Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Tailoring an alien ferredoxin to support native-like P450 monooxygenase activity

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Experimental

Enzymes and molecular biology

General reagents were from Sigma-Aldrich or Merck, UK. NADH was from Roche Diagnostics, UK. General DNA and microbiological experiments were carried out by standard methods.[1] Production of CYP199A2, PuR, Pux and PuxB in *Escherichia coli* and enzyme purification were carried out as previously described.[2-4] Enzymes were stored at –20 °C in 50 mM Tris, pH 7.4, containing 50% *v*/*v* glycerol. Glycerol was removed immediately before use by gel filtration on a 5-mL PD-10 column (GE Healthcare, UK) by eluting with 50 mM Tris, pH 7.4. UV/Vis spectra and spectroscopic assays were recorded at 30 ± 0.5 °C on a Varian CARY-50 or 1E spectrophotometer. Gas chromatography analysis was performed on a ThermoFinnegan TRACE instrument equipped with a CP-SIL 8CB fused silica column (15 m × 0.32 mm, Agilent Technologies) using helium as the carrier gas and flame ionisation detection. Both the injector and detector were held at 250 °C.

Mutagenesis of PuxB-5 was carried out using the Stratagene QuikChange mutagenesis kit. The oligonucleotides (codon for the relevant residue underlined), each used with their reverse complement, were 5′-cggtgatggaagcggcgatcagcaacgccatccccggcg-3′ (R29S), 5′-ccccggcgttgtagcagagtgcggcgg-3′ (E36V), 5′-ccg**gac**gaagaagacttgctcgac**ggc**ggc-3′ (M70L; existing M66D/F73G mutations in bold [4]), 5′-gaagacatgctcgacggcggctacgacgtgcgc-3′ (F73G). Mutations were confirmed by sequencing of the *RPA3956* gene encoding PuxB (GeneService, UK).

Activity and product assays

NADH turnover rate assays were performed with mixtures (1.2 mL) containing 50 mM Tris, pH 7.4, 0.5 μM CYP199A2, 5 μM ferredoxin, 0.5 μM palustrisredoxin reductase (the standard set of conditions with the components in a 1:10:1 ratio) and 100 μg mL^{-1} bovine liver catalase. The mixtures were oxygenated and then equilibrated at 30 °C for 2 min. 4-Methoxybenzoate was added as a 100 mM stock solution in ethanol to a final concentration

of 1 mM. NADH was added to *ca*. 320 μM (A_{340} = 2.00) and the absorbance at 340 nm was monitored. The rate of NADH consumption was calculated using ε_{340} = 6.22 mM^{-1} cm^{-1}. To determine the K_m and k_{cat} for the first electron transfer from the ferredoxin to CYP199A2 the assays were carried out as above but with a lower concentration of CYP199A2 (0.1 μM) and the ferredoxin concentration was varied from 1 to 30 μM. K_m and k_{cat} values were obtained by fitting the rate of NADH consumption (k_{obs}) against the ferredoxin concentration [Fdx] to a hyperbolic function for Michaelis-Menten kinetics using the Origin 8 software (Origin Labs).

$$k_{obs} = \frac{k_{cat} \times [Fdx]}{K_m + [Fdx]}$$

On completion of NADH consumption, 990 μL of the incubation mixture was mixed with 10 μL of internal standard solution (25 mM 9-hydroxyfluorene in ethanol) and 2 μL of concentrated HCl. The mixture was extracted with 3 × 400 μL of ethyl acetate and the organic extracts were combined and dried over $MgSO_4$. Volatiles were removed *in vacuo* and the residue dissolved in 200 μL CH_3CN. Excess (25 μL) BSTFA + TMCS (99:1) was added and the mixture left for 2 h to produce the trimethylsilyl ester of the carboxylic acid group and trimethylsilyl ether of the phenol. The mixtures were used directly for GC analysis. The oven temperature was held at 100 ºC for 1 min and then increased at 15 ºC min^{-1} up to 220 ºC. The retention times for the trimethylsilyl derivatives were 6.18 min for 4-methoxybenzoic acid and 7.00 min for 4-hydroxybenzoic acid while that of the internal standard eluted at 8.25 min.

