Howard Hughes Medical Institute

Published as: *Chem Commun (Camb)*. 2016 December 20; 53(1): 224–227.

Probing the Role of the Backbone Carbonyl Interaction with the Cu_A Center in Azurin by Replacing the Peptide Bond with an Ester Linkage

Kevin M. Clark[a], **Shiliang Tian**[b], **Wilfred A. van der Donk**[a,b], and **Yi Lu**[a,b]

[a]Department of Biochemistry University of Illinois at Urbana-Champaign 600 South Mathews Avenue Urbana IL 61801

[b]Department of Chemistry University of Illinois at Urbana-Champaign 600 South Mathews Avenue Urbana IL 61801

Abstract

The role of a backbone carbonyl interaction with an engineered Cu_A center in azurin was investigated by developing a method of synthesis and incorporation of a depsipeptide where one of the amide bonds in azurin is replaced by an ester bond using expressed protein ligation. Studies by electronic absorption and electron paramagnetic resonance spectroscopic techniques indicate that, while the substitution does not significantly alter the geometry of the site, it weakens the axial interaction to the Cu_A center and strengthens the Cu-Cu bond, as evidenced by the blue shift of the near-IR absorption that has been assigned to the Cu-Cu $\psi \rightarrow \psi^*$ transition. Interestingly, the changes in the electronic structure from the replacement did not result in a change in the reduction potential of the Cu_A center, suggesting that the diamond core structure of Cu_2S_{Cys2} is resistant to variations in axial interactions.

Exploring the structure and function of metalloproteins requires intimate knowledge about the roles of residues around the metal-binding sites. Such knowledge forms a strong basis for successful design and engineering of novel metalloproteins with tunable functional properties.[1-6] The most common way to acquire such knowledge is site directed mutagenesis (SDM). Because SDM is limited to only ~20 proteinogenic amino acids, determining the precise role of the residues is difficult, as SDM often simultaneously changes more than one factor, such as electronic and steric effects, at the same time. Therefore, it is nearly impossible to de-convolute the contributions of each individual factor in such instances. To overcome this limitation, non-proteinogenic analogs of natural amino acids have been incorporated into metalloproteins.[7-11] For example, we have previously employed Expressed Protein Ligation (EPL) to replace conserved metal-binding amino acids with their iso-structural nonproteinogenic analogs.[12,13] Such replacements allowed us to de-convolute steric factors from other factors such as electronic effects and hydrophobicity, thereby firmly establishing the roles of these residues in metalloprotein function.[12, 14-16]

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

While most residues use side chains to interact with metal centers, increasing numbers of examples have appeared in the literature showing backbone carbonyl oxygens as metal-coordinating ligands. One such example is the Cu_A center found in cytochrome c oxidase (CcO) and nitrous oxide reductase (N_2OR).[17-20] Cu_A contains a dinuclear copper center bridged by two cysteine thiolates wherein each copper is also coordinated by a histidine (Figure 1). More interestingly, perpendicular to this "diamond core" structural plane, are three carbonyl oxygens of the backbone peptide linkage that are within 2.17 to 4.07 Å of the two copper centers, suggesting that these backbone carbonyl oxygens may play a key role in the formation and fine-tuning of the Cu_A center.[21] However, their roles in CcO, N_2OR, and other metalloproteins remain a mystery, as it is not possible to replace the backbone carbonyl oxygens using SDM or most other methods that introduce non-proteinogenic amino acids into proteins. Here we describe the first investigation of the role of backbone carbonyls in the structure and function of the Cu_A center by developing a method of synthesis and incorporation of a depsipeptide where one of the amide (Ψ-CONHR-) bonds in azurin is replaced by an ester (Ψ-COOR-) bond by EPL, and its subsequent characterization using electronic absorption (UV-vis) and electron paramagnetic resonance (EPR) spectroscopy and cyclic voltammetry. The method developed in this work can be applied to studying backbone carbonyl groups in other proteins.

