The XAS Model of Dissolved Cu(II) and Its Significance to Biological Electron Transfer

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Abstract: The standard model for dissolved Cu(II) portrays the complex ion as an axially elongated, equatorially planar octahedron. Using EXAFS and MXAN analyses of copper K-edge XAS spectra, new structural models for dissolved [Cu(aq)]2+ and [Cu(amm)]2+ have been determined. These structures uniformly depart from the octahedral model in favour of an axially elongated square pyramidal core. MXAN results also indicate that the equatorial ligands need not be coplanar with copper. Further structural elements include a ~3 Å axially localized scatterer. Even more distant scatterers imply second shell solvent organization, which can vary with the medium. Preliminary results from new extended, k = 18 Å−1, higher resolution copper K-edge XAS data sets are reported. The low symmetry of dissolved Cu(II) ion contradicts the central thesis of the rack-induced bonding hypothesis of copper electron transfer proteins. The asymmetry of biological copper is not a frozen vibronic excited state enforced by a rigid protein scaffold, but is entirely in harmony with the structural ground state of the dissolved aqueous Cu(II) complex ion.

1. Introduction:

The solution structure of cupric ion has again become an open question since the provocative molecular dynamics study of Pasquarello, et al. 1, which predicted a 5-coordinate ligand core about dissolved aqueous copper(II). Prior to this time it was widely accepted that the six-coordinate Jahn-Teller axially elongated equatorially planar octahedron was the immediate solution-phase complexation environment around copper(II). The only experimental contradiction to the majority view was the LEFE EPR results of Peisach and Mims 2,3, indicating that dissolved low molecular weight Cu(II) complexes are not centrosymmetric, but these results were widely ignored.

The structural prediction of Pasquarello, et al., was tested by analyzing the K-edge x-ray absorption (XAS) spectrum of aqua cupric ion using the full multiple scattering theory
MXAN method\textsuperscript{4-6}. EXAFS and MXAN analysis of the K-edge XAS spectrum of dissolved $[\text{Cu(aq)}]^{2+}$ indicated a preferred 5-coordinate equatorially distorted square pyramidal geometry for this complex, thus $[\text{Cu(H}_2\text{O)}_5]^{2+}\text{.}\text{7,8}$

In a continuing study, we have been using the EXAFS and MXAN methodologies conjointly to re-examine the question of the solution structure of the dissolved complex ions of Cu(II). Here we report results testing prior Cu(II) EXAFS and MXAN solution structural models using new high-resolution XAS data sets, and comparing the $1s\rightarrow3d$ pre-edge transition intensities of crystalline and dissolved copper(II) complexes.

2. Materials and Methods:

All glassware was pre-washed with dilute HCl and thoroughly rinsed with 18 MΩ doubly de-ionized water. Low-zinc solutions were made using Chromasolv LC-MS water (Riedel-de-Haen). Preparation of dissolved aqueous copper(II) perchlorate used 99.995% CuO (0.26 ppm Zn, 403.6 mg, 5.07 mmoles) (Aldrich Chemicals), sonicated for three days in a 50 mL glass mixing-cylinder. The final light blue solution was 0.10 M $[\text{Cu(aq)}]^{2+}$ in 1 M HClO$_4$.

The stock solution of low-zinc used Cu(NO$_3$)$_2\cdot2.5\text{H}_2\text{O}$ 99.999% (Aldrich Chemicals , 0.04 ppm Zn) 2.0 M in 0.1 M HNO$_3$, and was diluted to 5.0 mL. Copper(II), 0.10 mM in 4 M aqueous ammonia, was prepared using the stock copper(II) nitrate with 2.90 mL of 14 M aqueous ammonia, followed by dilution to 10 mL (pH 11.8). Copper imidazole solution used the 0.10 M stock Cu(II) perchlorate diluted to 5.0 mL with 1.36 gm (20 mmoles) of imidazole.

