Supporting information for

Porphyrin Triplet State as a Potential Spin Label for Nanometer Distance Measurements by PELDOR Spectroscopy

Marilena Di Valentin*, Marco Albertini, Enrico Zurlo, Marina Gobbo and Donatella Carbonera

Dipartimento di Scienze Chimiche, Università di Padova, via Marzolo 1, 35131 Padova, Italy

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Sample Preparation

General Methods. All chemicals were commercial products of the best grade available and, unless otherwise indicated they were used directly without further purification. 5-(4-Carboxyphenyl)-10,15,20-triphenylporphyrin (TPP-OH) and 9-Fluorenylmethoxycarbonyl(Fmoc)-4-amino-1-oxyl-2,2,6,6,-tetramethylpiperidine-4-carboxylic acid (Fmoc-TOAC) were prepared according to literature procedures.[1,2] Fmoc-amino acids and all other chemicals for the solid phase synthesis were supplied by Sigma-Aldrich. H-Ala-2-Chlorotrityl resin was purchased from Novabiochem (Merck Biosciences). Analytical HPLC separations were carried out on a Dionex Summit Dual-Gradient HPLC, equipped with a four-channel UV-Vis detector, using a Vydac 218TP54 C18 or Phenomenex Jupiter C4 column (250 x 4.6 mm, 5 μm, flow rate at 1.5 mL/min). The mobile phase A (aqueous 0.1% trifluoroacetic acid (TFA)) and B (90% aqueous acetonitrile containing 0.1% TFA) were used for preparing binary gradients. All crude peptides were purified to 95% or more homogeneity for analytical and other experimental purposes. Semi-preparative HPLC was carried out on a Shimadzu series LC-6A chromatographer, equipped with two independent pump units, an UV-Vis detector, and a Vydac 218TP1022 column (250 x 22 mm, 10 μm, flow rate at 15 mL/min) or Phenomenex Jupiter C4 column (250 x 10 mm, 10 μm, flow rate at 5 mL/min). Elutions were carried out by the same mobile phases described above, without TFA as modifier. Mass spectral analyses were performed on a Mariner API-TOF Workstation (PerSeptiveBiosystemsInc), operating with ESI techniques in positive mode. UV-Vis spectra were recorded at rt on a Shimadzu UV-2501PC spectrophotometer or on a Lambda 5 spectrophotometer (Perkin-Elmer), in 1 cm quartz cells. CD measurements were carried out on a Jasco-715 spectropolarimeter, using a quartz cell of 0.02 or 0.1 cm path length. The spectra were recorded at 298 K and were the average of a series of six scans made at 0.1 nm intervals in the 190-250 nm and 350-550 nm regions. Sample concentrations in methanol were in the range 0.1-0.01 mM. Ellipticity is reported as mean residue ellipticity $[\theta]_R$(deg x cm^2 x dmol^{-1}) or differential molar circular dichroic extinction coefficient $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (cm^2 dmol^{-1}). Solution FT-IR spectra were recorded at 293 K using a Perkin-Elmer model 1720X FT-IR spectrophotometer, nitrogen flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Solvent (baseline) spectra were recorded under the same conditions. For spectral elaboration the software SpectraCalc provided by Galactic (Salem, MA) was employed. Cells with path lengths of 1.0 and 10 mm (with CaF_2 windows) were used. Spectrograde deuterated chloroform (99.8%, d_2) was purchased from Merck.

Peptide Synthesis. The peptide sequences were assembled on an automated Advanced Chemtech348Ω Peptide Synthesizer, on 0.05 mmol scale, starting from H-Ala-2-Chlorotrityl resin (substitution 0.77 mmol/g resin). Fmoc deprotection was achieved with 20% piperidine in DMF (5 + 15 min). Couplings were performed in the presence of O-(7-azabenzotriazol-1-yl)*N,N,N',N'*-tetramethyluroniumhexafluorophosphate/*N,N*-diisopropylethylamine (DIPEA) (reaction time 45-60 min), using an excess of 4 equivalents of the carboxyl component. After the coupling of the last amino acid and removal of the Fmoc group, the resin was washed with DMF and CH_2Cl_2 and then dried under vacuum. The peptide-resin was split in two: one portion was acetylated by reaction with 0.5 M acetic anhydride/ 0.12 M DIPEA in DMF for 45 min. The remaining H-peptide-resin(0.025 mmol) was reacted with the porphyrin(0.05 mmol in DMF-CH_2Cl_2, 1:1 v/v) in the presence of diisopropylcarbodiimide/1-hydroxybenzotriazole as coupling reagents. The reaction mixture was shaken overnight and the resin was repeatedly washed with DMF and CH_2Cl_2, until the filtrate was colorless.

