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LICRED: A Versatile Drop-In Vector for Rapid Generation of Redox-Self-Sufficient Cytochrome P450s

Federico Sabbadin,[a] Ralph Hyde,[b] Aelig Robin,[c] Eva-Maria Hilgarth,[c] Marie Delenne,[a] Sabine Flitsch,[c] Nicholas Turner,[c] Gideon Grogan,*[b] and Neil C. Bruce[a]

Cytochromes P450 (P450s) are a family of haem-containing oxidases with considerable potential as tools for industrial biocatalysis. Organismal genomes are revealing thousands of gene sequences that encode P450s of as yet unknown function, the exploitation of which will require high-throughput tools for their isolation and characterisation. In this report, a ligation-independent cloning vector "LICRED" is described that enables the high-throughput generation of libraries of redox-self-sufficient P450s by fusing a range of P450 haem domains to the reductase of P450RhF (RhF-Red) in a robust and generically applicable way. Cloning and expression of fusions of RhF-Red with the haem domains of P450cam and P450-XplA resulted in soluble, active, redox-self-sufficient, chimeric enzymes. In vitro studies also revealed that electron transfer from NADPH to haem was primarily intramolecular. The general applicability of the LICRED platform was then demonstrated through the creation of a library of RhF-Red fusion constructs by using the diverse complement of P450 haem domains identified in the genome of *Nocardia farcinica*. The resultant fusion-protein library was then screened against a panel of substrates; this revealed chimeric enzymes competent for the hydroxylation of testosterone and methyltestosterone, and the dealkylation of 7-ethoxycoumarin.

Introduction

Cytochromes P450 (P450s) are a superfamily of haem-containing monooxygenases that catalyse a wide range of regio- and stereospecific reactions of industrial relevance, including the hydroxylation of nonactivated carbon centres and aromatics, sulfoxidation, epoxidation, N- and O-dealkylation, deamination and dehalogenation[1–4] and thus have great potential as industrial biocatalysts for the synthesis of fine chemicals and drug metabolites.

As many as ten classes of cytochrome P450s have been identified,[3] with this classification largely dependent on the structural organisation of the electron-transport chain required to deliver electrons from the requisite nicotinamide cofactor NAD(P)H through to the catalytic haem. Most prokaryotic P450s require auxiliary electron-transport proteins for this role, such as the extensively studied P450cam from *Pseudomonas putida*, a prototypical class I P450 system, which requires an Fe_2S_2 protein (putidaredoxin, Pd) and a reductase (putidaredoxin reductase, PdR) in order to transfer electrons from NADH and catalyse the 5-*exo* hydroxylation of (1*R*)-(+)-camphor.[5] In class I P450s, redox partners are often rate-limiting for reaction and are thus required in a high molar excess in vitro in order for the P450 to display high turnover rates.[6]

By August 2009, more than 11 000 cytochrome P450 sequences had been identified, mainly through genome sequence analysis (source: http://drnelson.utmem.edu/cytochromeP450.html). However, the cognate electron-transfer partners for most of these P450s have not yet been identified; neither has their biological function, nor the type of chemical reaction that they catalyse. Surrogate redox partners, notably the Pd and PdR from *P. putida*, have been used in the past to characterise these "orphan" P450s, with variable results, probably because of the high uncoupling of electron transfer that occurs between cofactor and haem.[7,8] Alternative approaches, in which P450 haem domains are fused with reductase domains, might therefore present many advantages, such as improved electron transfer, turnover rate and process suitability, as well as the requirement to express only one gene product rather than three. Over the years, various artificial fusion proteins of bacterial P450s have been engineered in attempts to generate self-sufficient biocatalysts. The PdR-Pd-cytochrome P450cam triple-fusion protein was the first successful attempt to link together the separate domains of a class I P450, by trying artificial peptide linkers and domain arrangements. The authors were also able to demonstrate that the resulting chimera exhibited intramolecular electron transfer and was, therefore, self-sufficient.[9]

[a] *F. Sabbadin, M. Delenne, Prof. N. C. Bruce*
Centre for Novel Agricultural Products
Department of Biology, University of York
YO10 5YW York (UK)

[b] *Dr. R. Hyde, Dr. G. Grogan*
York Structural Biology Laboratory
Department of Chemistry, University of York
YO10 5YW York (UK)
Fax: (+44) 1904-328266
E-mail: grogan@ysbl.ac.uk

[c] *Dr. A. Robin, E.-M. Hilgarth, Prof. S. Flitsch, Prof. N. Turner*
School of Chemistry and Manchester Interdisciplinary Biocentre (MIB)
University of Manchester
M1 7ND Manchester (UK)

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However, much of the research in recent years has focused on the exploitation of naturally occurring P450 fusion proteins or derivatives thereof.

