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Supplementary Information

Structural Information from Orientationally Selective DEER Spectroscopy

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Sample Preparation

1. ***Preparation of 3-phenyl diporphyrin dicopper complex***

BOP (benzotriazole-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate) (0.118 g, 0.267 mmol) was added to a stirred solution of 3-phenyldiacid (0.050 g, 0.103 mmol), $TPPNH_2$[1,2] (0.133 g, 0.211 mmol), DIPEA (0.15 mL, 0.822 mmol) in anhyd. DMF (2.5 mL) at room temperature under nitrogen atmosphere. The resultant mixture was stirred at room temperature for four days, during which time a spot of higher R_f was found by tlc analysis (2:3 EtOAc/$CHCl_3$). The mixture was then concentrated under reduced pressure, before being purified by flash chromatography using 1:1 Et_2O/$CHCl_3$ to afford, in order of elution, the desired 3-phenyl diporphyrin (0.041 g, 23 %) as a dark purple coloured solid and recovered $TPPNH_2$ (59 mg, 44% recovery). υ_{max}(KBr)/cm^{-1} 1681(CO); δ_H($CDCl_3$, 500 MHz) 0.90 (6H, t , *J* 6.5 Hz, 2×CH_3), 1.27 (12H, m, 6×CH_2), 1.58 (4H, m, 2×CH_2), 2.68 (4H, dd, *J* 8.0 and 8.0 Hz, 2×ArCH_2), 7.27 (2H, s, Ph), 7.63 (4H, d, *J* 7.5 Hz, Ph), 7.78 (18H, m, Ph), 8.11 (4H, d, *J* 8.0 Hz, Ph), 8.15 (4H, d, *J* 8.0 Hz, Ph), 8.25 (18H, m, Ph), 8.87 (8H, s, Ph), 8.89 (4H, d, *J* 4.8 Hz, pyrrole), 8.94 (4H, d, *J* 4.8 Hz, pyrrole), 13.21 (2H, s, 2×NH); δ_C($CDCl_3$, 125.8 MHz) 14.1, 22.5, 29.2, 31.5, 31.6, 32.7, 118.4, 119.4, 120.2, 126.7, 127.0, 127.7, 129.9, 130.9, 133.4, 134.6, 135.3, 137.7, 138.5, 140.2, 140.2, 142.2, 145.9, 165.9; *m/z* (MALDI-TOF LD$^+$) 1710.59 $C_{120}H_{96}N_{10}O_2$ requires 1710.78. The dicopper species was prepared by the addition of $Cu(OAc)_2{\cdot}H_2O$ (0.014g, 0.0701 mmol) in MeOH (5 mL) to a stirred solution of 3-phenyl diporphyrin (0.029 g, 0.0170 mmol) in $CHCl_3$ (10 mL) at room temperature. The resultant mixture was heated at reflux for 15 h and then cooled to room temperature. The mixture was then concentrated to dryness *in vacuo* to generate a dark red/purple solid. This residue was taken up in $CHCl_3$ (15 mL) and washed with

H_2O (3×10 mL). The organic phase was dried over anhyd. Na_2SO_4, filtered and concentrated to dryness *in vacuo* to afford the desired dicopper product as a pink/purple solid (0.022 g, 71 %). υ_{max}(KBr)/cm^{-1} 3431, 2952, 2924, 2853, 1675(CO), 1599, 1004, 799, 753, 701. λ_{max}($CHCl_3$)/nm 539 (ε/dm^{3}mol^{-1}cm^{-1} 3698), 417 (105,5337), 400sh (6976), 296 (5759); *m/z* (MALDI-TOF LD$^+$) 1833.45 $C_{120}H_{92}Cu_2N_{10}O_2+H^+$ requires 1833.61. All other characterisation methods and instrumentation are the same as those described previously[3].

2. Production and purification of PuR and Pux-B

Mutant genes were fully sequenced on an automated ABI 377XL Prism DNA sequencer by the DNA sequencing facility at the Department of Biochemistry, University of Oxford. PuR and its mutants, and Pux-B, were produced and purified by similar methods. The pET26 or pET28 plasmids (Merck Biosiences, UK) containing the gene of interest was transformed into *Escherichia coli* BL21(DE3) and grown at 37 °C in Luria-Bertani media containing 30 μg/mL kanamycin. When A_{600} reached ~1.0, the temperature was reduced to 25 °C and gene expression was induced by the addition of 100 μM IPTG. The growths were continued for at least another 6 h. The cells were harvested and the cell pellet of Pux-B was resuspended in 50 mM Tris, pH 7.4, whilst that of PuR was resuspended in 50 mM Tris, pH 7.4 containing 50 mM KCl. Cells were lysed by sonication and the mixture centrifuged at 38000 g, 4 °C for 20 min to remove the cell debris. The supernatant was loaded onto a DEAE Fast Flow Sepharose Column (40 mm × 200 mm). PuR was eluted with a linear gradient of 50 – 200 mM KCl and Pux-B with a linear gradient of 100 – 400 mM KCl. Coloured fractions were collected, desalted and loaded onto a Source Q column (50 mL bed-volume, GE Healthcare) attached to a Fast Protein Liquid Chromatography

instrument. The PuR and Pux-B proteins were eluted with 50 – 150 mM and 62.5 – 250 mM KCl salt gradients, respectively. PuR fractions with $A_{280}/A_{454} < 7$, and Pux-B fractions with $A_{325}/A_{280} > 0.6$, were collected and concentrated *via* ultrafiltration (MW cut-off 30,000 Da for PuR and MW cut-off 10,000 Da for Pux-B). An equal volume of glycerol was added and the proteins stored at –20 °C. The presence of the mutations was confirmed *via* electrospray mass spectrometry (ES-MS) analysis using a Micromass Platform II instrument coupled to an Agilent 1100 HPLC and calibrated using myoglobin.

