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Rationally Tuning the Reduction Potential of a Single Cupredoxin Beyond the Natural Range

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Abstract

Redox processes are at the heart of numerous functions in chemistry and biology, from long-range electron transfer (ET) in photosynthesis and respiration to catalysis in industrial and fuel cell research. Nature accomplishes these functions by employing only a limited number of redox-active agents. A long-standing issue in these fields is how redox potentials are fine-tuned over a broad range with little change to the redox-active site or ET properties. Resolving this issue will not only advance our fundamental understanding of the roles of long-range, non-covalent interactions in redox processes, but also allow for design of redox-active proteins having tailor-made redox potentials for applications such as artificial photosynthetic centers^{1,2} or fuel cell catalysts³ for energy conversion. We have shown here that two important secondary coordination sphere interactions, hydrophobicity and hydrogen-bonding, are capable of tuning the reduction potential of the cupredoxin azurin (Az) over a 700 mV range, surpassing the highest and lowest reduction potentials reported for any mononuclear cupredoxin, without perturbing the metal binding site beyond what is typical for the cupredoxin family of proteins. We also demonstrate that the effects of individual structural features are additive and that redox potential tuning of Az is now predictable across the full range of cupredoxin potentials.

Cupredoxins are redox-active copper proteins that span several kingdoms in biology and play crucial roles in many important processes, including photosynthesis, respiration, cell signaling, and many other reactions in oxidases and reductases⁴⁻⁸. Despite these many functions, cupredoxins employ a single redox active center, whose reduction potential (E°) must be tunable to match that of a given redox partner without compromising the structural and ET properties of the protein. The protein environment provides not only a means to overcome the large ($> 1\text{eV}$) reorganization energies of Cu complexes in aqueous solution, but also the limited range of E° attainable by Cu ions under physiological conditions ($<200\text{ mV}$). The mononuclear type 1 (T1) cupredoxins provide a stunning example, as they all

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Author contributions N.M. performed most of the experimentation, lead the study and authored most of the manuscript. D.G. and T.W. contributed intellectually and assisted in experimentation and paper editing. Crystal diffraction patterns were collected by H.R. and refined into PDB structures by Y.G. M.N. assisted with ERP data collection and simulation. Y.L. is the principle investigator and also assisted with paper editing.

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contain a single Cu ion with similarly low (0.7 eV) reorganization energy, yet E° from various members of this ubiquitous family span an ~500 mV range⁴⁻⁸.

Despite their vastly different E° , the overall and active site structures are very similar in all T1 cupredoxins,⁹ which contain a Cu ion coordinated by a Cys and two His residues in a trigonal planar geometry, and a weak axial ligand, such as Met121 in Az (Figure 1A). A weak interaction between the Cu and a backbone carbonyl oxygen is also present in some cupredoxins, such as Gly45 in Az (Figure 1A). The similar structure of various cupredoxins results in similar spectroscopy, characterized by a ligand to metal charge transfer (LMCT) absorbance at ~600 nm and very small EPR hyperfine coupling constants ($A_{\parallel} \sim 60$ G). That the highly similar Cu-binding site structure exhibits such a wide range of E° suggests that Nature likely utilizes subtle interactions outside the primary coordination sphere to attain the required E° in cupredoxins.

A number of studies have been performed to elucidate structural features responsible for controlling the E° of cupredoxin⁴⁻⁸. Factors identified in other proteins as crucial for tuning E° , such as electrostatics¹⁰ and solvent^{11,12} effects, have thus far shown minimal effect in cupredoxins, suggesting that there is still a significant lack of knowledge about other long-range, non-covalent interactions responsible for tuning E° . Mutational studies on rusticyanin (Rc), the mononuclear cupredoxin with the highest E° of 680 mV at pH 2.0, resulted in a lowest E° of ~470 mV, still higher than the highest E° of other cupredoxins⁴⁻⁸. Similarly, numerous mutations in other cupredoxins always reach a E° ceiling, with the highest E° being ~400 mV, still lower than that of Rc. So far, replacement of the axial copper ligand (Met121 in Az) has resulted in the largest changes to the E° of several cupredoxins⁴⁻⁸. Studies using unnatural amino acids as isostructural analogs of Met established a linear correlation between the E° and the hydrophobicity of the axial ligand in cupredoxin variants (Figure 2A)^{13,14}. Other interactions, such as π - π interactions between aromatic residues and the copper ligands, have been shown to affect cupredoxin E° to a lesser degree^{15,16}. While these studies exemplify the importance of long range (> 3 Å) interactions in tuning the properties of metalloproteins, the level of change to the E° is relatively small (< 250 mV)^{15,16}, and no report has shown control of the E° of a single cupredoxin across the entire range of native cupredoxin potentials at similar pH values. As there are significant pH effects on the reduction potential of every cupredoxin protein, all potentials in this paper will be compared at pH 7.0, unless otherwise noted.

