

Designing Novel Blue Copper and Purple Cu_A Centers Into Azurin With Natural and Unnatural Amino Acids

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Final Defense

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Designing proteins with novel functions or selectively modulating protein active sites has been a long-standing goal of the protein chemistry field. Given the estimate that up to 50 % of proteins in biological systems utilize metal ions¹ and that many of these are catalytic metal ions, the design of metal binding sites into proteins is an important goal. Advancements of molecular biology techniques, such as site-directed mutagenesis, have provided powerful tools for fine-tuning the activity and probing the roles of residues in proteins. Many new metal binding sites have been designed into existing proteins in order to understand metal site design.² Particularly interesting is loop-directed mutagenesis which has been used to successfully engineer metal binding sites into protein scaffolds, including the design of a dinuclear copper center into the blue copper protein azurin (Figure 1).³ Developing new aspects of protein active site design is the focus of this work, with a goal of addressing unanswered questions about the dinuclear Cu_A site, the type 1 copper site of azurin, and the design of an iron sulfur cluster binding site.

The Cu_A center is a dinuclear, $\text{Cu}_2\text{S}_2(\text{Cys})$ electron transfer center found in cytochrome *c* oxidase and nitrous oxide reductase.⁴ The Cu_A site, structurally unknown until 1995 is now known to be a dinuclear, class III mixed valence copper center, efficient at electron transfer. We probed the role of His120 in Cu_A azurin by mutating it to Asp, Asn, Ala, and Gly.^{5,6} Remarkably, the His120X azurin variants bind two copper ions. UV-visible absorption, CD, MCD, resonance Raman, EXAFS, and ENDOR data of the mutants strongly resembled the parent Cu_A azurin spectra and suggest that the mutants resemble valence delocalized Cu_A centers.⁷ However, four line EPR spectra of the mutants show that the site is valence trapped. The possible ligands replacing His120 were ruled out by titrations with exogenous ligands and by mutating a possible internal residue. The results indicate that, while His120 is important for modulating the geometric and electronic structure of the center, an unknown replacement ligand helps

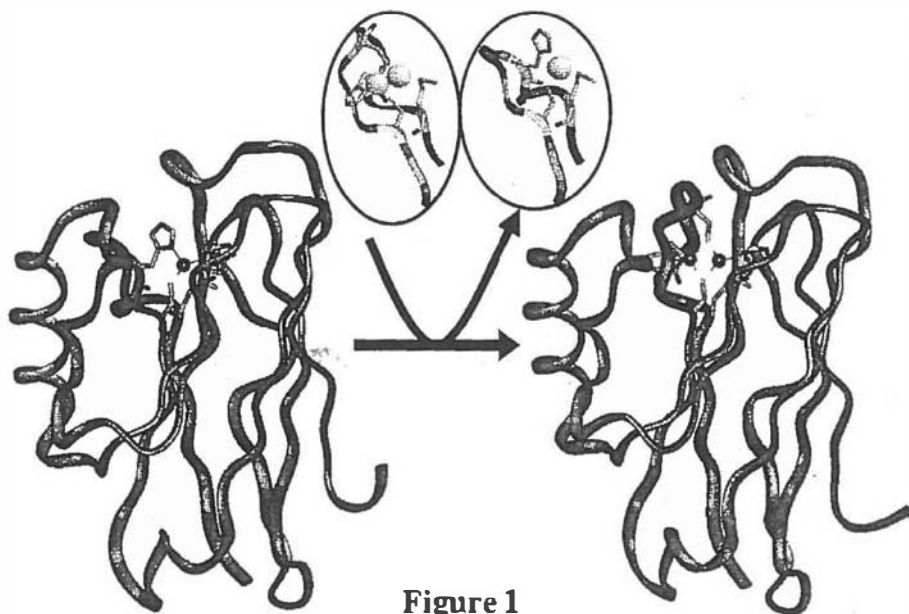


Figure 1

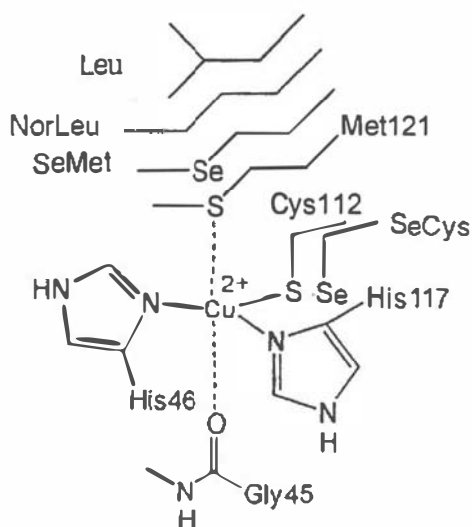


Figure 2

maintain the dinuclear structure of the site. Future work will attempt to identify the replacement ligand by x-ray crystallography and by the modification of active site peptide carbonyl bonds through expressed protein ligation.

A limitation of the site-directed mutagenesis approach is the availability of only the 20 amino acids naturally occurring in the genetic code. The more recent technique of expressed protein ligation (EPL) provides an alternative route for protein synthesis that allows the incorporation of unnatural amino acids into proteins.⁸ The use of EPL to overcome this limitation is demonstrated (Figure 2). The highly conserved methionine residue was replaced with the isostructural amino acids norleucine (Nle) and selenomethionine (SeMet) in azurin, thereby fine-tuning the blue copper site characteristics.⁹ It was successfully demonstrated that EPL could be used to incorporate unnatural amino acids into the active site of a metalloprotein while preserving the proper protein fold. The similar UV-vis, EPR, and EXAFS spectroscopies for the mutants pointed to a small role for the axial ligand in influencing these properties. In contrast to the small spectroscopic changes, the reduction potentials of M121SeMet, M121Leu, and M121Nle are 25, 135, and 140 mV higher than that of WT azurin, respectively. The use of unnatural amino acids allowed deconvolution of different factors affecting the reduction potentials of the blue copper center. A careful analysis, including the plot of observed reduction potentials of the WT azurin and its variants with the corresponding hydrophobicity of the axial ligand side chain, revealed hydrophobicity as the dominant factor in tuning the reduction potentials of blue copper centers by axial ligands.

An azurin variant containing selenocysteine in place of cysteine at the blue copper center was also synthesized using EPL.¹⁰ The variant displays spectroscopic properties that are consistent with a decrease in covalency of the Cu-Se bond, compared to the Cu-S bond. Remarkably, the general type 1 copper characteristics are maintained, including similar reduction potentials. The selenolate selenoether complex of SeMet121 SeCys112 azurin was also prepared. The minimal changes in copper spectroscopies of the SeCys azurin with SeMet incorporation mirrored the results obtained with the WT derivative. This study illustrates that iso-structural substitution using EPL can fine-tune the structural and functional properties of a metal binding site without loss of its characteristics or metal binding properties. Further application of the EPL method to this and other metalloproteins will make it possible to tune protein active site structure and function in an unprecedented fashion.

Finally, the loop directed mutagenesis technique was used to incorporate a 2Fe-2S cluster binding loop from human ferrochelatase (HFC) into azurin. The appropriate 2Fe-2S cluster binding motif was determined by analysis of 2Fe-2S cluster protein structures. Three classes were identified, the 2&2, the 3&1 loop, and the 1&3 inverted loop motifs, and were classified according to the order of appearance of the iron ligands. The class best suited for incorporation into azurin, the 1&3 inverted loop motif, was found in HFC. The amino acid sequence for the final FeS azurin model will be discussed. Preliminary iron sulfur cluster binding studies are presented.

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