Natural redox-self-sufficient P450s, in which the requisite electron-transport chain is fused within a single polypeptide, have therefore attracted interest as potentially more robust and efficient oxidation catalysts.[10] One example of such an enzyme, P450M3 from *Bacillus megaterium* (CYP101A2)[11] is a soluble, self-sufficient P450 hydroxylase in which the catalytic haem domain (BMP) is C-terminally fused to a FAD/FMN reductase (BMR). Both the very high efficiency of electron transfer from NADPH to the haem iron and the high turnover rate of P450M3 with its fatty acid substrates (17100 min^{-1} for arachidonic acid[12]) are thought to be attributable primarily to the proximity and orientation of the protein domains engendered by their fusion.[13] Another P450 exhibiting a natural fusion of haem domain and redox partners, P450RhF (CYP116B2), was identified by our groups in the soil bacterium *Rhodococcus* sp. NCIMB 9784.[14] In P450RhF, the C-terminal reductase partner (RhF-Red) has a FMN- and NADPH-binding motif and a Fe_2S_2 ferredoxin-like component, and is fused to the haem-containing oxygenase domain at the N terminus[15,16] (Figure 1). The

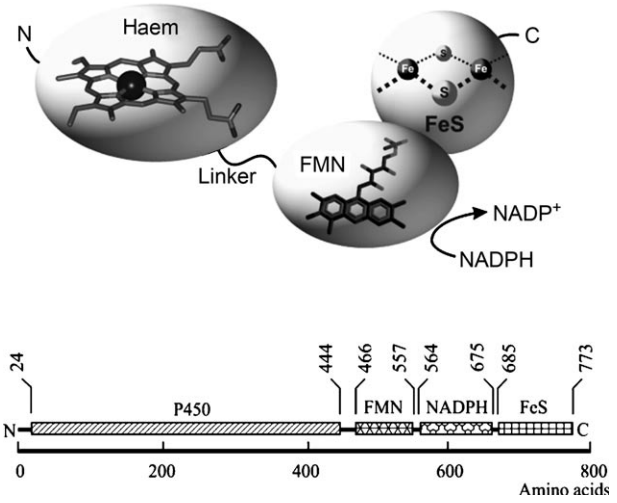


Figure 1. Schematic representation of the architecture of P450RhF (CYP116B2) spanning from the N-terminal haem domain to the C-terminal reductase domain.

physiological role and the natural substrate of P450RhF remain elusive, although both in vivo and in vitro assays showed that it catalysed the dealkylation of 7-ethoxycoumarin to 7-hydroxycoumarin. Recent work has demonstrated that different bacterial P450 haem domains can be swapped with the haem domain of P450RhF and be thus fused to its reductase domain, resulting in active chimeric constructs with substrate specificities conserved from that of the wild-type proteins.[17–22] However, the distinct haem domains that have been used as part of the RhF-Red fusion strategy are still small in number.

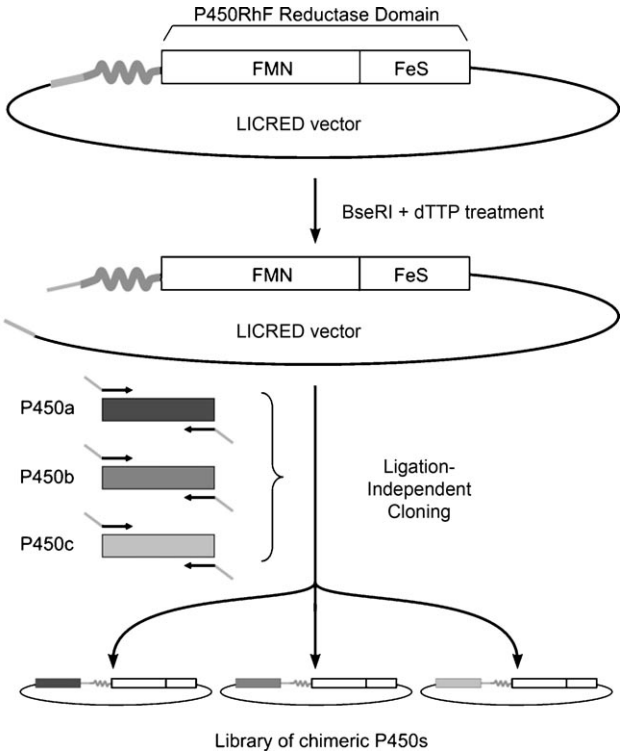
In order to realise the potential of the vast numbers of P450s in the genomic resource, high-throughput generic tools for the isolation, characterisation and screening[23] of these enzymes will be required. As part of our investigation of genome-encoded biocatalyst libraries,[24,25] we have been interested in developing robust cloning strategies suitable for application within the automated robotics infrastructure in our laboratory. In this report, we describe the engineering of a new platform for the high-throughput generation of libraries of redox-self-sufficient P450s by using a generic protocol that combines the highly adaptable reductase domain of P450RhF with a recently developed ligation-independent cloning (LIC) vector.[24] The LICRED platform eliminates the need for custom restriction enzyme sites to be engineered within the RhF-Red fusion gene sequence and eliminates the cost of DNA ligase, restriction enzymes and the extended incubation times required for ligation protocols, each consonant with application in high-throughput automated strategies. As part of the study, we generated a novel self-sufficient RhF-Red fusion of the explosive-degrading enzyme XplA and a library of sequence-diverse chimeric P450s that includes a fusion protein that selectively hydroxylates testosterone. We also demonstrate for the first time that, in contrast to systems such as P450-BM3, the electron transfer in these RhF-Red systems is predominantly intramolecular along the electron-transport chain from NAD(P)H to the catalytic haem, consonant with process suitability in future applications.