Transmission x-ray absorption spectra of copper(II) in liquid aqueous solutions were measured and processed as described previously\textsuperscript{9}. For low-zinc Cu(II), data collection was extended to 10247 eV. The XAS of crystalline $[\text{Cu(ImH)}_4\text{SO}_4]$ was described previously\textsuperscript{10}, and that of crystalline $[\text{Cu(H}_2\text{O)}_6]\text{(ClO}_4)\text{$_2$}$ was measured at 10 K for this work. Copper(II) pre-edge features were fit over the energy range 8975 eV to 8985 eV using a single pseudo-Voigt, a small variably positive linear offset (0.008±0.004), and a single pseudo-Voigt to represent the rising edge. The intensities, mean positions, and half-widths of the pseudo-Voigts were floated, with 1:1 Lorentzian-Gaussian ratio. The energy positions of the rising K-edge absorption maxima were determined from second derivative XAS spectra. The pre-edge features of the copper K-edge XAS spectra were fit using the program EDG_FIT, which is part of the EXAFSPAK software suite, written by Prof. Graham George, University of Saskatchewan, and is available at http://ssrl.slac.stanford.edu/~george/exafspak/exafs.htm.

3. Results and Discussion:

3.1. Fits to XAS pre-edges: The XAS K-edges of transition metals is preceded by a low-intensity pre-edge feature that reflects the quadrupole-allowed $1s\rightarrow3d$ transition\textsuperscript{11,12}. The intensity of this transition is sensitive both to the ligation symmetry of the absorber and to the covalence of the metal-ligand bond\textsuperscript{12-15}. In non-centrosymmetric ligation symmetries, such as $D_{2d}$, $T_d$, or $C_{4v}$, 3d-4p mixing can add dipole intensity into the otherwise quadrupolar excited state. However, metal-ligand covalence can reduce 3d-4p mixing even in low-symmetry ligation environments\textsuperscript{14}. Although comparison of the pre-edge intensities of the XAS spectra of structurally known and unknown complexes can reveal information about the absorber ligation symmetry within the unknowns\textsuperscript{16}, caution is needed because of the opposing effects of bond covalence and ligation symmetry. The intensities of pre-edge transitions can be estimated by fitting XAS spectra with pseudo-Voigt line-shapes. Here, the single pre-edge transition of Cu(II) complexes was fit with a single pseudo-Voigt line. The rising edge
background was fit with a broadened pseudo-Voigt, representing the sum of the remaining rising edge transitions (see the Materials and Methods Section).

Figure 1 shows the result of a pseudo-Voigt fit to the pre-edge transition of the crystalline imidazole complex [Cu(ImH)4SO4]17. This complex is an axially distorted octahedron with crystallographic distances of 4×Cu-Neq=2.000, 2.020 Å and 2×Cu-Oax = 2.574 Å. Similar core distances were found using EXAFS fits to the copper K-edge XAS spectrum of [Cu(ImH)4]2+ in liquid aqueous solution (k = 2-12.5 Å⁻¹), to be fully reported elsewhere, which revealed 4×Cu-NImH = 2.02 Å and 1×Cu-Oax = 2.15 Å. Although the best fit indicated that a square pyramidal structure dominated the solution complex, the octahedral variant could not be statistically eliminated. In Figure 1, the integrated areas of the fitted pre-edge pseudo-Voigt lines were 0.023 and 0.034 normalized area units (nau), and the energy positions of these pseudo-Voigts were 8979.0 eV and 8979.9 eV, respectively.

Figure 2 shows the analogous pseudo-Voigt fits to the pre-edge energy region of the K-edge XAS spectrum of crystalline [Cu(NH3)4(H2O)]SO4 and of 0.1 M Cu(II) in pH 11.2 liquid-phase 4 M aqueous ammonia. The crystalline complex is square pyramidal (C4v) 18. The energy positions and integrated areas of the fitted pre-edge pseudo-Voigt lines were 8980.5 eV and 0.027 nau, and 8980.0 eV and 0.031 nau, respectively.

