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Inner- and Outer-Sphere Metal Coordination in Blue Copper Proteins†

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Abstract

Blue copper proteins (BCPs) comprise classic cases of Nature's profound control over the electronic structures and chemical reactivity of transition metal ions. Early studies of BCPs focused on their inner coordination spheres, that is, residues that directly coordinate Cu. Equally important are the electronic and geometric perturbations to these ligands provided by the outer coordination sphere. In this tribute to Hans Freeman, we review investigations that have advanced the understanding of how inner-sphere and outer-sphere coordination affects biological Cu properties.

Keywords

blue copper; electron transfer; reorganization energy

1. Introduction

Blue copper (Type 1 or T1) sites in proteins, which are found in a remarkable variety of organisms ranging from bacteria to humans [1,2], have long fascinated chemists and biologists. Single T1 Cu centers are encountered in small proteins such as plastocyanin and azurin that shuttle electrons between various donors and acceptors. T1 Cu centers also are found in larger proteins such as multicopper oxidases and nitrite reductases, where they participate in intramolecular electron transfer (ET) reactions. The spectroscopic signatures of T1 Cu are an intense (ε = 2,000-6,000 M^{-1} cm^{-1}) ligand to metal charge transfer (LMCT) absorption around 16,000 cm^{-1} (conferring a striking blue color) and a very small ^{63,65}Cu hyperfine splitting in their EPR spectra ($A_{||}$ = 60-285 MHz). The latter feature in particular distinguishes T1 from type 2 (T2) Cu centers, which have large ^{63,65}Cu hyperfine splittings ($A_{||} \geq$ 450 MHz). The spectroscopic properties of blue copper proteins have been discussed in depth by Solomon and coworkers [3,4].

T1 Cu is one of Nature's ET workhorses. Its versatility is manifested by polypeptide-based control over standard reduction potentials ($E°$); $Cu^{II/I}$ potential tuning is a fascinating and

†Dedicated to the memory of Hans Freeman, a great scientist and dear friend.

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vibrant realm of blue copper redox chemistry. $Cu^{II/I}$ reduction potentials in BCPs span a large window ($E°'$ = 0.18 to >1 V versus NHE (normal hydrogen electrode)), suiting these proteins to redox with a wide variety of ET partners. Blue copper protein (BCP) reduction potentials also are notably higher than the standard aqueous reduction potential, $E°(Cu^{II/I})$ = 0.161 V [5]. It has been recognized for over a century that its inner coordination sphere most directly affects the redox properties of metal ions, and Lever has provided a clear illustration of such inner-sphere control in his parameterization of some metal complex reduction potentials [6]. Despite a wealth of data, an understanding of the effects of coordination on many other reduction potentials remains elusive. For example, $E°$ values have been reported for a great many Cu coordination complexes [7-10], but a predictive model has yet to emerge.

It also has been recognized for more than a century that ligands not directly coordinated to metal ions can control reactivity [11]. Werner's definition [12] that the outer coordination sphere is “a group coordinated directly to a ligand in the first sphere” is among the most intuitive, but is perhaps too limiting. For example, steric clashes within the peptide matrix can confer different geometries upon coordinated ligands. With this in mind, we propose a broadening of the term “outer coordination sphere” to include those groups that in any way influence the orientation and/or electronic properties of ligands directly coordinated to a metal center. Also included in this definition are electrostatic contributions and hydrophobicity or hyrophilicity effects that are particularly important in metalloprotein redox chemistry.

Outer-sphere coordination is an important determinant of the reorganization energy for an ET reaction. Nuclear reorganization of a metal center and coordinated ligands within a peptide can cause a “ripple” effect of other structural changes throughout a protein. All of these geometry changes contribute to the outer-sphere reorganization energy. Likewise, outer-sphere coordination exerts control over transition metal reduction potentials ($E°$). Hydrogen bonding (H-bonding) to or from coordinated residues, in particular, can tune the electronic properties of the metal center. Furthermore, positioning charged or uncharged hydrophilic residues can modulate potentials to favor more oxidized or more reduced metal centers.

Well documented cases of outer-sphere effects in biological redox chemistry include reduction potential tuning in Mn- and Fe-superoxide dismutases [13-15], oxygen binding in globins [16] and oxygen binding, activation and heme redox tuning in peroxidases [17]. Of special interest here is how inner- and outer-sphere coordination environments exert control over the spectral and redox properties of BCPs. We will pay a great deal of attention to *Pseudomonas aeruginosa* azurin, which within the present context is among the most studied BCPs.

2. Historical Perspective

BCPs came on the scene in the 1950s and 1960s as their striking blue color led to their observation and isolation from bacteria. These proteins had spectroscopic and chemical properties that could not be reproduced in model complexes, and their structures were not known. In the 1970s Hans Freeman turned his attention to structural BCP biology, and his target was plastocyanin. After several false starts, he found that poplar leaves could provide the right protein for crystal structure analysis. Many kilograms of poplar leaves were harvested to obtain the plastocyanin for structural work. He and Mitchell Guss reported preliminary structural data in 1977 [18], and in 1978 the 2.7 Å structure was published [19] At the time, poplar plastocyanin was one of only 41 protein structures available in the Protein Data Bank; there are now around 80,000 [20]. It would be hard to overstate the

critical importance of this structure to bioinorganic chemistry, as until the 1990s, it was the starting point for most, if not all, studies of BCPs and related model compounds.

3. Inner-Sphere Coordination

Blue copper sites are characterized by three strongly interacting ligands: one cysteine thiolate and two histidine imidazoles. Additional weak interactions are frequently encountered, typically involving an axial methionine thioether or glutamine amide. In the azurins, a backbone carbonyl provides a second axial ligand. Many BCPs have been characterized crystallographically: selected examples are shown in Figure 1. Multiple structures with better than 1.5 Å resolution are available, facilitating detailed structural analyses. The three strong ligands are arranged in a pseudo-trigonal manner; and the most commonly encountered coordination modes are trigonal pyramidal, trigonal bipyramidal and distorted tetrahedral. The Cu^{II}-S(Cys) bonds are remarkably short (2.1-2.3 Å) as a consequence of high Cu-S π-covalency [3]. Cu^{II}-N(His) bond lengths are 1.9-2.1 Å and typical of such interactions. When Met coordinates, the Cu^{II}-S(Met) bonds range from 2.6 to 3.2 Å. Where reduced (Cu^{I}) protein structures are available, the bonds are elongated by 0.1 Å or less and the structures of the oxidized and reduced proteins are nearly superimposable. One notable exception is amicyanin, where one of the ligating histidines (His95) is not bound to Cu^{I}. pH influences copper coordination in some wild-type (WT) proteins, but the effect in other cases is small, as shown for *P. aeruginosa* azurin. Metrical parameters for copper coordination in several representative blue proteins are set out in Table 1 [21-29].

