Role of the Axial Ligand in Type 1 Cu Centers Studied by Point Mutations of Met148 in Rusticyanin†

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ABSTRACT: Type 1 Cu centers in cupredoxins, nitrite reductases, and multi-copper oxidases utilize the same trigonal core ligation to His-Cys-His, with a weak axial ligand generally provided by a Met sulfur. In azurin, an additional axial ligand, a carbonyl oxygen from a Gly, is present. The importance of these axial ligands and in particular the Met has been debated extensively in terms of their role in fine-tuning the redox potential, spectroscopic properties, and rack-induced or entatic state properties of the copper sites. Extensive site-directed mutagenesis of the Met ligand has been carried out in azurin, but the presence of an additional carbonyl oxygen axial ligand has made it difficult to interpret the effects of these substitutions. Here, the axial methionine ligand (Met148) in rusticyanin is replaced with Leu, Gln, Lys, and Glu to examine the effect on the redox potential, acid stability, and copper site geometry. The midpoint redox potential varies from 363 (Met148Lys) to 798 mV (Met148Leu). The acid stability of the oxidized proteins is reduced except for the Met148Gln mutant. The Gln mutant remains blue at all pH values between 2.8 and 8, and has a redox potential of 563 mV at pH 3.2. The optical and rhombic EPR properties of this mutant closely resemble those of stellacyanin, which has the lowest redox potential among single-type 1 copper proteins (185 mV). The Met148Lys mutant exhibits type 2 Cu EPR and optical spectra in this pH range. The Met148Glu mutant exhibits a type 2 Cu EPR spectrum above pH 3 and a mixture of type 1 and type 2 Cu spectra at lower pH. The Met148Leu mutant exhibits the highest redox potential (∼800 mV at pH 3.2) which is similar to the values in fungal laccase and in the type 1 Cu site of ceruloplasmin where this axial ligand is also a Leu.

Rusticyanin is a small type 1 "blue copper" protein and is thought to be a principal component in the iron respiratory electron transport chain of *Thiobacillus ferrooxidans* (*1*, *2*). Crystallographic studies (*3*, *4*) have confirmed that the Cu coordination is very similar to that of other blue copper proteins, namely, a distorted trigonal planar geometry with the three strong planar ligands His85 $N^{\delta 1}$, Cys138 S^{γ}, and His143 $N^{\delta 1}$ and a relatively weaker Met148 S^{δ} ligand in an axial position. The protein possesses the highest redox potential of the single-blue copper proteins (680 mV compared to more typical values of ∼300 mV) and is stable and active at pH 2. We have previously suggested that Ser86 (the residue which is adjacent to the Cu ligand His85), rather than conserved Asn as found in all other structurally characterized single-blue copper proteins, may provide some protection for the metal in highly acidic media. Asn is also fully conserved in the type 1 sites of copper-containing nitrite reductases. In azurin, mutation of Asn to Leu results in an increase in redox potential of 110 mV (*5*). Site-directed mutagenesis (SDM) with rusticyanin has confirmed that Ser86 has a significant impact on the acid stability and redox potential primarily arising from the reduction in the number of hydrogen bonds due to this mutation (*6*). A study of the equivalent Asn residue in poplar plastocyanin has been reported where Asn was mutated to Gln, Thr, and Leu, confirming its importance in H-bonding and its role in suppressing side reactions of the cysteine ligand (*7*). We suggest that the substitution of Asn with Ser in plastocyanin would lead to a more stable Cu site.

The nature of the axial ligands has also been discussed extensively in terms of fine-tuning of the redox potential of cupredoxins (*8*, *9*), and SDM studies with azurin, for example, have tended to support this view (*10*). The electronic properties of the type 1 Cu site have also been the subject of a number of studies (*11*, *12*). NMR measurements have suggested that in azurin the Cu−S(Met) bonding interaction is very weak but is somewhat more significant for amicyanin, where about 1% of the spin density lies on the S(Met) atom (*13*). The possible importance of the axial ligand was also emphasized by the finding that stellacyanin, which has a low redox potential of 184 mV, has a glutamine residue in place of the usual methionine. Furthermore, the type I centers in fungal laccase and ceruloplasmin, where the methionine ligand is replaced by a leucine, have redox potentials in excess of 500 mV. A recent crystal structure of a laccase from *Coprinus cinereus*, for which the redox potential is 550 mV, has revealed a Leu at about 3.5 Å (*14*). Leu is expected to be the axial ligand in most of the fungal