The two aqua copper(II) complexes exhibited pre-edge pseudo-Voigts at the same energy, but with about a 3σ dissimilarity in intensity favoring the solid complex. This result, taken by itself, implies that the copper absorber in the solution complex occupies a ligand environment of comparatively higher symmetry, with less 3d-4p mixing and a concomitantly lower transition intensity.

The results from pseudo-Voigt fits to the pre-edge feature of the copper K-edge XAS of crystalline and dissolved complex ions [Cu(H2O)5]2+, [Cu(NH3)4]2+, and [Cu(ImH)4]2+ are shown in Table 1 with
the crystal structure or solution EXAFS equatorial copper-ligand bond lengths, and the energy separation between the fitted pre-edge feature and the XAS maximum.

Figure 2: Pseudo-Voigt fits (—) to the pre-edge energy region of the K-edge XAS spectra (○) of: a. crystalline [Cu(NH₃)₄(H₂O)]SO₄, and; b. 0.1 M Cu(II) in 4 M liquid aqueous ammonia pH 11.2 solution. Insets: the second derivatives. Each pre-edge pseudo-Voigt appears on the respective abscissa and the dashed line shows the total pseudo-Voigt background.

However, the solid complex also exhibited a greater energy difference between the pre-edge transition and the XAS absorption maximum. K-edge XAS transitions reflect the relative energy difference between the absorber 1s orbital and the valence orbitals, not the absolute energy difference between the 1s level and the continuum. Therefore, shifts in the energy position of the 1s→3d transition, following absorber redox, need not reflect the actual direction of change in potential energy of the 1s orbital itself. Within a given complex ion, all the filled and partially filled orbitals are raised or lowered in energy with decreases or increases, respectively, in absorber oxidation state, and the 1s→3d transition energy reflects only this relational orbital energy difference. Rather counter-intuitively, density functional theory (DFT) calculations show that the change in orbital potential energy with oxidation state is virtually equivalent in both 1s and valence orbitals ²³,²⁴. In contrast to the filled orbitals, the energies of unfilled virtual orbitals, such as the 4p orbital of first row transition metals, are not strongly affected by changes in absorber oxidation state, because no electrons are present to be influenced by the increase in uncompensated nuclear charge. Therefore the comparative XAS K-edge energy difference between the pre-edge transition and the absorption maximum reflects a 3d–4p energy difference, and can give a measure of the relative energies of the respective 3d orbitals in closely related 3d transition metal complexes.

In Table 1, the Δ(eV)_{max} entries for the aqua copper(II) complexes show that the 3d₂₋₂γ₂ orbital is about 0.7 eV higher in energy in the dissolved pentaaqua complex (i.e., is closer to the absorption maximum), than is that of the crystalline hexaaqua complex. The equatorial bonding is otherwise invariant, and so the shift to higher energy implies greater electronic repulsion between the half-filled 3d₂₋₂γ₂ orbital and the oxygen 2p orbitals in the dissolved
complex. That is, the energy of the half-filled valence orbital has increased in the solvated copper(II) aqua complex, implying the appearance of a greater ligand-metal repulsion following dissolution of crystalline hexaaqua Cu(II) in water.

Figure 3 compares the XANES spectra of the crystalline and dissolved aqua copper(II) complexes, showing their otherwise great similarity. The increased metal-ligand repulsion implied by the observed shift of the 1s→3d transition is suggestive that the oxygen 2p electrons are relatively more delocalized onto the copper(II) ion of the solution-phase aqua complex. It thus appears that the equatorial copper-oxygen bond has become relatively more covalent following dissolution in water. The source of any increased covalence may stem from hydrogen-bonding interactions between the ligand hydrogens and second shell water oxygens.