Results and Discussion

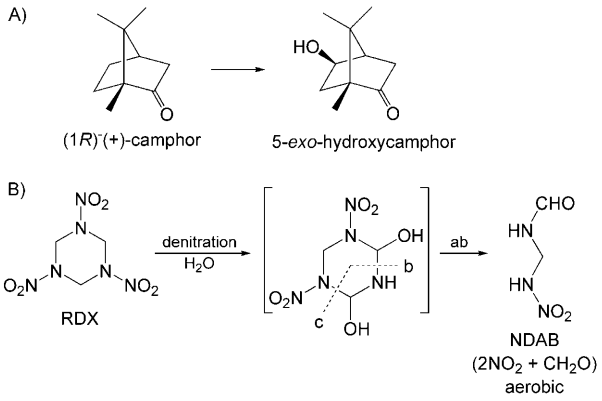
Construction of the LICRED vector

P450RhF, identified in the soil bacterium *Rhodococcus* sp. NCIMB 9784, is a natural fusion of haem domain and redox partners. In P450RhF, the C-terminal reductase has a FMN- and NADPH-binding motif and a Fe_2S_2 ferredoxin-like component, and is fused to the N-terminal haem-containing oxygenase domain (Figure 1),[14–16] which has 55% amino acid sequence identity with the thiocarbamate-inducible CYP116 from *Rhodococcus erythropolis* NI86/21.[26] It was decided to exploit the properties of the pET-YSBLIC 3C vector[24] to accommodate the sequence of the P450RhF reductase domain, thereby permitting the consequent LIC-based insertion of haem domains of choice.

In order to construct the LICRED vector, the reductase domain of P450RhF itself required several preliminary modifications. First, BseRI and NdeI restriction sites were added at the 5′- and 3′-ends, respectively, in order to ligate the reductase domain beside the LIC site (Scheme 1). Second, the first five amino acids of the original linker of the reductase domain were modified in order to incorporate both the BseRI restriction site and the standard LIC sequence CGCGCCTTCTCCTCA. Site-directed-mutagenesis of the P450RhF reductase was then performed in order to remove the BseRI restriction site naturally present in the gene sequence. Modified reductase (insert) and pET-YSBLIC 3C (vector) were then cut with the restriction enzymes BseRI and NdeI and ligated by using the procedures detailed in the Experimental Section to give the final plasmid construct, which was named LICRED.



Scheme 1. Scheme of the general strategy used to clone P450 haem domains in the LICRED platform to generate libraries of self-sufficient P450s.



Scheme 2. A) Hydroxylation of (1*R*)-(+)-camphor catalysed by P450cam-RhF-Red; B) double denitration of RDX by XplAP450-RhF-Red and spontaneous ring cleavage under aerobic conditions.

Validation of the LICRED vector with P450cam and XplA haem domain

In order to first validate the LICRED platform, it was decided to create the RhF-Red fusion constructs of two P450 haem domains of which we had extensive experience, the 5-*exo*-camphor hydroxylase from *P. putida*[5] and the haem domain of the P450 XplA, which catalyses the reductive degradation of nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).[27,28] The relevant PCR primers with LIC-specific overhangs were designed, and the relevant genes were amplified and cloned into the LICRED vector, and the consequent fusion proteins (P450cam-RhF-Red and XplAP450-RhF-Red) were expressed in *E. coli* strain Rosetta 2 (DE3). Initially, resting-cell assays were carried out to assess the hydroxylation of (1*R*)-(+)-camphor by P450cam-Rhf-Red and the degradation of RDX by XplAP450-RhF-Red (Scheme 2).

In the case of cells expressing P450cam-RhF-Red, GC analysis of samples taken at different time points showed efficient conversion of 10 mM substrate to the expected product 5-*exo*-hydroxy camphor. Cells expressing XplAP450-RhF-Red were shown to rapidly degrade RDX from an initial concentration of 150 μM over time, as revealed by HPLC analysis. Negative-control experiments showed that cells transformed with empty LICRED plasmid displayed no activity in either case.

Characterisation of purified RhF-Red chimeric P450s

Encouraged by these results, we then purified the fusion proteins in order to investigate their physical and catalytic properties. P450cam-RhF-Red and XplAP450-RhF-Red were purified by nickel affinity chromatography followed by gel filtration. CO difference spectra were recorded for both chimeric proteins and showed the signature Soret peak at 450 nm upon reduction with sodium dithionite and bubbling with carbon monoxide (Figure 2). The calculated dissociation constant of