We chose an engineered Cu_A center in azurin from *Pseudomonas aeruginosa* (hereafter "Cu_A azurin"), achieved by using loop directed mutagenesis.[22] Spectroscopic and x-ray crystallographic studies have confirmed that the Cu_A site thus engineered in azurin is nearly identical to that in CcO and N_2OR, including the "diamond core" structure and the three carbonyl oxygens of the backbone peptide linkage.[23-25] It also possesses the same functional properties as the native Cu_A, such as similar reduction potential and low reorganization energy during electron transfer. Cu_A azurin is an ideal model to probe the role of the backbone carbonyl oxygens involved in metal coordination. Cu_A azurin is much smaller, more stable and easier to express and purify with higher yield than CcO and N_2OR.[22, 26, 27] The backbone carbonyl of Glu114 is only 2.17 Å from one of the copper ions in Cu_A azurin (Figure 1).[22, 26] This Glu residue would be accessible through EPL using Cys^{112} as a ligation point for transthioesterification.

Linear synthesis of depsipeptides containing ester linkages has been reported previously in the literature.[28-30] However, surprisingly, application of depsipeptides in deciphering protein function is limited primarily to the study of α-helical hydrogen bonding networks or hydrogen bonds to substrates in catalysis, since esters display different hydrogen bonding properties than their amide counterparts.[31-33] Depsipeptides are also known for their increased susceptibility to base hydrolysis compared to their peptide counterparts, and some studies have utilized this property within proteins.[34-37] Conversely, because of their lability most reported Fmoc-based solid phase peptide synthesis (SPPS) methods for depsipeptides typically involve extensive optimization to minimize hydrolysis and reduce racemization observed when the standard protocols are used for ester synthesis. The synthesis of esters also requires longer reaction times that can further complicate the preparation of complex depsipeptides.

To investigate the interaction between the copper of the Cu_A center and carbonyl oxygen of Glu^{114}, a 19-mer peptide (H_2N-CSE[ΨCOO]LCGINHALMKGTLTLK-OH) containing an ester between residues Glu^{114} and Leu^{115} (called "19-mer depsipeptide" hereafter) was required. We initially adopted a standard Fmoc-based SPPS route to access the 19-mer depsipeptide (Scheme 1). Installation of the ester moiety was completed using DIC/DMAP chemistry to minimize racemization and the reaction was monitored using a hydroxyl specific test similar to the Kaiser Test.[30] Using piperidine to deprotect the Fmoc group on the residues following ester incorporation resulted exclusively in a truncated peptide spanning residues Leu114-Lys130. This truncation is likely a result of diketopiperizine formation upon deprotection of the Fmoc from Ser113 using piperidine. To reduce or eliminate diketopiperizine formation, an optimized deprotection strategy using 1% DBU (3 × 1 min) was employed. While the full length 19-mer yield was increased, truncated peptide was still prevalent necessitating an alternative route to obtain the 19-mer depsipeptide.

Accordingly, we synthesized the depsipeptide using Boc-based SPPS on PAM resin, thereby eliminating deprotection with a base. Removing exposure of the peptide to bases during synthesis resulted in greater peptide yields and little-to-no truncated peptide (Figure S1). Cys^{116} was protected with StBu to prevent inter- and intramolecular disulfide formation that otherwise reduced the efficiency of transthioesterification with Cys^{112} during EPL.[38, 39]

After successful synthesis and purification of the 19-mer depsipeptide, it was used in EPL reactions according to previously published procedures.[12, 14-16] During the EPL reaction, we noticed poor protein yields with depsipeptides lacking a Cys^{116}-StBu protection, which was attributed to disulfide bond formation with the Cys^{116} thiol. Furthermore, both the 19-mer depsipeptide and the ester Cu_A azurin protein product demonstrated high sensitivity of the ester linkage to hydrolysis at any pH above 7.5. MALDI-TOF MS analysis of EPL elutions in buffer at pH > 7.5 showed the primary product consisted of protein hydrolyzed between residues 113 and 114. Therefore, to increase the protein yield and reduce hydrolysis, buffers at pH 7.2 were used for all EPL reactions and protein storage.