Structures of apoproteins from azurin, plastocyanin and pseudoazurin are nearly identical with those of the respective holoproteins. However, the active site must have some conformational flexibility in order to bind copper in solution, as demonstrated in recent NMR investigations of apocupredoxins [30]. Once formed, outer-sphere interactions lock the active site in a geometry intermediate between that for Cu^{II} and Cu^{I} coordination.

Another feature of the binding site is the ability to accommodate other transition metal ions, including Co^{II} [31], Ni^{II} [32], Zn^{II} [33], Hg^{II} [34], and Au^{I} [35]. The Co^{II} and Ni^{II} proteins are redox-inactive within the aqueous window, but the structural features are similar to those of the Cu^{II} proteins. Metrical parameters for several metal-substituted azurins as well as one plastocyanin are given in Table 2. The bond lengths are all similar to those in Cu azurin, albeit with shorter M-O(Gly45) and longer M-S(Cys112) bonds. Notably, Hg^{II} plastocyanin shows an additional weak interaction with the backbone carbonyl of Pro46 that is absent in the WT Cu^{II} protein, consistent with the ~0.3 Å difference in ionic radii of Hg^{II} and Cu^{II}.

4. Reduction Potentials

Many small molecule BCP-mimics fall short of reproducing T1 structure, spectroscopy and reactivity [1,7,36-40]. It has been found that, in general, ligand sets that constrain complexes to (pseudo)tetrahedral symmetry raise reduction potentials, presumably owing to weak ligand fields. A few generalizations can be distilled [41] from the many factors that influence the reduction potentials of protein embedded metal ions [42,43]. Since nonpolar protein cavities disfavor charged species (as in organic solvents), the T1 $[Cu\text{-}Cys]^{+}$ state is disfavored and $Cu^{II/I}$ reduction potentials (Table 1) are higher than that of aquo Cu^{II} ($E°$ ($Cu^{II/I}$) = 0.161 V [5]). Residues near T1 Cu also can modulate reduction potentials. To cite one example, the surface proximal to T1 Cu in rusticyanin is hydrophobic/neutral, while the analogous region of azurin is more hydrophobic/negatively charged, thereby accounting for the relatively high rusticyanin $Cu^{II/I}$ potential [44]. Mutations that delete or introduce charged surface residues can substantially alter reduction potentials, as discussed below for azurin.

Reduction potentials for several BCPs are given in Table 3 [45-52]. Many of these reduction potentials have been obtained by several different methods, including solution redox titrations, optically transparent thin layer electrochemistry (OTTLE) and direct electrochemistry. The potentials in Table 3 are "best values" and in our view are accurate to ±10 mV. These BCPs exhibit a 0.5 V range in $Cu^{II/I}$ potentials, which is remarkable given the similarities in inner-sphere coordination and protein tertiary structure. Also, most BCPs have pH dependent reduction potentials, thereby indicating that reduction/oxidation is coupled to protonation/deprotonation of a residue near the Cu center, though the exact origin of the pH dependence is not always clear.

5. Inner-Sphere Effects on Blue Copper Properties

The spectroscopic and redox properties of BCPs depend strongly on the geometrical arrangement of ligands around the Cu center, which is intermediate between the preferences for Cu^{II} and Cu^{I}. Positioning the ligands in such a manner incurs some energetic penalty; as the active site is in an activated or "entatic" state [53,54]. This energetic cost is overcome in the case of BCPs by outer-sphere interactions between the Cu-ligating amino acids and surrounding residues [55]. These networks, including H-bonding interactions, have been referred to as "the rack," and as such the rack may be said to induce the entatic state [56], which we will take up again below.

Many investigators have probed the effects of altering the primary coordination sphere in BCPs. Substitution of Met121 in *P. aeruginosa* azurin with a hydrophobic residue without a donor atom (Ala, Val, Leu, Ile) increases the $Cu^{II/I}$ potential by 60-140 mV, while substitution with Glu markedly lowers the potential (His, Lys and Asp variants show little change) [45,57]. The single-pH measurements in the original report masked more complex behavior is some variants. Later pH dependent spectroscopic studies of the Met121Glu protein showed that Glu is probably not coordinated at pH 4 and the Cu has T1 character [58]. Glu121, which is deprotonated at higher pH, coordinates to Cu^{II}, lowering the $Cu^{II/I}$ potential and imparting T2-character to Cu^{II}. Likewise, several studies of Met121His azurin have shown that ligation of His121 is pH-dependent, strongly affecting the spectroscopic properties of the Cu^{II} center. Studies of these proteins led to the proposal of "type 1.5" Cu, which has features intermediate between those of T1 and T2 centers [59].

Met121Ala [60] and Met121Glu [61] variants have been structurally characterized (Figure 2). In the Met121Ala structure, the Cu-S(Cys112) bond contracts by 0.2 Å versus WT and the Cu-N3(His) bonds are elongated by about 0.1 Å versus WT. The Cu-S(Cys112) bond shows the same contraction in Met121Glu, while Cu-N3(His) is even more elongated and there is a Cu-O(Glu121) interaction (2.17 Å). In comparison to WT azurin, the Cu^{II} ion moves noticeably toward Glu121, while the rest of the ligating residues do not move. In the Ala121 variant, the Cu^{II} moves little with respect to WT azurin, but the ligating residues are displaced substantially based on an overlay of α-carbons. For reference, the RMS deviation between the ligating residues in WT azurin and Met121Ala is 0.213 and between WT and Met121Glu is 0.187.

Lu and coworkers extended Met121X mutations in azurin to include unnatural amino acids that are roughly isostructural with Met [62,63]. A key result was that the $Cu^{II/I}$ reduction potentials, which vary from 0.220 to 0.450 V, scale linearly with the water/octanol partition coefficient (log *P*); increasing hydrophobicity raises reduction potentials. Others have made Met → X mutations in amicyanin [64], cucumber basic protein [65] and rusticyanin [66] with similar results, confirming that variations in hydrophobicity affect BCP reduction potentials. Likewise, in stellacyanin [67] and umecyanin [65], which have no natural Met (see below), Gln → Met mutations increase reduction potentials.

