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High-Potential C112D/M121X (X = M, E, H, L) *Pseudomonas aeruginosa* Azurins

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Site-directed mutagenesis of *Pseudomonas aeruginosa* azurin C112D at the M121 position has afforded a series of proteins with elevated $Cu^{II/I}$ reduction potentials relative to the Cu^{II} aquo ion. The high potential and low axial hyperfine splitting (Cu^{II} electron paramagnetic resonance $A_{||}$) of the C112D/M121L protein are remarkably similar to features normally associated with type 1 copper centers.

The capacity for precise tuning of active site reduction potentials in folded polypeptide environments has afforded nature the freedom to access a myriad of chemistries despite its limited repertoire of incorporated elements.[1−3] The reduction potential of Cu^{II} in *Pseudomonas aeruginosa* azurin has long been known to be sensitive to substitutions within both the inner and outer type 1 copper coordination spheres.[4−12] This sensitivity makes it possible to tune the potential over 0.5 V higher than the aqueous redox couple.[8]

We have exploited this sensitivity to obtain robust azurins with relatively high reduction potentials in the absence of soft ligands. Here we report work on the C112D protein

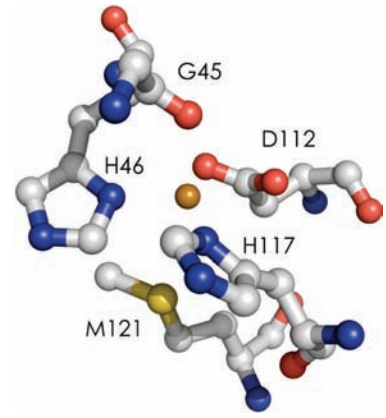

Figure 1. PyMol rendering of the active site of C112D azurin (PDBID: 1AG0).[13]

(Figure 1), where the C to D substitution is made to avoid the unwanted irreversible oxidation of the cysteine thiol group.[13−15] We have generated mutants possessing charged axial ligands as well as one lacking axial coordination from position 121 to evaluate the potential range of these robust scaffolds.

We constructed plasmids encoding azurins C112D, C112D/M121E, C112D/M121L, and C112D/M121H via site-directed mutagenesis of a T7-controlled plasmid encoding the wild-type protein. We expressed all four proteins in *Escherischia coli* BL21(DE3). Periplasmic fractions were passed through Q-Sepharose media in 50 mM Tris pH 7.8 containing 100 mM NaCl to remove anionic contaminants. The proteins were desalted into 10 mM CHES at pH 9.0 and purified to homogeneity on a MonoQ column. Purity was assessed by silver-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry. Apoazurin concentrations were determined using $\varepsilon_{280} = 8800\ M^{-1}\ cm^{-1}$. For well-resolved spectra and for electrochemistry, a slight excess of $CoCl_2$ or $CuSO_4$ was added to the apoprotein solution, which was subsequently desalted to remove surface-bound metal ions.

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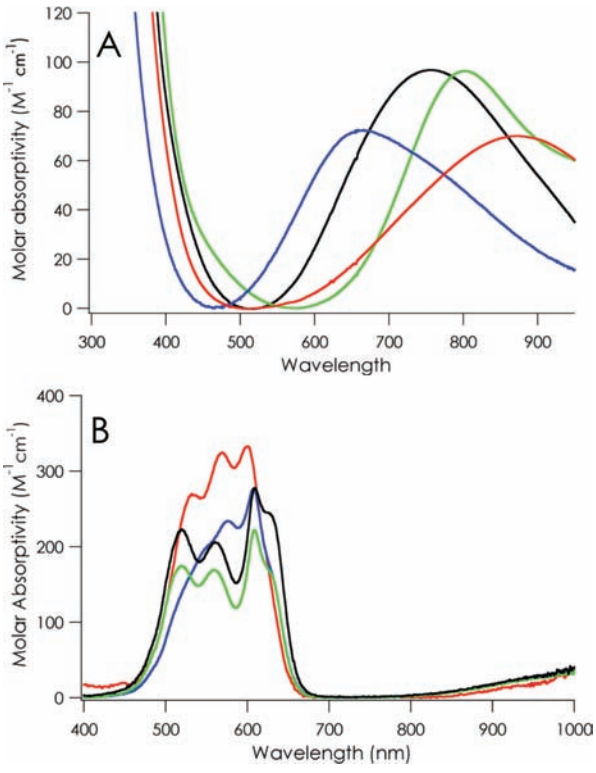

Figure 2. Electronic absorption spectra of Cu^{II} (A) and Co^{II} (B) C112DM121X azurins in 10 mM sodium phosphate at pH 7.0. X = M (black), L (green), E (red), H (blue).