With its moderate E° of 265 ± 19 mV at pH 7.0, Az is an ideal system to test rational tuning of cupredoxin E° , since it represents an intermediate step between the potential extremes. Inspection of several cupredoxin structures against that of Rc revealed a Ser residue (Ser86 in Rc) at a position corresponding to a highly conserved Asn in other cupredoxins. This Asn residue is ~5.5 Å away from the copper in Az, but forms hydrogen bonds between two of the ligand containing loops. Mutating Ser86 in Rc to Asn lowered the E° by 77 mV¹⁷. Ser86 in Rc has been proposed to raise the Cu E° by strengthening the hydrogen bonding interactions between two ligand-containing loops, adding rigidity to the copper containing loops and influencing the direct hydrogen bonds between the backbone of the protein and the thiolate of Cys112¹⁷⁻¹⁹. We therefore incorporated these interactions into Az by making the N47S mutation. The UV-vis spectroscopy of N47S is very similar to that of native Az (Figure S3),

suggesting that the core cupredoxin structure is preserved. Interestingly, the mutation resulted in an ~130 mV increase in E° , from 265 ± 19 mV to 396 ± 25 mV at pH 7.0.

Since the axial ligand, Met121, and the N47S mutation influence different aspects of the copper binding site, we reasoned that their individual effects on E° would be additive. Consequently, we constructed both N47S/M121Q and N47S/M121L Az. The linear dependence of the redox potential on the hydrophobicity of the axial residue, previously identified in cupredoxins, was still observed, with N47S mutation exerting similar effects on their E° (Figure 2A), suggesting that the two influences are additive. Due to this additive nature, the highest E° of this series of Az mutants, seen in N47S/M121L Az, was observed to be 496 ± 13 mV at pH 7.0. As shown by a crystal structure of N47S/M121L Az (Figure 1B), the N47S mutation in Az introduces a hydroxyl group 4.13 Å from the sulfur of Cys112 and 3.42 Å from the backbone amide nitrogen of Thr113, reproducing a hydrogen bonding pattern similar to that of native Rc (PDB code: 1rcy)²⁰, which shows distances of 4.04 Å, and of 2.88 Å, respectively, at the equivalent positions.

Despite N47S/M121L Az having the highest redox potential of any known Az variant, its E° still falls short of Rc's. Therefore, other factors that tune the cupredoxins E° to high values must still be missing. Another examination of cupredoxin crystal structures reveals the presence of a backbone carbonyl oxygen, from Gly45, near the copper ion in Az, which is missing in other cupredoxins, such as Rc^{6,7}. This ionic interaction in Az results in more electron density near the copper, preferentially stabilizing the Cu(II) form of the protein and, therefore, lowering the redox potential²¹. We thus reasoned that manipulating the electronic properties of the carbonyl oxygen in Az may enable us to close the gap between the high and low potential cupredoxins.

Since the carbonyl oxygen itself cannot be changed by site-directed mutagenesis, altering its properties represents a new challenge for protein design and engineering. To meet this challenge, we searched for residues in Az that could affect the carbonyl through either steric repulsion or hydrogen bonding. Phe114 was chosen as a suitable candidate because of its proximity to Gly45 and since its side chain points toward the carbonyl. To observe the effect of introducing a hydrogen bonding group at position 114 in Az, F114N Az was constructed and a potential of 398 ± 4 mV at pH 7.0 was measured for this variant.

Since both the N47S and F114N mutations individually perturb separate areas of the protein, they were combined by constructing the N47S/F114N variant of Az. The redox potential of N47S/F114N Az was observed to be 494 ± 11 mV at pH 7.0 (Figure 2). Again, the position and extinction coefficient of the LMCT band in this double mutant are similar to wild type Az (Figure S5). Combining the axial Met mutations with N47S and F114N also yielded a linear dependence of reduction potential on axial ligand hydrophobicity, resulting in a reduction potential for N47S/F114N/M121L Az of 640 ± 1 mV at pH 7.0 (Figure 2). The redox potential of Rc at the same pH is reported to be 550 mV²². Its axial ligand variant, M148L Rc exhibits a potential of 613 mV vs. NHE at pH 6.2²³, which may be directly compared to 668 ± 1 mV at pH 6.0 for N47S/F114N/M121L Az. Therefore, N47S/F114N/M121L Az has a redox potential exceeding that of any known Rc variant at the same pH.

Following the observed trend of increasing redox potential with decreasing pH, a maximum redox potential of 706 ± 3 mV was observed for N47S/F114N/M121L Az at pH 4.0.

