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UNIVERSITÀ DEGLI STUDI DI TORINO

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Identification of Mutant Asp251Gly/Gln307His of Cytochrome P450 BM3 for the Generation of Metabolites of Diclofenac, Ibuprofen And Tolbutamide

Georgia E. Tsotsou,^[a] Anastasia Sideri,^[a] Abhineet Goyal,^[a] Giovanna Di Nardo,^[a] and Gianfranco Gilardi*^[a]

The soluble, catalytically self-sufficient cytochrome P450 BM3 from *Bacillus megaterium* is a good candidate as biocatalyst for the synthesis of drug metabolites. To this end, error-prone PCR was used to generate a library of P450 BM3 mutants with novel activities toward drugs. A double mutant Asp251Gly/Gln307His (A2) with activities towards diclofenac, ibuprofen and tolbutamide was identified by screening with the alkali method. This is based on the detection of NADPH oxidation during enzymatic

turnover on whole *Escherichia coli* cells heterologously expressing the P450 BM3 mutants in the presence of the target substrates. The three drugs screened for are marker substrates of human liver cytochromes P450 belonging to the 2C subfamily.

Keywords: P450 BM3 • directed evolution • diclofenac • ibuprofen • tolbutamide

Interestingly mutations Asp251Gly / Gln307His are located on the protein surface and they are not directly involved in substrate binding and turnover.

Dissociation constants and K_M values of mutant A2 for diclofenac, ibuprofen and tolbutamide are in the μM range. Catalysis leads to hydroxylations in specific positions, producing 4'-hydroxydiclofenac, 2-hydroxyibuprofen and 4-hydroxytolbutamide.

Introduction

Cytochromes P450 are a widely distributed super-family of heme-enzymes, catalyzing the oxidation of a broad variety of endogenous and exogenous organic substrates. They catalyze the insertion of an oxygen atom from atmospheric dioxygen into an unreactive C-H bond.^[1] The enzymes from human liver are responsible for the metabolism of xenobiotics, including drugs that are converted in one or more metabolites. The toxicity and biological activity of such metabolites have to be fully explored during the development of new drugs as they can cause adverse reactions in the human body.^[2-4]

The possibility that the metabolites themselves can be reactive or toxic makes it necessary to synthesize preparative amounts of these reaction products, a process that is sometimes difficult and expensive to achieve by classical synthetic methods. The use of human cytochromes P450 to produce drug metabolites is mainly

limited by the fact that they are difficult to handle and unstable,^[5] as they are membrane-bound and they need a redox partner, cytochrome P450-reductase, for catalysis. Furthermore, they waste most of the electrons donated by the cofactor NADPH *via* the reductase in the so-called uncoupled reactions that generate oxygen reactive species bypassing the catalytic cycle that leads to the drug products.

By contrast, cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* is a self-sufficient fatty acids monooxygenase^[6,7] with an excellent capability of selective C-H oxidation^[8,9] and it shares a 30% degree of homology with the human enzymes. The polypeptide chain of the catalytic heme domain of P450 BM3 is fused to its reductase, a mammalian-like diflavin reductase. Furthermore, the wild type enzyme has already been demonstrated to be able to recognize some drugs in different reactions such as hydroxylation, N-dealkylation and dehydration.^[10] For these reasons, it is a good template for protein engineering aimed at improving the substrate specificity and the catalytic performances of the enzyme toward compounds of interest.^[11,12]

In the last years, either through rational design or directed evolution, P450 BM3 has been engineered to oxidize a variety of substrates, including drugs.^[13-19] In particular, the use of directed evolution has led to the development of mutants able to generate the metabolites typically produced by human liver P450s.^[20-21]

Directed evolution approaches can be limited by the method used for screening the library of mutants able to oxidise the compounds of interest. Often catalysis towards new substrates is detected through coupled reactions or by the generation of