The peptides were cleaved from the resin upon 3-4 treatments with 30% 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) in DCM for 30 min. The filtrates were combined and evaporated to dryness providing the crude peptides, both presenting the intact nitroxylic radical. The crude peptides were purified by semi-preparative HPLC to afford the desired products with a purity ≥95% as determined by analytical HPLC. The peptides were characterized as follow:

Ac-(Ala-Aib)$_4$-Ala-TOAC-Ala-(Aib-Ala)$_2$-OH: yield 41%; HPLC (C18, isocratic 3 min 10% B; linear gradient 10-90% B in 30 min) t^R 24.0 min; IR ($CDCl_3$): cm^{-1} 3694, 3605, 3311, 2985, 1658, 1600, 1538. ESI-MS: calcd for $C_{60}H_{105}N_{16}O_{18}$ 1337.78 $[M+H]^+$, found: 1337.75.

TPP-(Ala-Aib)$_4$-Ala-TOAC-Ala-(Aib-Ala)$_2$-OH: yield 49%; HPLC (C4, isocratic 50% B 3 min; linear gradient 50-100% B in 30 min) t^R 20.7 min; IR ($CDCl_3$): cm^{-1} 3689, 3605, 3313, 2987, 1660, 1600, 1540. UV-vis (methanol): λ_{max} /nm (log ε/ M^{-1} cm^{-1}) 414(5.14), 511(3.76) , 548(3.47), 590(3.32), 644 (3.10); ESI-MS: calcd for $C_{103}H_{131}N_{20}O_{18}$ 1935.99 $[M+H]^+$, found: 1935.96.

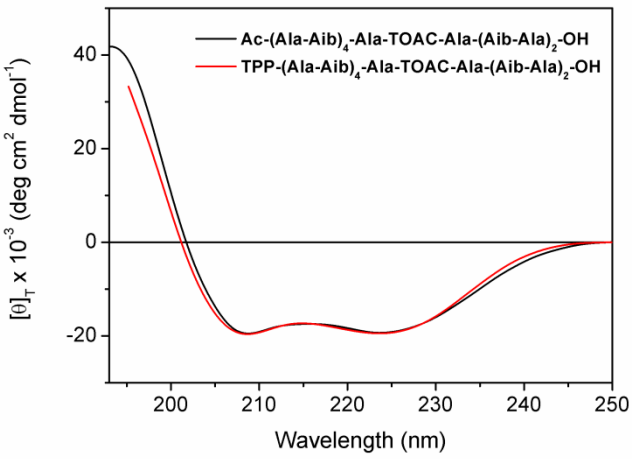

Figure S1. CD spectra of the peptide and the porphyrin-peptide conjugate in methanol. Peptide concentration 0.1 mM.

EPR spectroscopy

Sample preparation. *The bis-labeled model peptide TPP-*(Ala-Aib)$_4$-Ala-TOAC-Ala-(Aib-Ala)$_2$-OH was dissolved in a mixture of 98% deuterated methanol (Sigma Aldrich) and 2% D_2O (Cambridge Isotopes) to reach a final concentration of approximately 200 μM. The sample inserted into a quartz EPR tube, which was sealed after several freeze-thaw cycles. Removal of oxygen assures long-term stability of the porphyrin label.

Pulsed EPR. Pulsed EPR was performed on a BrukerElexsys E580 pulse EPR spectrometer equipped with a Bruker split-ring resonator ER4118X-MS3 (microwave frequency = 9.55 GHz) and an Oxford CF935 cryostat. The measurements were performed at a temperature of 20 K. The sample was photoexcited with the second harmonic of a pulsed Nd:YAG laser (532 nm) with an average power of 5 mW and a repetition rate of 10 Hz.

For the electron spin-echo experiments a standard Hahn echo sequence (laser flash – DAF – π/2 – τ – π – τ – echo) was employed with a nominal length of 16 ns for the π/2 pulse, a delay after the laser flash (DAF) value of 50 ns and a τ value of 300 ns. For the two-pulse ESEEM tau was incremented of 12ns. Data were collected with a single scan and 20 shots per point.