More recently, Lu and coworkers replaced Met121 with Cys or homocysteine (Hcy) [68]. At low pH the Met121Cys variant exhibited many features of T1 Cu, while at high pH it became a “green copper” center with blue shifted spectral features and somewhat larger $A_{||}$. The Met121Hcy derivative showed radically different spectroscopic features and a low reduction potential, consistent with the T2, red Cu^{II} protein nitrosocyanin in which Cu^{II} is ligated by 2 His residues, as well as by Cys, Asp and H_2O in a nearly ideal trigonal bipyramidal geometry [69,70].

The roles of the key His-His-Cys residues in BCPs also have been elucidated. Sulfur π(Cys) → Cu $3d_x^2–y^2$ LMCT is responsible for the blue color of these proteins as well as certain other spectroscopic features [3]. Thus, it is not surprising that the Cys112Asp variant of azurin is comparatively colorless [71]. Broad bands in the visible spectrum attributable to d-d transitions appear near 15,000 cm^{-1} ($\varepsilon \sim 100\ M^{-1}\ cm^{-1}$) [71]. The EPR spectrum shows a much larger $A_{||}$ than WT azurin [71,72]. Both of these observations are consistent with conversion from T1 to T2 Cu [3]. Cys112Asp $E°'$ is 0.180 V; and there are noticeable geometry changes in the solid state [73].

The only other metalation-competent Cys112X azurin is the selenocysteine-substituted variant [74], which has been extensively characterized using a host of spectroscopic techniques [75,76]. The main band in the optical spectrum is red shifted to 14,700 cm^{-1} and the EPR spectrum shows a 2-fold increase in $A_{||}$ (to 310 MHz) versus WT azurin. The Cu-Se bond is elongated compared to Cu-S in WT azurin as determined by K-edge EXAFS ($d(Cu^{II}\text{-Se})$ = 2.30(2) Å) (EXAFS = extended X-ray absorption fine structure). Like WT azurin, the Cu-Se bond undergoes little change upon reduction ($d(Cu^{I}\text{-Se})$ = 2.33(2) Å), in further support of a rack-constrained coordination geometry [75].

Work from our laboratory has probed Met121X substitutions in the Cys112Asp scaffold [72,77]. As with WT azurin, replacement of Met with hydrophobic or positively charged residues raises the $Cu^{II/I}$ reduction potential. Notably, the Met121Glu variant displays anomalous behavior. Accompanying the raised $E°'$ values, replacement of Met121 with Leu (Figure 3), Phe or Ile results in proteins whose features are not those of either T1 or T2 Cu. The proteins lack intense charge transfer bands in the visible region and exhibit $A_{||}$ near 300 MHz. Moreover, they exhibit a 100-fold increase in ET reactivity relative to a Cys112Asp single mutant. We adopted the term “type zero” for this class of Cu^{II} proteins.

His117 is a key residue in ET reactions of azurin [78]. Canters and coworkers investigated azurin variants where His117 is replaced with glycine [79,80], which creates a “hole” in the azurin coordination sphere owing to a large rearrangement in the protein backbone [81]. The unsaturated coordination sphere allows for addition of exogenous ligands including halides, azide and imidazoles to the copper center. From UV-visible (UV-vis), Raman and EPR data it was concluded that addition of imidazole “rescues” the T1 Cu center, while addition of anionic ligands enhances T2-character [79]. The open coordination site also allows ET and structural investigations using imidazole-modified complexes [81,82,83].

His46 has been replaced with the other 19 amino acids [84]. All of the His46X variants were expressed in *E. coli* based on Western blot analysis, but only His46Asp has been extensively investigated. The $Cu^{II/I}$ reduction potential (0.266 V, pH 7) and $A_{||}$ (66 MHz) are characteristic for BCPs, but shifted somewhat, reflecting the introduction of an anionic aspartate ligand. NMR analysis suggests subtle structural changes to the Cu-binding pocket, as well as decreased Cu-S(Cys112) and increased Cu-S(Met121) interactions [85]. Intramolecular ET reactions between Cu^{I}-His46Asp azurin and a surface tethered Ru-oxidant are slower compared to those in WT azurin [86], owing to weaker electronic coupling associated with the His to Asp mutation.

Stellacyanin, and closely related protein, umecyanin [87], are unique among BCPs because the WT proteins do not have active-site methionine residues. Stellacyanin has a relatively low $Cu^{II/I}$ reduction potential (Table 3) and its EPR spectrum is distinct from those of other BCPs (it has substantial anisotropy in $g_{\perp}$: $g_x = 2.018$, $g_y = 2.077$) [88]. X-ray structures of the proteins showed that the axial ligand is the oxygen from a Gln amide (Gln99 in stellacyanin and or Gln95 in umecyanin) [29,87]. Importantly, the Cu atom is displaced below the His-His-Cys plane (toward Gln) by about 0.4 Å, making the geometry closer to tetrahedral than trigonal planar. By analyzing several spectroscopic features of WT, Gln99Met and Gln99Leu stellacyanin, DeBeer and coworkers showed that the axial ligand modulates the Cu geometry and also the $[Cu\text{-}SCys]^+$ covalency. They also concluded that the lowering of the $Cu^{II/I}$ reduction potential in stellacyanin is predominantly a result of decreased active-site charge (Z_{eff}, owing to the presence of Gln99). The Gln99Met mutation produces a stellacyanin with spectroscopic and redox properties similar to those of plastocyanin, as would be expected (both possess a His-His-Cys-Met ligand set).

pH can strongly affect coordination of the inner-sphere residues in BCPs. Guss and Freeman first conclusively illustrated this property in the analysis of poplar plastocyanin crystallized at 6 pH values [25]. The structure of the reduced proteins showed that the solvent exposed His87, which moves away from Cu^I as the pH is lowered, is fully dissociated below pH 4. Similarly, surface exposed His residues in related plastocyanins titrate with pK_as between 4.5 and 5.5 [89-91]. Dissociation of the surface exposed His also occurs in Cu^I amicyanin from two different sources, with pK_a values between 7.2 and 7.5 [27,92]. In contrast, the exposed His residues in azurin and rusticyanin remain coordinated to Cu across a wide range of pH [21,23]. The variability of surface His pK_as among BCPs likely is a result of very different outer-sphere interactions in the vicinity of that residue. Finally, BCPs where the exposed His is dissociated from Cu are less ET-active, probably because of their unfavorable reduction potentials [27,89,92]. Such behavior could play a role in regulating interprotein ET, as suggested for reaction of amicyanin with cytochrome c_{551} [27].

