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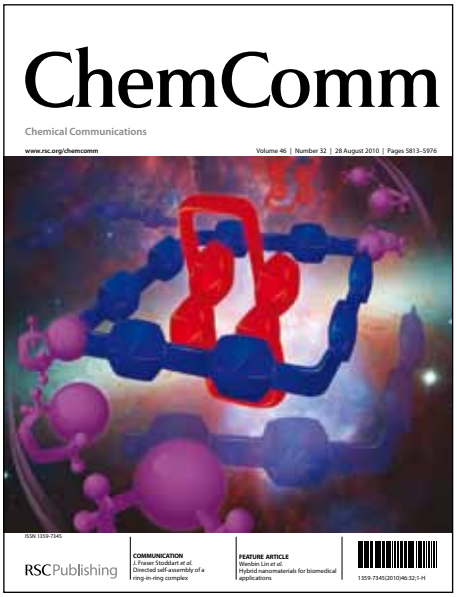

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COMMUNICATION

Redox Tuning of Two Biological Copper Centers through Non-covalent Interactions: Same Trend but Different Magnitude†

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The same non-covalent interactions previously found to affect the redox potential (E_m) of the mononuclear T1 Cu protein azurin (Az) are shown to also fine-tune the E_m of the dinuclear Cu_A center in the same Az protein scaffold. The effects of these mutations are in the same direction but with smaller magnitude in the Cu_A site, due to dissipation of the effects by the dinuclear Cu_A center.

Redox processes and associated electron transfer (ET) reactions are at the heart of most chemical and biological functions.[1] Redox active centres play a key role in these reactions. Two primary examples of such redox sites are the Type 1 (T1) copper centres and purple Cu_A centres, which are both well known for their low reorganization energy and high ET efficiency. Fine-tuning of the E_m of these redox active centres is essential in conferring and regulating their functions, as well as harnessing such sites for bio-inspired devices such as solar and fuel cells.[2] While the E_m of copper centres may be changed over a wide range through changing ligands, ligand geometries or solvents around the copper centre, these changes often result in dramatic changes in reorganization energy and thus ET efficiency. Therefore a significant challenge is to fine-tune the E_m of redox active centres without major perturbation of the physical properties of the redox active centre. Toward this goal, several successes have been reported by making mutations that alter non-covalent interactions near the metal centre.[3] For example, in a recent publication, we showed that hydrogen bonding and hydrophobic interactions around the T1 blue copper centre in Az (Figure 1a) can tune the E_m across the entire range of potentials attainable to native blue copper proteins and beyond.[3d] Despite this success, a critical question remained as to whether such interactions apply to other types of copper centres and whether the influence is in the same direction and with the same scale. Directly comparing the effects of individual mutations across different redox active centres is very difficult because the different amino acid sequences of different proteins make it impossible to de-convolute the exact effect of an individual mutation.

The purple Cu_A site has been shown to play a critical role in long range ET in complex proteins such as cytochrome *c* oxidase (C*c*O), the terminal oxidase in the respiratory chain responsible for energy production in all aerobic organisms.[4] The Cu_A centre consists of two copper ions coordinated by two bridging cysteine (Cys) residues and two histidine (His) residues, one for each copper, as well as several longer range ligands and ionic interactions (Figure 1b).[5] Cu_A has also been shown to be one of the most efficient ET sites in biology, exhibiting exceptionally low reorganization energies, even lower than that of the blue copper centre.[6] While blue T1 copper proteins are widely employed along the ET chain due to their wide range of E_m, purple Cu_A sites are only found at the terminal position in the respiratory chain, which may be due to a greater resistance to mutational changes. Previously, we and others reported engineering a Cu_A centre into blue copper Az by loop-directed