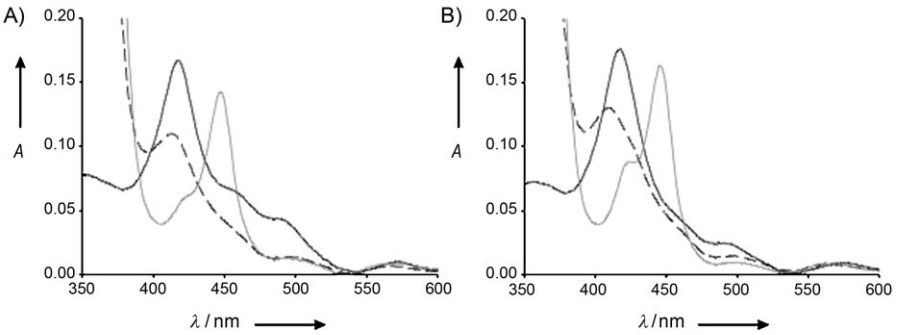


Figure 2. UV-visible absorbance spectra of A) P450cam-RhF-Red and B) XplAP450-RhF-Red fusion proteins in the oxidised (——), sodium dithionite reduced (-----) and reduced, CO-bound (——) forms.

P450cam-RhF-Red with camphor, 1.09 μM, was similar to the value previously reported for isolated P450cam of 1.59 μM.[5] Similarly, the K_D of XplAP450-RhF-Red with RDX, 4.88 μM, was consistent with data collected in our laboratory by using the isolated haem domain of XplA of 6 μM (Figures S4 and S5 in the Supporting Information). These spectral characteristics suggest that, in both P450cam-RhF-Red and XplAP450-RhF-Red, neither the 16-amino-acid linker nor the reductase domain in-

terfered significantly with the folding of the haem domain or with its ability to bind the relevant substrate with high affinity.

Incubations of the isolated P450cam-RhF-Red and XplAP450-RhF-Red with NADPH plus (1*R*)-(+)-camphor and RDX, respectively, demonstrated that the chimeric P450s were able to catalyse transformations of those substrates in vitro. For the XplAP450-RhF-Red fusion, activity assays followed by Griess assay and Hantzsch assays also revealed that RDX degradation was followed by the release of nitrite and formaldehyde in ratios 2:1 and 1:1, as previously reported for the degradation of RDX by XplA under aerobic conditions.[27] Measurements of the rate of NADPH oxidation, both in the absence and presence of increasing substrate concentrations (Figure 3) revealed

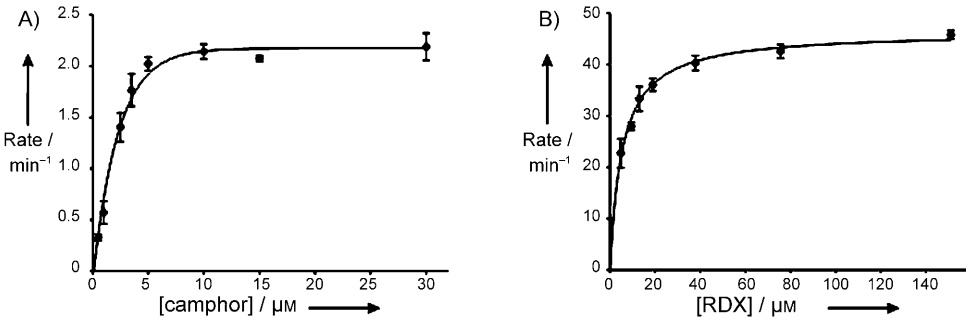


Figure 3. Rate of NADPH oxidation by A) P450cam-RhF-Red and B) XplAP450-RhF-Red.

that the coupling levels between NADPH consumption and product formation were 34 and 82% for P450cam-RhF-Red and XplAP450-RhF-Red, respectively (Table 1). The high coupling level of the XplAP450-RhF-Red chimera was unexpected, because in the bacterium *R. rhodochrous* 11Y, from which it was originally identified, the XplA haem domain is naturally fused at the N terminus with a flavodoxin domain. In the XplAP450-RhF-Red chimera, the FeS domain, which has the same function as the flavodoxin, is located at the C terminus. Based on these observations, the LICRED vector appeared to be reasonably versatile as an electron-transfer partner for classical bacterial P450s (such as P450cam) as well as for more unusual P450s, such as XplA.

Previous work has shown that haem domains can be successfully fused to the reductase domain of P450RhF;[17–19,21,22] however, in the majority of those cases, the resultant fusion proteins were not purified, the spectral characterisation was performed on the crude cell lysate, and activity assays were performed as whole-cell experiments. The only previous example of an in vitro characterisation of an RhF-Red chimera similar to the fusions described here was reported by Sherman and co-workers, who showed that the fusion of RhF-Red with the P450 PikC could hydroxylate the YC-17 and narbomycin macrolactones upon addition of NADPH.[21] Gel filtration analysis of PikC-RhF-Red indicated that PiKC-RhF-Red formed dimers in storage buffer even in the presence of the reducing agent dithioerythritol (DTE). The authors thus recognised that it was unclear if the electron transfer occurred in an intermolecular or intramolecular fashion.

