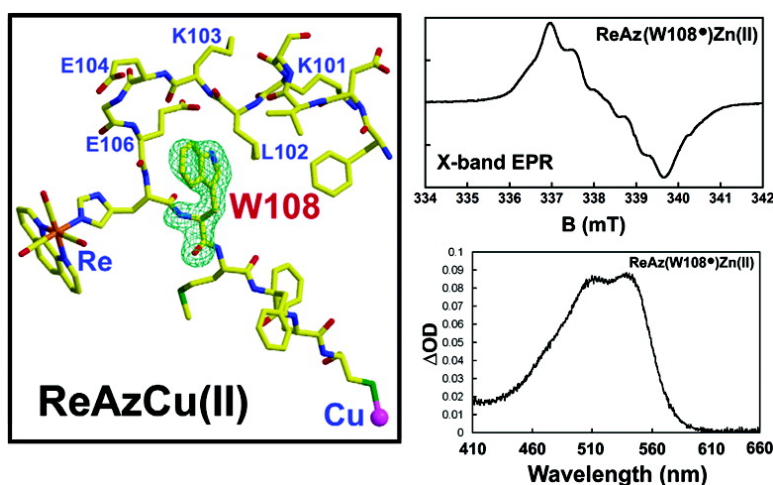Communication

Spectroscopy and Reactivity of a Photogenerated Tryptophan Radical in a Structurally Defined Protein Environment

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Spectroscopy and Reactivity of a Photogenerated Tryptophan Radical in a Structurally Defined Protein Environment

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The lifetimes of tryptophan radicals in biological systems span a wide range:[1–7] in short peptide chains, they live only ∼400 ns,[4] but in DNA photolyase (∼10 ms)[3] and a ribonucleotide reductase (RNR) mutant (49 s),[5] they persist much longer, thereby facilitating spectroscopic characterization. Here we report both the EPR and optical spectra of an exceptionally long-lived tryptophan radical in a structurally characterized *Pseudomonas aeruginosa* azurin (Az),[7] [Re(I)(CO)$_3$(1,10-phenanthroline)(Q107H)](W48F/Y72F/H83Q/Y108W)AzM(II) [M = Cu, Zn]. In this protein, the single tryptophan (W108) is in an unusual environment (Figure 1);[8] part of the indole ring is exposed to solvent, but much of the residue is encapsulated by the surface loop comprising residues 101 to 107. Significant conformational variability in the crystal structure for this entire region indicates heightened mobility of W108, the surrounding polypeptide, and the solvent. W108 interacts with the peptide backbone and K101 or E106, depending on the loop conformation; notably, the 330 nm fluorescence maximum also indicates a polar environment[9] (Supporting Information).

EPR signals attributable to uncoupled tryptophan radicals in RNR mutants have been reported.[10,11] In certain other proteins, however, electronic coupling of the radical with paramagnetic centers or the presence of other radicals obscures the signals.[12–14] The EPR spectrum of the W108 radical in frozen solution (generated by an irreversible flash/quench method under anaerobic conditions)[6] is shown in Figure 2; the spectrum is independent of the metal center (Cu(II) or Zn(II)), indicating that the electronic coupling between the radical and Cu(II) is negligibly small (Cu−C$_γ$(W108) distance is 16.7 Å). The 285 GHz EPR spectrum (Figure 2, lower left inset) yielded accurate g values (g_x = 2.00355; g_y = 2.00271; and g_z = 2.00221) that agree with those reported for other tryptophan radicals analyzed by high-frequency EPR.[10] Density functional calculations on characterized protein radicals as well as model systems support the assignment of W108 as a neutral species;[15] moreover, the value of g_x indicates that the indole nitrogen is near an H-donor. At neutral pH, the EPR signal[16] can be detected for over 5 h at room temperature (Figure 2, upper right inset); indeed, in regard to kinetic stability, the W108 radical in Re(I)AzM(II) is rivaled only by the tyrosine radicals in photosystem II (TyrD)[17] and RNR.[18]

