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A Highly Active Single-Mutation Variant of $P450_{BM3}$ (CYP102A1)

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Cytochrome P450 (CYP) enzymes catalyse the oxidation of a wide variety of endogenous and exogenous organic substrates, most commonly by the haem-dependent insertion of an oxygen atom from atmospheric dioxygen into an unreactive carbon–hydrogen bond.[1] There is widespread interest in harnessing this valuable catalytic activity, particularly in $P450_{BM3}$ (CYP102A1) from *Bacillus megaterium*, which is catalytically self-sufficient. Applications include drug development and metabolism,[2] the production of fine chemicals,[3] the degradation of environmental contaminants[4] and the hydroxylation of short-chain alkanes.[5] Site-specific mutagenesis and directed evolution have been extensively employed as methods of redesigning P450s to accept non-natural substrates and influence product profiles.[6] Several of the resulting variants have contained proline mutations. Although proline is structurally disruptive and can cause misfolding when incorporated, significant successes have been forthcoming when this has been avoided. Mutation A330P enhanced activity relative to wild-type $P450_{BM3}$ (WT), but also altered selectivity;[7] an important contribution to the evolution of $P450_{BM3}$ into a propane hydroxylase was made by mutation L188P;[5b] and in CYP101A1 ($P450_{cam}$) the L358P substitution[8] helped to increase the activity of variants towards propane and ethane, substrates towards which the wild-type enzyme was inactive.[5a]

Against this background, we introduced proline residues at a selection of targeted $P450_{BM3}$ sites in the expectation that this would create a number of inactive variants, but also some with unexpected capabilities. One of these was Ile401, the residue to the C-terminal side of the thiolato ligand to the haem iron. Proline occurs naturally at this position in chloroperoxidase and P450s such as CYP7A1, CYP101B1[9] and CYP121.[10] The resulting mutant was approximately 50% high-spin in Tris buffer (50 mM, pH 7.4) and showed a 650 nm absorbance band characteristic of type I substrate-bound spectra as well as the usual α/β bands (Figure S1 in the Supporting Information). I401P gave a similar k_{cat} value to WT in the oxidation of lauric acid (71 vs. 77 s^{-1}, Table 1), but there was a tenfold reduction in K_M from 230 to 21 μM (Figure S2). This was reflected in the in vitro NADPH consumption rate under steady-state turnover conditions (100 nM enzyme, 1 mM substrate), which was 3812 nmol min^{-1} (nmol P450)$^{-1}$—henceforth abbreviated to min^{-1}—as compared to 2777 min^{-1} for WT. Coupling and regiospecificity were little changed, while peroxide formation fell slightly.

Table 1. Kinetic and potentiometric parameters for I401P and WT $P450_{BM3}$.

In vitro oxidation of lauric acid	I401P	WT
K_M [μM]	21	230
k_{cat} [s^{-1}]	71	77
NADPH rate [nmol min^{-1} per nmol P450]	3812	2777
coupling [%]	53	52
PFR [nmol min^{-1} per nmol P450]	2012	1439
peroxide [%]	9	14
(ω-1) hydroxylation [%]	36	34
(ω-2) hydroxylation [%]	31	29
(ω-3) hydroxylation [%]	33	37
leak rate [nmol min^{-1} per nmol P450]	455	28
reduction potential vs. SHE (pH 7.4) [mV]	−303	−445

Coupling: percentage of NADPH utilised for product formation; PFR: product formation rate. All data are means of at least three experiments with standard deviations less than 5% of the mean. The leak rate is the NADPH rate in the absence of substrate.

To study the effect of the mutation in greater detail, we truncated the I401P gene at residue 481 to detach the reductase domain. The reduction potential of the haem domain was determined by equilibrium spectroelectrochemistry in the substrate-free form at (−303 ± 10) mV (Figure S3). This was 142 mV more oxidising than the (−445 ± 15) mV potential determined for WT under the same conditions—a difference comparable to the shift induced in WT by arachidonic acid binding (135 mV).[11] The crystal structure of I401P[12] showed a number of unexpected features. The iron atom lay slightly to the proximal side of the porphyrin ring rather than in-plane (Figure 1A) and was essentially 5-coordinate. The nearest water molecule was 3.65 Å distant, as compared to 2.6 Å in WT, consistent with the partially high-spin character of the new variant, and at an S–Fe–O angle of 161.5°. In this respect, the structure resembled that of the *N*-palmitoylglycine complex with WT.[13] The residues immediately to the proximal side of the haem occupied positions similar to those found in WT (Figure 1A). On the distal side, conformational changes in the I-helix close to the dioxygen binding site led to His266 adopting a position similar to that found in palmitoleic acid-bound WT (Figure 1B).[14]

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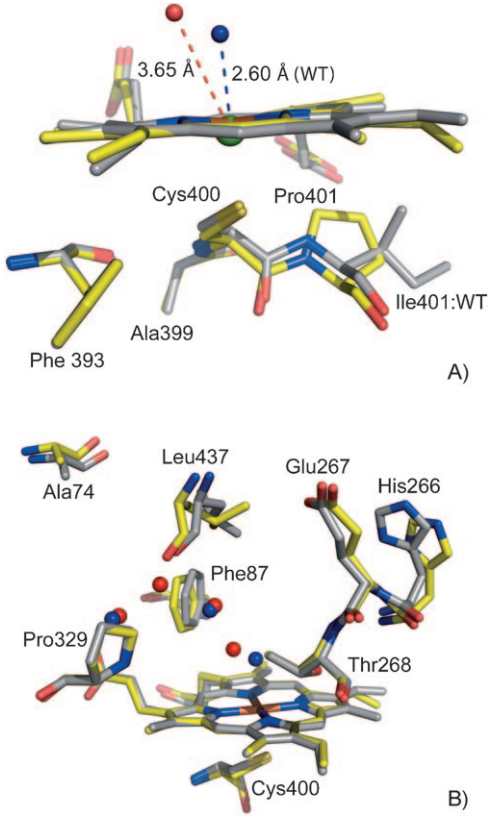