Spin labelling

Glycerol was removed from PuR stocks and the buffer exchanged with 50 mM Tris, pH 7.2, containing 1 mM EDTA (TE buffer) by use of a 5-mL PD-10 column. A 10-fold excess of the nitroxide radical spin label (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate (Toronto Research Chemicals Inc.) was added as a 10 mM stock in DMSO and the mixture left for 2 h at 4 °C. The solution was filter-sterilised, and excess spin label was removed by concentrating the solution using a Millipore Amicon Ultra-4 centricon (MW cut-off 30,000 Da), 7000 g and 4 °C, followed by elution through a 5-mL PD-10 gel filtration column with TE buffer. Attachment of spin-label was confirmed by EPR and ES-MS analysis.

Sample preparation for DEER measurements

Glycerol was removed from stock solutions of Pux-B and the spin-labelled PuR mutants by elution with TE buffer through a 5 mL PD-10 column inside a nitrogen-filled glove box (<1 ppm O_2). The eluted proteins were placed in a Millipore Amicon Ultra-4 centricon, sealed and reduced to <50 μL by centrifuging at 7000 g, 4 °C. The Pux-B and PuR proteins were quantitated and mixed in a 1:1 stoichiometry. One equivalent of NADH (from a 20 mM stock) was then added to generate the reduced,

EPR-active form of Pux-B complexed to a spin-labelled PuR mutant. The concentration of the protein-complex was adjusted to 1.2 – 1.4 mM using TE buffer. An equal amount of glycerol was then added to the protein solution, and a 50 μL aliquot was placed in a quartz EPR tube which was then sealed. Samples were frozen and stored in liquid nitrogen.

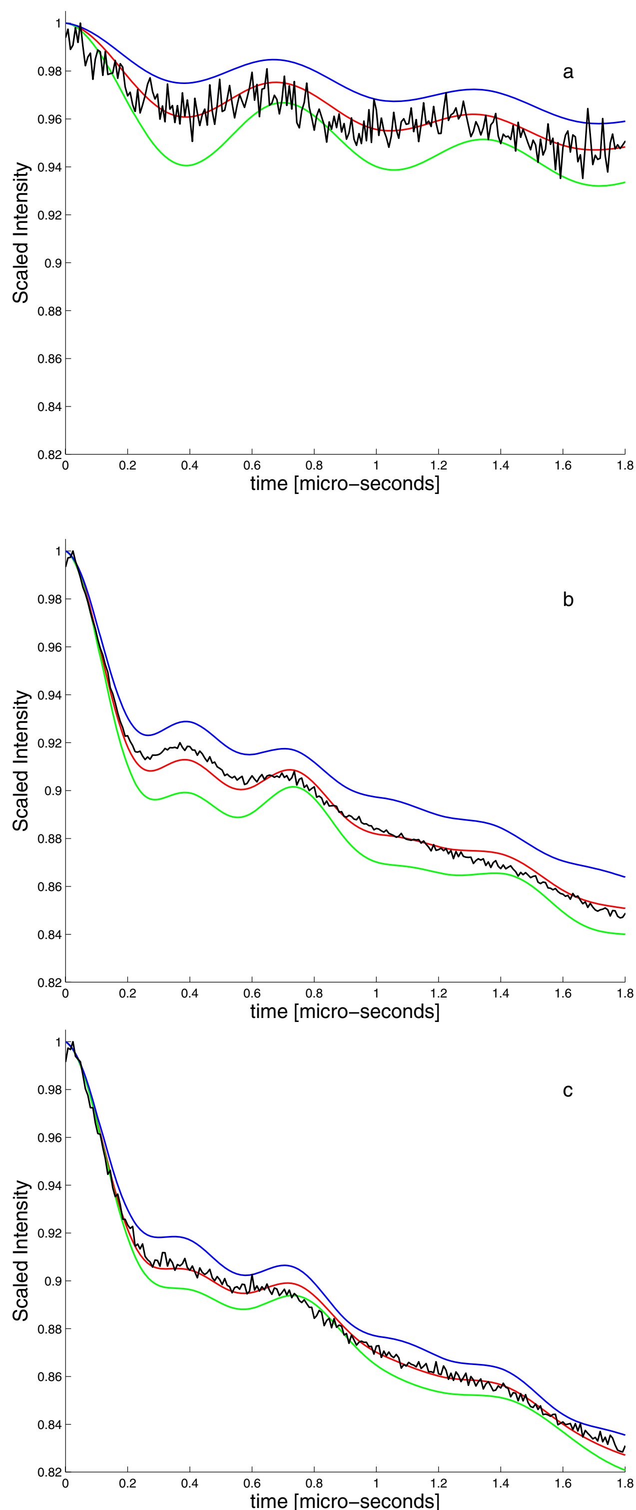

Fig. S1: DEER traces (black) at the observer positions indicated in Fig. 6B and simulations for three different conformer models. Blue line - computed with a single CuTTP − CuTTP orientation. Green line - a restricted CuTTP − CuTTP conformer set where one CuTPP ligand is rotated by ±50° around the long axis of the molecule (0° corresponds to both TTP ligands lying in the same plane). Red line – DFT model having one CuTPP ligand rotated through 360° around the long axis of the molecule. Data highlight the importance of the conformer model on the DEER simulation and in particular the modulation depth.

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