Electronic absorption spectra of the Cu^{II} and Co^{II} C112D/M121X (X = M, E, H, L) azurins in a pH 7 aqueous solution are shown in Figure 2 (data are set out in Table 1). The M121L substitution results in a red shift of the Cu^{II} ligand-field (LF) absorption to 800 nm; consistent with this decrease in LF splitting, there also is a small red shift of the imidazole to Cu^{II} ligand-to-metal charge-transfer band. The Cu^{II} LF band of C112D/M121L Cu^{II} azurin is much sharper than that of C112D, which could indicate decreased site reorganization if a single electronic transition is responsible for each of the observed absorptions. The Co^{II} C112D/M121L LF system near 600 nm is virtually identical with that of the single mutant, confirming that the inner-sphere electronic interactions involve mainly the equatorial ligands.

The Cu^{II} LF band in the spectrum of C112D/M121E azurin is red-shifted to 875 nm. The Co^{II} LF absorption system exhibits structure characteristic of tetrahedral coordination, indicating that the E121 carboxylate is ligated to the metal and that this interaction forces the metal out of the plane of the H46, D112, and H117 donor atoms.[16] The Cu^{II} LF band in the spectrum of the C112D/M121H protein is blue-shifted to 680 nm. On the basis of the profile of its 600-nm absorption system, the Co^{II} geometry appears intermediate between five- and four-coordinate, raising the possibility that the H121 imidazole does not interact strongly with the metal center.

The coordination geometries that can be inferred from examination of the Co^{II} spectra of C112D/M121X (X = M, E, H) are supported by 77 K X-band EPR data of Cu^{II} analogues (Table 1 and Figure 3).[17] The C112D/M121E and C112D/M121H proteins possess axial hyperfine splittings comparable to that of the single mutant. Concomitant with the lack of significant anisotropy in the perpendicular components of the **g** tensors, these parameters suggest an axial coordination environment similar to that of the C112D mutant in which the X121 side chain weakly coordinates the metal.

The C112D/M121L EPR spectrum is particularly interesting. The spin Hamiltonian parameters represent a departure from the other azurins, displaying a substantial increase in g_z and a decrease in $A_{||}$, which in turn suggests that there is a shift toward tetrahedral site geometry.[18] Furthermore, there is in-plane **g**-tensor anisotropy that may be attributed to d_{z^2} mixing into a d_{xy} ground state.[19,20] Together these features suggest that C112D/M121L Cu^{II} is not in a type 2 electronic environment.

Cyclic voltammograms (CVs) of Cu^{II} C112D/M121X (X = M, L) azurins adsorbed onto [$CH_3(CH_2)_8SH$ and $HO(CH_2)_8SH$] mixed self-assembled-monolayer (SAM)-modified gold electrodes[21,22] are shown in Figure 4, with midpoint potentials appearing in Table 1. Azurin C112D/M121E is coupled poorly to the electrode, as indicated by weak CV signals (Figure S1 in the Supporting Information); however, square-wave voltammetry (SWV) provides sufficient current for midpoint potential determination. The C112D/M121L and C112D/M121E $Cu^{II/I}$ midpoint potentials are both higher than that of C112D, likely a result of weaker overall LFs. From the position of the LF band in the C112D/M121E spectrum, we would have predicted an even larger upshift were it not for the negative charge of the carboxylate. Earlier work has shown that a carboxylate interaction with Cu^{II} in azurin leads to a ~100 mV decrease in the reduction potential.[8]

We were not able to determine the reduction potential of C112D/M121H azurin from electrochemical experiments because the CV was very broad and asymmetric (Figure S2 in the Supporting Information). On the basis of a redox titration with *P. aeruginosa* cytochrome c_{551} (Figure S3 in the Supporting Information), we estimate a reduction potential near 305 mV. An optical absorption pH titration suggests that the pK_a of the H121 imidazole is ~8 (Figure S4 in the Supporting Information), so the high potential is likely attributable to relative destabilization of Cu^{II} by the nearby protonated side chain. The elevated pK_a suggests that there is a favorable electrostatic interaction between the D112 carboxylate and protonated H121.