Stopped-flow studies

The rate constant for the formation of the $Fe^{II}(CO)$ complex, k_f by electron transfer from reduced PuxB mutants to substrate-bound CYP199A2 was determined at 25.0 ± 0.1 °C by monitoring the absorbance at 450 nm using an Applied Photophysics SX20 stopped-flow spectrophotometer housed inside a Belle Technology glove box ([O_2] ≤5 ppm). Solution components were prepared in 50 mM Tris, pH 7.4, which was saturated with CO by bubbling gently for 5 min and then purging the headspace for a further 5 min. One syringe contained

different concentrations of a PuxB mutant with 100 nM PuR and one equivalent of NADH. The other syringe contained 1 μM CYP199A2 and 1 mM 4-methoxybenzoic acid. The components were mixed in a 1:1 ratio in the spectrometer. The data for all mutants were biphasic with a minor (<10%) component while the major component was assigned to PuxB-to-CYP199A2 electron transfer. The time courses were fitted to a double exponential function using Pro-data Viewer software (Applied Photophysics). The k_f and K_S values for each PuxB mutant were obtained by fitting k_{obs} for the major component to the PuxB concentration using a hyperbolic function. Simple hyperbolic behaviour was observed in all experiments, with no evidence for cooperativity.

$$k_{obs} = \frac{k_f \times [\mathrm{PuxB}]}{K_S + [\mathrm{PuxB}]}$$

Spectroelectrochemical titrations

Reduction potentials were determined by spectroelectrochemical titrations as described previously.[5] All experiments were performed at 25 °C in 50 mM Tris, pH 7.4. The spectral data were fitted to the Nernst equation using absorbance changes at 450, 465 and 550 nm for CYP199A2, and at 415 and 460 nm for the ferredoxins.

Fig. S1. (a) Structure-based sequence alignment of PuxB [4] with the P450-associated ferredoxins Pux from *Rhodopseudomonas palustris* CGA009,[2] HaPux from *Rhodopseudomonas palustris* HaA2,[6] putidaredoxin (Pdx) from *Pseudomonas putida* [7] and adrenodoxin (Adx) from the mitochondria,[8] and the *isc* ferredoxins Fdx from *Escherichia coli* [9] and FdVI from *Rhodobacter capsulatus*.[10] (b) Structure of the A105R mutant of PuxB (pdb code: 3HUI) highlighting in green residues in the P450 recognition region. (c) Electrostatic potential surface of the A105R mutant of PuxB from the same view as in (b) with negatively charged areas in red and positively charged areas in blue. (d) Electrostatic potential surface of the likely ferredoxin recognition, heme proximal surface of CYP199A2 (pdb code: 2FR7) with negatively areas in red and positively charged areas in blue, showing the apparent charge complementarity between the recognition surfaces of the two interacting proteins.

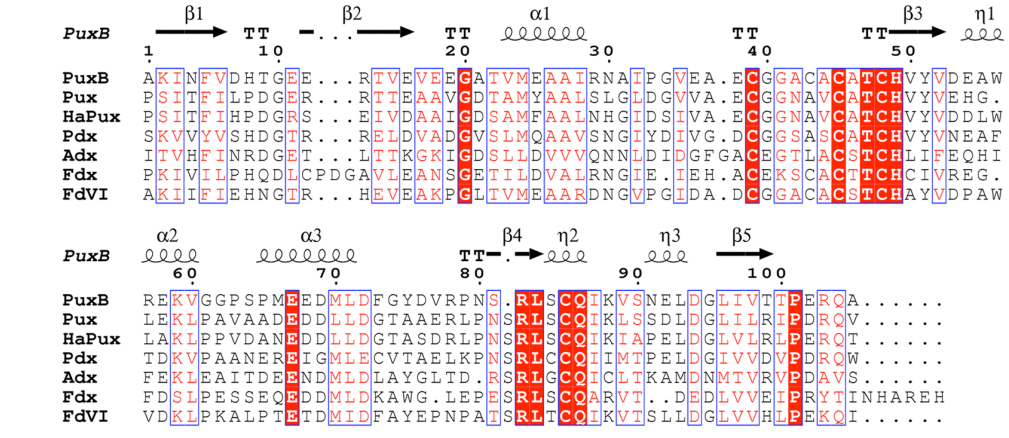
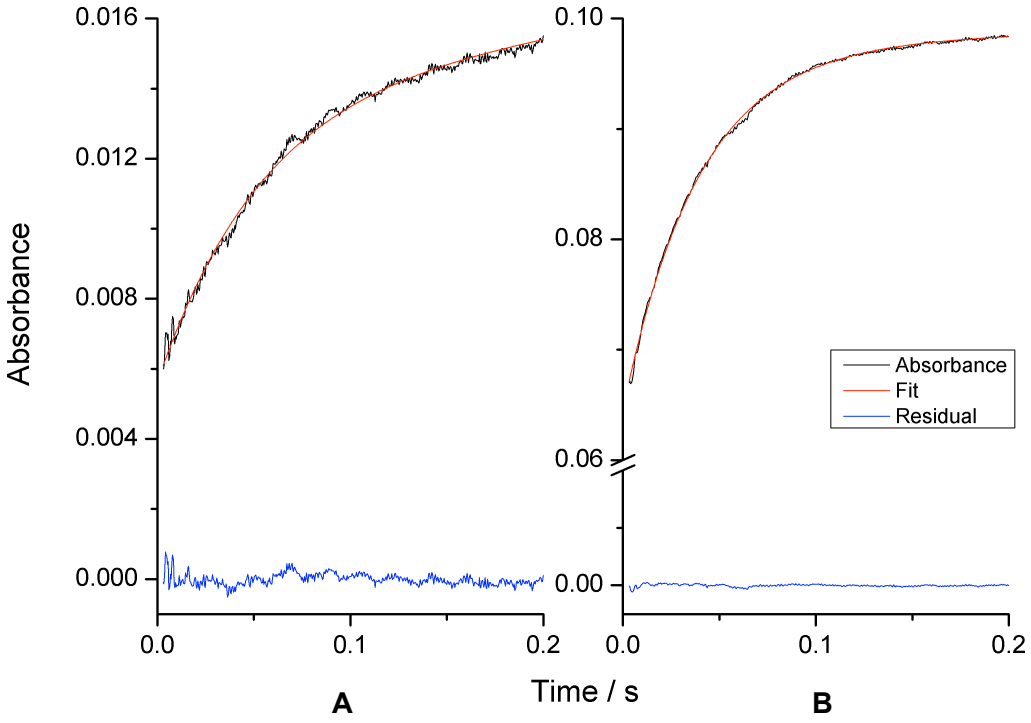
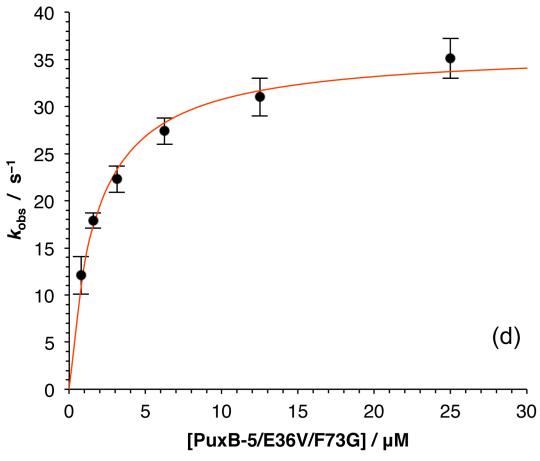