In sum, multiple studies have shown that the three strong ligands (His, His, Cys) in BCPs are largely responsible for the bright blue color and small $^{63,65}Cu$ hyperfine coupling of T1 Cu. Substitution of even one of those ligands typically results in dramatic electronic structural changes. The one notable exception to this rule is His46Asp azurin. Axial ligand substitution can strongly alter reduction potentials and (to a lesser extent) spectroscopic properties.

6. Outer-Sphere Coordination

The key role of outer-sphere coordination in BCPs was widely appreciated in the early 1990s. Once x-ray structures of several BCPs were available (Figure 4) it became apparent that residues not directly bound to the Cu center could affect spectroscopic and/or redox properties [21,45]. In azurin, the Cys112 thiolate accepts hydrogen bonds (H-bonds) from backbone amides of Asn47, and Phe114, and His46 donates a hydrogen bond to the backbone carbonyl of Asn10. In rusticyanin, Cys138-S accepts H-bonds from backbone amides of Ser86 and Ile140. Also, His85 donates an H-bond to the sidechain carbonyl of Asn80. The Cys84 thiolate of plastocyanin accepts one H-bond from a backbone NH (Asn38), and His37 interacts strongly with backbone carbonyls of Ala33, and more weakly with the backbone carbonyls of Leu5, Gly 34, and the backbone amide NH of Phe35. In amicyanin, the Cys92 thiolate weakly interacts (d(S-N) = 3.64 Å) with the backbone NH from Asn54. His53 interacts with the backbone carbonyl of Met51, but also forms a strong H-bond with the carboxylate of Glu49. The Cu-ligating Cys89 in stellacyanin accepts H-bonds from the sidechain of Asn47 and the backbone amide NHs of Asn47, and Val91. Notably, the NH_2 group of the ligating Gln89 interacts with N3 of the ligated His46 and the

indole NH of nearby Trp13. Finally, The Cu-ligating His imidazoles of cucumber basic protein do not interact with the peptide, but the Cys79 thiolate weakly interacts with backbone NHs of Asn40 and Phe81.

The above description is only part of the picture. Consider, for example, the H-bond network that orients Cys112, His117 and Met121 around the copper center in azurin (Figure 5). This network, which is coupled to other H-bonds that extend throughout much of the protein, is thought to mediate long-range intramolecular ET [93,94].

In sum, T1 Cu centers display not only similar inner-sphere coordination, but also share several common outer-sphere coordination motifs. The ligating Cys-thiolate accepts 1-2 H-bonds from backbone carbonyls, modifying Cu-Cys covalency, and thus reduction potentials. One of the ligating His residues donates an H-bond to a nearby backbone carbonyl or another sidechain; this interaction could orient His around the metal center, or could impart partial histidin*ate* character to the ligand, stabilizing higher oxidation states as in His-ligated peroxidases [17].

7. Outer-Sphere Effects on Blue Copper Properties

Perturbations to their outer coordination spheres can strongly affect the spectroscopic properties and reactivities of BCPs. In early work, azurin ET self-exchange reactions were shown to involve the hydrophobic patch near the H117-Cu site, as indicated by kinetics study of Met44Lys and WT azurin [95]. It was found that mutation of the surface residue Met64 to Glu decreases the rate constant by 10^2-fold when Glu is deprotonated [96]. The spectroscopic features of the Met64Glu variant resemble WT, while the reduction potential drops by *ca*. 25 mV. The lowered reduction potential likely results from electrostatic stabilization of Cu^{II} by the additional negative charge at the protein surface. Likewise, the Met64Lys mutation slows the self-exchange reaction and raises the reduction potential [95].

Phe114 is another key residue in the hydrophobic patch of azurin; its replacement affects the spectroscopic and structural features of the Cu center. The Phe114Ala and Phe114Val variants show distortions in Cu coordination, but only small changes in spectroscopic features [97]. The amide NH of Phe114 forms an H-bond with the Cys112 thiolate, so the structural changes probably are related to the additional conformational freedom when Ala or Val occupy position 114.

Dennison and coworkers have produced a Phe114Pro azurin variant, a mutation that eliminates the amide-Cys hydrogen bond entirely [98]. Like the Phe114Val and Phe114Ala variants, this mutation substantially changes how the backbone interacts with the ligating Cys112. Unlike WT azurin, the Cu atom position and Cu-L bond lengths change significantly (≥ 0.1Å) upon cycling between Cu^I and Cu^{II}. In the Cu^{II} mutant, the characteristic ~16,000 cm^{-1} band blue shifts by ~800 cm^{-1}, and the $Cu^{II/I}$ reduction potential is 90 mV lower than the WT protein. Removal of the Phe114-NH···Cys112 H-bond results in rotation of the Cu-Cys dihedral (7° in WT azurin and 34° in Phe114Pro azurin), while Cu moves away from Gly45 and toward Met121. In short, removal of one H-bond from the backbone to the Cys112 thiolate causes substantial changes in Cu coordination.

Dennison and coworkers also have systematically investigated the role of the loop region between Cys112 and Met121 on BCP properties [99-101]. Interestingly, changing the native azurin loop sequence (C^{112}TFPGH^{117}SALM^{121}) to the shorter plastocyanin loop (C^{112}SPH^{115}QGAGM^{120}, azurin numbering) results in an azurin-like global fold, but plastocyanin-like Cu properties (λ_{max}, $E°'$, $A_{||}$) [100]; and the same effect is found when the azurin loop is replaced with that of amicyanin, or when the plastocyanin loop is replaced

with that of azurin [99]. It is apparent that the loop region is a critical determinant of the geometric arrangement of ligands around Cu, and thus the properties of the active site.

Type zero Cys112Asp/Met121X (X = Leu, Phe, Ile) azurins represent cases where the rack controls electronic structure and reactivity. Removal of axial ligation from the Cys112Asp framework drives Cu^{II} toward the Gly45 carbonyl, and rotation of the Asp112 carboxylate attends this migration; in its resulting orientation, the coordination of this sidechain becomes monodentate. The nondonating (to Cu) O is positioned to H-bond with Phe114 and Asn47, restoring the rack. As in T1 Cu proteins, these H-bonds constrain Cu^{II} and Cu^{I} states to similar geometries, lowering the ET reorganization energy [102]. Moreover, the electronic structure undergoes a “coupled distortion” [103], where the singly occupied molecular orbital rotates such that Cu^{II} experiences a dramatically weaker ligand field, thereby giving rise to the A_z values characteristic of type zero copper [104].