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products easily identified in a high throughput assay. This

requires the set up

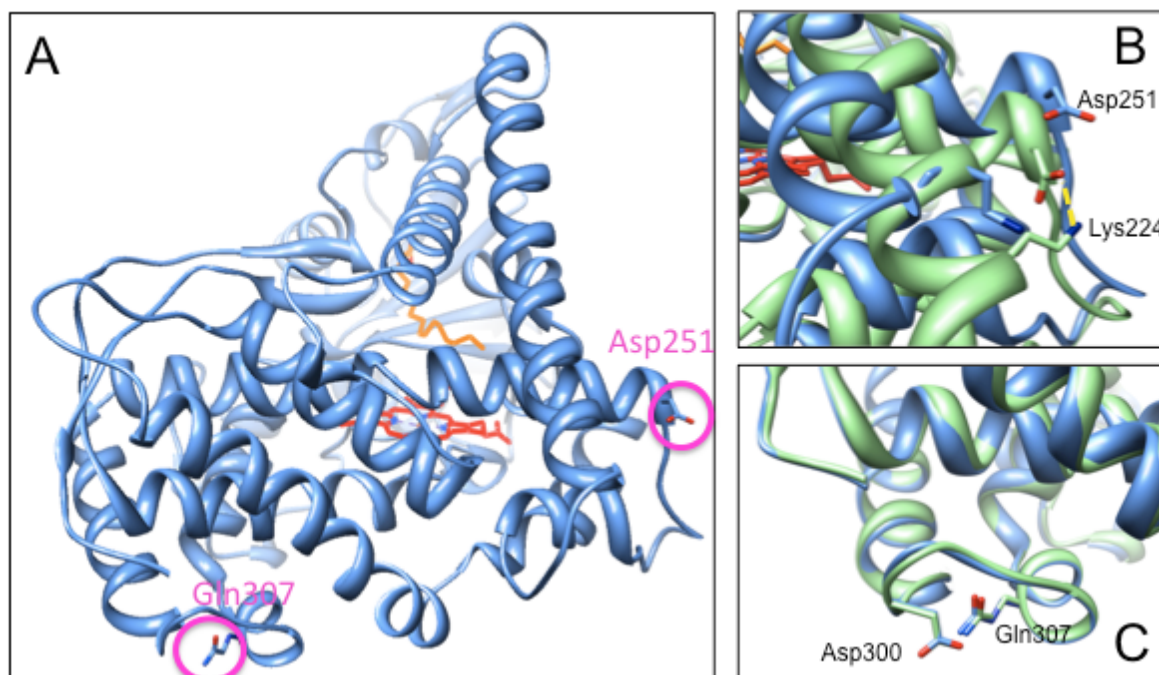


Figure 1. Analysis of the crystal structure of P450 BM3. A) Overall structure of P450 BM3 in complex with palmitoleic acid (PDB 1FAG). Heme is shown in red, palmitoleic acid in orange. The magenta circles indicate the location of the residues Asp251 and Gln307, found to be mutated in mutant A2. B-C) Superimposition of the crystal structures of the substrate-free (green, PDB 2IJ2)^[30] and palmitoleic-bound (blue, PDB 1FAG)^[31] forms of P450 BM3. B) The salt bridge between Asp251, mutated in A2, and Lys224 is indicated by the dashed black line in the substrate-free structure. C) Gln307, mutated in A2, forms a hydrogen bond with Asp300 (black dashed line).

of a different screening procedure each time a new target substrate is selected, and this is not always universally possible for all target molecules. On the other hand, our laboratory has developed and described a method for screening activity of NAD(P)H-dependent oxidoreductases on perspective substrates with an assay that is widely applicable, independently from the substrate considered^[22]. The method can be performed in whole *E.coli* cells where the mutants of the P450 of interest are heterologously expressed, and it has been adapted to a microtitre-plate format. It is based on the detection of a fluorescent product derived by alkali degradation of the NAD(P)⁺ generated during P450 enzymatic turnover when the target molecule is recognized and turned into a product.^[22]

In this work, we used the alkali assay for screening a library of P450 BM3 random mutants, derived from error prone PCR, for their ability to oxidise diclofenac, ibuprofen and tolbutamide. A double mutant called A2, containing the Asp251Gly/Gln307His mutations, was found to catalyse specific hydroxylations of diclofenac, ibuprofen and tolbutamide. Interestingly these drugs are marker substrates for the activity of human P450s 2C. Mutant A2 is here characterized in terms of binding constant and kinetic parameters that make it an interesting target for biocatalytic applications.

Results and Discussion

The sequence chosen for error prone PCR included amino acids 1-324 (initial Met excluded) and contained four out of the six regions that aligned to the substrate recognition sites (SRS), proposed by Gotoh^[23] for the P450 family 2, as well as the entire helix I, important in binding and catalysis.^[24-27] The conserved haem binding sequence (amino acids 392-405) and the so-called meander region (369-378) were deliberately excluded from mutagenesis to avoid impairing the heme-binding ability of the mutants. The whole sequence of the reductase (amino acids 479-1049) was also excluded to avoid interfering with the electron transfer from the NADPH to the flavins (FAD and FMN) and ultimately to the heme.