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laccases, where the highest recorded redox potential is 767 mV (*15*). A redox potential of 580 mV has been assigned to one of the three mononuclear Cu centers in ceruloplasmin, where again Leu is the axial ligand. Rusticyanin has the second highest redox potential known for type 1 centers, but in this case, the axial ligand is Met. Consequently, the role of the axial ligand in determining these high redox potentials remains unknown. In this paper, SDM studies of Met148 in rusticyanin are reported to provide new insight into the role of the Met ligand. Unlike azurin (the only other single-blue Cu protein to be extensively studied by SDM and crystallography), rusticyanin lacks the second axial interaction and thus provides a better framework for studying the role of the Met ligand and its effect on the type 1 Cu site. Rusticyanin is also a superior model for the type 1 Cu centers in multi-copper proteins which also lack the additional axial interaction. Therefore, the Met148 residue was replaced by glutamine to mimic stellacyanin and by leucine to mimic fungal laccase and the high-redox potential Cu site of ceruloplasmin. Previous SDM work with azurin where glutamic acid and lysine were substituted for the Met ligand revealed an interesting pH dependency of the properties of the copper center (*10*). Equivalent mutations in rusticyanin were carried out, and their properties are discussed in terms of the requirement for a type 1 Cu site.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Escherichia coli strains XL-2 Blue (Stratagene) and BL21(DE3) (Novagen) were used in this study. Electroporation was used routinely for transforming cells. Plasmids were obtained from Novagen (pET21d), Promega (pGem Z), and Stratagene (Bluescript).

Cloning and Site-Directed Mutagenesis. SDM was performed using the PCR-based method of Higuchi et al. (*16*). Briefly, primer 1 was used with primer 3 (or the corresponding primer for the appropriate amino acid) and primer 2 with primer 4 in the first round of PCR. The products from this were purified and used together in the second round of PCR with primers 1 and 2 to complete the entire gene containing the desired mutation. The resulting mutants were cloned into pMCS-3 (a modified pGEM 3Z vector containing an *Nco*I site). Sequences were verified, using the Thermo Sequenase II kit (USB, Amersham), prior to ligating into pET21d. The primers used to introduce the mutations are given below.

Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer PCR Mate: primer 1, 3′-TAAGCTCGAGGCAGCT-5′; primer 2, 5′-TTGTTTAACTTTAAGAAGGA-3′; primer 3, 5′-ACCGGTAAGTTCGGCAAGA-3′; and primer 4, 3′-GCGGTGGCCATTCAAGCCG-5′ lysine. The underlined codons were replaced with TTG/AAC, GAG/CTC, CAG/GTC, and TAA/ATT for leucine, glutamic acid, glutamine, and STOP, respectively.

Cell Culture and Expression. The conditions used for cell culture and expression were as reported previously (*6*) with the exception that the cells were stored in 50 mM acetate buffer (pH 4.0) at −20 °C prior to extraction.

Protein Purification. The majority of the protein produced when Met148 was replaced with Gln, Glu, Leu, or Lys or when the C-terminus was deleted was found in the cell debris after sonication, presumably as inclusion bodies. Consequently, the cell debris was treated with 5 M urea in 50 mM acetate buffer (pH 4.0) and dialyzed against the same buffer. Copper sulfate (5−20 mM) was added during dialysis, and the characteristic colors developed over the course of several minutes. The extract was then centrifuged at 15000*g* to remove the precipitate and filtered through a 0.2 μm filter prior to ion exchange and gel filtration chromatography as described previously (*6*).

pH Stability Tests. Protein samples at approximately 1.0 mg/mL were fully oxidized with K_2IrCl_6 in 100 mM acetate buffer (pH 6.2). Following removal of excess oxidizing agent with PD 10 columns (Pharmacia), the absorbance at 600 and 399 nm was monitored for 30 min at 6 min intervals utilizing the autorate assay program on a UVIKON 930 UV−vis spectrophotometer fitted with a sample changer. The pH of the solution was then lowered by adding predetermined volumes of 0.5 M H_2SO_4 and the absorbance monitored for 30 min at each pH value. In other experiments, the pH was set at 2.0 for 30 min, after which the solution was exchanged for 100 mM acetate buffer (pH 6.2). The samples were monitored for 15 min, and then 2.5 μL of oxidant was added and monitoring continued for 15 min. Copper sulfate (5 μL, 20 mM) was then added, and the response was followed for a further 15 min.