Our investigation of the Cys112Asp/Met121X azurin scaffold not only resulted in the discovery of type zero copper, but also exposed subtler effects of outer-sphere coordination that would have otherwise been masked by the dominant Cu-S(Cys) interaction. Of special interest is Cys112Asp/Met121Glu, which has an anomalously high $Cu^{II/I}$ reduction potential ($E°'$ = 0.270 V, pH 7) that is pH dependent (pK_a near 8). It is likely that the high potential is attributable to the protonation of His35, which profoundly affects Cu coordination. At low pH, His35 is protonated and clashes with the ligating His46, effectively pushing the Cu away from Glu121, enforcing unfavorable three-coordination (Figure 6). At high pH, His35 is deprotonated, allowing Cu to “sink” toward Glu 121. The signature absorption in the optical spectrum red shifts and $A_{||}$ increases as the ligand field (LF) strength of the Cu center increases. Commensurate with these changes, the $E°'$ plummets to 120 mV, an example where steric clash, not H-bonding, exerts an outer-sphere effect. These seemingly subtle structural changes result in drastic changes in the properties of the copper center and should be considered in structural analyses of redox proteins.

Investigations of *P. denitrificans* amicyanin have shown that replacement of key surface residues distal to the Cu atom can result in changes in ET reactivity most likely attributable to perturbations to electrostatic interactions with redox partners [105]. Amicyanin is unusual because in the solid state His95 is not ligated to Cu^{I} [27]; the solution pH dependence of $E°$ ($Cu^{II/I}$) tracks with protonation/deprotonation of this residue. Interestingly, the amicyanin potential is shifted ($E°'(Cu^{II/I})$ = 0.22 V [106]) and pH independent in the presence of cytochrome c_{551} [27]. It is thought that when complexed to a protein redox partner, His95 must be (at least partially) coordinated to Cu^{I} due to steric clash with residues in the other protein. Conformational specificity is also though to play a role in rapid ET between azurin and cytochrome c_{551} [107]. It is possible that these types of protein-protein outer-sphere interactions play critical roles in redox function, as in the reactions of ribonucleotide reductases [108].

Replacement of amicyanin Pro94 with Ala or Phe results in substantial changes in $Cu^{II/I}$ reduction potentials, as well as absorption red shifting (16,700 to 16,400 cm^{-1}) and increasing $A_{||}$ (222 to 285 MHz). These mutations also strongly affect the pH dependence of the $Cu^{II/I}$ reduction potential [109], which could be understood when x-ray structures became available [110]. Replacement of Pro94 allows a backbone amide NH to donate an H-bond to the Cys92 thiolate, making the $[Cu\text{-}S]^{+}$ less electron rich and easier to reduce. Interestingly, His95 remains ligated to Cu^{I} in the solid state, perhaps because the pK_a shift induced by the mutation to Pro94 disfavors protonation of His95 [109]. Analysis of x-ray structures showed that Phe94 is oriented “in” toward the body of the protein, thereby causing steric clash and rearrangement of Met71; the backbone around Phe94/His95/Pro96 also shifts, promoting a conformation that pushes His95 toward Cu. Notably, the shift from

0.385 (Pro94Ala) to 0.415 V (Pro94Phe) is consistent with azurin work that showed that introduction of Phe residues near the Cu center increases $E°'(Cu^{II/I})$ by about 30 mV per Phe [111].

In related work on plastocyanin, Spiro and coworkers found that replacement of plastocyanin Asn38 with Gln, Thr or Leu produced a protein that would irreversibly lose Cu over time [112]. While the position 38 mutants were not structurally characterized, it is known from the WT structure that the Asn38 amide NH H-bonds to the Cys84 thiolate. Also, the terminal amide of Asn38 H-bonds with Ser85, which could contribute to T1 Cu site stability. Spiro concluded that these outer-sphere interactions protect the T1 sites from misligation and Cys84 from oxidation.

8. Concluding Remarks

Great strides have been made in understanding the remarkable properties of T1 Cu centers. We now know that it is not only the primary coordination sphere that modulates the $Cu^{II/I}$ reduction potential and spectroscopic features, but key contributions also come from the interplay between the Cu ligands and the surrounding protein matrix. The three strong ligands (His-His-Cys) are necessary for blue copper features, as replacement of even one of these ligands produces nonblue properties. In some cases T1 Cu can be rescued by exogenous ligands, or new behavior (type zero) can be introduced with additional mutations.

We also have highlighted a set of common features for outer-sphere coordination in blue copper [11]. First, the ligating Cys thiolate accepts H-bonds from backbone amides, contributing to changes in T1 Cu-Cys covalency. In most BCPs one of the ligating His residues donates H-bonds to backbone carbonyls or other sidechains. These interactions may orient the His or impart histidin*ate* character on this ligating residue, as has been suggested in studies of CueO [113] and Fet3p [114]. Abolishing histidinate-promoting interactions by changing outer-sphere ligands destabilizes Cu^{II}, raising the $Cu^{II/I}$ reduction potential. Beyond these outer-sphere interactions are extensive H-bond networks throughout the protein. Some of these interactions are part of the rack that defines the spectral features and ET reactivities of T1 sites.

In sum, studies of blue copper proteins have elucidated many of the features that tune reduction potentials and promote redox reactions. It is of interest that Lu and coworkers rationally designed azurins with reduction potentials that span a 700 mV range using only a handful of inner- and outer-sphere mutations [115]. Related investigations of metal coordination in proteins have the potential to yield artificial enzymes for catalysis of very challenging chemical transformations.

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Highlights

- We present a focused review of metal coordination in blue copper proteins.
- Inner- and outer-sphere interactions tune properties of copper sites.
- Electronic structure and chemical reactivity of blue copper centers are discussed.

