P450cin Purification

The plasmid with the P450cin gene inserted into pCWori vector (pCW-P450cin) with amino acids 2-7 deleted was provided by Dr. James J. De Voss. The deletion does not affect activity and circumvents the accumulation of heterogeneous N-terminal cleavage products during protein purification.[1] Protein expression for substrate-bound and substrate-free P450cin were identical. The pCW-P450cin construct was transformed into *E. coli* DH5α purchased from Invitrogen™ and plated overnight at 37 °C on Lauria Bertani agar plates supplemented with 100 μg/mL ampicillin. A single colony was used to inoculate 100 mL of 2x YT media overnight at 37 ˚C. Ten mL of overnight culture were used to inoculate each 1 L of TB media. Cells were allowed to grow at 37 ˚C with shaking at 220 rpm until an OD_{600} of 0.6 and then induced with 0.5 mM IPTG. The temperature was then lowered to 27.5 ˚C and shaking set to 80 rpm. After 17 hours the cells were harvested and stored at -80 ˚C.

For preparation of substrate-free P450cin cell pellets were resuspended in buffer A (50 mM potassium phosphate pH 7.4, 50 mM KCl, 750 μM 1,8-cineole). DTT was added to a final concentration of 0.5 mM. Protease inhibitors were added to final concentrations of 1 μg/mL apostatin and pepstatin, 2 μg/mL leupeptin and 0.1 mM PMSF. Resuspended cells were passed three times through a microfluidizer at 18,000 psi and centrifuged at 14,000 rpm for 40 minutes. The supernatant was then loaded onto a Q sepharose column pre-equilibrated in buffer A. The protein bound column was washed with 500 mL of buffer A followed by a stepwise increase in KCl from 200 mM to 300 mM where the protein eluted from the column. Buffer was exchanged by dialysis into buffer B (50 mM

potassium phosphate pH 6, 100 mM KCl and 750 μM 1,8-cineole). The dialyzed protein was loaded onto a second Q sepharose column (50 mL XK16 column) at 5 mL/min. The column was washed with 10 column volumes of buffer A plus 100 mM KCl and eluted with 6 column volumes of 100-200 mM KCl gradient in buffer A. Fractions with an A_{392}/A_{280} ratio > 1 and similar purity as determined by SDS-PAGE were pooled and dialyzed into buffer A. The dialyzed fractions were concentrated in a 30 kDa Amicon® ultrafiltration device before loading (1 mL/min) onto a 300 mL XK26 prep grade Superdex75® column equilibrated in the same buffer. Fractions were pooled according to purity as judged by the A_{392}/A_{280} ratio and SDS-PAGE. Heme incorporation was determined to be 98% using the alkaline pyridine hemochromogen assay[2] and an ε_{red557} = 34.7 mM^{-1} cm^{-1} and known protein concentrations. Routine concentrations were determined using the extinction coefficient $\varepsilon_{450-490}$ = 91 mM^{-1} cm^{-1} for the carbon monoxide bound ferrous heme.[3]

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A slightly different procedure was used for preparation of substrate-bound P450cin. The initial cell free extract was loaded onto a 30 mL Q HiTrap® column (GE Lifesciences) equilibrated in buffer A2 (50 mM potassium phosphate pH 7.4, 50 mM KCl and 2.5 mM 1,8-cineole). The protein was washed with 3 column volumes of buffer A with 100 mM KCl followed by elution in 250 mM KCl. Red fractions were pooled, dialyzed into buffer A and loaded onto a 40 mL XK16 DEAE column. The column was washed with 3 column volumes of buffer A2 followed by elution in 100 mM KCl. Fractions with the best A_{392}/A_{280} were pooled and diluted with 3 parts 50 mM Tris pH 7.5, 1.5 M ammonium sulfate, 2 mM cineole (buffer B2) and loaded onto an 40 mL XK16 phenyl sepharose fast

flow HiTrap® column. The protein was washed with 5 column volumes of the diluted buffer followed by 10 column volumes of 40% diluted buffer B2 (50 mM Tris pH 7.5, 2 mM cineole) and eluted in 50% diluted buffer B2. Fractions with an A_{392}/A_{280} ratio > 1 were dialyzed into 50 mM Tris pH 7.5, 20 mM NaCl, 2 mM cineole. After concentration in an Amicon® with 30 kDa membrane the protein was loaded at 1 mL/min onto a 300 mL prep grade XK26/60 Superdex75® column equilibrated in the same buffer. Eluted fractions with $A_{392}/A_{280} \geq 1.3$ were pooled and concentrated. Concentration and heme content were determined as above giving approximately 98% heme incorporation.

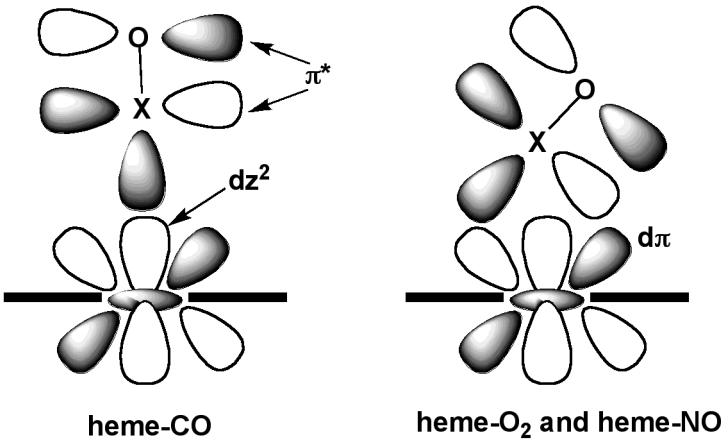

Figure S1 - Standard molecular orbital diagram of diatomic ligands bound to ferrous heme iron. In the CO complex the ligand antibonding π* orbitals (only one is shown, the other one is perpendicular to the shown orbital) are empty. The electrons from the filled iron dπ orbitals (two of them with one shown) back donate to the ligand π* orbitals so that CO favors a linear geometry. The occupied ligand π* orbitals with O_2 and NO cause bending which enables the π* orbitals to overlap (mix) with the iron d_{z2} orbitals, resulting in less antibonding character. Thus NO is a good geometric mimic of O_2.

1. Hawkes, D. B., Adams, G. W., Burlingame, A. L., Ortiz de Montellano, P. R., and De Voss, J. J. (2002) Cytochrome P450cin (CYP176A), Isolation, expression, and characterization, *J. Biol. Chem. 277*, 27725-27732.

2. Berry, E. A., and Trumpower, B. L. (1987) Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra, *Anal. Biochem. 161*, 1-15.

3. Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. Ii. Solubilization, purification, and properties, *J. Biol. Chem. 239*, 2379-2385.