The apparently increased covalence of the Cu-O bond in the dissolved aqua complex ion is suggested to be the source of the decreased intensity of the pre-edge transition relative to that of the crystalline complex (Table 1). This analysis can be extended to the XAS spectra of the remaining complexes listed in Table 1. Thus, the rising edge intensities of the two ammine complexes are about 1σ (pH 11.2) and 2σ (pH 7.1) more intense than that of the crystalline complex. At the same time, the transitions are 1.7 eV and 2.2 eV lower in energy relative to the respective rising edge maxima. These energy positions suggest that the equatorial Cu-NH₃ bonds are less covalent in the solution complexes than in the crystalline complex. EXAFS and MXAN analysis of Cu(II) in 4 M pH 11.2 ammonia solution indicated the majority dissolved complex ion has a square pyramidal, [Cu(NH₃)₅]²⁺, core structure. The comparable pre-edge intensities are compatible with this structure, with the difference in intensities consistent with the relative bond covalency implied by the respective Δ(eV)max values.

For copper(II)-imidazole, the intensity of the solution complex ion is about 3σ more intense than that of the crystalline Jahn-Teller distorted octahedral complex. At the same time, the 1.2 eV disparity in the respective Δ(eV)max values implies that the equatorial bonding in the crystalline complex is again the more covalent. It is difficult to infer any judgment concerning the symmetry of the solution complex from these data, however both the apparent increased solid-state covalency and a lower solution symmetry should shift the relative pre-edge intensities in favor of the solution-phase complex ion, as observed.
3.2. Extended XAS Analysis: The use of low-zinc conditions allowed measurement of unusually high-resolution XAS spectra of copper(II) in water and ammonia solutions. Figure 4 compares the EXAFS and Fourier transform XAS spectra of 0.1 M Cu(II) ion in liquid aqueous perchloric acid or ammonia solution. Under low-zinc conditions, good quality EXAFS spectra extended to at least $k = 18 \text{ Å}^{-1}$.

The EXAFS are very similar, as expected for the relatively simple aqua and ammine complex ions. The respective waves fall out of phase around $k = 8 \text{ Å}^{-1}$ but are again nearly in-phase after $k = 16 \text{ Å}^{-1}$. The main feature in the Fourier transform (FT) spectrum reflects a slight difference in first shell bond lengths, and the intensity difference indicates that the ligation environments are inequivalent despite the structural similarity of the water and ammonia molecules. The FT features between $R = 2-4 \text{ Å}$ are well above the noise, and are derived from single scattering from more distant atoms as well as multiple scattering. In what follows, we show the results of testing the previously published structural models for dissolved liquid aqueous $[\text{Cu(aq)}]^{2+}$ and $[\text{Cu(amm)}]^{2+}$, as derived from conjoint EXAFS and MXAN analyses, against these extended XAS data sets.

![Figure 4](image_url)

**Figure 4:** a. K-edge EXAFS spectra, and b. EXAFS Fourier transform spectra of: (—), 0.1 M Cu(II) in liquid aqueous 1 M HClO$_4$ solution, and; (····), 0.1 M Cu(II) in 4 M pH 11.8 liquid aqueous ammonia solution. The vertical dashed line in part ‘a’ shows the Å$^{-1}$ position of the zinc cut-off that typically plagues the collection of copper EXAFS.

3.2.1. The $[\text{Cu(aq)}]^{2+}$ model: The previous EXAFS and MXAN studies of the K-edge XAS spectrum of solution-phase aqua copper(II) indicated a best-fit five-coordinate axially-elongated square pyramidal structure $^7,8$. The MXAN model also included a semi-$D_{2d}$-like distortion of the equatorial ligands, featuring two 85° O$_{ax}$-Cu-O$_{eq}$ angles, and two of 107°.

The previously published EXAFS model was tested against the new $k = 18 \text{ Å}^{-1}$ data set, and the results of this test are shown in Figure 5. The fit metrics appear in Table 2, along with the metrics from the original fit.

In Figure 5a, the square pyramidal model is shown to account for virtually all of the data. The fit residual (Figure 5a, bottom) includes only some low intensity periodic features at small $k$-values. In the Fourier transform of the EXAFS and the fit, the first shell feature at 1.55 Å (non phase-adjusted) is well-fit. The two less intense Fourier features at 2.2 Å and 2.5 Å were only fully fit when equatorial hydrogens were added to the model (Table 2). Absent these hydrogens, the fit produced an almost featureless curve passing through the center of the structured Fourier region at 2-4 Å.