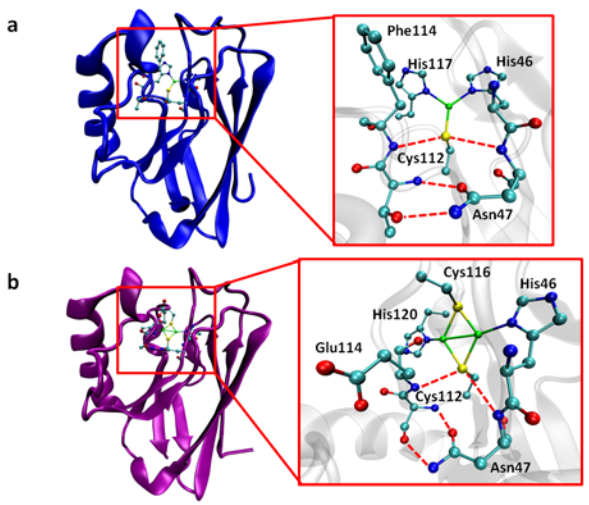

Fig. 1 Crystal structures of blue copper Az and purple Cu_A Az. (a) Blue copper Az with the active site contains a single thiolate bound copper (PDB ID: 4AZU).[5] (b) Purple Cu_A Az shows similar protein scaffold as blue copper Az, with the active site consists of dinuclear copper in a diamond core geometry (PDB ID: 1CC3).[7] The mutation sites, Asn47 and Glu114 in purple Cu_A Az, as well as Asn47 and Phe114 in blue copper Az, are shown.

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Table 1. Comparison of E_m (mV *vs.* NHE) of Asn47Ser and Glu114Pro in Cu_A Az with Asn47Ser and Phe114Pro in blue copper Az. For the mutated proteins, the difference in E_m compared to non-mutated protein is shown in parenthesis.

Purple Cu_A				Blue Cu Az		
Variants	E_m (pH 7.0)	E_m (pH 5.1)	E_m from spectrochemical titration (pH 5.1)	Variants	E_m (pH 7.0)	E_m (pH 5.1)
Cu_A Az	277 ± 21	264 ± 4	253 ± 1	WT Az	286 ± 8	347 ± 8
Asn47Ser	307 ± 7 (+30)	337 ± 4 (+73)	275 ± 14 (+22)	Asn47Ser	385 ± 3 (+99)	439 ± 2 (+92)
Glu114Pro	235 ± 3 (-42)	-	214 ± 8 (-39)	Phe114Pro	219 ± 8 (-67)	327 ± 3 (-20)

mutagenesis.[8] Therefore Cu_A Az provides a unique system to determine if the same mutations that fine-tune the E_m of blue copper Az will generate the same effect in Cu_A centre in the same protein scaffold. In a previous study, we showed that the Cu_A site in Az was relatively unaffected by the identity of the axial ligand to one of the copper, an interaction that has been shown to be critical to the T1 site.[9] However, the effect of non-covalent interactions such as hydrogen bonding networks is more subtle than that of the axial ligand. Herein, we report that the same two representative mutations (Asn47Ser and Glu114Pro) in Cu_A Az resulted in a similar trend of change in reduction potentials of Cu_A in blue copper Az, but the magnitude of the change less than that observed in Az. In the blue copper site, both the Asn47Ser and Phe114Pro mutations alter hydrogen bonding interactions near one of the copper ligands (Cys112), but one (Asn47Ser) increases the reduction potential while the other (Phe114Pro) decreases it (Table 1).[3d] The same trend is observed with the Asn47Ser and Glu114Pro mutations in Cu_A Az, but with a smaller magnitude. The reason for such a difference is discussed and a comparison is made with the effect of axial ligand mutations on the E_m of the same two Cu centers.

The Asn47Ser and Glu114Pro variants of purple Cu_A Az were prepared following the reported procedures.[8b,8c,10] The electrospray ionization mass spectrometry (ESI-MS) results of purified variants showed that the experimentally determined molecular weights of metal-free apo proteins match the corresponding calculated molar mass for both variants within the standard deviation of ESI-MS (See Table S1 and Figure S3). Upon addition of Cu^{2+} into the apo proteins, both variants displayed a strong purple colour, with UV-vis spectra very similar to that of unmodified Cu_A Az (see Figure S1). Both samples show characteristic intense charge transfer (CT) bands at ~480 and 530 nm, corresponding to $S_{cys}(p_x)\rightarrow Cu\ \psi^*$ (HOMO) and $S_{cys}(p_y)\rightarrow Cu\ \psi^*$ (HOMO), and a weaker band at ~760 nm, assignable to Cu–Cu $\psi\rightarrow\psi^*$ transitions.[4d] These results indicate that the two mutations did not perturb the structure of the Cu_A centre significantly.