Transformation of *E. coli* BL21(DE3) with the plasmid pT7Bm3Z containing genes resulting from the error-prone PCR led to a library of 834 colonies; these were screened using the alkali assay^[22] to detect the enhancement in NADPH oxidation relative to the WT upon addition of the target substrates, diclofenac, ibuprofen and tolbutamide. Background levels of NADPH oxidation were measured without the addition of any substrates, and control

experiments were carried out with the addition of the known substrate lauric acid.

A mutant named A2, with improved activity toward a mixture of polyaromatic hydrocarbons,^[28] showed a significant increase in NADPH oxidation when incubated with diclofenac, ibuprofen and tolbutamide; this was selected for further studies and DNA sequencing. DNA sequence analysis of A2 revealed four point mutations, two of which are silent (Val127Val and Thr268Thr), whereas another two introduce significant variation in amino acids, namely Asp251Gly and Gln307His. The positions of Asp251 and Gln307 in the wild type structure are shown in Figure 1. Gln307His is one of the four mutations present in a variant of cytochrome P450 BM3 reported to have improved activity toward p-cymene.^[29]

Mutant A2 was successfully over-expressed in *E. coli* BL21 (DE3) and purified following the same protocols used for the wild type protein^[22] with minor modifications. Typical yields for the mutants were 16-20 mg of pure protein per g of wet cell paste, that is in the same range as that of the wild type, and it migrated to the expected molecular weight of 119 kDa on SDS-PAGE gels. Elution of A2 from Q-Sepharose at around 470 mM NaCl, led to a protein with the maximum of the visible absorbance spectrum at 392 nm that is typical of the high spin form (hs). Upon desalting by ultra-filtration, the peak of the spectrum partially shifted to 419 nm, typical of the low spin state form (ls). Reduction with sodium dithionite resulted in the disappearance of the 455-485 nm flavin shoulder, followed by the appearance of a neat peak at 450 nm when carbon monoxide was added to the fully reduced protein (Figure 2A).

Titration of A2 with diclofenac, ibuprofen and tolbutamide resulted in a low-to-high spin shift (Figure 2B); the plot of the absorbance changes calculated from the difference spectra versus the concentration of the substrate added allowed the calculation of the K_D values. Results of the fitting of the binding curves to a single site saturation binding curve are reported in table 1. All the three values are in the micromolar range with the highest affinity found for diclofenac and the lowest for ibuprofen. Mutant A2 also showed a lower dissociation constant toward lauric acid when compared to the wild type protein. When the same experiment was performed on the wild type protein, the three drugs were not able to induce a spin state change.

The rate of NADPH oxidation was calculated from the decrease of its absorbance at 340 nm following substrate addition, rates were measured for lauric acid on both wild type and A2, and for diclofenac, tolbutamide and ibuprofen on A2. The NADPH oxidation rates upon addition of lauric acid were found to be lower for A2 (703 min^{-1}) when compared to the wild type (1832 min^{-1}), but the significant turnover found in A2 indicates that the mutations did not radically changed its ability in oxidizing the fatty acid substrate. All the three drugs tested showed significant NADPH oxidation rates for A2 (table 1).

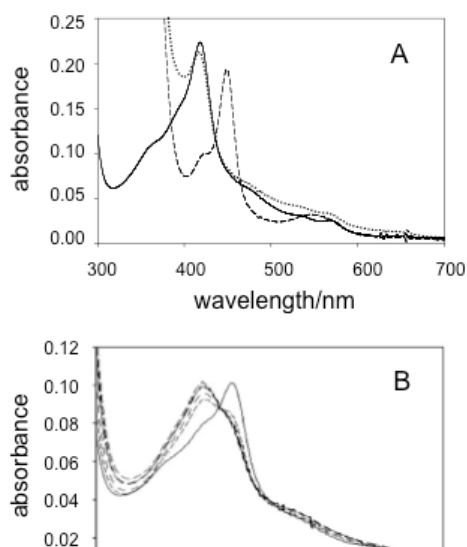


Figure 2. A) Visible spectrum of mutant A2 (1.7 μM) in the oxidized (solid line), reduced (dotted line) and reduced-CO-bound (dashed line) forms. B) Spectral transition observed during the titration of A2 (0.75 μM) with diclofenac (from 0.2 to 15 μM). Substrate binding is detectable as a shift from the low (418 nm) to the high (392 nm) spin form.