In order to clarify the nature of the electron transfer in chimeras generated by using the LICRED platform, SEC-MALLS analysis was performed on the purified chimeras P450cam-RhF-Red and XplAP450-RhF-Red. This revealed that both chimeras exist predominantly as monomers in buffer solution at pH 7.0, even in the absence of reducing agents. This, together with the observation that the protein concentration did not significantly affect either the turnover rates of the two (Figure 4) chimeras or the background level of uncoupling in the absence of substrate per enzyme unit, strongly suggests that electron transfer within the chimeric systems was intramolecular.[29] The natural fusion protein P450M3 is only active as a dimer and requires specific interactions between the domains of the two monomers, including mostly intermolecular transfer, in order to maximise catalytic activity.[30,31] Attempts to generate chimeras by attaching noncognate haem domains to the reductase of P450M3 often resulted in uncoupling. Low coupling efficiencies have also been observed when CYP haem domains have been artificially fused to yeast[32] or rat[33] NADPH-P450 reductases.

Table 1. Kinetic constants determined for P450cam-RhF-Red and XplAP450-RhF-Red chimeric P450s by using (1*R*)-(+)-camphor and RDX as substrate, respectively.

Enzyme	K_D[a]	K_M[b]	k_{cat}[b] [min^{-1}]	k_{cat}/K_M (×10)[b] [μM^{-1} min^{-1}]	Coupling[c] [%]
P450cam-RhF-Red	1.09 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	9.56	34
XplAP450-RhF-Red	4.88 ± 0.1	5.4 ± 0.4	46.3 ± 0.7	85.7	82

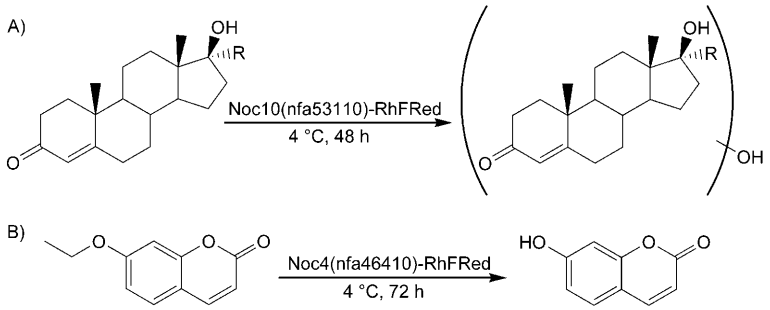
[a, b] The K_D and K_M are given in μM. [b] The data of NADPH oxidation represent the averages of three experiments after subtraction of the background. [c] Percentage of NADPH consumed that was channelled to product formation.

Application of LICRED to the generation of a library of chimeric P450s

In order to test the generic applicability of the LICRED platform, it was decided to create a library of fusion constructs by using a family of P450s from a single prokaryotic genome as an initial and easily accessible illustration of its applicability. The genome of the soil actinomycete *Nocardia farcinica* IFM 10152 was described as containing multiple genes that encode

putative P450s;[34] this suggested that it would be a good model organism from which to draw a P450 family, as some hydroxylating activity had been described for the associated haem domains previously.[35] Twenty-two of those genes (listed in the Supporting Information) were amplified from genomic DNA and cloned into the LICRED vector by using a robotics-based multiwell plate facility available in our laboratories. These genes encoded a sequence-diverse group of P450s with mostly low amino acid sequence identity ranging from, for example, 16% between *nfa*56380 and *nfa*34990 up to the two most closely related of the group, *nfa*24320 and *nfa*25870 with 72% identity.

Preliminary expression trials were carried out by using *E. coli* cells Rosetta 2 (DE3) grown in Terrific Broth medium and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG). SDS-PAGE and Western blot analysis with an anti-His tag antibody suggested that at least eleven constructs of this library of chimeric P450s were expressed in the soluble fraction, each generating a band around 80 kDa (Figure 5). A second band, corresponding to a molecular weight of approximately 45–50 kDa, was also detected for some of the constructs and most probably corresponded to the product that results from cleavage of the peptide linker between the His-tagged N-terminal haem domain and the reductase. The activity of resting cells expressing the family of chimeric P450s that had been grown on M9 medium with induction by IPTG and 5-aminolevulinic acid (5-ALA) was subsequently tested against a small panel of 14 potential substrates (Figure S9). GC/MS analysis revealed that Noc10-RhF-Red, containing the haem domain encoded by gene *nfa*53110, was able to hydroxylate testosterone (Scheme 3) and 17-methyl-testosterone, consistent with observations on the relevant haem domain made previously.[35] Noc4-RhF-Red, containing the haem domain encoded by *nfa*46410, was shown to dealkylate 7-ethoxycoumarin to 7-hydroxycoumarin, and was the first oxidative activity to be described for this cytochrome P450 (Scheme 3).



Scheme 3. Whole-cell activity. A) Hydroxylation of testosterone and 17-methyltestosterone catalysed by the Noc10-RhF-Red chimera. B) Dealkylation of 7-ethoxycoumarin catalysed by the Noc4-RhF-Red chimera.

