation of 1,8-cineole **1** to produce 6-β-hydroxycineole **2** (Fig. 3) [36,37]. $P450_{cin}$ does not possess the conserved active site threonine but instead contains an asparagine (Asn242). Initially, it was proposed that the asparagine could be a functional replacement for the threonine [36]. However, X-ray structural and mutagenic studies revealed that Asn242 was not important in oxygen activation in $P450_{cin}$ [38,39] but it was crucial in controlling the regiospecificity of the substrate oxidation. It is still not known what controls oxygen protonation in $P450_{cin}$ but recent structural studies have suggested that H-bonding from a hydroperoxy intermediate to a backbone carbonyl (Gly238) may be important in controlling the regiochemistry of protonation [40]. Interestingly, $P450_{cin}$ still has a conserved active site acid (Asp241; Fig. 2) but no role for it was proposed.

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