As the most accurate way to determine the result of P450 catalysis is the measurement of the reaction products following the catalysis, HPLC analysis of the reaction products generated by catalysis of A2 on diclofenac, ibuprofen and tolbutamide was carried out. Standards of the hydroxylated products of three drugs, 4'-hydroxydiclofenac, 2-hydroxyibuprofen and 4-hydroxytolbutamide were used to set up the HPLC separation methods. Analysis of the chromatograms of the reaction mixture with diclofenac shows the presence of a peak at retention time $t_R=53.8$ minutes corresponding to 4'-hydroxydiclofenac, whereas the peak of diclofenac was detected at a t_R of 60.0 minutes. A series of reactions were carried out in four replicates with 1 μM A2 and increasing amount of diclofenac in the presence of excess NADPH (5 mM) and catalase (200 U) in 100 mM KPi pH 7.4 at 37°C for 30 min to enable the determination of the A2 kinetic parameters.

The K_M and k_{cat} values derived from fitting to the Michaelis-Menten model (Figure 3A) are reported in table 1.

Incubations of ibuprofen with A2 led to three peaks in the chromatograms; the first peak shows a t_R of 14.0 minutes and it corresponds to 2-hydroxyibuprofen, the second with t_R of 15.5 minutes corresponds to a second metabolite derived from the oxidation in a different position, and the third at t_R 22.0 minutes corresponds to the substrate not consumed. The substrate concentration was then varied in the range 0-2 mM and the metabolite 2-hydroxyibuprofen was quantified. The amount of the product formed was plotted as function of substrate concentration. In this case, a sigmoid function was found to better fit the experimental data (Figure 3B), indicating a cooperative effect in substrate binding. The Hill equation was therefore used to calculate the kinetic parameters shown in table 1. Furthermore, the Hill equation provided the number of allosteric sites that resulted 2.1 ± 0.3 . This is consistent with a homeotropic cooperative effect for ibuprofen binding to A2. This kind of effect has already been reported for mutants of the bacterial P450 BM3 able to turn over drugs.^[19]

For the drug tolbutamide, the HPLC analysis of the product formed during enzymatic turnover showed the presence of a peak at a t_R of 7.0, corresponding to the metabolite 4-hydroxytolbutamide. Also in this case, the amount of substrate was varied in a series of reactions and the product quantified. The experimental data were adequately fitted by a hyperbolic equation (Figure 3C) leading to the determination of the catalytic parameters shown in table 1.

The catalytic parameters determined show that A2 is able to bind the three drugs with high affinities if compared to those reported for the human P450 enzymes. The K_M measured for diclofenac ($156 \pm 32 \mu\text{M}$) is comparable to one reported for human

P450 2C18 ($170 \pm 30 \mu\text{M}$).^[32] The other human enzymes involved in diclofenac metabolism are 2C9, 2C8 and 2C19 and the measured K_M values are 15 ± 8 , 630 ± 30 and $440 \pm 50 \mu\text{M}$ respectively.^[32]

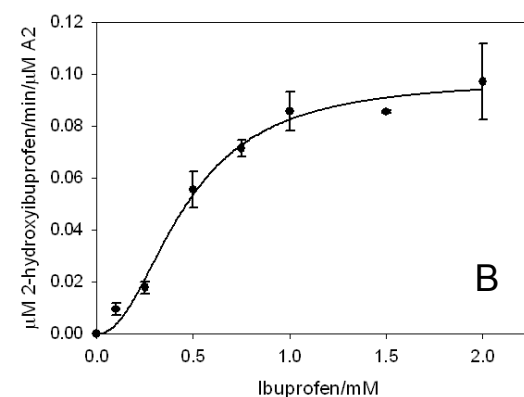
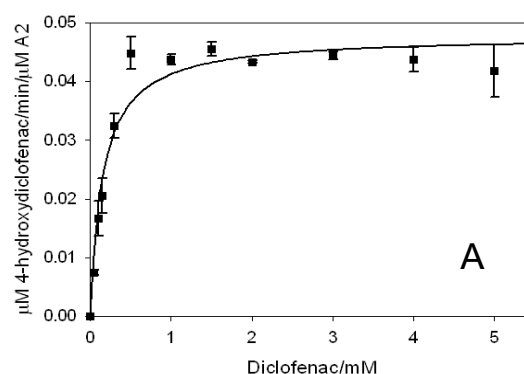
In the case of tolbutamide the K_M of A2 resulted $70.91 \pm 19.12 \mu\text{M}$, lower than the one reported for P450 2C9 ($255 \pm 38 \mu\text{M}$)^[33] whereas for ibuprofen, the K_M values reported in the literature are in the range 29 - $292 \mu\text{M}$.^[34] The new binding abilities found in A2 are also accompanied to a decrease of both the K_D and K_M values in comparison to WT for the known substrate lauric acid suggesting