Protein Characterization. The midpoint redox potential of the copper center of the expressed protein and mutants was determined by redox potentiometry. Redox titrations were performed according to the method of Ingledew and Colby (*17*). The solutions were poised at approximately 100 mV above the anticipated potential with K_2IrCl_6 in alanine sulfate buffer (pH 3.2) or acetate buffer (pH 4.2−6.2) at 20 °C with 100 μM $FeCl_3$, thionin, TMPD, or $FeCl_3$/EDTA as the mediator. Electrodes were calibrated against 2,6-dichlorophenolindophenol. Reduction of proteins was achieved with ascorbic acid. SDS−PAGE was performed as described by Hall et al. (*6*). Electronic absorption spectra were taken on a Perkin-Elmer lambda 16 double-beam spectrometer, and X-band EPR spectra were recorded at −150 °C on a JEOL JES RE2X spectrometer.

RESULTS

Expression and Purification of the Met148 Mutants. The constructs in pET21d were successfully expressed in large quantities from *E. coli* BL21(DE3). The mutant proteins exhibited chromatographic behavior identical to that of recombinant wild-type rusticyanin. SDS−PAGE analysis demonstrated that the mutant proteins were highly pure. The STOP protein (C-terminus deleted) was also successfully expressed in *E. coli* BL21(DE3) and was located in the cell debris. However, following urea extraction, precipitation occurred upon dialysis unless the pH of the buffer was greater than 7.0. Therefore, dialysis was performed in Tris-HCl buffer (pH 8.0). The purified protein has the expected M_r on SDS−PAGE and also binds copper to give very weak absorbance at 400, 618, and 718 nm. However, it passes through 10 000 and 3000 molecular weight ultrafiltration membranes, probably as a result of incorrect folding. It has been noted previously that C-terminal deletions have an adverse effect on production of pseudoazurin (*18*) which in this case was attributed to proteolytic attack.

Characterization of Met148 Mutants. Except for the STOP mutant, each of the mutant proteins fold correctly as

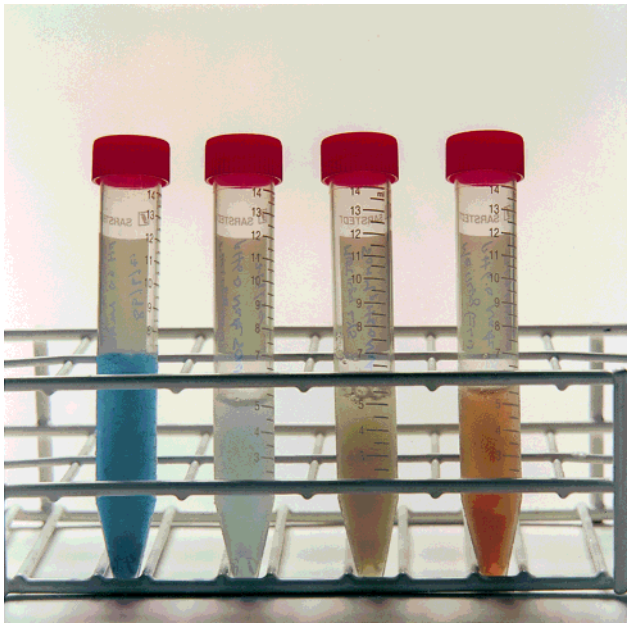

FIGURE 1: Color of Met148 mutants at pH 6.0. From left to right, Gln (deep blue), Leu (light blue), Lys (pale green), and Glu (pale brown).

evidenced by the detailed characterization given below. Like the native protein, each possesses some color (Figure 1), with the Met148Gln mutant exhibiting an intense blue color and Met148Glu a brown color at pH ~6.

