The 21st Amino Acid and Beyond: Novel Copper Sites in Azurin

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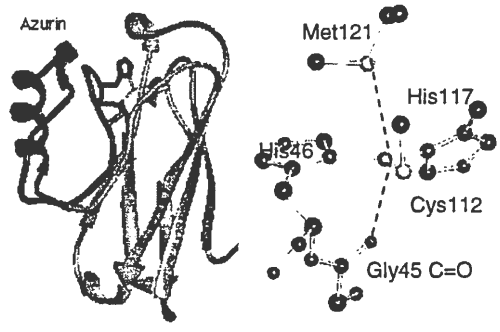
The design of metal binding sites into smaller protein frameworks for more facile study is an important means of modeling metalloenzymes. For example, the loop-directed mutagenesis technique[1,2] allowed the successful incorporation of the dinuclear Cu_A active site into the native, mononuclear copper site of *Pseudomonas aeruginosa* azurin.[1] However, further study and creation of this and other metalloenzymes is limited by normal site-directed mutagenesis with only 20 naturally occurring amino acids. In an effort to overcome this hurdle, we have utilized a new method, called expressed protein ligation (EPL),[3] to create azurin variants with unnatural amino acids as metal ligands. For the first time, unnatural amino acids were successfully incorporated into the active site of a metalloprotein using EPL.



Azurin (figure left) was chosen for EPL due to its stability and well studied spectroscopic properties. The structure of azurin from *Pseudomonas aeruginosa* is known.[4] The copper is in a pseudo trigonal bipyramidal coordination environment (figure right). Three of the copper ligands, Cys112, His117, and Met121, are in the last 17 amino acids and form a loop, separate of the main body. Azurin has been extensively characterized by a number of spectroscopic techniques.[5-7] The protein has an intense absorption peak at 625 nm, assigned to a S(Cys) to Cu(II) charge transfer band.[8,9] Also, the EPR spectrum is axial with $g_{||} = 2.260$ and a very narrow $A_{||}$ of 60×10^{-4} cm^{-1}.[8,9]

The axial methionine is highly conserved in blue copper proteins. The role of Met121 on the structure and function of the blue copper center, particularly the spectroscopic properties and reduction potential, has been proposed.[7,10-12] The shorter S(Met)-Cu(II) bond distance is believed to cause either a more tetrahedral or a more tetragonal distortion in the trigonal blue copper center, resulting in varied absorption spectra, different rhombicity of the EPR signals, altered Cu(II)-S(Cys) covalency, and different reduction potentials.[7,10-13] Mutations of Met121 to natural amino acids have been carried out in blue copper proteins.[14,15] Despite much progress, no clear trend in modulating the redox potentials and electronic structures of blue copper proteins has been established because many properties were altered with mutagenesis using natural amino acids.

The cysteine residue is known to play key roles in defining the structure and function of the blue copper site in azurin.[5,7] It has been shown that the Cu(II)-S(Cys) covalency is responsible for the intense blue color, the small hyperfine coupling constant in EPR spectra and the efficient electron transfer.[16] Mutation of Cys112 to Asp resulted in a "normal" type 2 copper center and a dramatic decrease in electron transfer rates.[17] However, with only natural amino acids for mutagenesis, the Cu-S covalency could not be modulated in a systematic way.

The role of the axial Met121 and the Cys112 ligands covalency in azurin was probed with the unnatural amino acids norleucine (Nle), selenomethionine (SeM),[18] and selenocysteine[19,20] (Sec). Recent spectroscopic data of these azurin variants containing unnatural amino acids will be presented. The successful employment of EPL in azurin marks the beginning of this technique in bioinorganic chemistry.

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