that the two mutations introduced in A2 have caused an increase of the active site accessibility.

Table 1. Summary of binding constants and kinetic parameters calculated for P450 BM3 WT and A2 for lauric acid, diclofenac, ibuprofen and tolbutamide.

	Lauric acid		Diclofenac		Ibuprofen		Tolbutamide	
	WT	A2	WT	A2	WT	A2	WT	A2
K_D (μM)	83.8 ± 6.1	28.6 ± 2.5	-	1.3 ± 0.4	-	78.9 ± 18.7	-	26 ± 9
High spin (%)	88 ± 1	100 ± 3	-	97 ± 8	-	30 ± 8	-	26 ± 11
NADPH oxidation rate (min^{-1})	1832 ± 95	703 ± 37	0	428 ± 19	0	146 ± 13	0	231 ± 8
K_M (μM)	1318 ± 23	44 ± 9	-	156 ± 32	-	457 ± 41	-	71 ± 19
k_{cat} (min^{-1})	1810 ± 89	697 ± 30	-	0.048 ± 0.002	-	0.10 ± 0.01	-	0.94 ± 0.06

Mutant A2 is able to bind and turn over tolbutamide with rate constants higher than those measured for ibuprofen and diclofenac. The turnover rates for the bacterial enzyme are low when compared to the values calculated for lauric acid (a known substrate of P450 BM3) and to those reported for the human enzymes. Furthermore, it should be noted that the turnover rates for product formation are much lower than the ones calculated for NADPH oxidation, indicating that most of the electrons derived from NADPH are wasted by the enzyme in the so-called uncoupled reactions leading to the production of ROS. However, two considerations have to be made: the first is that new binding and catalytic abilities have been introduced in a bacterial enzyme by mutating only two amino acids; the second one is that the known substrates of the enzyme, the fatty acids, are structurally very different from the three drugs investigated and therefore the catalytic pocket of the protein, that has not been changed in A2, is not optimized for the oxidation of these new substrates. This consideration is supported by the fact that mutants of P450 BM3 able to generate the metabolites of diclofenac have been produced and characterized and they carry at least 6 mutations, located also within the active site.^[14] Furthermore, this is the one of the first mutant of P450 BM3 reported to have new catalytic activity toward ibuprofen and tolbutamide. A chimeric enzyme of P450 BM3 was reported to perform tolbutamide oxidation^[35] whereas, recently, a mutant able to perform ibuprofen oxidation was engineered.^[36]



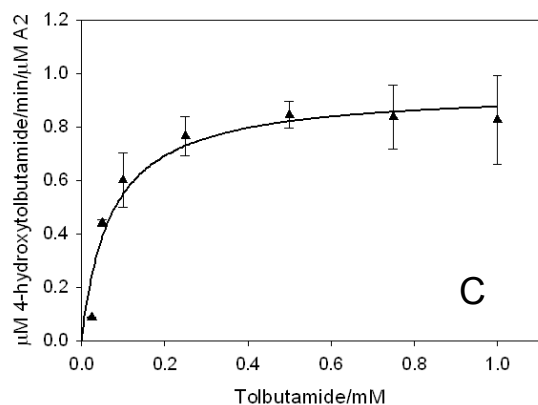
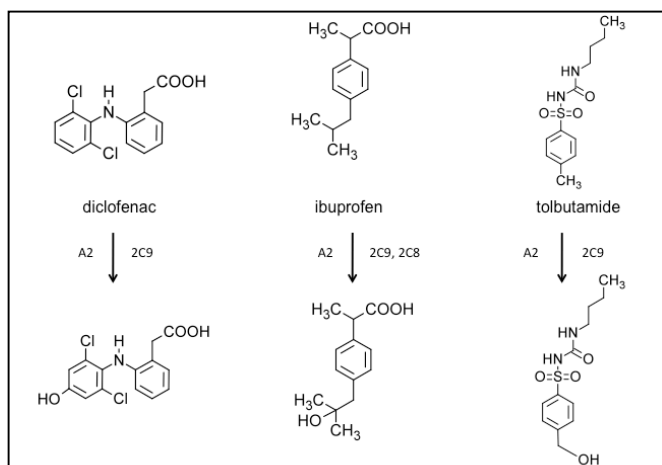