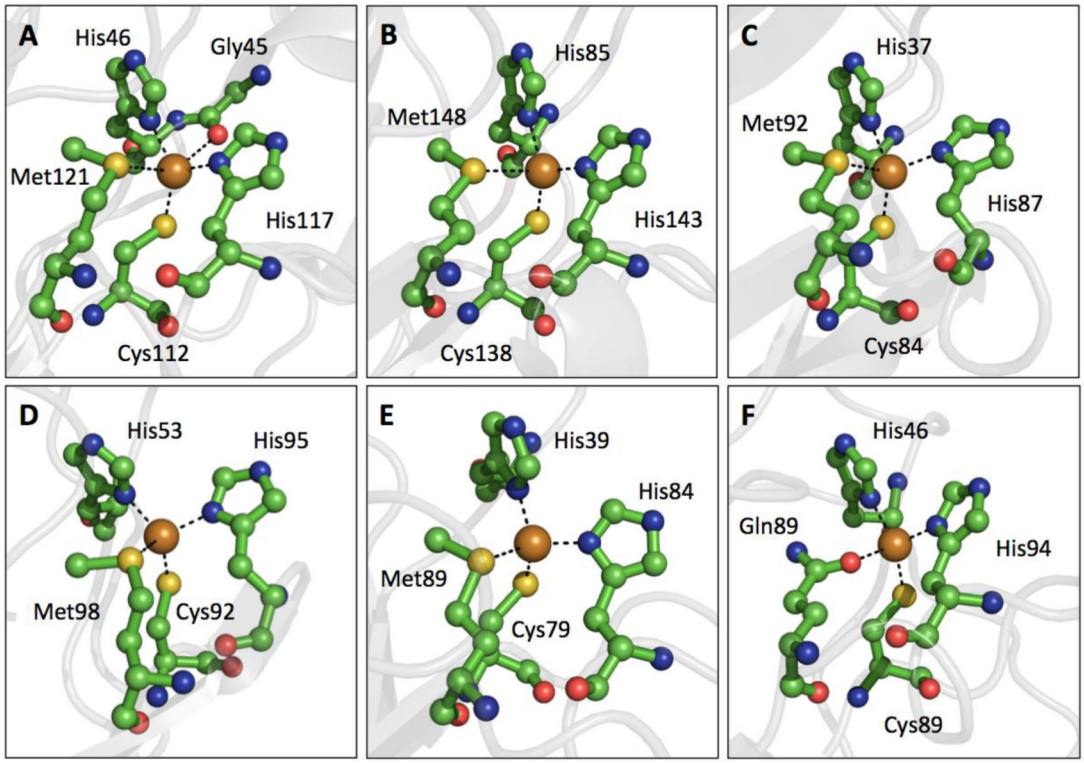

Figure 1.

Copper coordination in representative blue copper proteins in their oxidized forms. (A) *P. aeruginosa* azurin; (B) *T. ferrooxidans* rusticyanin; (C) *P. nigra* plastocyanin; (D) *P. denitrificans* amicyanin; (E) *C. sativus* cumber basic protein; (F) *C. sativus* stellacyanin. Spheres indicate carbon (green), oxygen (red), nitrogen (blue) and copper (tan). The protein tertiary structure is shown as a partially transparent grey ribbon.

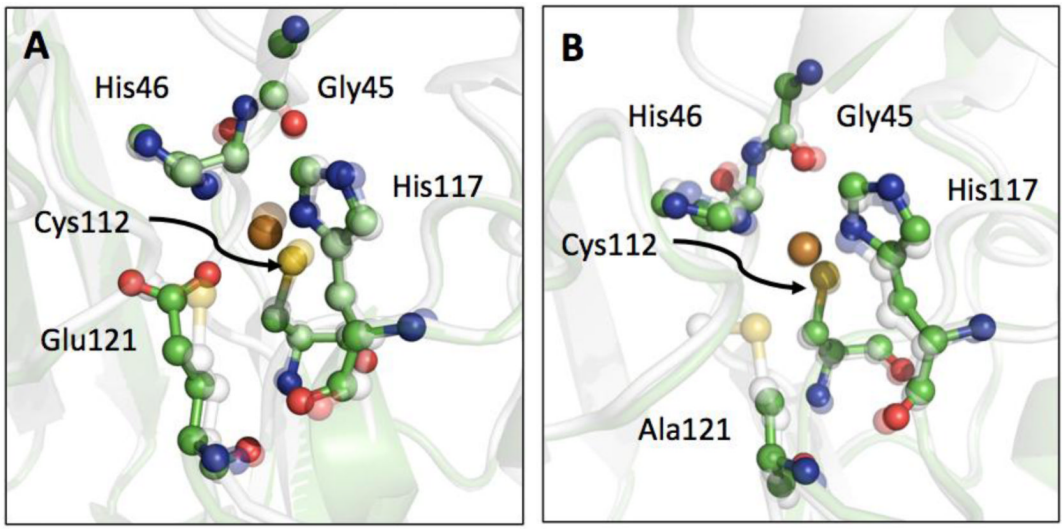

Figure 2.
Comparison of Met121Glu (A) and Met121Ala (B) with the structure of WT *P. aeruginosa* azurin. The WT structure is shown in white and is partially transparent. Spheres indicate carbon (green), oxygen (red), nitrogen (blue) and copper (tan). The protein tertiary structure is shown as a partially transparent grey or green ribbon.