Following the EPL reaction using the 19-mer depsipeptide, the product retained the Cys^{116}-StBu protection. A refolding step was necessary to obtain the Cu_A azurin with the intact metal site. Full-length metal–free apo protein from EPL reactions was refolded in ammonium acetate buffer containing 4 M guanidinium chloride and 0.9 mM dithiothreitol (DTT) immediately following ligation. The protein was then exchanged into ammonium acetate buffer containing 0.9 mM DTT before a final exchange into DTT free ammonium acetate buffer. Upon titration with Cu(II)SO_4, ester Cu_A azurin was formed. During the titration the protein turned purple in color, which is the same as observed for wild type (WT) Cu_A azurin.

To verify the ester Cu_A azurin contained the ester moiety instead of the normal amide moiety, base hydrolysis of the protein was performed by exposing the protein to 1 M NaOH. As shown in Figure 3, exposing the WT Cu_A azurin to 1 *M* NaOH for 20 min at room temperature resulted in no change of the protein band by SDS-PAGE (lanes 1 and 2). This result is not surprising, as native protein amide bonds are not susceptible to base hydrolysis. In contrast, exposure of the ester Cu_A azurin to 1 *M* NaOH showed partially hydrolyzed

product (lanes 4 (20 min) and 5 (60 min)) that was not found in the same protein that was not exposed to 1 *M* NaOH (lane 3). These results strongly suggest correct incorporation of the ester moiety.

After confirming successful preparation of Cu_A azurin containing an ester linkage at position 114, we collected an electronic absorption spectrum in the UV-vis range (UV-vis). As shown in Figure 3 the ester Cu_A azurin had absorption bands blue shifted to 475 nm, 530 nm, and 750 nm, compared to WT Cu_A azurin (Figure 3).[26] Specifically the near-IR band, assigned to a Cu-Cu $\psi \rightarrow \psi^*$ transition, shifted from 760 to 750 nm. Since the amide carbonyl group is a stronger base than the ester carbonyl, a weaker interaction would be expected between Cu and the ester carbonyl. Such a weakened Cu carbonyl interaction would result in a stronger Cu-Cu interaction in Cu_A azurin and a blue shift in the UV-vis spectrum. Previous mutations of the axial Met to Glu in both Cu_A azurin and the *Thermus thermophilus* Cu_A center resulted in a red-shift of the near-IR band from 760 to 855 nm, which was attributed to an elongated Cu-Cu distance due to a strong interaction between the Glu and the Cu.[24, 40] The weaker interaction as a result of the change from amide to ester carbonyl reported in this work produced an opposite effect of strengthening the Cu-Cu bond.

To investigate the effect of the ester bond on the Cu_A center further, we collected an X-band EPR spectrum of the ester Cu_A azurin. A 7-line hyperfine splitting, similar to WT Cu_A azurin was observed (Figure 4). Both Cu_A sites exhibit the same g values and parallel hyperfine couplings, $g_x = 2.02$, $g_y = 2.01$, $g_z = 2.17$ and averaged $A_z = 56 \times 10^{-4}$ cm^{-1} respectively, as indicated by the simulation. The major difference between the spectra of WT Cu_A and ester Cu_A azurins is the presence of a type 2 copper species in the ester Cu_A azurin, which accounts for 60 % of all Cu signal; a type 2 copper species is not expected to have contributed significantly to the electronic absorption spectrum in Figure 2, as it displays very low extinction coefficient due to the forbidden d-d transitions. Interestingly, the EPR parameters (see parameters in Table S1) is very similar to those of a C112S/C116S mutant of Cu_A azurin, suggesting that a portion of the ester Cu_A azurin contains no Cys coordination to the copper ion. This is not surprising, as the ester CuA azurin was synthesized by EPL and refolded in a high concentration of guanidinium chloride. As a result, a portion of the protein may not fold properly around the metal-binding site to allow Cys coordination to the copper ion. The interaction of the ester with the copper is similar to the native amide and therefore appears not to significantly alter either the structural or electronic integrity of the Cu_A site. Substitution of the amide with an ester appears to only mildly affect the Cu_A site and thus likely does not appear to play a significant role in maintaining the site geometry.