Figure 1. Overlay of the crystal structures of the active-site regions of variant I401P (in yellow, with water molecules in red) and WT (in grey, with water molecules in blue; PDB ID: 1bu7); A) proximal side, B) distal side. The water molecules in the I401P variant, unlike those in WT, form a hydrogen-bonded chain.

Because the NADPH consumption rate of WT with lauric acid was already close to the k_{cat}, the new variant gave a relatively modest 37% increase under steady-state conditions. With non-natural substrates, by contrast, NADPH rate enhancements of at least 400% were recorded. Coupling efficiency also improved significantly. With propylbenzene,[15,7] the NADPH rate was 4476 min^{-1} as compared to 894 min^{-1} for WT, and coupling rose from 71 to 80% to give a product-formation rate (PFR) of 3578 min^{-1}—one of the highest reported for this enzyme with any substrate (Table 2). 3-Methylpentane, a small branched alkane, gave a PFR of 1378 min^{-1} versus 28 min^{-1} for WT, while (+)-α-pinene,[16] a substrate not metabolised by WT, was oxidised at 238 min^{-1} with 73.5% conversion to verbenol.

Table 2. Activity data for the in vitro oxidation of non-natural substrates by I401P and WT $P450_{BM3}$.

	WT			I401P		
	N	C	PFR	N	C	PFR
(+)-α-pinene	41	<0.1	<0.05	1229	19	238
fluorene	7.9	0.9	0.1	1057	18	188
3-methylpentane	126	22	28	2763	50	1378
propylbenzene	894	71	635	4476	80	3578

N: NADPH turnover rate; C: coupling; PFR: product-formation rate; rates in nmol min^{-1} per nmol P450.

The PFR for the oxidation of the polyaromatic hydrocarbon, fluorene to fluoren-9-ol[17] was 188 min^{-1} as compared to 0.1 min^{-1} for WT. The I401P mutation therefore functioned as a powerful rate accelerator across a range of hydrophobic non-natural substrates, while leaving product distributions broadly similar to those given by WT (Tables S1–S5).

Partially high-spin iron centres are uncommon in substrate-free bacterial P450s, but occur, for example, in CYP107H1 ($P450_{Biol}$),[18] CYP164A2[19] and the 1-12G variant of $P450_{BM3}$.[20] Single mutation $P450_{M3}$ variants with substrate-free reduction potentials and K_M values for fatty acid substrates similar to those of I401P are also known, F393A being an example,[11b,21] as are variants with low K_M values and mixed spin-states, for example, A82W.[22] I401P is unusual, however, in combining all these attributes with an ability to deliver dramatic activity enhancements. To set its performance into context, the highest previously reported PFR for a substrate not related to fatty acids appears to be 2688 min^{-1} for our seven-mutation variant RLYF/KT2 with propylbenzene.[7] The conformational features that I401P shares with the substrate-bound form of the wild-type enzyme, for example, the positioning of the axial water molecule, might be significant in this regard, as they suggest that substrate binding could require relatively little structural reorganisation in the new variant. Crystal structures of Ala264 variants that resemble the substrate-bound conformation of the enzyme have been published,[23] but these are inefficient catalysts because ligation of the mutated residue to the haem iron compromises k_{cat} values. It seems unlikely that the lost hydrogen bond between the amide proton of the substituted residue and the sulfur atom of the proximal thiolato ligand[24] is central to the activity increase, given that the $P450_{cam}$ homologue of I401P (L358P) scarcely altered NADH consumption rates and had a more negative reduction potential than WT $P450_{cam}$ (in the substrate-bound form).[8]

Although $P450_{BM3}$ is one of the most active P450 cytochromes known, the usefulness of the wild-type enzyme is limited by the fact that turnovers involving non-natural substrates often have low NADPH consumption rates and are poorly coupled. Activity-enhancing mutations allow the considerable catalytic potential of this self-sufficient system to be more fully exploited, particularly those that are effective across a range of substrates. I401P has little impact on product profiles, and contains only a single mutation, on the proximal side of the haem. As such, it appears well-suited for combination with known selectivity-directing mutations, which potentially opens new routes for the production of synthetically desirable end-products.

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[12] Crystals of the I401P mutant belonged to the space group $P2_1$, with unit cell dimensions: $a=58.7$ Å, $b=145.8$ Å, $c=63.2$ Å, $\beta=97.3^\circ$. A total of 182040 reflections were measured with R_{merge} of 5.9% for 53030 unique reflections and 99.2% completeness (50–52.2 Å). Data were collected to 94.1% completeness in the highest resolution shell. The structure was solved by molecular replacement, based on the crystal structure of the haem domain of wild-type $P450_{BM3}$ (PDB ID: 1bu7). The final refinement parameters were $R_{work}=18.5\%$ and $R_{free}=24.4\%$. More details will be published in due course.
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