The absorption spectrum obtained 20 μs after 355 nm laser excitation of Re(I)AzZn(II) is shown in Figure 3. The spectrum changes only very slightly over the pH range 4.0−9.8; it also is very nearly the same in deuterated buffer (see Supporting Information). The absorption maxima (512, 536 nm) fall between reported values for protonated and deprotonated tryptophan radicals,[19] which seems reasonable for electronic excitation of W• in a polar (H-
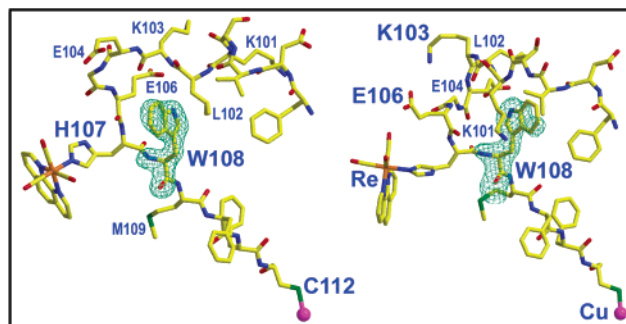
Figure 1. Two different W108 environments in the crystal structure of Re(I)AzCu(II): pdb code 1R1C. The four independent azurin molecules contained in the asymmetric unit show two dominant conformations (left and right) for W108 (F_{obs}−F_{calcd} 1.9 Å resolution omit-electron density in green). Rearrangement of the polypeptide in the surrounding loop (101−107) accompanies the change in W108 conformation. In the two molecules not shown, W108 has mainly the left conformation, but in each case, the W108 indole ring has less definition in the electron density than depicted above.
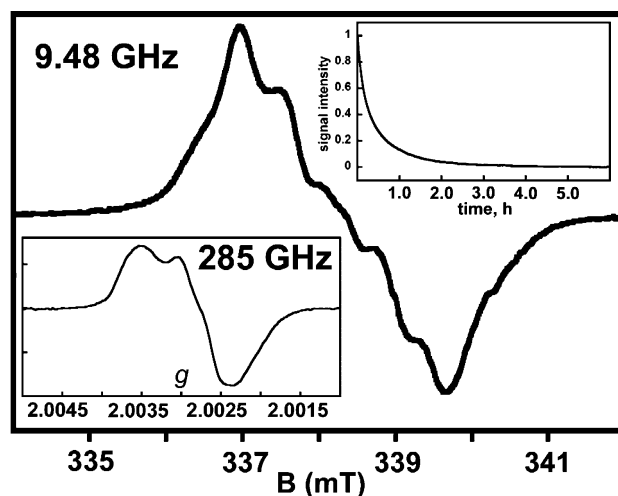

Figure 2. X-band EPR spectrum of ReAz(W108•)Zn(II) under anaerobic conditions (77 K, pH 7.2 KP$_i$, ν = 9.4753 GHz, modulation amplitude = 0.2 mT, microwave power ≈ 200 μW). Lower left inset: 285 GHz EPR spectrum under nonsaturating conditions (50 K, modulation amplitude = 0.1 mT); for a description of the high-field spectrometer, see: Un, S; Dorlet, P.; Rutherford, A. W. *Appl. Mag. Res.* **2001**, *21*, 341−361. Upper right inset: room-temperature decay of the EPR signal (monitored at g = 2.011, ν = 9.7972 GHz).

donor) environment. More cannot be said, as the positions of these radical transitions depend strongly on the nature of outer-sphere interactions.[7] Single-wavelength monitoring[20] of Re(I)Az(W108•)-Zn(II) at pH 7.2 confirmed that there was no transient absorption

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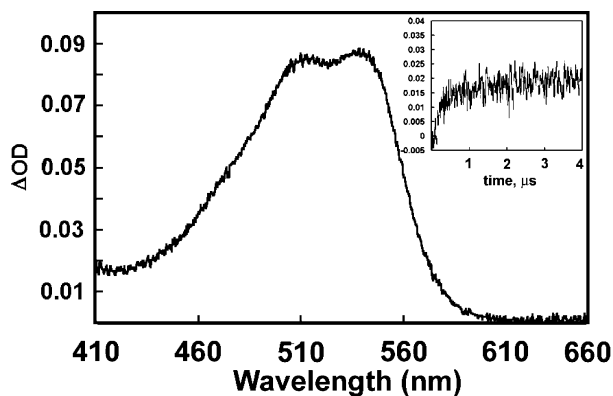
Figure 3. Absorption spectrum recorded 20 μs after flash/quench of 63 μM Re(I)Az(W108)Zn(II)/5 mM [Co(NH$_3$)$_5$Cl]Cl$_2$ in 50 mM KP$_i$ (pH 7.2) at room temperature. Inset: single-wavelength monitoring of Re(I)Az-(W108$^\bullet$)Zn(II) formation (500 nm). The trace was produced by photoexcitation of a solution of 42 μM Re(I)Az(W108)Zn(II) with 5 mM [Co(NH$_3$)$_5$Cl]Cl$_2$ in 50 mM KP$_i$ pH 7.2 at room temperature. The slight bleach immediately after excitation is due to emission from the rhenium complex.

at 600 nm (a signal expected if a protonated species had formed).[3,19,21] The rate constant for formation of Re(I)Az(W108$^\bullet$)-Zn(II) is 2.8×10^6 s^{-1} (Figure 3, inset).

