

Understanding the blue copper proteins

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There are several copper-containing proteins which have a beautiful intense blue color and contain so-called Type I copper ions, which, in the +2 oxidation state, exhibit unusual electronic paramagnetic resonance spectra (EPR). In this issue, Antholine, Hanna, and McMillin report a notable advance in characterizing the Type I copper in these blue copper proteins. They have carried out a tour de force of the EPR spectroscopy and spectral analysis of the electron-transfer protein azurin from *Pseudomonas aeruginosa*, achieving unusually high spectral resolution and definition of some of the magnetic and electric parameters which describe this copper site.

The blue copper proteins, exemplified by plastocyanin, ceruloplasmin, azurin, and stellacyanin, are intriguing and have been subjects of investigation over many years. Some have only Type I copper sites; others have other types of copper, as well. The blue color reminds one of the blue color of many square-planar copper (II) chelates but is much more intense; the optical transition responsible for it is attributed to a sulfur-metal charge transfer band. The EPR technique is excellent for probing such copper sites, but may suffer from lack of resolution. The EPR signal from the Cu (II) of the Type I copper sites is special, as the largest hyperfine coupling between the unpaired electron and the copper nucleus is much smaller than that found in the usual square-planar copper(II) complexes. This indicates a special coordination geometry, which x-ray crystallography results show to be a distorted and very irregular tetrahedron or trigonal pyramid. In the case of the azurin from *Pseudomonas aeruginosa*, isolated over three decades ago (Horio, 1958), there is just one copper, Type I. The structure (see Nar et al., 1991, and the review by Adman, 1991) can be described as a distorted trigonal pyramid with nitrogen atoms from each of two histidine residues and sulfur from a cysteine residue forming a triangle roughly coplanar with the copper atom, each about 2–2.2 Å from it. Two more atoms (a methionine sulfur and a carbonyl oxygen) occupy much more distant (~3 Å) roughly axial positions. Despite the fact that the coordination geometry is quite unsymmetrical, the conventional EPR spectra appeared to display nearly axial symmetry, with one branch of the resonance (corresponding to an axial direction) lying at low magnetic field (high *g*-factor, 2.26) and exhibiting copper nuclear hyperfine splitting and the other branch, corresponding to the equatorial directions, occurring at a higher field (*g*-factor, 2.05, closer to the free-electron value). EPR at higher

microwave frequency (35 GHz) showed some evidence of nonaxiality, or rhombicity. Otherwise, there were no hyperfine or other features to help one understand molecular bonding in this site. Electron-nuclear double resonance (ENDOR), which is a concerted NMR and EPR experiment, succeeded in adding information on some of the hyperfine couplings of ligand and copper atoms (Roberts et al., 1984; Werst et al., 1991).

With the picture of electronic structure at the copper site considerably improved, Antholine et al., 1993, now have revisited the conventional EPR spectroscopy of *Pseudomonas aeruginosa* azurin. In this painstaking work, a carefully crafted combination of techniques is employed to enhance spectral resolution and wring extra information from this system. First of all, copper, in its natural abundance, is a mixture of two isotopes, ⁶³Cu and ⁶⁵Cu, which have the same spin but slightly different nuclear magnetic moments and nuclear quadrupole moments. To prevent the overlap of spectra from these two isotopes from spoiling spectral resolution, they stripped out the copper from the azurin and reconstituted it with isotopically pure ⁶³Cu. Secondly, these workers not only used low temperatures to minimize line widths, but recognized that strain effects in both electronic Zeeman interaction and hyperfine coupling must contribute significantly to line broadening in the EPR spectra of azurin. (In this context, "strain" is simply the term for a distribution of magnetic parameters among individual molecules caused by varying microconformations or environments.) They invoked the power of multifrequency EPR spectroscopy, tuning the spectrometer frequency to a range of low values (1 to 2.5 GHz in this case) at which the Zeeman strain is reduced and the partly correlated Zeeman and hyperfine strains (*g*- and *A*-strains) act to counteract each other. This line-sharpening technique had been described in earlier works (see for example Froncisz and Hyde, 1980, and Aqualino et al., 1991). New copper and ligand (¹⁴N) hyperfine resolution appeared in the region of the spectrum corresponding to magnetic field oriented perpendicular to the principal magnetic axis, and presumably in the equatorial plane of copper coordination sphere. Finally, they used a powerful spectral simulation method (see for example Belford and Duan, 1978, and Liczwek et al., 1983) with many iterations to fit these better resolved spectra at all frequencies with spin-Hamiltonian parameters. The parameters required for good fits indicated an extremely low symmetry site, with the principal axes of the hyperfine and Zeeman interactions being far from coincident with

each other and the resolved equatorial-plane copper hyperfine coupling being on the order of half the values previously deduced from higher frequency ENDOR and EPR experiments. We now have much more detailed information about the copper site in this azurin as a result of this and the other recent EPR and ENDOR work. We also can learn some lessons from this work; among them is that metal sites in nature may be of very low symmetry, with spectroscopic consequences which may be very large and should not be ignored.

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