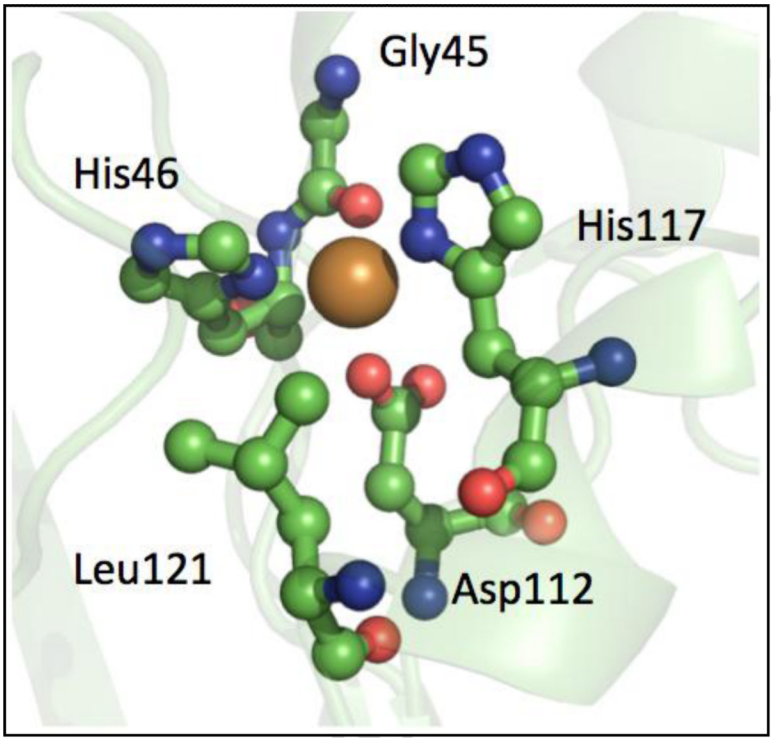

Figure 3.
X-ray structure of Cys112Asp/Met121Leu (type zero) azurin [77] (PDB ID 3FPY). Spheres indicate carbon (green), oxygen (red), nitrogen (blue) and copper (tan). The protein tertiary structure is shown as a partially transparent green ribbon. Cu-ligand distances (Å) are: His46-N3 (1.94); His117-N3 (2.04); Asp112-O (1.92); Gly45-O (2.35) and Leu121-C (3.84).

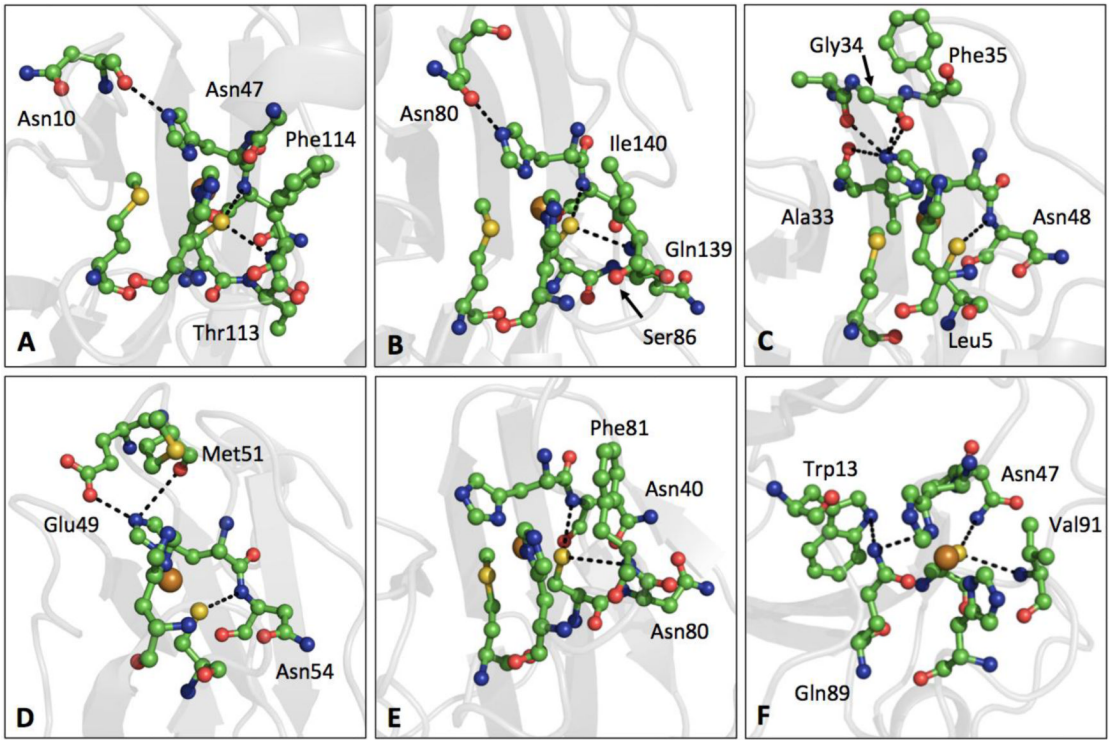

Figure 4.

Outer-sphere coordination in representative blue copper proteins in their oxidized forms. (A) *P. aeruginosa* azurin; (B) *T. ferrooxidans* rusticyanin; (C) *P. nigra* plastocyanin; (D) *P. denitrificans* amicyanin; (E) *C. sativus* cumber basic protein; (F) *C. sativus* stellacyanin. Cu-ligating residues are the same as in Figure 1.

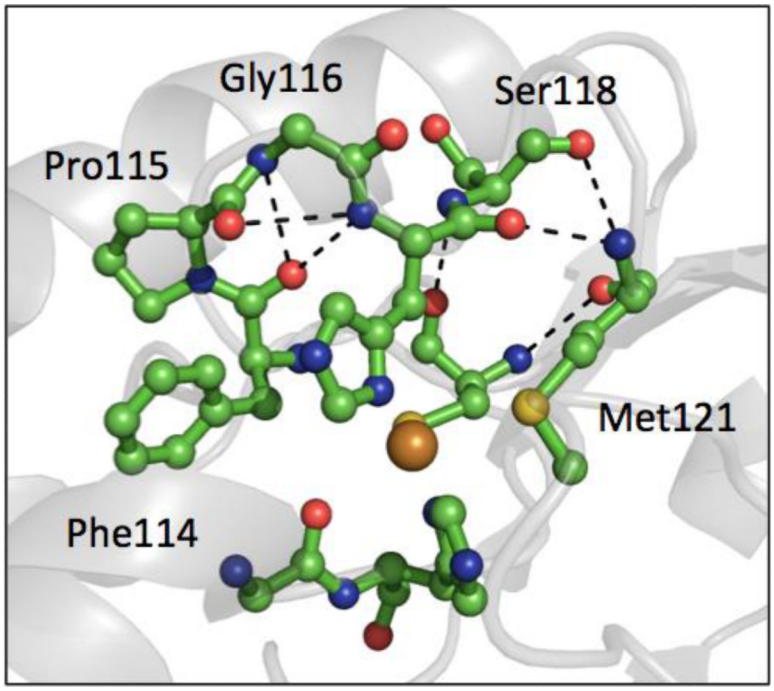

Figure 5.
View of the H-bond network surrounding Cu in *P. aeruginosa* azurin. Cu-ligating residues are the same as in Figure 1.