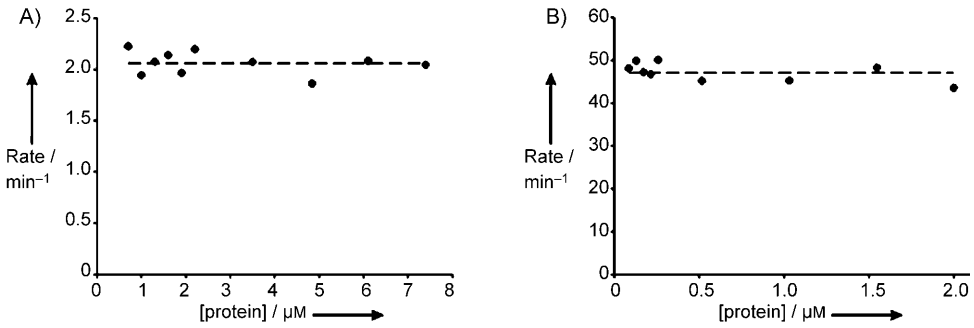


Figure 4. Effect of varying protein concentration on the rate of NADPH oxidation by A) P450cam-RhF-Red and B) XplAP450-RhF-Red. The average rates (-----) are 2.06 ± 0.1 min^{-1} and 47.1 ± 2.2 min^{-1}, respectively.

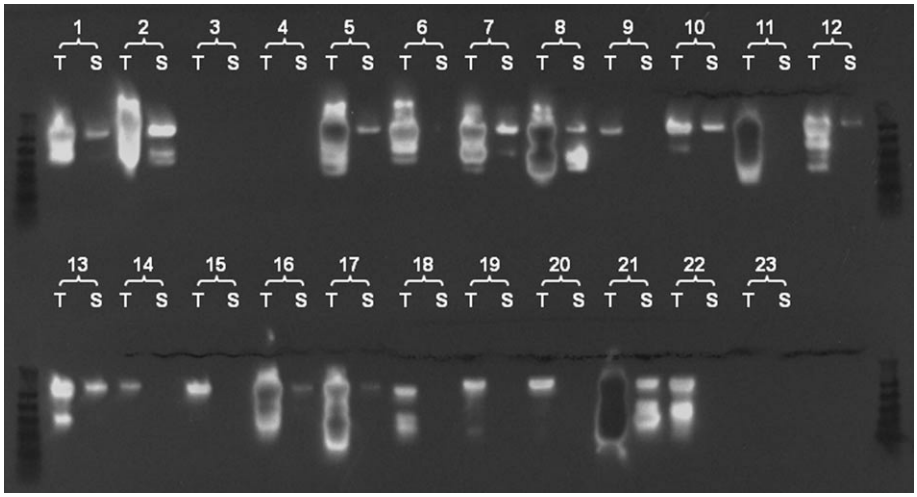


Figure 5. Composite image of anti-his Western blots of protein expression following IPTG induction and overnight incubation at 16 °C for the 22 chimeric P450 constructs from *N. farcinica* (1–22) plus the negative control (23). T is total protein, S is soluble protein.

Although the other genes did not yield productive chimeric hydroxylases as tested against the substrates screen, this in itself was revealing of the difficulties associated with screening naturally encoded P450 sequences, given that not only can the substrate specificity of individual prokaryotic P450s be very narrow; some like XplA, are not hydroxylases at all.[36] Notwithstanding the low "hit-rate" of productive catalysts, the exercise was useful in demonstrating the

applicability of the vector to a sequence-diverse library of P450 genes.

Conclusions

A new platform for the rapid construction of redox-self-sufficient P450s was established and validated through the expression of at least 13 soluble fusion proteins by using the versatile reductase system from P450RhF. Detailed characterisation of the purified chimeras P450cam-RhF-Red and XplAP450-RhF-Red generated by using the LICRED platform indicated that they were active, redox-self-sufficient monomers employing a predominantly intramolecular electron-transfer chain with a coupling efficiency that can exceed 80%. The system was readily applied to the creation of families of chimeric P450s based on sequence-diverse prokaryotic P450 haem domains. The application of the LICRED platform to the expression, characterisation and application of a variety of orphan eukaryotic P450s from both mammals and plants is currently underway. This new technology platform, combined with a rapid colorimetric activity screening, represents a robust and cost-effective strategy for identifying new P450 activities and generating redox self-sufficient oxygenases for industrial biocatalysis.

Experimental Section

Design and construction of the LICRED vector: First, site-directed mutagenesis was performed on the DNA encoding the reductase domain of P450RhF by using overlap extension PCR to remove the BseRI recognition site naturally present in the reductase gene (CTCCTC→CTACTC). The sequence coding for the first five amino acids of the linker was then modified in order to include the sequence for ligation-independent cloning and also the recognition site for BseRI (GTG CTG CAC CGG CAT→CGC GCC TTC TCC TCA). The LICRED plasmid was then constructed by ligating the modified P450RhF reductase gene between the BseRI and NdeI restriction sites in the pET-YSBLIC 3C plasmid.[24] The vector also included a short sequence coding a hexahistidine tag at the N terminus of the LIC site in order to facilitate further purification of the protein by nickel affinity chromatography and its visualisation through Western blotting analysis.

Cloning, overexpression and purification of the chimeric proteins: Primers for each gene target encoding a P450 haem domain were designed with standard 5′ LIC ends, for example, in the case of P450cam-RhF-Red: P450cam_LIC_Forward (5′-CCA GGG ACC AGC AAT GAC GAC TGA AAC CAT ACA AAG CAA CGC-3′) and P450cam_LIC_Reverse (5′-GAG GAG AAG GCG CGT ACC GCT TTG GTA GTC GCC GGA TC-3′). A full list of the primers used to create the fusion proteins in this paper is available in the Supporting Information. In each case, in order to obtain the fusion construct, the stop codon in all haem-domain sequences was removed.