The remaining unfit Fourier features at $R > 2.7$ Å reflect more distant scatterers and a full exposition of these data will be given elsewhere. Here, it is demonstrated that the previous
square pyramidal structural model for aqua copper(II) retains its validity with a significantly longer EXAFS data set.

**Figure 5:** Copper K-edge of 0.1 M Cu(II) in 1 M HClO₄ liquid aqueous solution: a. (○), EXAFS, and; (—), square pyramidal fit. Bottom: the EXAFS fit residual. b. (○), Fourier transform of the EXAFS, and; (—), Fourier transform of the fit.

| Table 2: Test of the [CuX₅]¹⁺ Square Pyramidal Solution Structural Model |
|-------------|-----------------|-----------------|
| Scattering | Prior Model      | Final Model      |
| O            | 1.95 ± 0.05 Å²  | 1.96 ± 0.05 Å²  |
| S            | 2.36 ± 0.05 Å²  | 2.32 ± 0.05 Å²  |
| N            | --              | 4.98 ± 0.05 Å²  |
| H            | --              | 2.28 ± 0.05 Å²  |
| L            | --              | 2.47 ± 0.05 Å²  |

Note that with use of the longer data set, the axial oxygen distance was found at 2.52 Å. A crystal structure for pentaaquacopper(II) perchlorate has been recently reported, but which exhibits the usual planar equatorial.

3.2.2. The [Cu(amm)]²⁺ model: Prior EXAFS and MXAN analysis of K-edge XAS data for the complex ion of copper(II) in liquid aqueous ammonia solution resulted in an unusual model featuring an axially elongated square pyramidal core plus a distant −z axial N/O scatterer thought to represent a localized water molecule. MXAN analysis further found that the four equatorial nitrogens were depressed 12° below the equatorial plane of Cu(II). Small Fourier features in these data, also evident in Figure 4 above, were found to primarily reflect a durable environment of second shell scatterers.

**Figure 6:** Copper K-edge of 0.1 M Cu(II) in 4 M pH 11.8 liquid aqueous ammonia solution: a. (○), EXAFS, and; (—), the fit with the square pyramidal core plus solvent shell. The EXAFS fit residual is shown at the bottom. b. (○), Fourier transform of the EXAFS, and; (—), of the fit.

This model was tested against the low-zinc EXAFS data set. Figure 6 shows the fit to the EXAFS and to the Fourier transform EXAFS spectra, which fit strictly adhered to the prior core model.
Table 3 presents the metrics from the prior fit and from the test fit discussed here. The two copper(II)-ammonia samples were prepared at pH 11.2 or pH 11.8, respectively, and are not expected to sustain identical coordination environments.

| Scatterer | d (Å) | DW-value (σ²) | d (Å) | DW-value (σ²) |
|-----------|-------|---------------|-------|---------------|
| 4=N(H)    | 2.62  | 0.08673       | 2.02  | 0.00537       |
| 1=O(H₂O)  | 2.16  | 0.01789       | 2.18  | 0.00402       |
| 1=O(H₂O)  | 2.77  | 0.00404       | 2.72  | 0.00958       |
| 4=N(H)₄   | 4.22  | 0.035588      | 3.71  | 0.00968       |
| 7=H(H₂O)  | ---   | ---           | 2.22  | 0.00822       |
| 9=O(H₂O)  | ---   | ---           | 2.79  | 0.00928       |
| Cu-O₄     | 3.40  | 0.03029       | 3.82  | 0.03537       |