Figure 1C. Analysis of the reactions of mutant A2 with A) diclofenac, B) ibuprofen and C) tolbutamide. Each data point represents the average of at least 4 independent experiments. Fitting of the data was performed by using Sigma Plot 10.0.

It is notable the fact that not only A2 recognizes and oxidises drugs that represent marker substrates of the human P450 belonging to the 2C family, but also that the reactions performed led to the production of the same metabolites generated by these human enzymes (scheme 1). In fact, 4'-hydroxydiclofenac, 5-hydroxydiclofenac, 4',5-dihydroxydiclofenac, 3'-hydroxydiclofenac, and 3'-hydroxy-4'-methoxy-diclofenac have been identified as diclofenac metabolites produced by P450 2C9, 2C8, 2C19 and 2C18.^[37, 38] However, the hydroxylation of diclofenac in the position 4' is attributed to P450 2C9.^[39] Tolbutamide, a drug used to treat type-II diabetes, is converted by human P450 2C9 to 4-hydroxytolbutamide.^[40] In human liver, the cytochromes P450 responsible for the regio- and stereoselective 2- and 3-hydroxylation of the chiral non-steroidal antiinflammatory drug ibuprofen are 2C9 and 2C8.^[41]

Analysis of the 3D X-ray crystal structure of the wild type protein shows that Asp251 is located in the helix I and Gln307 in the helix J' (Figure 1A). Asp251 participates in a salt bridge with Lys224 of helix G (Figure 1B, green). Upon substrate binding the distance between the pair Asp251-Lys224 is significantly altered (Figure 1B, blue); these two residues move further apart breaking the salt bridge.^[31] Replacement of Asp251 with Gly in A2 prevents the formation of the salt bridge and this is likely to confer a higher degree of local flexibility in this region of the protein known to undergo structural rearrangements upon substrate binding.

Gln307 is the last residue of helix J' and is also located on the protein surface, at the distal site of the haem (Figure 1A). The structure of the substrate-free enzyme shows that the N_ε of Gln307 is likely to participate in H-bond with carboxylate oxygen O_{a2} of Asp300 (Figure 1C). Replacement with the charged His may cancel the hydrogen bond and perturb the local structure. Helix J' is



absent in other bacterial P450s, but it is present in microsomal cytochromes P450 where it has a role in recognition and binding of the reductase.^[42] However, the structure of the complex of the P450 BM3 heme domain with the FMN reductase domain^[37] shows that Gln307 is located far from the reductase docking site; Gln307 does not belong to the postulated pathway of electron transfer from the reductase to the heme.^[43]

Scheme 1. Products performed by the catalysis of mutant A2 on diclofenac, ibuprofen and tolbutamide.

Conclusion

A new biocatalyst, able to mimic the substrate specificity and the metabolite profile of human P450 2C, has been generated through random mutagenesis applied to P450 BM3. The mutant shows new catalytic abilities toward diclofenac, ibuprofen and tolbutamide respect to the WT protein.

The data shown give evidence to the fact that new biocatalytic capabilities can be introduced through directed evolution, an approach that allows the production of protein variants with mutations in random positions, unpredictable by rational methods, but important in maintaining the structural scaffold of the protein optimal for a certain range of substrates. In this specific case, only two mutations in positions not directly involved in substrate binding or turnover in the catalytic site are found to be sufficient to generate new specific reactions of biotechnological interest.

Experimental Section

Library generation.