Genes encoding P450cam and XplAP450 were amplified in 50 μL PCRs by using Phusion DNA polymerase (New England Biolabs) under the following cycle parameters: one cycle of 98 °C for 5 min, then 35 cycles at 98 °C for 10 s, 68 °C for 30 s and 72 °C for 60 s. The PCR product was subcloned into the LICRED plasmid and transformed into expression strains of *E. coli*, as previously described for the pET-YSBLIC 3C vector.[24] Single colonies of transformants were used to inoculate cultures of Luria–Bertani broth (10 mL) with antibiotics (100 μg mL^{-1} kanamycin, 34 μg mL^{-1} chloramphenicol). After overnight incubation at 37 °C with shaking, the culture was used to inoculate M9 minimal medium (1 L plus 100 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol). When the OD_{600} reached 0.8, protein expression was induced with IPTG (1 mM), 5-ALA (1 mM), $FeCl_3$ (0.5 mM) and riboflavin (5 μg L^{-1}). After overnight incubation at 20 °C with shaking, the cells were harvested by centrifugation, resuspended in potassium phosphate buffer (50 mM, pH 7.0, "buffer") and disrupted by ultrasonication.

After centrifugation, the crude supernatant was filtered and loaded onto a nickel–agarose affinity column (5 mL, GE Healthcare). The N-terminally His_6-tagged proteins were eluted with an increasing gradient of imidazole (30–500 mM) in buffer. Fractions containing the fusion protein were concentrated and applied to a Superdex 200 (GE Healthcare) gel filtration column that had been pre-equilibrated with the buffer. Protein purity was assessed by using SDS-PAGE on a Mini*Protean 3 system (BioRad). Protein samples were diluted in 4× SDS-PAGE loading buffer comprising glycerol (1 mL), dH_2O (2 mL), Tris-HCl (1 mL, 0.5 M, pH 6.8), SDS (1.6 mL 10% *w*/v), β-mercaptoethanol (0.4 mL) and bromophenol blue (20 mg). Samples were then boiled for 5 min at 100 °C. The gels consisted of a top layer of 4% (*w*/v) acrylamide stacking gel and a bottom layer of 12% (*w*/v) acrylamide separating gel. The protein samples were run at a constant voltage of 200 V.

Resting-cell biotransformations: Resting-cell assays were carried out to assess the hydroxylation of (1*R*)-(+)-camphor by P450cam-RhF-Red and the degradation of the explosive compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by XplAP450-RhF-Red. A single colony of *E. coli* Rosetta 2 (DE3) (Novagen/Merck) transformed with LICRED containing XplA haem domain or P450cam was used to inoculate LB medium (5 mL plus 100 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol). After overnight incubation at 37 °C with shaking, a culture (1 mL) was used to inoculate M9 minimal medium (100 mL) with antibiotics (100 μg mL^{-1} of kanamycin and 34 μg mL^{-1} of chloramphenicol). When the OD_{600} reached 0.8, protein expression was induced with IPTG (1 mM), 5-ALA (1 mM), $FeCl_3$ (0.5 mM) and riboflavin (5 μg L^{-1}). Cultures were incubated overnight at 20 °C with shaking, the cells were harvested by centrifugation and a portion of the wet cell paste (300 mg) was resuspended in potassium phosphate buffer (3 mL, 50 mM, pH 7.0) in a sterile 15 mL Falcon tube. D-Camphor and RDX were added to final concentrations of 10 mM and 150 μM, respectively, and the tubes were put in a shaking incubator at 20 °C. Analysis of camphor hydroxylation was performed with an Agilent 5975C GC/MS. Helium was used as the carrier gas at flow rate of 1.2 mL min^{-1}. The GC was equipped with an Agilent HP5 column (30 m, 250 μm i.d., 0.25 μm film thickness). The temperature program for camphor was: 120 °C for 3 min, then 120–160 °C at 10 °C min^{-1}. The temperatures were 250 °C for the injection and for the detector. A sample of 1 μL was injected onto the column. The retention times of camphor and 5-*exo*-hydroxy camphor were 3.5 and 6.5 min, respectively. RDX degradation was measured by HPLC, as previously described.[28]

Size-exclusion chromatography–multiangle laser light scattering (SEC–MALLS) analysis: The oligomeric state of the P450cam-RhF-Red and XplAP450-RhF-Red fusion proteins was determined by using SEC-MALLS measurements performed on a UFLC purifier system (Shimadzu) connected to a UV/Vis detector (SPD-20 A, Shimadzu), a multiangle light-scattering detector (DAWN HELEOS II, Wyatt Technology, Santa Barbara, CA, USA) and a refractive index detector (OPTILAB rEX, Wyatt Technology). A SuperdexTM 200 (GE Healthcare) gel filtration column was pre-equilibrated with buffer at a flow rate of 0.5 mL min^{-1}. Sample volumes of 100 μL were

injected at a protein concentration of 1 mg mL^{-1}. The data were recorded and processed by using ASTRA software (Wyatt Technology) to yield a molecular mass of 81.87 kDa for P450cam-RhF-Red and 85.5 kDa for XplAP450-RhF-Red.

