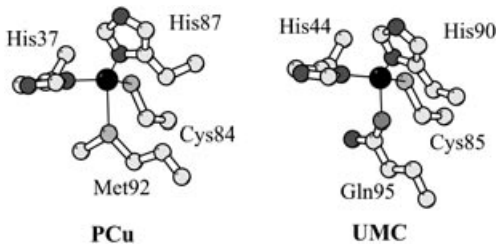


***Figure 1.*** *The active site structures of the type 1 copper sites of plastocyanin (PCu) and umecyanin (UMC).*

# An Axial Met Ligand at a Type 1 Copper Site is Preferable for Fast Electron Transfer

Mark D. Harrison[a, b] and Christopher Dennison*[a]

The structure, spectroscopy and electron transfer (ET) reactivity of the type 1 copper sites of cupredoxins have been extensively studied by site-directed mutagenesis.[1–4] All of these investigations have highlighted the importance of the ligating residues, in particular the equatorial Cys ligand,[1] at the typically distorted tetrahedral metal centre (see Figure 1). The axially coordinating amino acid, which is usually a Met located ~3 Å from the metal, is the only nonconserved type 1 copper site residue.[5] In the stellacyanin subfamily of the cupredoxins, a Gln residue is found in the axial position and coordinates in a monodentate fashion through its side-chain amide oxygen atom (see Figure 1).[6–8] The influence of this natural variation at a type 1 copper site on ET reactivity is not well understood and the exact physiological function of the stellacyanins is not known. Replacement of the axial Met ligand with a Gln has been carried out in two cupredoxins and has been found to result in a decrease in ET reactivity.[2,3] Herein we describe the influence of the axial Gln to Met mutation on the ET reactivity of a stellacyanin.

The Gln95Met variant[9,10] of umecyanin (UMC), the stellacyanin from horseradish roots, has spectroscopic properties[11] for the $Cu^{II}$ form that are consistent with the substitution of the axial oxygen ligand with the thioether sulfur of the introduced Met (see Figure 2). This is particularly apparent from the EPR parameters,[12] since, due to coordination by an axial oxygen,[3,7] the large $g_z$ and small $A_z$ values for the wild-type

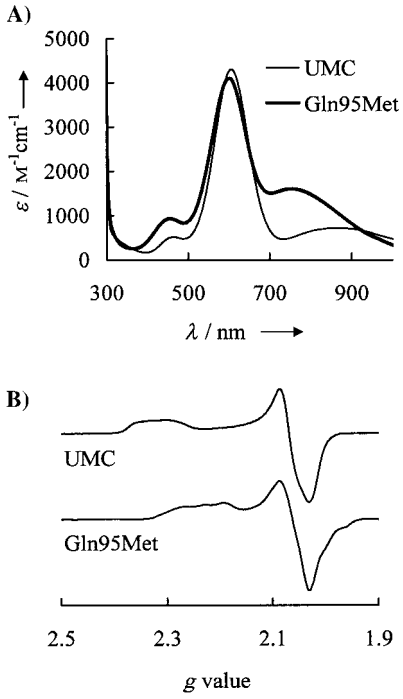


***Figure 2.*** *The A) UV/Vis and B) EPR spectra of oxidised WT UMC and the Gln95Met variant.*

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(WT) protein are replaced with more typical values for a type 1 copper site in the Gln95Met variant. (Type 1 copper sites with a weak axial Met ligand usually possess EPR spectra with $g_z \approx 2.24$ and $A_z \approx 5$ mT.[3]) The low-energy UV/vis band is shifted from 860 nm in WT UMC to 750 nm in the variant. This band has been assigned to d–d transitions, and the increased energy in Gln95Met UMC is consistent with a decreased axial interaction.[4] The relatively weak axial bond is confirmed by the paramagnetic $^1$H NMR spectrum of Cu$^{II}$ Gln95Met UMC[13] in which one of the C$^{\gamma}$H proton resonances of this residue is observed at 22.4 ppm. The isotropic shift of this signal is very similar to that seen in other cupredoxins with a long axial Cu–S(Met) bond.[14,15] The reduction potential[16] of Gln95Met UMC (420 mV) is significantly larger than the value of 290 mV for the WT protein.[10] This is also consistent with the substitution of a hard axial oxygen ligand with a softer sulfur donor. The reduction potential of Gln95Met UMC is a little higher than that typically found for a type 1 copper site with a weak axial Met ligand, but is almost identical to that found for the Gln99Met *Cucumis sativus* stellacyanin variant.[4]