For the PELDOR experiments a standard four pulse sequence (laser flash – DAF – π/2 – τ – π – τ_1 – π_{pump}– τ_2 – π – τ_2 – echo) was applied at a DAF of 50 ns; the microwave power was adjusted to obtain an observer sequence of 16/32/32 ns and a pump pulse of 12 ns. The difference between the pump (nitroxide) and observer (porphyrin triplet-state) frequency was set to 240 MHz. A two-step phase cycle was applied for base-line correction while deuterium nuclear modulations were suppressed using an 8 step τ cycle from a 180 ns starting value with 56 ns increment steps. Data were collected with a single scan and 50 shots per point for a total of 300 points. Measurement time was approximately 7 hours at a repetition frequency of 10 Hz.

DEER time traces as obtained from the spectrometer were treated using the *DeerAnalysis2013* routine.[3] A phase correction was performed prior to correct the traces for the background decay. Fourier Transform was then applied.

Time-Resolved EPR. Time-resolved EPR was carried out on the same spectrometer equipped with a critically coupled Bruker dielectric resonator ER4118X-MD5 (microwave frequency = 9.71 GHz) and an Oxford CF935 cryostat. The measurements were performed at a temperature of 20 K. The sample was photoexcited with the same laser equipment previously described. Spectrometer resolution was of about 150 ns. No field modulation or phase sensitive detection was applied. The signal output from the preamplifier was digitized via the internal oscilloscope (4ns of resolution) for a direct detection of the transient signal. At each magnetic field position, an average of 50 transient signals was recorded. Microwave power has been set to 6.3 mW (equivalent to a 15dB of attenuation).

Simulation of the time-resolved EPR spectrum, to obtain the triplet-state parameters, was performed using Easyspin routine in Matlab®.[4] The routine computes the time-resolved EPR spectrum via the full diagonalization of the spin hamiltonian of the system, comprehensive of the Zeeman and magnetic dipole–dipole interactions. The EPR spectrum is calculated assuming a powder-like distribution of molecular orientations with respect to the magnetic field direction. Triplet state input parameters are the relative population probabilities at zero field and the zero field splitting parameters *D* and *E*.[5]

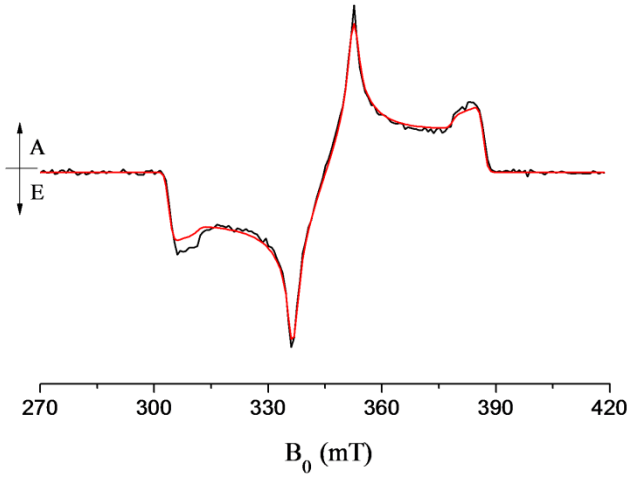

Figure S2. X-band spin-polarized time-resolved EPR spectrum of TPP-(Ala-Aib)$_4$-Ala-TOAC-Ala-(Aib-Ala)$_2$-OH at 20 K and corresponding simulation (red line). Simulation parameters are reported in Table S1. A = enhanced absorption, E = enhanced emission.

Table S1. Spectral parameters for the simulation of the time-resolved EPR spectrum of TPP-(Ala-Aib)$_4$-Ala-TOAC-Ala-(Aib-Ala)$_2$-OH spectrum shown in figure S2. *D*, *E* are the zero field splitting parameters of the triplet state, p_i is the relative intersystem crossing population of the *i*-th triplet sublevel.

Simulation parameters	
D	41.4 mT
E	-8.5 mT
[p_x, p_y, p_z]	[0.33, 0.41, 0.26]
Linewidth	2 mT