To investigate the effect of the amide to ester replacement on the redox properties of the Cu_A center, cyclic voltammetry was employed following the reported procedure for WT Cu_A azurin.[24, 41] In our hands, the reduction potential of WT Cu_A azurin was determined to be 277 ± 3 mV vs NHE (Figure S2), matching the reported value.[24, 41] Interestingly, the redox potential for the ester Cu_A azurin under the same condition was 278 ± 5 mV (Figure S2). Previous studies of Cu_A azurin has shown that mutations of the axial Met to Glu, Gln or Leu resulted in only ~ 20 mV changes of the reduction potentials of the Cu_A azurin.[24] The lack of change in reduction potential observed for the amide to ester carbonyl change in another

axial position is consistent with previous reports of mutations at the other axial Met123 position[24,42] and leads us to conclude that the diamond core structure of Cu_2S_{Cys2} shown in Figure 1B is resistant to variations of axial interactions.

In summary, a Cu_A azurin variant consisting of an amide-to-ester replacement in the backbone has been prepared to investigate the properties of the carbonyl interaction with the Cu_A center. This work marks the first such study to investigate backbone carbonyl interactions with bound metal ions in a metalloprotein. While the substitution does not significantly alter the geometry of the site, it weakens the axial interaction to the Cu_A center and strengthens the Cu-Cu bond, as evidenced by the blue shift of the near-IR absorption that has been assigned to Cu-Cu $\psi \rightarrow \psi^*$ transition. Interestingly, the changes in the electronic structure from the replacement did not result in a change in the reduction potential of the Cu_A center, suggesting that the diamond core structure of Cu_2S_{Cys2} is resistant to variations of axial interactions.

Acknowledgments

We wish to thank Dr. Yang Yu for helpful discussions. This work was supported by grants from CHE 14-13328 (to Y.L.) and the Howard Hughes Medical Institute (to W.A.V.). The authors would like to thank the Illinois EPR facilities including Dr. Mark Nilges for help in collecting EPR spectra.

Notes and references

1. De Grado, William F., Summa, CM., Pavone, Vincenzo, Nastri, Flavia, Lombardi, A. Annu Rev Biochem. 1999; 68:779–819. [PubMed: 10872466]
2. Lu Y, Berry SM, Pfister TD. Chem Rev. 2001; 101:3047–3080. [PubMed: 11710062]
3. Reedy CJ, Gibney BR. Chem Rev. 2004; 104:617–650. [PubMed: 14871137]
4. Lu Y, Yeung N, Sieracki N, Marshall NM. Nature. 2009; 460:855–862. [PubMed: 19675646]
5. Zastrow ML, Pecoraro VL. Coord Chem Rev. 2013; 257:2565–2588. [PubMed: 23997273]
6. Yu F, Cangelosi VM, Zastrow ML, Tegoni M, Plegaria JS, Tebo AG, Mocny CS, Ruckthong L, Qayyum H, Pecoraro VL. Chem Rev. 2014; 114:3495–3578. [PubMed: 24661096]
7. Lu Y. Curr Opin Chem Biol. 2005; 9:118–126. [PubMed: 15811795]
8. Clark, KM., van der Donk, WA., Lu, Y. Methods Enzymol. Tom, WM., John, NA., editors. Vol. 462. Academic Press; 2009. p. 97-115.
9. Minnihan EC, Nocera DG, Stubbe J. Acc Chem Res. 2013; 46:2524–2535. [PubMed: 23730940]
10. Hu C, Chan SI, Sawyer EB, Yu Y, Wang J. Chem Soc Rev. 2014; 43:6498–6510. [PubMed: 24699759]
11. Lewis JC. Curr Opin Chem Biol. 2015; 25:27–35. [PubMed: 25545848]
12. Garner DK, Vaughan MD, Hwang HJ, Savelieff MG, Berry SM, Honek JF, Lu Y. J Am Chem Soc. 2006; 128:15608–15617. [PubMed: 17147368]
13. Clark KM, Yu Y, Marshall NM, Sieracki NA, Nilges MJ, Blackburn NJ, van der Donk WA, Lu Y. J Am Chem Soc. 2010; 132:10093–10101. [PubMed: 20608676]
14. Berry SM, Gieselman MD, Nilges MJ, van der Donk WA, Lu Y. J Am Chem Soc. 2002; 124:2084–2085. [PubMed: 11878940]
15. Berry SM, Ralle M, Low DW, Blackburn NJ, Lu Y. J Am Chem Soc. 2003; 125:8760–8768. [PubMed: 12862470]
16. Ralle M, Berry SM, Nilges MJ, Gieselman MD, van der Donk WA, Lu Y, Blackburn NJ. J Am Chem Soc. 2004; 126:7244–7256. [PubMed: 15186162]
17. Iwata S, Ostermeier C, Ludwig B, Michel H. Nature. 1995; 376:660–669. [PubMed: 7651515]

18. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S. Science. 1995; 669:1069–1074.
19. Vila, AJ., Fernández, CO. Handbook on metalloproteins. Bertini, I.Sigel, A., Sigel, H., editors. Dekker; New York: 2001. p. 813-856.
20. Morgada MN, Abriata LA, Zitare U, Álvarez-Paggi D, Murgida DH, Vila AJ. Angew Chem Int Ed. 2014; 53:6188–92.
21. Robinson H, Ang MC, Gao YG, Hay MT, Lu Y, Wang AHJ. Biochemistry. 1999; 38:5677–5683. [PubMed: 10231517]
22. Hay M, Richards JH, Lu Y. Proc Natl Acad Sci U S A. 1996; 93:461–464. [PubMed: 8552661]
23. Berry SM, Wang X, Lu Y. J Inorg Biochem. 2000; 78:89–95. [PubMed: 10714710]
24. Hwang HJ, Berry SM, Nilges MJ, Lu Y. J Am Chem Soc. 2005; 127:7274–7275. [PubMed: 15898751]
25. Hwang HJ, Nagraj N, Lu Y. Inorg Chem. 2006; 45:102–107. [PubMed: 16390045]
26. Hay MT, Ang MC, Gamelin DR, Solomon EI, Antholine WE, Ralle M, Blackburn NJ, Massey PD, Wang X, Kwon AH, Lu Y. Inorg Chem. 1998; 37:191–198.
27. Hay MT, Lu Y. J Biol Inorg Chem. 2000; 5:699–712. [PubMed: 11128997]
28. Coin I, Beyermann M, Bienert M. Nat Protocols. 2007; 2:3247–3256. [PubMed: 18079725]
29. Coin I, Dölling R, Krause E, Bienert M, Beyermann M, Sferdean CD, Carpino LA. J Org Chem. 2006; 71:6171–6177. [PubMed: 16872202]
30. Kuisle O, Quiñoá E, Riguera R. J Org Chem. 1999; 64:8063–8075. [PubMed: 11674717]
31. Zickermann V, Wittershagen A, Kolbesen BO, Ludwig B. Biochemistry. 1997; 36:3232–3236. [PubMed: 9116000]
32. Lu W, Randal M, Kossiakoff A, Kent SBH. Chem Biol. 1999; 6:419–427. [PubMed: 10381402]
33. Desai BJ, Goto Y, Cembran A, Fedorov AA, Almo SC, Gao J, Suga H, Gerlt JA. Proc Natl Acad Sci U S A. 2014; 111:15066–15071. [PubMed: 25275007]
34. England PM, Lester HA, Dougherty DA. Biochemistry. 1999; 38:14409–14415. [PubMed: 10572015]
35. Guo J, Wang J, Anderson JC, Schultz PG. Angew Chem Int Ed. 2008; 47:722–725.
36. Watanabe T, Miyata Y, Abe R, Muranaka N, Hohsaka T. ChemBioChem. 2008; 9:1235–1242. [PubMed: 18418818]
37. Bindman NA, Bobeica SC, Liu WR, van der Donk WA. J Am Chem Soc. 2015; 137:6975–6978. [PubMed: 26006047]
38. Galonić DP, van der Donk WA, Gin DY. J Am Chem Soc. 2004; 126:12712–12713. [PubMed: 15469231]
39. Wade JD, Bedford J, Sheppard RC, Tregear GW. Pept Res. 1991; 4:194–199. [PubMed: 1823190]
40. Slutter CE, Gromov I, Richards JH, Pecht I, Goldfarb D. J Am Chem Soc. 1999; 121:5077–5078.
41. Hwang HJ, Lu Y. Proc Natl Acad Sci U S A. 2004; 101:12842–12847. [PubMed: 15326290]
42. Tsai ML, Hadt RG, Marshall NM, Wilson TD, Lu Y, Solomon EI. Proc Natl Acad Sci USA. 2013; 110:14658–14663. [PubMed: 23964128]