To estimate the Re(I)Az(W108$^\bullet$/W108)Zn(II) reduction potential, we photolyzed a solution containing 0.24 mM ReAz/12 mM [Co-(NH$_3$)$_5$Cl)]Cl$_2$ and trapped the radical-containing product at 77 K.[6] By spin integration, the concentration of W108$^\bullet$ was found to be ~60 μM. After warming of the sample to room temperature, addition of 160 μM K$_4$[Mo(CN)$_8$], and refreezing of the sample, the only EPR signal aside from Cu(II) was from a Mo(V) species, [Mo(CN)$_8$]$^{3-}$.[22] Since the reduction potential of the [Mo(CN)$_8$]$^{3-/4-}$ couple is 0.78 V vs NHE,[23] that of the W108 radical in ReAz ($\geq$0.8 V) is within the range (0.6$-$1.0 V vs NHE) estimated for the residue exposed in solution.[24-28] The time course of the EPR signal amplitude (Figure 2, upper right inset) clearly indicates that multiple pathways are associated with W108$^\bullet$ decay. Work aimed at elucidation of these pathways is underway.

Supporting Information Available: Details of the synthesis of *Re*-modified azurins, EPR spectra in various solvents, and details of the transient absorption apparatus. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Crystals of [Re(I)(CO)$_3$(1,10-phenanthroline)(Q107H)]$^+$(W48F/Y72F/H83Q/Y108W)AzCu(II) (space group *P*2$_1$, cell dimensions 58.947 × 56.933 × 7.296 Å^3; $\beta = 98.37°$, four molecules per asymmetric unit) grew from 2 μL drops made from equal volumes of 30 mg/mL ReAzCu-(II) in 25 mM HEPES pH 7.5 and reservoir. The drops were equilibrated against 500 μL of reservoir containing 20% PEG molecular weight 4000, 100 mM LiNO$_3$, and 100 mM imidazole pH 7.0. Diffraction data (40.0−1.9 Å resolution, 98.8% complete, $R_{sym} = \Sigma_j |I_j - \langle I \rangle|/\Sigma\Sigma_j I_j = 7.5\%$; overall signal-to-noise = $\langle I \rangle/\sigma I = 14.0$) were collected on a Quantum-210 CCD (Area Detector Systems Corporation) at the Cornell High Energy Synchrotron Source, beamline F2 (0.964 Å), and processed with DENZO (Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307−326). The structure of Re(I)AzCu(II) was determined by molecular replacement with EPMR (Kissinger, C. R.; Gehlhaar, D. K.; Fogel, D. B. *Acta Crystallogr.* **1999**, *D55*, 484−491) using a probe derived from the structure of Ru-labeled azurin (PDB code: 1BEX). Rigid-body, positional and thermal factor refinement with CNS (Brunger, A.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr.* **1998**, *D54*, 905−921), amidst rounds of manual rebuilding, Re(I)(phen)(CO)$_3$- incorporation, and water placement with XFIT (McRee, D. E. *J. Mol. Graphics* **1992**, *10*, 44−46), followed by further anisotropic refinement of all heavy atoms' temperature factors (S, Cu, and Re) with SHELX-97 (Sheldrick, G.; Schneider, T. *Methods Enzymol.* **1997**, *277*, 319−343) produced the final model (1.9 Å resolution, *R*-factor = 22.4%; *R*-free = 26.0%; against 5.0% of the free reflections removed from refinement). All residues have favored backbone dihedral angles. Stereochemical restraints were removed from the copper ligand bonds in the later stages of refinement.
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(15) Principal values of the *g* tensor for W$^{\bullet+}$, H-bonded (formic acid) deprotonated W$^\bullet$, and neutral W$^\bullet$ models were calculated by the method of Schreckenbach and Ziegler (Schreckenbach, G.; Ziegler, T. *J. Phys. Chem. A* **1997**, *101*, 3388−3399) as implemented in the ADF package (te Velde, G.; Bickelhaupt, F. M.; Baerends, E. J.; Fonseca Guerra, C.; Van Gisbergen, S. J. A.; Snijders, J. G.; Ziegler, T. *J. Comput. Chem.* **2001**, *22*, 931−967) using the Becke gradient correction (Becke, A. D. *Phys. Rev. A* **1988**, *38*, 3098−3100) and Perdew correlation term. The calculated *g* values were scaled according to a survey of a variety of radicals, which indicated that they should be linearly scaled to achieve better agreement with experiment (Un, S. Manuscript in preparation). Such scaling produced *g* values that were within ±0.0004 of experimental ones. The three models gave identical calculated g_z values (2.00247) and showed only slight variations in g_y (protonated 2.00271; hydrogen-bonded 2.00296; deprotonated 2.00299) and g_x (protonated 2.00345; hydrogen-bonded 2.00359; deprotonated 2.00380).
(16) EPR spectrum of the W108 radical shows small variations in the pH range 7−10 and in D$_2$O/KP$_i$ buffer. A tryptophan peroxyl radical forms in the presence of O$_2$ (EPR spectrum $g_z = 2.034$ and $g_{x,y} = 2.006$; Sahlin, M.; Cho, K.-B.; Pötsch, S.; Lytton, S. D.; Huque, Y.; Gunther, M. R. G.; Sjöberg, B.-M.; Mason, R. P.; Gräslund, A. *J. Biol. Inorg. Chem.* **2002**, *7*, 74−82).
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JA037203I