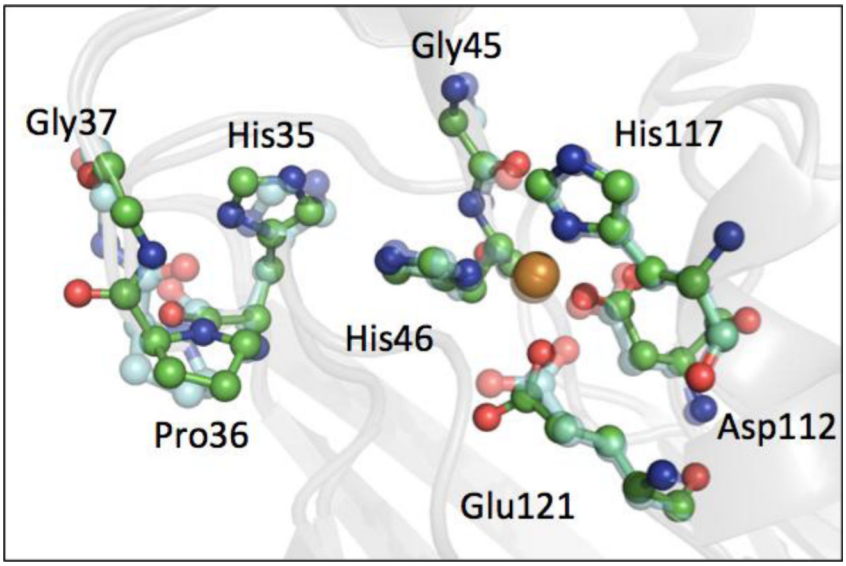

Figure 6.
Structural changes in Cys112Asp/Met121Glu *P. aeruginosa* azurin at pH 10 (green, solid) and pH 7 (cyan, partially transparent). The PDB IDs are 3OQR and 3NP3, respectively. Spheres indicate carbon (green/cyan), oxygen (red), nitrogen (blue) and copper (tan). The protein tertiary structure is shown as a partially transparent grey ribbon.

Table 1

Cu—ligand bond lengths in selected blue copper proteins.

***P. aeruginosa* azurin**	**pH**	**Cu-N3 (His46)[a]**	**Cu-S (Cys112)[a]**	**Cu-N3 (His117[a]**	**Cu-S (Met121)[a]**	**Cu-O (Gly45)[a]**	**Res (Å)**	**PDB ID**
Cu(II)	5.5	2.08(6)[a]	2.24(5)	2.01(7)	3.15(7)	2.97(10)	1.9	4AZU [21]
Cu(I)	5.5	2.14(9)	2.29(2)	2.10(9)	3.25(7)	3.02(8)	2.0	1E5Y [22]
Cu(II)	9.0	2.06(6)	2.26(4)	2.03(4)	3.12(7)	2.94(11)	1.9	5AZU [21]
Cu(I)	9.0	2.20(11)	2.30(23)	2.21(12)	3.16(9)	3.11(11)	2.0	1E5Z [22]
T. ferrooxidans rusticyanin		Cu-N3 (H85)	Cu-S (Cys138)	Cu-N3 (H143)	Cu-S (Met148)	--	Res (Å)	PDB ID
Cu(II)	4.6	2.04	2.26	1.89	2.88	--	1.9	1RCY [23]
Cu(I)	4.6	2.22	2.25	1.96	2.75	--	2.0	1A3Z [22]
P. nigra plastocyanin		Cu-N3 (His37)	Cu-S (Cys84)	Cu-N3 (His87)	Cu-S (Met92)	--	Res (Å)	PDB ID
Cu(II)	6.0	1.91	2.07	2.06	2.82	--	1.33	1PLC [24]
Cu(I)	7.0	2.13	2.17	2.39	2.87	--	1.80	5PCY [25]
P. denitrificans amicyanin		Cu-N3 (His53)	Cu-S (Cys92)	Cu-N3 (His95)	Cu-S (Met98)	--	Res (Å)	PDB ID
Cu(II)	6.0	1.95	2.11	2.03	2.90	--	1.31	1AAC [26]
Cu(I)	7.7	1.95	2.12	Unbound	2.91	--	1.30	2RAC [27]
C. sativus cucumber basic protein		Cu-N3 (His39)	Cu-S (Cys79)	Cu-N3 (His84)	Cu-S (Met89)	--	Res (Å)	PDB ID
Cu(II)	6.0	1.93	2.16	1.95	2.61	--	1.80	2CBP [28]
C. sativus stellacyanin		Cu-N3 (His46)	Cu-S (Cys89)	Cu-N3 (His94)	--	Cu-O (Gln89)	Res (Å)	PDB ID
Cu(II)	7.0	1.96	2.18	2.04	--	2.21	1.60	1JER [29]

[a]Average of distances for four molecules in the asymmetric unit. Errors are one standard deviation.

Table 2

M—ligand bond lengths in selected blue copper proteins.

***P. aeruginosa* azurin**	**pH**	**M-N3 (His46)[a]**	**M-S (Cys112)[a]**	**M-N3 (His117)[a]**	**M-S (Met121)[a]**	**M-O (Gly45)[a]**	**Res (Å)**	**PDB ID**
Co(II)	5.7	2.39(13)	2.34(3)	2.27(8)	3.56(12)	2.23(8)	1.90	1VLX [31]
Ni(II)	5.7	2.15(10)	2.49(7)	2.07(9)	3.34(7)	2.35(16)	2.20	1NZR [32]
Cu(II)	5.5	2.08(6)	2.24(5)	2.01(7)	3.15(7)	2.97(10)	1.9	4AZU [21]
Zn(II)	5.7	2.07(5)	2.30(2)	2.01(3)	3.38(9)	2.32(9)	2.14	1E67 [33]
P. nigra plastocyanin		M-N3 (His37)	M-S (Cys84)	M-N3 (His87)	M-S (Met92)	M-O (Pro36)	Res (Å)	PDB ID
Hg(II)	6.0	2.38	2.34	2.38	3.02	3.46	1.90	3PCY [34]

[a]Average of distances for 4 molecules in the asymmetric unit. Errors are 1 standard deviation.

Table 3

$Cu^{II/I}$ reduction potentials.

	pH	*E*°' (V vs. NHE)	Ref.
P. aeruginosa azurin	7.5	0.310	[45]
S. oleracea plastocyanin	7.5	0.384	[46]
P. nigra plastocyanin	7.5	0.380	[47]
T. ferrooxidans rusticyanin	2.0	0.680	[48]
P. denitrificans amicyanin	6.7	0.294	[49]
C. sativus stellacyanin	7.0	0.260	[50]
R. vernicifera stellacyanin	7.1	0.184	[51]
C. sativus cucumber basic protein	7.0	0.306	[52]