Optical Spectra. The spectroscopic properties of the Met148 mutants are considerably different from those of the wild-type, recombinant[1] protein (Figure 2a). The Gln mutant most resembles RWT with an absorbance maximum at 601 nm and a second band at 453 nm (Figure 2b,c). The absorbance maxima have moved by 9 and 3 nm, respectively, to longer wavelengths relative to that of the RWT. The Leu mutant is light blue (Figure 1b) with maxima at 600 and 400 nm (Figure 2d). The Lys mutant is light green (Figure 1c) and absorbs maximally at 400 nm with a much weaker absorbance at 497 nm and little absorbance at around 600 nm (Figure 2g,h). Met148Glu absorbs strongly at 396 nm with a second peak at 518 nm and is brown (Figures 1d and 2f). There is some variation with pH (Figure 2). The least affected by pH was that of the Gln mutant which exhibits a shift in the maximum absorbance from 450 and 600 nm at pH 2.8 to 454 and 603 nm at pH 8.0, respectively, with only minor changes in the absorbance ratio between these two bands. This movement continues as the pH is further increased until the protein becomes purple. The properties of the Glu mutant are very much pH-dependent and change dramatically as the pH is reduced (Figure 2e). The absorbance maxima change to 440 and 594 nm, and the intensities reverse so that the protein becomes deep blue (somewhat similar to Met148Gln) when the pH is below 3.0. The effects of pH on the Lys and Leu mutants are significant but less extreme. Thus, the absorbance maximum of Met148Lys drifts as the pH is raised (maximum absorbance at pH 8.0 = 400 nm, at pH 9.0 = 396 nm, at pH 11.0 = 391 nm, and at pH 12 = 388 nm) with some loss of color at pH 12. There is a transient reversal of intensities of the peaks in the Met148Leu mutant as the pH is reduced; at pH 4.0, the initial ratio (600 nm/400 nm) was 0.93, but after 6 min, this increased to 1.15. There is a steady decline in the intensities of both the 600 and 400 nm bands at pH 6, to 90 and 77% of the original absorbance, respectively, after 60 min and at pH 4.0 to 58 and 48%, respectively. The situation as the pH is raised is more complex as the intensities reverse and then slowly decline. However, after 35−40 min, the intensities stabilize and there is little or no decline for at least a further 20 min.

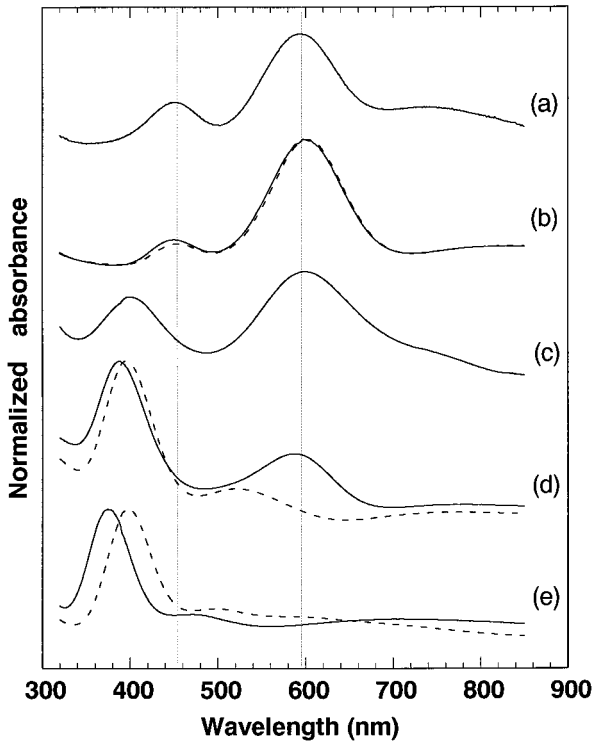

FIGURE 2: pH-dependent optical spectra of the recombinant wild type (a) and Met148 mutants: (b) Gln at pH 8 (- - -) and 2.8 (—), (c) Leu at pH 6, (d) Glu at pH 8 (- - -) and 2.8 (—), and (e) Lys at pH 8 (- - -) and 4 (—). A vertical line is drawn to indicate the position of the peaks in the wild-type protein.