For the generation of the library of variants containing random mutations within the haem domain of P450 BM3, the recombinant construct pT7Bm3Z,^[44] which contains the 3,147 bp gene translating to P450 BM3, was used. Part of the gene extending from -24 bp to 1,000 bp and incorporating the *Bam*HI and *Ball* sites was amplified by PCR using the 27-mer primers 5'-CTTAACAAGTGAAGGAGGGATCCTATG-3' (forward primer) and 5'-GGGAAAACGCAGGAGCAGTTGGCCATA-3' (reverse primer). The amplified gene was then subjected to error-prone PCR. The reaction mixture (40 µl) contains: 10 mM Tris-HCl pH 8.0, 50 mM potassium chloride, 3.5 mM magnesium chloride, 0.5 mM manganese chloride, 0.4 mM of each primer, 1.0 mM of three dNTPs with the fourth at 0.2 mM, 3.1-6.2 ng of template DNA, 2 U Taq-Polymerase. Four reactions were run in parallel, each having a different dNTP triplet in excess. Reactions were pooled and the PCR product was purified from an agarose gel. It was subsequently digested with *Bam*HI and *Msc*I (an isoschisomer of *Ball*). The digested insert was purified subsequently ligated to the similarly digested pT7Bm3Z vector, replacing the WT sequence.

Alkali assay.

The alkali assay was carried out as described before.^[22] Briefly, the colonies containing the randomised pT7Bm3Z library were inoculated into 170 µl of LB/amp (100 µg/ml) in a microtitre-plate well. As a control, cells carrying the WT plasmid were also inoculated in a separate well. The microtitre plate was incubated at 37 °C, and when the culture reached an OD₆₀₀ of ~0.7, protein expression was induced by the addition of 1.0 mM isopropyl β-D-1-thiogalattopyranoside (IPTG). After growth, the cells were harvested by centrifugation and the pellet resuspended into 100 mM KPi pH 8.0 to give OD₆₀₀ of about 0.4-0.5. By correcting cell density before performing the assay, equal background NADP⁺ levels were achieved.

The compounds to be screened for turnover were added to the cells for 2 h before the reaction was started by the addition of 1.5 mM NADPH. The final concentration of diclofenac, ibuprofen and tolbutamide was 0.5 mM. After incubation for 4 h at room temperature, the alkali product of NADP⁺ was developed, as previously described.^[22] The 340-450 nm absorbance spectrum of each well was read on a SPECTRAMax® 340PC microplate spectrophotometer (Molecular Devices, California).

Controls reactions were performed where no compound was added. With the exception of lauric acid that was dissolved in 50 mM potassium carbonate, stocks of all compounds were in ethanol. The final ethanol concentration in the assay incubation did not exceed 2%. The alkali assay was also repeated on the purified mutant. The protein

concentration, determined by the CO binding assay, was $\geq 1 \mu\text{M}$. A $\epsilon_{450\text{ nm}}$ of $91000 \text{ M}^{-1} \text{ cm}^{-1}$ was used to quantify the protein.^[45]

Expression and purification of P450 BM3 WT and mutant A2.

P450 BM3 WT and mutant A2 were expressed in the *E. coli* BL21(DE3) strain and purified essentially as described previously for the WT enzyme.^[22]

Substrate binding and determination of the high spin species.

Substrate binding was monitored using spectrophotometric titrations in 1 cm path-length cuvette by following the characteristic low-to-high-spin transition, as indicated by the shift in the main Soret band from 418 to 392 nm. The reaction mixture contained 0.5-1 μM concentrations of enzyme in 100 mM KPi pH 8.0. Spectra were recorded from 360 to 460 nm after each addition to the sample cuvette. Substrates were always freshly prepared. Controls with the same volume of the corresponding solvent added to the enzyme-containing solution were also carried out. All spectral titrations were carried out at 20°C. Difference spectra were generated by subtraction of the substrate-free spectrum to the ones corresponding to substrate-bound after each addition. K_D values were determined by plotting the maximal absorbance changes calculated from each difference spectrum against the concentration of drugs and fitting the data using SigmaPlot 8.0.

The percentage of high spin species was calculated assuming that the P450 BM3 ferric iron was 100% low spin in the resting state of the enzyme ($\epsilon_{418\text{ nm}} = 105,000 \text{ M}^{-1} \text{ cm}^{-1}$),^[46] and 100% high spin when saturated with arachidonic acid.

NADPH oxidation assay.