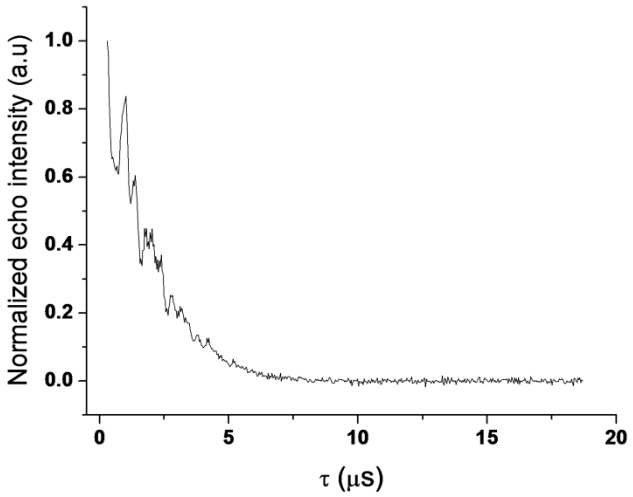

Figure S3. X-Band 2-pulses ESEEM trace of TPP-(Ala-Aib)$_4$-Ala-TOAC- Ala-(Aib-Ala)$_2$-OH recorded under photoexcitation at 20 K, at a field position corresponding to the PELDOR observer position (TPP triplet state). Phase memory time T_2 = 1.9 μs from monoexponential fit.

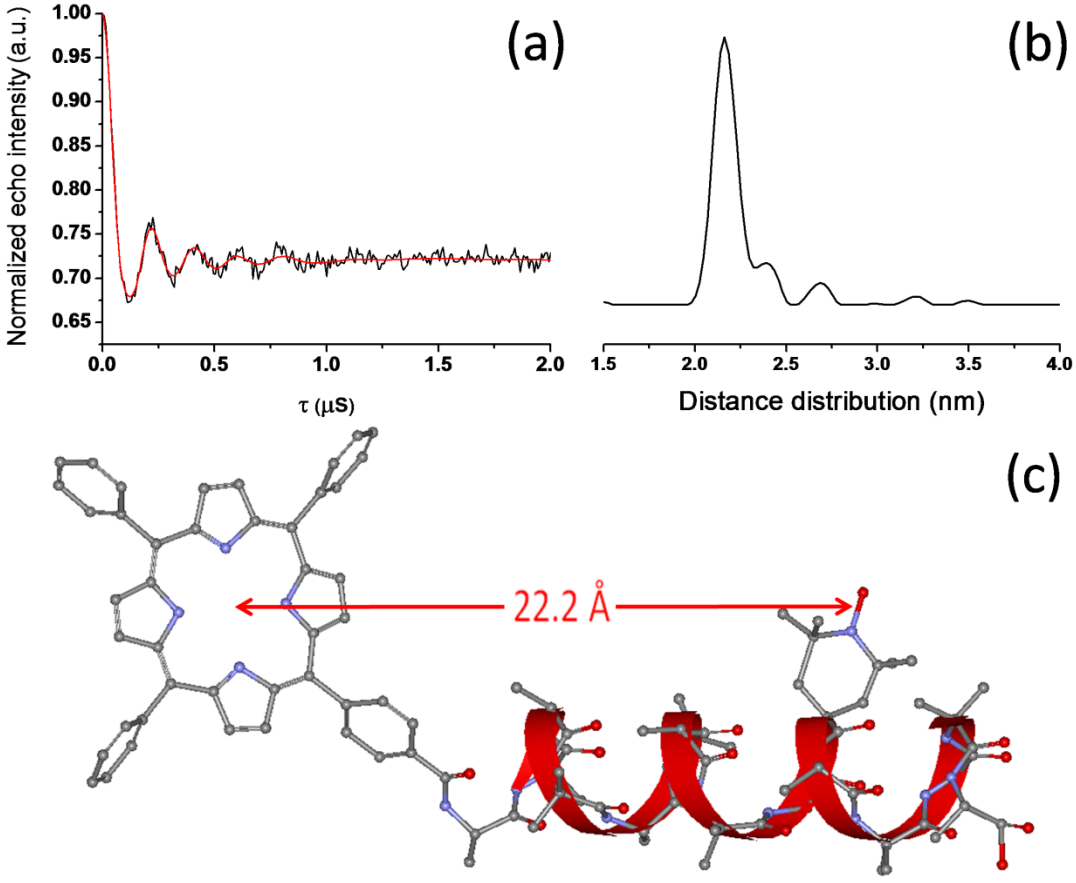

Figure S4. (a) DEER traces after background removal (black) and best fit with Tikhonov regularisation as calculated by DeerAnalysis2013. (b) Distance distribution corresponding to a Tikhonov regularization parameter α = 0.1 . (c) Structural model (WebLab Viewer Pro 3.20, Molecular Simulations) of TPP-(Ala-Aib)$_4$-Ala-TOAC- Ala-(Aib-Ala)$_2$-OH based on structural data reported in refs 6 and 7. The reference points for the interspin distance calculated in the point-dipole approximation are indicated.

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