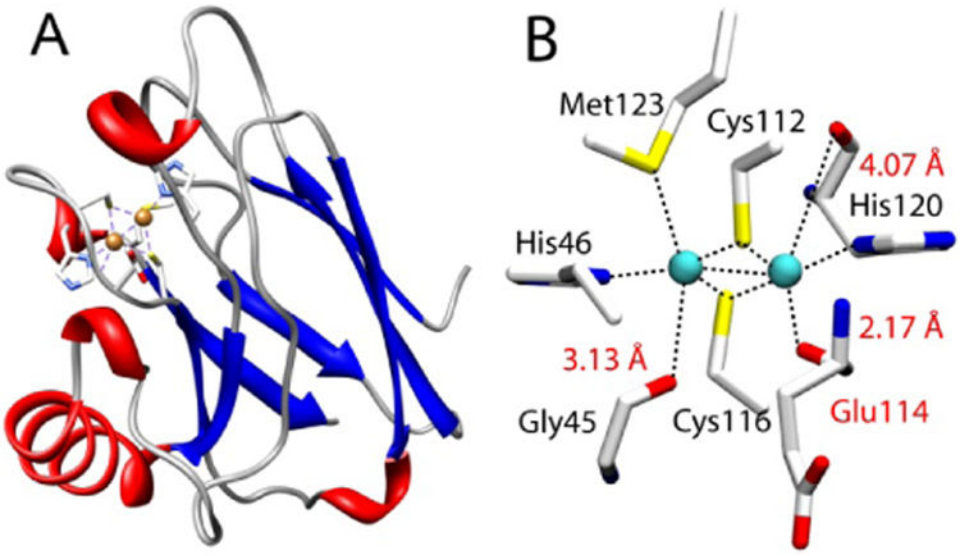


Figure 1.

Cu_A Azurin (PDB ID: 1CC3). A) The full-length Cu_A azurin scaffold. B) The Cu_A site in engineered purple Cu_A Azurin.

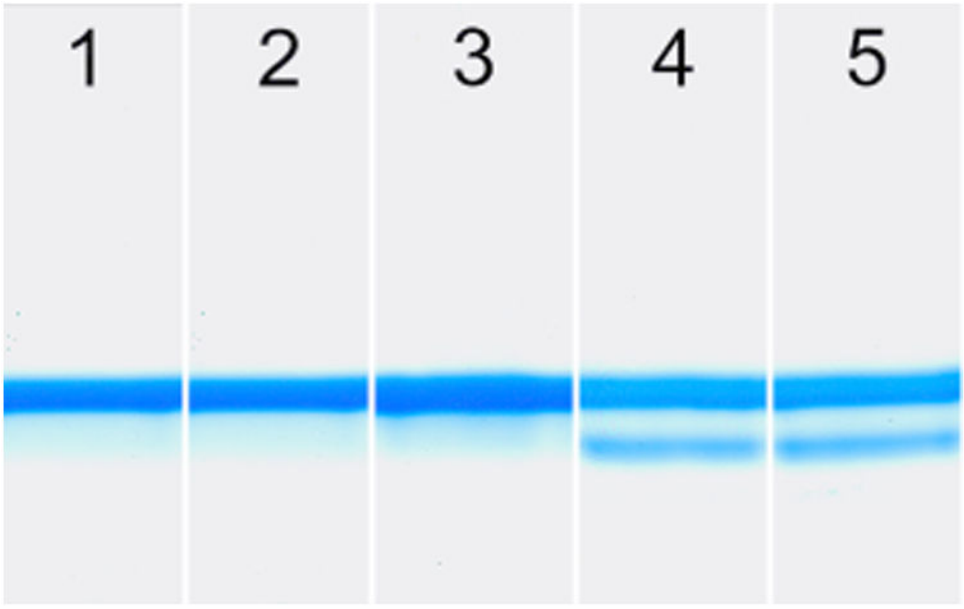


Figure 2.