EPR Spectra. The EPR spectra of the mutants are very different from each other and from that of the RWT protein (Figure 3). No EPR spectrum of Leu is given as it was more difficult to keep it fully oxidized at high concentrations in view of its very high redox potential. There is a distinct pH dependence for each mutant. The spectrum of Met148Gln is the least affected by pH, showing a rhombic type 1 Cu EPR signal between pH 8 and 2.8 (Figure 3b,c), which is very similar to the spectrum of stellacyanin. The EPR of the Met148Glu mutant is axial at high pH (6−8) with a type 2 Cu hyperfine splitting of 11.7 mT (Figure 3d and Table 1). The spectrum is similar to that recorded at pH 6 for the Met121Glu mutant of azurin from *Pseudomonas aeruginosa* (*19*). At lower pH (2.8), the spectrum is more complex (Figure 3e) and clearly consists of contributions of a mixture of at least two magnetically distinct Cu centers. The spectrum of the Met148Lys mutant possesses a type 2 Cu hyperfine splitting (13.9−13.6 mT) at all pH values (Figure 3f,g) and becomes slightly less axial at lower pH. At pH 4, the hyperfine splitting decreases by 0.03 mT, and this is accompanied by an increase of 0.043 in g_{zz}. This implies a more tetragonal Cu environment for Met148Lys at pH 4. The EPR parameters obtained for the best simulation are summarized in Table 1.

[1] Wild-type recombinant rusticyanin is termed RWT.

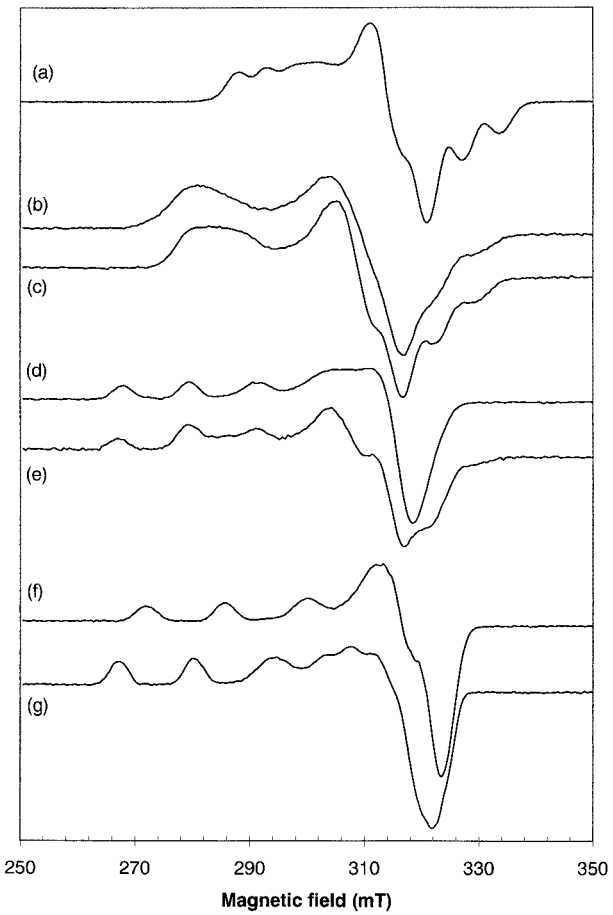

FIGURE 3: X-band EPR spectra of the recombinant wild type (a) and Met148 mutants: (b and c) Gln at pH 8 and 2.8, (d and e) Glu at pH 8 and 2.8, and (f and g) Lys at pH 8 and 4, respectively.

Table 1: Parameters Obtained by Simulation of EPR Spectra for Met148 Mutants of Rusticyanin[a]

mutant	pH	g_{xx}	g_{yy}	g_{zz}	A_{xx}	A_{yy}	A_{zz}
Met148Gln	2.8	2.045	2.077	2.285	6.8	1.2	4.2
Met148Glu	8	2.070	2.070	2.290	2.00	2.00	11.7
Met148Lys	8	2.054	2.054	2.227	2.05	2.05	13.9
	4	2.045	2.095	2.270	1.20	4.80	13.6
native protein	4	2.015	2.052	2.211	6.5	1.3	5.0

[a] Hyperfine values are given in milliteslas.