In vitro activity assays: In vitro activity was assayed in reactions containing buffer (200 μL), NADPH (150 μM), different concentrations of fusion protein (2.2 μM P450cam-RhF-Red or 86 nM XplAP450-RhF-Red) and different concentrations of substrate (0–30 μM (1*R*)-(+)-camphor for P450cam-RhF-Red, 0–150 μM RDX for XplAP450-RhF-Red). All reactions were performed aerobically at room temperature (20 °C). Camphor hydroxylation and RDX degradation were measured as described above (Resting-cell biotransformations). For kinetic measurements, NADPH concentration was measured at 340 nm, and the following formula was used to convert the difference in absorbance into the amount of NADPH oxidised per time unit [Equation (1)]:

$$\frac{\Delta C}{\Delta t} = \frac{K}{\varepsilon} \quad (1)$$

ΔC: difference in [NADPH] after x minutes; K: change of absorbance over time; ε: extinction coefficient of NADPH at 340 nm. All spectroscopic and kinetic data were processed with the Enzyme Kinetics software from SigmaPlot. Percentage couplings were determined from the ratio between NADPH consumption and product formation after 5 min at 20 °C in a reaction with P450cam-RhF-Reductase (2.2 μM protein, 150 μM NADPH, 10 μM (1*R*)-(+)-camphor) or XplAP450-RhF-Reductase (86 nM protein, 150 μM NADPH, 150 μM RDX). The effect of protein concentration on k_{cat} was determined by measuring the initial NADPH oxidation rates using different amounts of the purified chimeras in the presence of substrate (30 μM camphor for P450-RhF-Red and 150 μM RDX for XplAP450-RhF-Red) and NADPH (150 μM).

Expression and activity assays of the chimeric P450 library from *N. farcinica*: A single colony of *E. coli* Rosetta 2 (DE3) (Novagen/Merck) transformed with a LICRED containing Noc-P450 haem-domain plasmid (e.g., Noc10-RhF-Red) was used to inoculate LB medium (5 mL) supplemented with antibiotics (100 μg mL^{-1} of both kanamycin and chloramphenicol). After overnight growth at 37 °C with shaking, the culture was centrifuged, the medium was removed, and the cells were resuspended in freshly prepared sterile M9 medium (500 μL). A portion of this suspension (250 μL) was then used to inoculate M9 medium (250 mL, containing 100 μg mL^{-1} of both kanamycin and chloramphenicol), glucose (0.4% *w*/v) and $FeCl_3$ (0.05%) in a 1 L flask. Cultures were incubated at 30 °C until they had reached an optical density of 0.8, and were then induced with IPTG (0.4 mM) and 5-ALA (0.5 mM). The cultures were then incubated overnight at 25 °C with shaking. The cells were harvested by centrifugation, and a portion of the wet cell paste (300 mg) was resuspended in buffer (3 mL) in a sterile 15 mL Falcon tube. To this was added a solution of 50% glycerol in water (25 μL) and substrate (e.g., 0.5 mM testosterone from a concentrated stock in DMSO or ethanol). The tubes were then placed in a shaking incubator at 4 °C for several hours (e.g., 48 h for P450-Noc10-RhF-Red plus testosterone; 72 h for P450-Noc4-RhF-Red plus ethoxycoumarin).

GC/MS analysis of the P450 library from *N. farcinica*: Analyses were performed on a Hewlett Packard GC/MS (HP 6890 Series/HP 5973) or on an Agilent 6850 GC/FID. Helium was used as the carrier gas at flow rate of 1.6 mL min^{-1}. The GC was equipped with an Agilent HP-1 column (30 m, 0.32 mm i.d., 0.25 μm film thickness). The temperature programs were: 200 °C for 1 min, 200–270 °C at 35 °C min^{-1}, 270–300 °C at 30 °C min^{-1}, 300 °C for 13 min for testosterone and 17-methyltestosterone; 100 °C for 1 min, 100–220 °C at 4 °C min^{-1} for ethoxycoumarin. The temperatures were 270 °C for the injection and 230 °C for the detector. A sample of 1 μL was injected in a 10:1 split ratio onto the column. For analysis of the reaction mixture for Noc10-RhF-Red with testosterone, an aliquot of reaction mixture (3 mL) was extracted with ethyl acetate (1.5 mL). The organic phase was separated and was evaporated to dryness. Then ethyl acetate (50 μL) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; 50 μL) were added, and the reaction mixture was heated at 70 °C for 30 min and then analysed by GC/MS. For analysis of the reaction mixture for Noc4-Rhf-Red with ethoxycoumarin and Noc10-RhF-Red with 17-methyltestosterone, reaction mixture (200 μL) was extracted with ethyl acetate (500 μL) and HCl (3 N, 30 μL). Then the organic phase (200 μL) was analysed by GC/MS.

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