NADPH oxidation was followed spectrophotometrically at 340 nm, using an extinction coefficient at 340 nm of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Kinetic assays were performed under steady-state conditions at 20°C in 100 mM KPi pH 8.0 in 1 cm path-length cuvettes. The enzyme concentration was in the μM range ($> 0.1 \mu\text{M}$), whereas NADPH was at saturating concentration (150 μM).^[47] Substrate was added up to saturating concentrations using a Hamilton syringe. The reaction was initiated by the addition of NADPH to the enzyme:substrate complex. Each data point was the mean of at least three measurements of initial rate. The rate was calculated from the linear part of the decrease in absorbance at 340 nm. Activity units versus substrate concentration data were fitted using the SigmaPlot software to obtain the K_M and k_{cat} parameters.

HPLC analysis of drug metabolites.

HPLC analysis was performed to detect the presence of drug metabolites deriving from enzymatic turnover and to quantify the amount of the metabolites formed. An Agilent 1200 quaternary pump system equipped with an autosampler and a diode array detector was used. The reactions were carried out at 37°C in 300 μl of reaction volume and contained 100 mM KPi buffer pH 7.4, 1 μM enzyme, different amounts of the substrate and 5 mM NADPH along with catalase (200 U) to decompose H_2O_2 (if generated). Substrates were dissolved in either methanol or acetonitrile. Nevertheless, the volume of methanol/acetonitrile added to the reaction mixture was below 3% of the total reaction volume to minimize effects of the solvent on the enzyme. The enzyme was incubated with the substrate for 5-10 min at 25°C prior to start the reaction by the addition of NADPH. The reaction samples were stopped after 30 minutes by adding 500 μl of ice-cold acetonitrile, passed through solid phase extraction columns (Strata X-sorbents, Phenomenex) and extracted in 100 μl of 50-50% methanol and acetonitrile for HPLC analysis.

The separation of metabolite was carried out on a C18 reverse-phase column (Lichrosphere; 25 cm x 4.6 mm) and eluted by different separation methods described below. The metabolites were identified by comparing the retention times of the chromatographic peaks with those of standards of the commercially available 4'-hydroxydiclofenac, 2-hydroxyibuprofen and 4-hydroxytolbutamide. 4'-hydroxydiclofenac and 4-hydroxytolbutamide were purchased from Cypex Ltd. Scotland UK. The only available metabolite 2-hydroxyibuprofen was purchased from US Biologicals (SynFine Research Inc. Ontario, Canada). Each reaction was run with the appropriate control checking that the peaks with the same retention times of the metabolites standards were not present in the control reaction performed in the absence of NADPH or the enzyme or the drug tested.

Standards of diclofenac, tolbutamide, ibuprofen, 4'-hydroxydiclofenac, 4-hydroxytolbutamide and 2-hydroxyibuprofen were diluted in methanol and passed through solid phase extraction column. Along with these compounds, caffeine was used as an internal standard for the quantification of the product formed. Caffeine was dissolved in methanol:acetonitrile (50:50). The flow rate was 1.2 ml/min in all cases.

The separation of diclofenac from its hydroxy-metabolite was carried out using a mobile phase composed by acetonitrile (A) and water + 1% acetic acid (B). The separation method was: 0-10 min: 5% (A):95% (B); 10-15min: linear gradient from 5% (A):95% (B) to 25% (A):75% (B); 15-45min 25% (A):75% (B); 45-60 min: linear gradient from 25% (A):75% (B) to 95% (B):5% (C).

For tolbutamide and ibuprofen and for their corresponding hydroxyl-metabolites separation, the mobile phase was composed by water + 0.5% ammonium acetate (A) and methanol + 0.5% ammonium acetate (B). The separation method for tolbutamide and its corresponding hydroxyl-metabolite was: 0-15 min: linear gradient from 95% (A): 5%(B) to 5% (A): 95% (B). The separation method for ibuprofen and its corresponding hydroxyl-metabolite was developed as: 0-25 min: linear gradient from 95% (A): 5% (B) to 5% (A): 95% (B).

For the quantification of the metabolites formed, a calibration curve was constructed by injecting different amounts of the standards of the metabolites together with the internal standard (caffeine). The area of the peak corresponding to the metabolite was normalized for that of the internal standard. The peak areas were then plotted as function of metabolite concentration and a linear regression performed. The equation was then used for the quantification of the metabolite produced in the reaction of A2 with different amounts of diclofenac, ibuprofen and tolbutamide. All the fittings were performed by Sigma Plot 10.0 software.

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