pH Stability. Table 2 provides details of the pH stability for all of the mutant proteins. The data given in Table 2 clearly show that the Met148Gln mutant is far more acid resistant than the other mutants and the order of stability for the oxidized site is as follows: Gln ≫ Glu > Lys > Leu. There is no alteration of the absorbance ratio of the Gln mutant with little or no loss of protein down to pH 2.1. The interpretation for the Glu mutant is somewhat complicated by the changes in spectroscopic properties that begin at ca. pH 3.0. Consequently, the values above pH 3.0 are expressed as a percentage of the initial absorbance at pH 6.2, and below pH 3.0, the percentages are expressed in terms of the initial value at that pH. This shows that there is relative stability around pH 2.7, but at pH ≤2.4, the protein−copper site is unstable and the color quickly fades. The ratio of absorbance at 600 nm/399 nm for the Glu mutant shows that the intensity of the 600 nm band increases rapidly as the pH decreases and the ratio will reach at least 6.9 but that at low pH values

Table 2: (a) Stability of Met148 Mutants Expressed as a Percentage of the Initial Absorbance[a] and (b) Recovery of Original Absorbance Expressed as a Percentage

(a) Stability

pH	Gln	Glu	Leu	Lys	RWT
6.2	100	100	100	100	
5.3	100	100	86	96	
4.5	100	93	53	82	100 (4.0)
3.0	100	100	58	52	97 (3.2)
2.7	99	94	7	24	98 (2.5)
2.4	88	65	0	0	−
2.1	87	65	0	0	86 (1.9)

(b) Recovery

Gln	Glu	Leu	Lys	
100	56	71	41	30 min at pH 2.1
100	100	100	78	after addition of copper
100	64	83	41	reoxidation at pH 6.2

[a] See the text for experimental details. For comparison, values for the RWT (from ref *6*) are given in the last column; in this case the pH values were slightly different and are given in parentheses.

Table 3: Redox Potentials (in Millivolts) of the Met148 Mutants of Rusticyanin as a Function of pH[a]

	pH 3.2	pH 4.1	pH 5.2	pH 6.2
Gln	563	−	−	472
Glu	551 (2.6)	−	−	363
Leu	798	777	711	613
Lys	550	−	−	406
RWT	667	623 (4.2)	−	580

[a] The numbers in parentheses show the actual pH that was used. In the case of the Glu mutant, the protein is still in the "brown" configuration at pH >3.0.

the protein−copper site is very unstable and the color is rapidly lost. Data in Table 2b indicate that the Leu mutant is largely recovered upon reoxidation, showing that the loss of color is due to reduction. In contrast, the Lys mutant does not exhibit any recovery upon reoxidation, showing that the loss of absorbance is a result of the loss of copper. The data in Table 2b also demonstrate that the Gln, Glu, and Leu mutants will recover fully from treatment at ca. pH 2.1 for 30 min upon addition of copper, but there is significant loss in the case of the Lys mutant. This loss is almost certainly a result of denaturation as confirmed by an analysis of the precipitated protein from the Glu, Leu, and Lys mutants treated at pH <2.0. Thus, the mutations exhibit a reducing stability for the copper site in the following order: Gln > Leu > Glu > Lys.

Redox Potentials. The redox potentials of the Met148 mutants range from 363 mV for the Glu mutant at pH 6.2 to about 800 mV for the Leu mutant at pH 3.2 (Table 3). The total span is some 430 mV, but apart from the Glu mutant at pH 6.2, the potentials remain high compared to those typically encountered for the blue copper proteins. The redox potentials of all the mutants decrease as the pH increases, an effect that is largest for the Glu mutant (by 188 mV) and smallest for Gln (by 91 mV). The value for the Leu mutant was difficult to assess at lower pH with any great accuracy due to the slow decay of the oxidized form at low pH. Nevertheless, the values obtained at pH 6.2 where the mutant is reasonably stable (10% loss in absorbance at 600 nm over the course of 60 min) show that the redox potential is ∼800 mV, substantially above that of the RWT rusticyanin.

DISCUSSION

The differences in the optical and EPR spectra of the mutants imply significant changes in their Cu site coordination. The redox potentials presented in Table 3 reveal large differences between the mutants and the RWT protein. The range of redox values from ~360 to ~800 mV encompasses those found for plastocyanin (360 mV) and fungal laccase (767 mV) (*15*). There is a significant change in redox potential with pH for the RWT. The values determined for the RWT at various pH values are reasonably close to those reported previously [Ingledew and co-workers (*20*, *21*) (670 mV at pH 2.2 and 620 mV at pH 5.3) and Haladjian et al. (*22*) (600 mV at pH 4.0 and 564 mV at pH 6.0)].