To further investigate the effects of the mutations on the geometric and electronic properties of the Cu_A centre, EPR spectra were also collected at 30K in either 50 mM ammonium acetate (NH_4OAc) at pH 5.1 or in a temperature-independent buffer at pH 7 (TIP 7) (see Table S1 and Figure S2).[11] All samples at pH 7 displayed well-defined seven-line EPR hyperfines, and similar *g* values to those of other Cu_A centres reported previously.[12] The observed EPR are consistent with a single unpaired electron that is delocalized over two mixed valent copper ions. These spectroscopic results show that, similarly to the blue copper site, these alterations in Cu_A Az have a minimal effect on overall site geometry. In fact, mutations to the axial ligand in Cu_A Az resulted in substantially larger changes to the electronic spectra of the Cu_A site than those observed here.

To determine the effects of the Asn47Ser and Glu114Pro mutations on the E_m of the Cu_A centre in Az, both cyclic voltammetry (CV) and redox titration were carried out. Using the same procedure of CV measurements as reported previously,[13] we observed an E_m for Cu_A Az at 264 ± 4 mV *vs.* the normal hydrogen electrode (NHE) in 50 mM NH_4OAc buffer at pH 5.1 (Table 1); this potential is similar to that of previously reported value (271 ± 7 mV *vs.* NHE).[13a] Under the same condition, the E_m of Asn47Ser was determined to be 337 ± 4 mV, ~70 mV higher than that of Cu_A Az. However, we could not observe CV signal for Glu114Pro using the same method, despite numerous attempts. Therefore, we performed the CV measurement using 100 mM potassium phosphate (KPi) buffer at pH 7.0, as the higher pH increases the electrostatic attraction between the protein and the didodecyldimethylammonium bromide (DDAB) modified electrode. Under this condition we were able to observe signals for all three proteins, with E_m values of the Asn47Ser variant (307 ± 7 mV) being consistently higher than that of unmodified Cu_A Az (277 ± 21 mV), while the E_m of Glu114Pro variant being lower (235 ± 3 mV) (see Table 1 and Figure S4). While the removal of the negatively charged Glu residue from close proximity to the copper site should also change the redox potential, the direction of the change is wrong if the effect is purely electrostatic in nature. Removing negative charge from nearby the metal site should destabilize the oxidized state, thereby raising the redox potential.

As an independent confirmation of the trend observed in CV, spectrochemical titrations of the Cu_A Az variants with $[Ru(NH_3)_5Py](ClO_4)_2$ were also performed. The results showed the identical trend in E_m as was seen with CV (see Figure S5 and S6). The effect of the Asn47Ser and Glu114Pro mutations on the Cu_A reduction potentials is therefore very similar to that observed in blue copper Az,[3d] indicating that the same fine-tuning of the hydrogen bonding network around the copper ligand Cys112 of blue copper center is also operational in the purple Cu_A centre. Interestingly, the magnitude of the change in E_m of Cu_A Az is significantly less than that displayed in the blue copper Az (Table 1). Since the Cu_A site has two Cys thiolate ligands, the same mutational effects on the hydrogen bonding network around Cys112 are not as dramatic as in blue copper Az. The dissipation can be explained by the spreading-out effect by the second Cys ligand in Cu_A site. In this study, the hydrogen bonding environment surrounding the second Cys (Cys116) was left unperturbed. Furthermore, the bridging thiolate moiety is electronically more electropositive than a thiolate bound to one single metal, which would likely make a bridging thiolate less affected by such hydrogen bonding interactions. While changes to the axial ligand in other Cu_A containing systems have been shown to have a larger effect on the redox properties as compared

to Cu_A Az,[14] we have previously reported that replacing the conserved axial ligand methionine had only a small effect on the Cu_A centre in Az as compared to blue copper Az.[9] We now show non-covalent interactions such as hydrogen bonding interactions can similarly influence the redox potentials. More importantly, we show that such effects from hydrogen bonding caused bigger changes (~50 mV) than the changes of axial ligand (~ 25 mV) in this system.

In summary, we show here that the same alterations to the hydrogen bonding network around a Cu_A site produce the same effects to the redox potential as were seen in the blue copper site. While the magnitude of the changes was not as large in the Cu_A site, the trend of the change is the same as compared to the blue copper site. When combined with various other studies on the blue copper site, iron-sulphur clusters and haem proteins,[3d,3h,15] this study further supports the conclusion that fine-tuning functional properties like E_m by altering the non-covalent hydrogen bonding interactions around metal-binding sites in ET proteins is a general approach to regulate functional properties of all metalloproteins. Such interactions were seen to have an even greater effect than the primary coordination sphere mutations to the axial ligand in Cu_A Az. While non-covalent interactions dictate the directions of the redox potential changes, the nature of redox centres influences the scale of the changes.

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