The equilibrium constants for Cu(II) in 4 M ammonia solution predict that at pH 11.8 about 49% of the copper(II) is engaged in the [Cu(NH₃)₃(OH)]⁺ complex ion, rather than in the pentaamminecopper(II) complex that dominates at pH 11.2 (63%). The hydroxide ion is certain to occupy an equatorial Cu(II) site, however, the current EXAFS data set is insufficient to resolve a net 0.5 equatorial hydroxide ligand. Under the solution conditions of 4 M NH₃, the axial position of [Cu(NH₃)₂(OH)]+ is likely to be occupied by an ammonia ligand. Including the pentaammine and the aquotetraammine complexes, [Cu(NH₃)₅]²⁺ (34%) and [Cu(NH₃)₄(H₂O)]²⁺ (15%) respectively, three homologous complexes with nearly equidistant equatorial ligands dominate the pH 11.8 solution composition. A full fit to the EXAFS spectrum of this solution sample (not shown) indicated that the axial coordination as represented in Table 3 is not entirely correct. Nevertheless, the Table 3 fit (Figure 6) testing the prior model shows that the axially elongated square pyramidal core plus second shell of solvent scatterers is able to explain virtually all of the core environment about Cu(II) and most of the distant environment. In the FT spectrum, only the region above R = 2.3 Å remains incompletely fit. This Fourier region can be strongly influenced by the solvation environment of small complex ions, and the EXAFS unfit residual (Figure 6a, bottom) includes low-intensity high-frequency features consistent with this interpretation.

As was the case with the fit to the new aqua copper data set, the extended EXAFS fit included the equatorial hydrogen atoms. Interestingly, in the k = 18 Å⁻¹ fit, the distant scatterer shell moved to 3.82 Å, which is closer to the second shell distance found from MXAN analysis of the prior pH 11.2 XAS data set. The results from the complete fitting experiments alluded to here will be fully reported elsewhere. Homologous octahedral models tested against the aqua copper(II) or the ammine copper(II) k = 18 Å⁻¹ extended EXAFS data sets produced fits that were nearly as good. Goodness-of-fit values for the three best models were: square pyramidal, Cu-Lₐₓ = 2.18 Å, F=0.1601; octahedral, Cu-Lₐₓ = 2.18 Å, 2.22 Å F=0.1705, and; sq. pyr. plus axial solvent, Cu-Lₐₓ = 2.18 Å, 2.72 Å, F=0.1566. However, a more definitive exploration of the model space requires a complementary MXAN study.

Prior findings and the new results reported here show that in liquid aqueous solution unconstrained Cu(II) can adopt structures that are not centrosymmetric. The same underlying square pyramidal core geometry is always found, along with a persistent but distant axial scatterer – presumably a water molecule. The equatorial ligands also need not be on a coplanar bisect. Thus, solution phase Cu(II) neither requires nor adopts the rigid symmetries that exemplify its crystalline complexes.

3.2.3. Relevance to Biological Electron Transfer: The rack-bonding theory of blue copper proteins proposes that the Franck-Condon barrier to biological electron transfer is greatly
reduced by a ~70 kJ mol\(^{-1}\) thermodynamically unfavourable geometry at the metal site, imposed by a rigid protein rack\(^{26-31}\). This unfavourable geometry is intermediate between the equatorial square plane that is considered the energetically favoured ground state for Cu(II), and the tetrahedral geometry favoured by the spherically symmetric Cu(I) ion.

However, a recent analysis showed that blue copper proteins do not have the thermodynamic stability to enforce a rack\(^{8,32}\). Instead, an unfavourable 70 kJ mol\(^{-1}\) would force blue copper proteins to spontaneously unfold when copper is present, or else would induce a new structural ground state at the active site thermodynamically favourable to the resident copper(II). In contrast, however, native blue copper proteins are structurally stable and neither spontaneously denature nor reorganize in either oxidation state.

Further this and prior work\(^{7-9}\) have shown that the ground state structure of unconstrained Cu(II) complexes need not include a strictly planar square of equatorial ligands. Thus, there is no necessity of supposing that a blue copper protein active site is energized if it does not conform to the square equatorial planarity so often observed in the crystal structures of Cu(II) complexes\(^{33}\).