The transition from brown to blue of Met148Glu at pH <3 is similar to that reported for the Met121Glu mutant of azurin where it occurs at a higher pH (*19*). At pH 6 (for azurin) and 8 (for rusticyanin), the blue color is lost in both mutants and the EPR spectrum becomes similar to the spectrum that is typical for type 2 Cu. At pH 4, the Met121Glu mutant of azurin is intensely blue in color and exhibits a single-component rhombic type 1 EPR spectrum. For the Met148Glu rusticyanin mutant, a blue color is present at pH <3, signifying the presence of a type 1 Cu center, but a proportion of the type 2 Cu EPR signal remains to give a complex overall EPR spectrum (Figure 3d). In the azurin mutant, the change from a type 1 to type 2 Cu site has been interpreted as a change due to the protonation of the glutamate side chain. At pH 8.0, it is deprotonated and a strong interaction between Glu and the copper atom results, whereas at low pH, the glutamate is protonated and interacts weakly with the copper. The similar behavior of the Met148Glu mutant at high pH is an indication that the same mechanism is operating. Thus, the transition from blue to brown and from type 1 to type 2 EPR is most probably associated with the coordination shifting from three strong and one weak ligand to four strong ligands as is the case in the analogous mutation in Met121Glu azurin (*19*, *23*). The lowering of the redox potential to 363 mV at pH 6.2 for the Glu mutant brings it within the range of the values of the other cupredoxins; however, this only occurs when there is a significant alteration in the properties of the Cu site such that the defining features of the type 1 site are lost.

The Met121Lys and Met121Glu mutants of azurin exhibited similar pH dependencies (*10*). This is not the case for the equivalent mutations of rusticyanin (Figures 2 and 3). The Met148Lys mutant remains a nonblue type 2 Cu site at all pH values. Thus, at pH 6.0 the absorbance is around 400 nm with a second less intense absorbance at 497 nm. These bands shift to shorter wavelengths as the pH is altered in either direction, and this is accompanied by a tendency toward a less rhombic EPR spectrum (Figure 3). The manner in which the EPR spectrum varies with pH suggests that the optical changes are unlikely to be the result of the formation of an additional copper bond but rather are a result of a reorganization of the coordination geometry at the Cu site. Thus, at the lower pH the type 2 spectrum indicates a more planar tetrahedral geometry, while the axial EPR at higher pH suggests a trigonal arrangement with a weak fourth ligand.

The substitution of Met148 with Gln mimics the axial ligand found in stellacyanin. The optical and EPR spectra

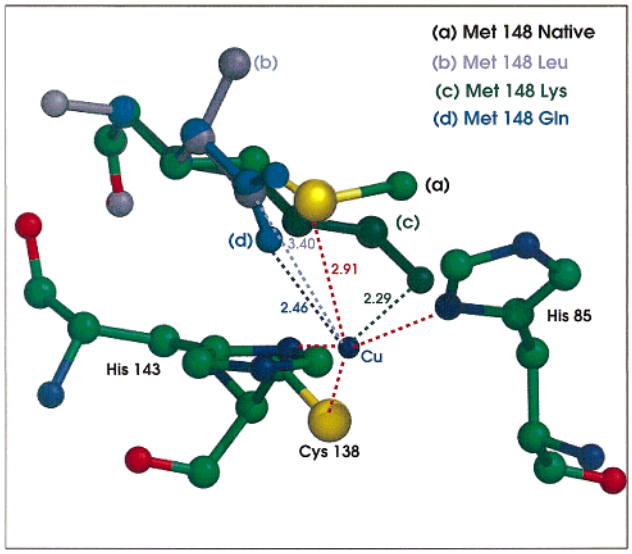

FIGURE 4: Copper site surrounded by its immediate ligands, His85, Cys138, His143, and Met148 (PDB file name 1a8z). The modeled Met148Gln, -Leu, and -Lys mutants are shown in different colors where (a) in the wild-type protein (black), the sulfur atom of Met forms a weak bond with copper (~2.9 Å); (b) in Met148Leu (gray), a longer distance between the Leu and Cu is found (~3.4 Å) for the most probable rotamer; (c) in Met148Lys (green), a stronger interaction (~2.3 Å) is possible; and (d) in Met148Gln (blue), the most probable rotamer forms a ~2.5 Å bond with the Cu. Although not shown in this figure, as one would expect, the Met148Glu has a very similar rotamer forming a similar bond (~2.5 Å).

of this mutant are very similar to those of stellacyanin and indicate that there is a stronger axial interaction with the Gln residue than with the Met found in the RWT. The stronger interaction implied by these data is fully consistent with the reduced redox potential of this mutant compared to that of the RWT. Stellacyanin has a short Cu−Gln distance of 2.2 Å demonstrated in the crystal structure (*24*) compared to ~2.8 Å for the Cu−Met bond in rusticyanin (*3*, *4*).