To investigate the combined effect of N47S and F114N on the structure of Az, a crystal structure of the N47S/F114N variant was obtained (Figure 1C). The structure showed that the F114N mutation puts a hydrogen bond donor near the backbone carbonyl of Gly45, but the distance between donor and acceptor is 3.63 Å and the presumed N-H...O angle is 92.7°; respectively, slightly longer and slightly smaller than what is typical for hydrogen bonding. X-ray crystallography is, however, not able to distinguish between oxygen and nitrogen atoms in a molecule. If the position of the oxygen and nitrogen on the sidechain of Asn114 are reversed, the amide nitrogen is in position to disrupt hydrogen bonding interactions between the copper ligand, His117, and other parts of the protein. Rc and other high potential cupredoxins achieve a similar effect by crowding the area around this His residue with Phe residues²⁴.

Having achieved predictable tuning of the redox potential of Az to the high end of cupredoxin potentials, we also wanted to tune its potential to the low end. It is known that replacing the axial ligand Met121 with Gln, the axial ligand found in low potential stellacyanin, lowers the potential of Az to ~190 mV^{25,26}. Another report has shown that mutation of Phe114 in Az to a Pro residue causes a reorganization of the hydrogen bonding network near the copper binding site of Az, which lowers the redox potential by ~90 mV. This decrease is due to the deletion of a direct hydrogen bond to Cys112, which increases the electron density on the sulfur ligand, thereby lowering the redox potential²⁷. Hence, we constructed F114P/M121Q and F114P/M121L Az, demonstrating that the effect of F114P is also additive with that of the axial ligand hydrophobicity (Figure 2A). With a E° of 90 ± 8 mV at pH 7.0, F114P/M121Q Az boasts the lowest E° reported for all cupredoxins and their variants. Following the same pH trend observed for the N47S/F114N/M121L Az variant, a minimum redox value of -2 ± 13 mV was observed at pH 9.0. We determined the crystal structure of F114P/M121Q Az (Figure 1D), which shows similar effects to those seen in F114P Az: a disrupted hydrogen bond between Phe114 and Cys112.

In summary, we have predictably and rationally tuned the E° of Az to the full range reported for native cupredoxins and beyond, as well as many E° in between (Figure 2B). This unprecedented level of control over an electron transfer protein was achieved by mapping out major interactions, an approach that applies to all other redox proteins. While redox agents for use in organic solvents are plentiful, water-soluble and stable redox agents are rare, and those available display a limited potential range. Previously, to cover a wide potential range, different compounds with different surface interactions or ET properties had to be used. Since point mutants of a given protein have almost identical structures and surface properties, such proteins will find a wide range of applications as redox agents in areas from fundamental biochemical and biophysical studies of enzymes, to tailor-made redox agents for artificial photosynthetic centers and fuel cell catalysts for energy conversion.

Methods

Az mutants were constructed, expressed and purified using a protocol described previously^{28,29}. Reduction potentials were recorded using either a pyrolytic graphite edge (PGE) or glassy carbon electrode assembled according to literature procedures³⁰.

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Figure 1. X-ray structures of Az and selected variants

a) native azurin (PDB: 4AZU). b) N47S/M121L azurin: N47S affects the rigidity of the copper binding site and, likely, the direct hydrogen bonds between the protein backbone and Cys112 c) N47S/F114N azurin: introducing a hydrogen bond donor at position 114 perturbs hydrogen bonding near the copper binding site, possibly disrupting donor-acceptor interactions to His117, or ionic interactions between the copper and the carbonyl oxygen of Gly45 d) F114P/M121Q azurin: F114P deletes a direct hydrogen bond to Cys112 resulting in a lower redox potential. The UV-vis spectroscopy of the F114P containing variants shows a significant increase in the copper $d \rightarrow d$ absorbance range around 800 nm. This increased absorbance suggests slight rearrangement of the copper binding site, but is consistent with F114P Az²⁷ and other T1 copper proteins, such as plastocyanin⁹. In all panels copper is shown in green, carbon in cyan, nitrogen in blue, oxygen in red and sulfur in yellow. Hydrogen bonding interactions are shown by dashed red lines.

Figure 2. Rational tuning of the reduction potential of Az

a) Plot of the E° at pH 7.0 vs. log P for the Az mutants from this study. The lowest reported E° (---) at pH 7.0 for any T1 cupredoxin prior to this study, M86Q Pseudoazurin, is indicated. The highest E° (---) at pH 6.2 of T1 cupredoxin variant prior to this study, M148L Rc, is also indicated; its potential was not measured at pH 7.0 due to protein instability. Considering the pH trend of the E° of all cupredoxins, this reported value for M148L Rc would be lower at pH 7.0. b) Plot showing the E° for each azurin variant at pH 7.0 unless otherwise noted. Not only is the redox potential of Az tunable to the extremes of the redox

potentials attainable by T1 cupredoxins, but to nearly any redox potential within the range. In both panels, standard deviations are shown as error bars.