The influence of the Gln95Met mutation on ET has been assessed by comparing the electron self-exchange (ESE) reactivity of this variant with that of the WT protein. The ESE rate constant is an intrinsic measure of the ET capabilities of a redox metalloprotein and is a convenient way to assess relative reactivities since the reaction has no driving force.[14] The ESE rate constant of Gln95Met UMC was determined by $^1$H NMR spectroscopy (at 40 °C) with the protein in 37 mM phosphate buffer at pH* 7.5 ($I = 0.10$ M) as described previously (pH* = pH uncorrected for the deuterium isotope effect).[14] Plots of $T_1^{-1}$ and $T_2^{-1}$ for the C$^{\varepsilon 1}$H protons of His44 and His90 in Cu$^{I}$ Gln95Met UMC against the concentration of Cu$^{II}$ protein are shown in Figure 3. The slopes of these plots, which provide the ESE rate constants are $1.2 \times 10^5$ M$^{-1}$s$^{-1}$ ($T_1$, □) and $8.2 \times 10^4$ M$^{-1}$s$^{-1}$ ($T_2$, ■) for one of the resonances and $9.8 \times 10^4$ M$^{-1}$s$^{-1}$ ($T_1$, ○) and $8.3 \times 10^4$ M$^{-1}$s$^{-1}$ ($T_2$, ●) for the other. These give rise to slope ratios ($T_2/T_1$) of 0.7 and 0.8 as expected for resonances in the slow-exchange regime.[14] The determination of $T_1$ values is more precise[14] and thus an average ESE rate constant of $1.0 \times 10^5$ M$^{-1}$s$^{-1}$ is obtained for the Gln95Met UMC variant.

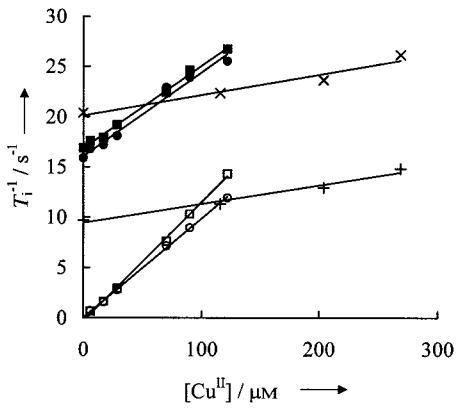


***Figure 3.*** *Plots (40 °C) of $T_1^{-1}$ (○, □) and $T_2^{-1}$ (●, ■) against [Cu$^{II}$] for the C$^{\varepsilon 1}$H protons of His44 and His90 in Gln95Met UMC in 37 mM phosphate buffer at pH* 7.5 ($I = 0.10$ M). Also shown are plots of $T_2^{-1}$ against [Cu$^{II}$] for the ligand His C$^{\varepsilon 1}$H (x) and Met43 C$^{\varepsilon}$H$_3$ (+) protons of WT UMC.*

The ESE rate constant of the WT protein was determined by using a similar approach and under identical conditions to those described for the Gln95Met variant.[17] The imidazole ring proton resonances of His44 and His90 in the Cu$^{I}$ WT UMC can be observed in a 1:1 mixture of the Cu$^{I}$ and Cu$^{II}$ proteins; this demonstrates that ESE is quite slow in the WT protein. This is confirmed by the lack of selection of resonances from protons close to the active site and arising from the Cu$^{I}$ protein in water-eliminated Fourier transform (WEFT) spectra[14] of partially oxidised samples (resonances arising from active site protons of reduced Gln95Met UMC are observed in the corresponding spectra of this variant). Only one of the His ligand (C$^{\varepsilon 1}$H) imidazole ring proton resonances is well resolved in the NMR spectrum of Cu$^{I}$ WT UMC. This signal and the C$^{\varepsilon}$H$_3$ resonance of Met43, which is situated very close to the copper site,[8] were used for determination of the ESE rate constant. From the plots of $T_2^{-1}$ against the concentration of Cu$^{II}$ protein shown in Figure 3, slopes of $1.8 \times 10^4$ M$^{-1}$s$^{-1}$ are obtained for both resonances. This ESE rate constant is confirmed by the analysis of the broadening of various resonances and also by coalescence studies on 1:1 samples of Cu$^{I}$ and Cu$^{II}$ protein. The ESE rate constant of WT UMC is consistent with a value of $6 \times 10^3$ M$^{-1}$s$^{-1}$ determined previously for the native protein from horseradish roots at a lower temperature (25 °C).[17]