The substitution of Leu for Met causes a large increase in the reduction potential (Table 3) to a value which is equivalent to the highest reported for fungal laccases. The lack of stability of the oxidized form of Met148Leu at pH <6 is fully consistent with its very high redox potential. Full recovery of the absorbance after addition of Cu at pH 2.1 suggests that this mutation does not cause any significant deterioration in the integrity of the protein or the Cu site. The elevation of the redox potential by ~130 mV at pH 3.2 is fully consistent with the increased potentials of the Leu-containing type 1 Cu sites of ceruloplasmin and fungal laccase.

The substitution of Met148 with Gln causes a drop in the redox potential of ~100 mV. Despite this reduction, the redox potential of this mutant is still substantially higher than that reported for stellacyanin at pH 6.0 (*25*). Consequently, it may be concluded that the outer-sphere environment exerts a major influence, probably as a result of the higher concentration of hydrophobic groups close to the copper center and the orientation of dipoles as discussed by Walter et al. (*3*), Botuyan et al. (*26*), and Harvey et al. (*4*). We have already demonstrated via point mutations of Asn86 that the redox potential of rusticyanin can be reduced by ~80 mV when Ser is substituted for Asn (*6*).

The structural consequences of these mutations have been modeled using the side chain rotamer library (*27*) and the

native rusticyanin crystal structure (*4*). The axial ligand in the native protein forms a ~2.9 Å bond from S(Met148) to the copper. All three rotamers for Met148Leu show that the Leu is significantly more distant (~3.4 Å) such that there is a much weaker interaction (Figure 4b). This is consistent with its elevated redox potential. The Gln and Glu mutants have very similar rotamers which are ~2.5 Å away from the copper. The stronger interaction in the case of the Gln mutant would result in a reduction in its potential, but its geometry is such that it would not necessarily cause the character of the type 1 site to change dramatically. Although Glu may occupy a position similar to that of Gln with respect to the Cu, the charged nature of this ligand accounts for a shift from type 1 character to a type 2 site at higher pH in a manner similar to that which has been observed for the Met121Glu mutant of azurin (*19*). The most probable rotamer of Lys would adopt a weak ~3.8 Å bond. However, the Met148Lys mutant may also be modeled using the second most probable rotamer. In this case, Lys comes very close to Cu (~2.3 Å), which is thus likely to force a change at the Cu site giving it its type 2 spectroscopic properties.

CONCLUSIONS

The role of the axial Met ligand in type 1 copper centers is complex. This ligand is important in ensuring a stable Cu^+ geometry and efficient conversion between the Cu^{2+} and Cu^+ redox states. The redox potential is elevated by ~100 mV if Met is replaced by Leu, while it is decreased by a similar amount if Met is replaced by Gln. These changes in redox potentials are consistent with the decreased or increased potential of stellacyanin or fungal laccase and ceruloplasmin where Gln or Leu is in the axial position, respectively. A similar elevation of the redox potential by ~100 mV was also reported for the Met121Leu mutant in azurin (*28*); however, a corresponding decrease in the value for Met121Gln was not observed for azurin (*29*). In the case of rusticyanin, the elevated redox potential must arise from several additional factors. We have already shown that Ser substitution (instead of the usual Asn) in rusticyanin increases both the acid stability and redox potential (by ~100 mV) (*6*). The redox potential of the Ser86Asn mutant, 590 mV, is still some 200 mV higher than the normal range for cupredoxins which must arise from the additional outer-sphere influences, including the increased hydrophobicity of the outer pocket of the Cu ligands. Nevertheless, the study presented here further confirms the importance of the axial ligand in defining the chemistry of the type 1 Cu centers.

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