Fig. S2: Time courses at 25 °C for the absorbance at 450 nm for the formation of the $Fe^{II}(CO)$ form of 4-methoxybenzoic acid-bound CYP199A2 via electron transfer from pre-reduced PuxB-5/E36V/F73G. The final concentration of CYP199A2 in the stopped-flow cell was 0.5 μM. Both datasets required a double exponential function to fit their biphasic behaviour. A: Time course for 1.56 μM ferredoxin concentration with k_1 = 1.37 s^{-1} and k_{obs} = 19.48 s^{-1} (78.5%), and B: 12.5 μM ferredoxin with k_1 = 5.78 s^{-1} and k_{obs} 25.14 s^{-1} (92.0%). C: Hyperbolic fit of the rate constant (k_{obs}) for the formation of the CYP199A2 $Fe^{II}(CO)$ species in stopped-flow experiments to the concentration of the PuxB-5/E36V/F73G mutant, giving k_f = 36.0 s^{-1} and K_s = 1.7 μM.





C

Fig. S3: Spectroelectrochemical titrations (25 °C) and (inset) sigmoidal fits of the absorbance change at 465 nm against the applied potential to the Nernst equation (n = 1) for A: substrate-free CYP199A2 (E_m = –445 ± 2 mV), B: 4-methoxybenzoate-bound CYP199A2 (E_m = –227 ± 3 mV).