SDS PAGE of recombinant WT Cu_A Azurin and EPL Ester Cu_A Azurin: **1)** WT Cu_A Azurin, **2)** WT Cu_A Azurin in the presence of 1 *M* NaOH for 20 min, **3)** Ester Cu_A Azurin, **4)** Ester Cu_A Azurin in the presence of 1 *M* NaOH for 20 min, 5) Ester Cu_A Azurin in the presence of 1 *M* NaOH for 60 min. The ester Cu_A Azurin protein hydrolyzes at the ester bond in the presence of NaOH, whereas the WT scaffold does not show hydrolysis.

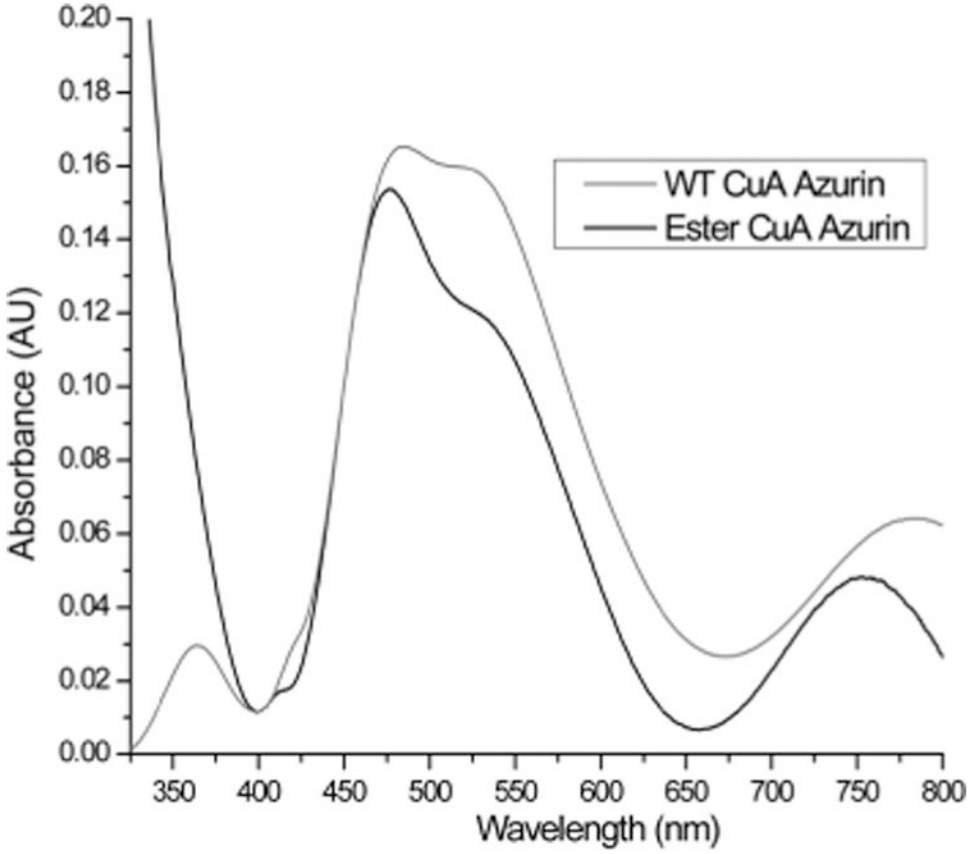


Figure 3.

UV-vis spectra of ester Cu_A azurin with absorption bands at 475 nm, 530 nm, and 750 nm and WT Cu_A azurin with absorption bands at 480 nm, 530 nm, and 775 nm.