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Table 1. Electronic Absorption,[a] Electron Paramagnetic Resonance (EPR),[b] and Electrochemical Data for Azurins C112D/M121X

X	Cu^{II} λ_{max} (nm)	Co^{II} λ_{max}(nm)	g_x	g_y	g_z	$A_\perp$ (10^{-4} cm^{-1})	$A_\parallel$ (10^{-4} cm^{-1})	$Cu^{II/I}$ $E^\circ_{1/2}$ (V vs NHE), pH 7
M	754 (100)	518 (210), 560 (200), 610 (280), 630 (650, sh)	2.07 (1)	2.05 (1)	2.31 (1)	1.58 (4)	151 (1)	180
E	875 (70)	569 (270), 531 (330), 600 (330)	2.06 (3)	2.070 (1)	2.32 (3)	2.15 (1)	151 (1)	270
H	657 (70)	549 (210, sh), 577 (240), 608 (280), 630 (170)	2.064 (2)	2.06 (5)	2.30 (3)	4 (2)	165 (1)	305
L	798 (100)	519 (170), 559 (170), 609 (220), 628 (170, sh)	2.11 (1)	2.05 (1)	2.385 (1)	15.07 (3)	101 (1)	280

[a] Parenthetical values represent molar extinction coefficients (M^{-1} cm^{-1}) calculated by titration of apoazurin with $CuSO_4$ or $CoCl_2$. These values are correct to within 5% uncertainty. [b] EPR parameters were simulated using SpinCount. Parenthetical values represent uncertainty due to *g* or *A* strain. See ref 17.

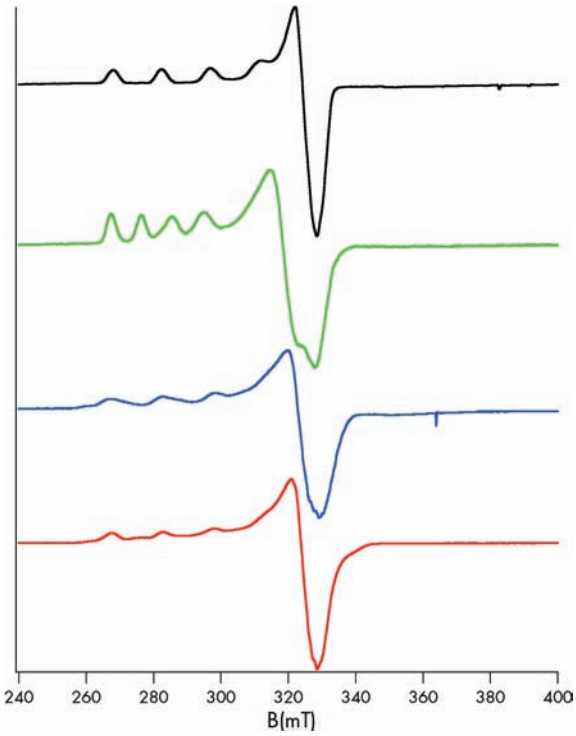

Figure 3. 77 K X-band EPR spectra of Cu^{II} C112DM121X azurins in 10 mM sodium phosphate at pH 7.0. X = M (black), L (green), E (red), H (blue).

Our work on C112D/M121L azurin demonstrates that type 2 copper can acquire type 1 character, even in the absence of sulfur ligation. Properties such as a relatively small $A_\parallel$ value and high reduction potentials (relative to C112D and, by extension, the Cu^{II} aquo ion) until now have been associated almost exclusively with blue copper centers.[10,11,23,24] One possibility is that electron donation from the equatorial ligands reduces the positive charge on Cu^{II} in the hydrophobic axial environment, effectively mimicking the covalency attributable mainly to cysteine thiolate ligation in a blue protein.

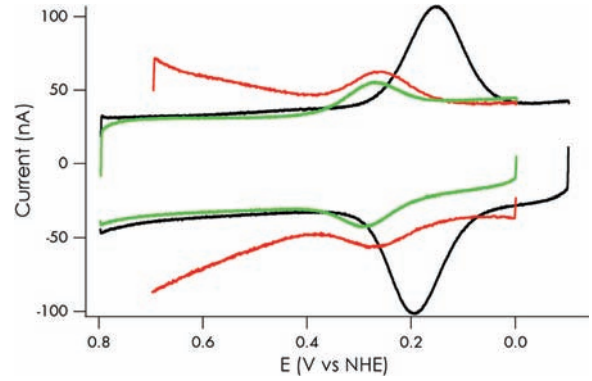

Figure 4. Electrochemistry of C112D/M121X azurins on SAM-modified Au electrodes. C112D (black) and C112D/M121L (green) potentials were measured by CV in 10 mM sodium phosphate at pH 7.0 at a scan rate of 50 mV/s referenced to Ag/AgCl in saturated KCl (197 mV vs NHE). Azurin C112D/M121E (red) was measured by SWV in 10 mM NaP_i with a square-wave frequency of 8 Hz.

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Supporting Information Available: pH-dependent UV/vis, CV and redox titration of C112D/M121H azurin, and CV of C112D/M121E azurin. This material is available free of charge via the Internet at http://pubs.acs.org.

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