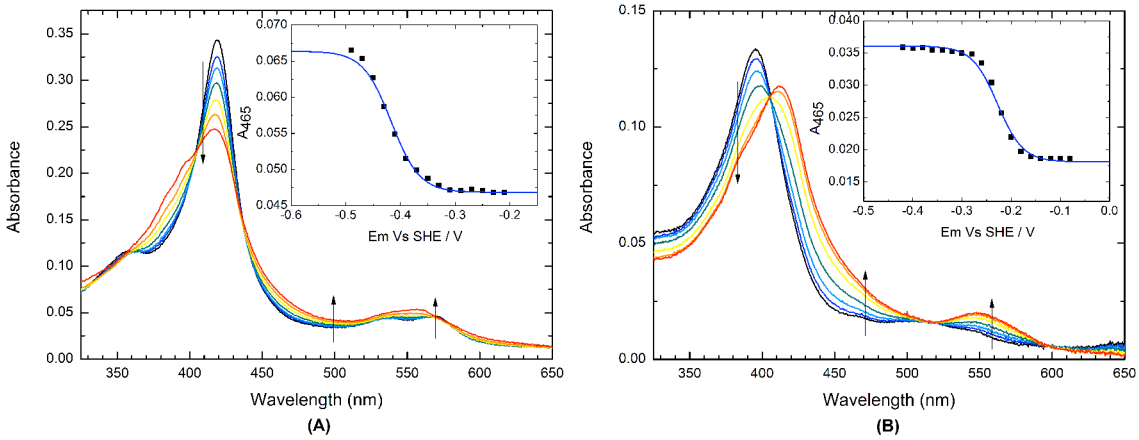
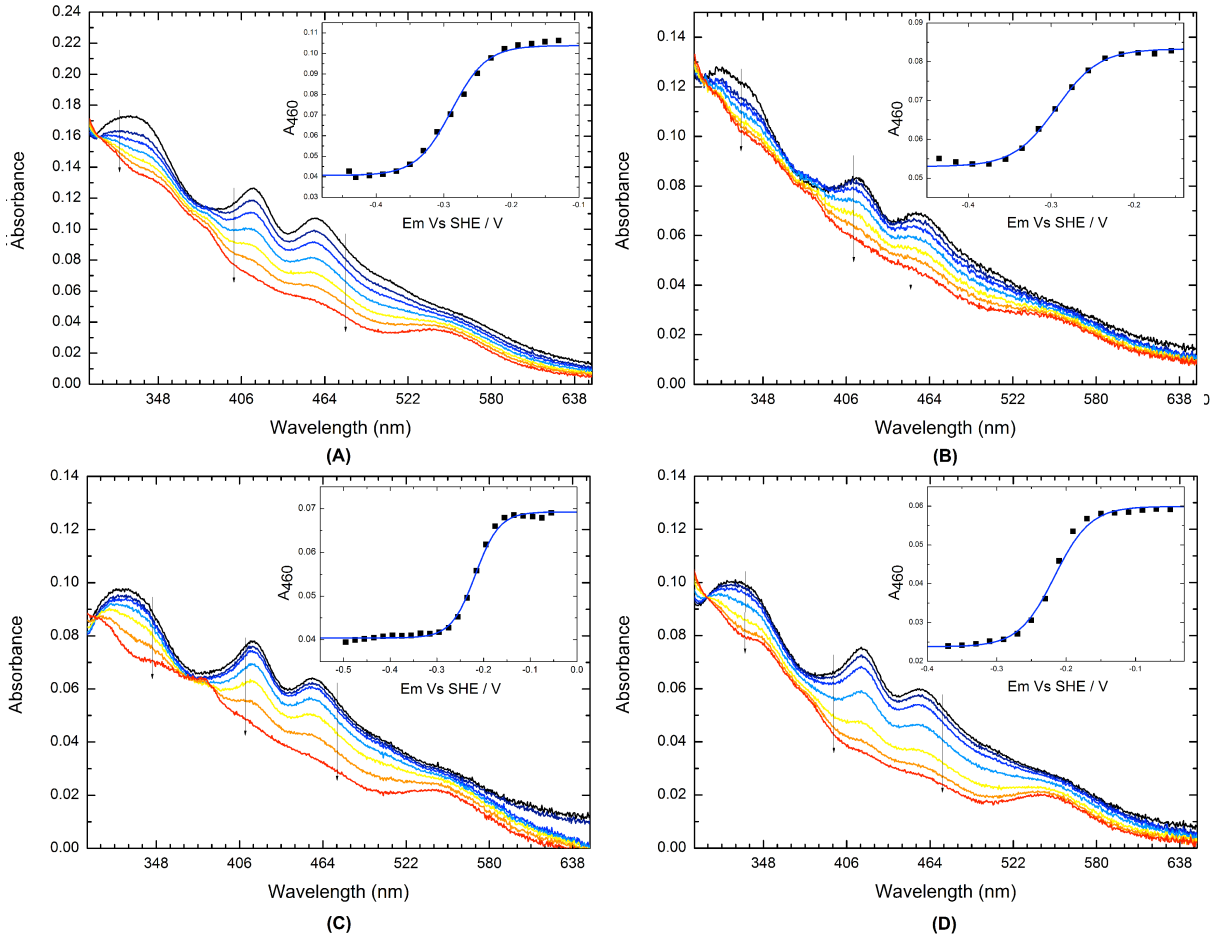


Fig. S4: Spectroelectrochemical titrations (25 °C) and (inset) sigmoidal fits of the absorbance change at 460 nm against the applied potential to the Nernst equation (n = 1) for A: wild type PuxB (E_m = –291 ± 4 mV); B: the A42N/C43A/A44V/M66D/A105V (PuxB-5) mutant (E_m = –293 ± 2 mV); C: the PuxB-5/E36V/F73G mutant (E_m = –225 ± 9 mV); D: the PuxB-5/E36V/M70L/F73G mutant (E_m = –202 ± 13 mV).



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