Figure 7, left, shows the EXAFS and MXAN consensus model for the core structure of solution-phase \([\text{Cu(NH}_3\text{)}_5\text{]}^{2+}\)\(^9\), including four ammonia nitrogens displaced about 12º below the copper-equatorial plane with Cu(II) about 0.45 Å above the mean N\(_4\) plane, an axial nitrogen at about 2.18 Å and a distant \(\sim z\) localized scatterer, most likely oxygen, at about 3 Å.

A thought experiment can be imagined, beginning with the \(C_{4v}\) core structure of solution-phase \([\text{Cu(NH}_3\text{)}_5\text{]}^{2+}\) (Figure 7; left). Two equatorial ammonia ligands may be removed from this structure, and replaced by one equatorial methylthiolate ligand, with the rest of the structure left unchanged. The new N\(_{ax}\)-Cu-S angle is set to 102º, which reproduces the original 12º equatorial depression. The result of this exercise is shown in Figure 7, right. The new arrangement is an equatorial N\(_3\)S ligand core that is very reminiscent of a blue copper protein site. One may expect that in a real system the high covalence of an equatorial Cu(II)-S\(_{\text{cysteinate}}\) bond would cause elongation of the axial ligand distance.

In the facsimile blue copper site structure, the 0.45 Å elevation of the copper(II) is very similar to the distance of Cu(II) from the mean equatorial plane in authentic blue copper proteins\(^{35}\). A dissolved Cu(II)-thiolate complex should be unstable only to bimolecular redox disproportionation into Cu(I) and disulfide. In blue copper proteins, this reaction is prevented by isolation of the active site cluster within a polypeptide sheath.

The redox behavior of the M121Q mutant of \textit{Alcaligenes denitrificans} azurin is also instructive and relevant. In the M121Q version of this azurin, the axial methionine 121 was
mutated to a glutamine, but retaining virtually all of the hydrogen bonding network previously offered as evidence of a rigid protein rack. However, reduction of Cu(II) M121Q A. denitrificans azurin induced a local rearrangement of the protein, producing a nearly linear Cu(I) site with only two remaining strong copper-ligand bonds, to the cysteinate sulfur and the imidazole of histidine. The large rearrangement of the copper active site geometry following reduction verifies the analysis that a $+\Delta\Delta G$ force induced by copper at the blue copper site will induce a protein rearrangement. The M121Q result is thus a strong experimental disproof of the predicted structural invariance entailed by a hypothetically rigid protein site. Instead, this result shows that azurin occupies the dynamic structural landscape typical of virtually all proteins.

Thus, the copper site in native blue copper proteins is structurally redox-invariant because equatorial asymmetry is preferred by Cu(II), and the small valence change at copper itself during redox turnover, guaranteed by the high covalence of the Cu(II)-S cysteinate bond means that the geometrical preference of copper does not greatly change during the redox turnover of blue copper proteins. These together are the primary factors sponsoring the uniform ground state geometric preference of Cu(II) and Cu(I) in blue copper proteins, providing a naturally graceful solution to the problem of the Franck-Condon barrier to electron transfer. This solution also avoids the violation of Evolutionary Theory implicit within the rack hypothesis, in that metabolically costly ATP need not be expended during holoprotein folding in order to impose an energetically unfavorable geometry on a blue copper active site.

The A. denitrificans M121Q experiment also showed that the active site ground state geometry of blue copper proteins is a function of the identity of the ligands, in that replacing just the methionine sulfur by a glutamine oxygen strongly modified the thermodynamics of Cu(I) ground state geometry. The chemical identity of the biological ligands is thus the third factor contributing to the invariant site structure. The appropriate geometry of the amino acid side chains in the empty active site of a waiting native apoprotein need only be favored by about $-6 \text{kJ mol}^{-1} \Rightarrow K_{\text{native}} \approx 10 \text{M}^{-1}$, an energy equivalent to about one hydrogen bond, in order that an accessing copper will encounter a coherent and predisposed array of ligands thermodynamically compatible with both Cu(II) and Cu(I). In this way the redox-facilitation of blue copper proteins, produced by an evolutionary teleonomy, is achieved by persuasion rather than by coercion.

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