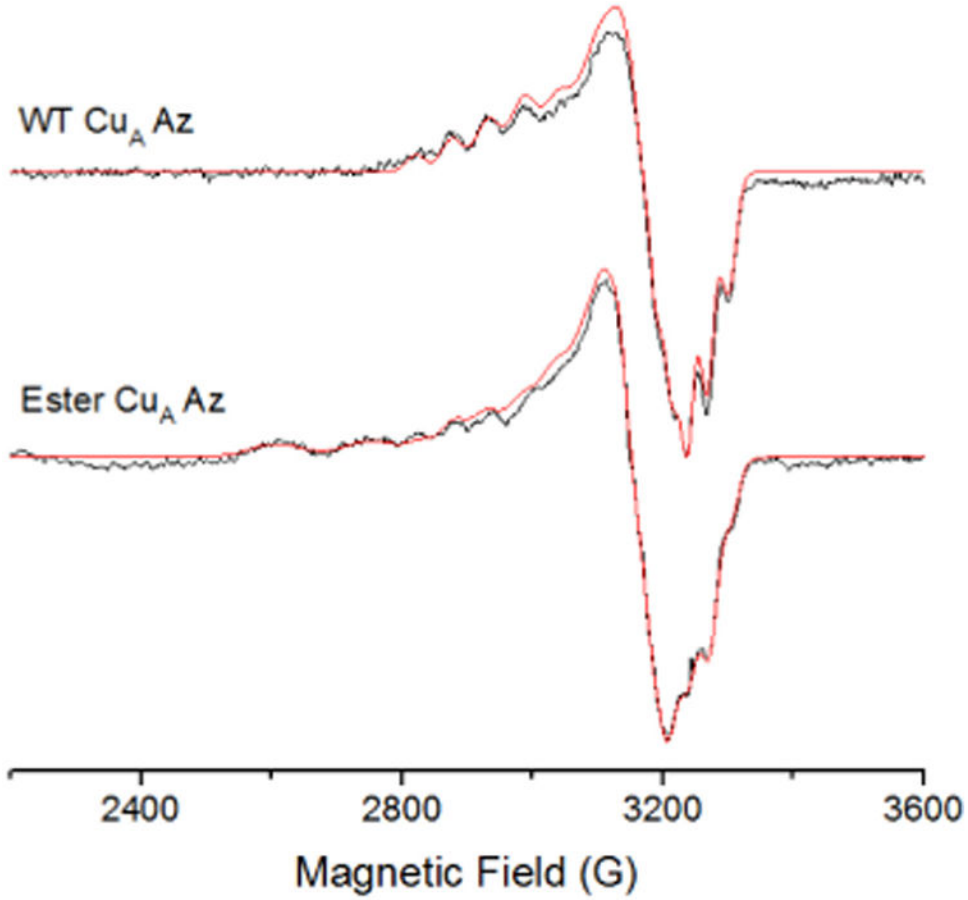
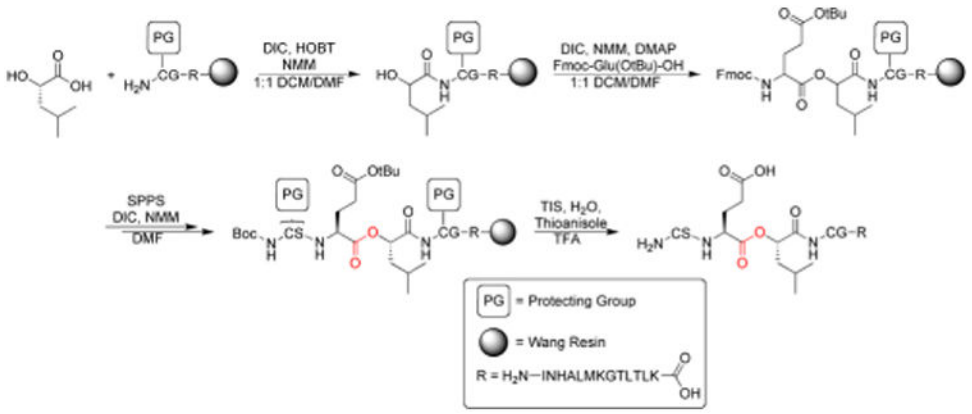


Figure 4.

X-band EPR of WT Cu_A and ester Cu_A azurin. The 7-line Cu hyperfine is present for ester Cu_A azurin with similar *g* values. The simulated spectra are shown in red. Parameters for the simulations are presented in the Supporting Information.



Scheme 1.
Synthesis of the 19-mer peptide for EPL.