The approximately fivefold increase in the ESE rate constant upon mutating Gln95 into a Met in UMC indicates that the replacement of the strong Cu–O(Gln) bond with a weaker axial Cu–S(Met) interaction results in a site with significantly enhanced ET reactivity. The ESE rate constant of the Gln95Met variant ($1.0 \times 10^5$ M$^{-1}$s$^{-1}$) is consistent with those of other cupredoxins with axial Met ligands.[14,18] Given that the axial Gln ligand is well away from the surface of the protein, that it is not in the purported ET pathway and that the Gln95Met mutation does not significantly alter the spin-density distribution onto the ligating residues,[13] the enhanced ET reactivity is most likely due to a decreased inner-sphere reorganization energy ($\lambda_i$) for the copper site in this variant compared to the WT protein. It should be noted that the Gln95Met mutation might have altered the outer-sphere reorganisation energy of UMC.

The crystal structures of Cu$^{II}$ and Cu$^{I}$ UMC have been determined recently at resolutions of 1.9 and 1.8 Å, respectively, and demonstrate an ~0.3 Å increase in the Cu–O(Gln95) bond length upon reduction (the Cu–S(Cys85) bond length increases by < 0.05 Å upon reduction).[8] The relatively small ESE rate constant for WT UMC can thus be attributed to a larger than normal $\lambda_i$ value for its active site. The Gln95Met variant would appear to possess a copper centre at which there is a significantly smaller structural change upon redox interconversion. Recent spectroscopic investigations of *C. sativus* stellacyanin and its Gln99Met variant have provided information about active-site differences between the Cu$^{II}$ and Cu$^{I}$ proteins.[4] The 0.5 Å increase in the Cu–O(Gln99) bond length in the WT protein upon reduction seems to be accompanied by a minimal alteration in the Cu–S(Cys89) bond. In the Gln99Met variant

  

there is a more significant change in the Cu–S(Cys89) bond, and it is assumed that there is very little alteration in the Cu–S(Met99) interaction (as seen for native cupredoxins with an axial Met ligand[5]). It has been suggested that this results in similar $\lambda_i$ values for the two proteins, but ET reactivities have not been measured.[4] The studies reported herein indicate that a type 1 copper site with an axial Met ligand, rather than a Gln ligand in this position, is preferable for fast ET. This is consistent with the finding that the Met-to-Gln mutation at the active sites of cupredoxins diminishes their ET reactivity.[2, 3] This study also represents the first example of an active-site mutation at a type 1 copper site that enhances ET reactivity.

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**Keywords:** cupredoxin · electron transfer · metalloproteins · mutagenesis · type 1 copper

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[12] EPR parameters, which were derived from simulations by using the program SIMFONIA (Bruker) are, WT UMC: $g_x = 2.033$, $g_y = 2.057$, $g_z = 2.320$, $A_z = 3.3$ mT, Gln95Met UMC: $g_x = 2.025$, $g_y = 2.060$, $g_z = 2.240$, $A_z = 4.7$ mT.
[13] Paramagnetic $^1$H NMR spectra were acquired on a JEOL Lambda 500 spectrometer with the protein in phosphate buffer (10 mM). The directly observed isotropically shifted resonances of WT UMC have been assigned previously[7] and are found at 51 and 48 ppm (His44/His90 $C^{\delta 2}H$), ~30 ppm (His44/His90 $C^{\varepsilon 1}H$), 13.7 ppm (Asp45 $C^{\alpha}H$), −4.2 ppm (His44/His90 $C^{\beta}H$) and ~−6 ppm (Cys85 $C^{\alpha}H$) at 40 °C with exchangeable resonances (observed in 90% $H_2O$/10% $D_2O$ at pH 4.6 and 25 °C) at ~47 and 28.5 ppm (His44/His90 $N^{\varepsilon 2}H$). In the case of Gln95Met UMC, the hyperfine shifted resonances are found at 57 and 48 ppm (His44/His90 $C^{\delta 2}H$), 33.5 ppm (His44/His90 $C^{\varepsilon 1}H$), 22.4 ppm (Met95 $C^{\gamma}H$), 16.0 ppm (Asp45 $C^{\alpha}H$), −3.5 ppm (His44/His90 $C^{\beta}H$) and ~−8.5 ppm (Cys85 $C^{\alpha}H$) at 40 °C. Exchangeable resonances are observed (in 90% $H_2O$/10% $D_2O$ at pH 4.8 and 25 °C) at 50.5 and 27.6 ppm (His44/His90 $N^{\varepsilon 2}H$).
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