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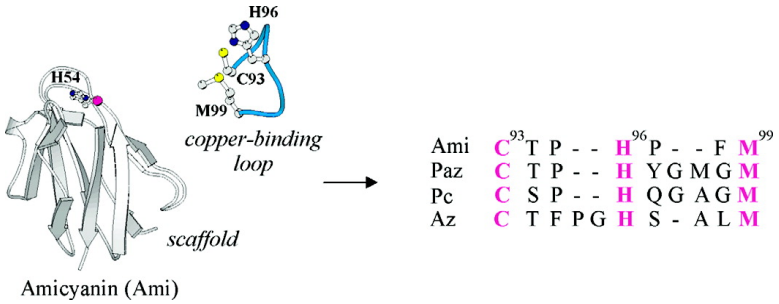


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# Loop-Contraction Mutagenesis of Type 1 Copper Sites

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***Abstract:*** The shortest known type 1 copper binding loop (that of amicyanin, Ami) has been introduced into three different cupredoxin $\beta$-barrel scaffolds. All of the loop-contraction variants possess copper centers with authentic type 1 properties and are redox active. The Cu(II) and Co(II) sites experience only small structural alterations upon loop contraction with the largest changes in the azurin variant (AzAmi), which can be ascribed to the removal of a hydrogen bond to the coordinating thiolate sulfur of the Cys ligand. In all cases, loop contraction leads to an increase in the p$K_a$ of the His ligand found on the loop in the reduced proteins, and in the pseudoazurin (Paz) and plastocyanin (Pc) variants the values are almost identical to that of Ami ($\sim$6.7). Thus, in Paz, Pc, and Ami, the length of this loop tunes the p$K_a$ of the His ligand. In the AzAmi variant, the p$K_a$ is 5.5, which is considerably higher than the estimated value for Az ($<$2), and other controlling factors, along with loop length, are involved. The reduction potentials of the loop-contraction variants are all lower than those of the wild-type proteins by $\sim$30$-$60 mV, and thus this property of a type 1 copper site is fine-tuned by the C-terminal loop. The electron self-exchange rate constant of Paz is significantly diminished by the introduction of a shorter loop. However, in PcAmi only a 2-fold decrease is observed and in AzAmi there is no effect, and thus in these two cupredoxins loop contraction does not significantly influence electron-transfer reactivity. Loop contraction provides an active site environment in all of the cupredoxins which is preferable for Cu(II), whereas previous loop elongation experiments always favored the cuprous site. Thus, the ligand-containing loop plays an important role in tuning the entatic nature of a type 1 copper center.

## Introduction

Engineering studies have been extensively used to determine the important features of proteins. In the case of metalloproteins, a variety of approaches have identified the key attributes of their active centers.[1−13] Many metal sites in proteins are fabricated from loop regions, and in some cases it is possible to model these using the loop alone,[3−5] but this is generally not possible.[12] The loop usually needs to be attached to a stable structure, and with "loop-directed mutagenesis" it is possible to graft active sites onto protein scaffolds.[9] The cupredoxins, a family of electron transfer (ET) metalloproteins, possess a rigid $\beta$-barrel structure which is an ideal scaffold for protein engineering studies, and loop-elongation mutagenesis has previously been used to modify the mononuclear type 1 copper site,[14−16] and to introduce a dinuclear $Cu_A$ center (naturally found in cytochrome *c* oxidase and nitrous oxide reductase).[17,18] We recently demonstrated that loop-contraction mutagenesis can also be carried out in a cupredoxin.[19]

The cupredoxins used in this present study are azurin (Az), pseudoazurin (Paz), plastocyanin (Pc), and amicyanin (Ami). The overall folds of these proteins are made up of 8 $\beta$-strands which form into a Greek key $\beta$-barrel topology.[20−27] The structures of Paz, Pc, and Ami are all similar, but Paz has a 30

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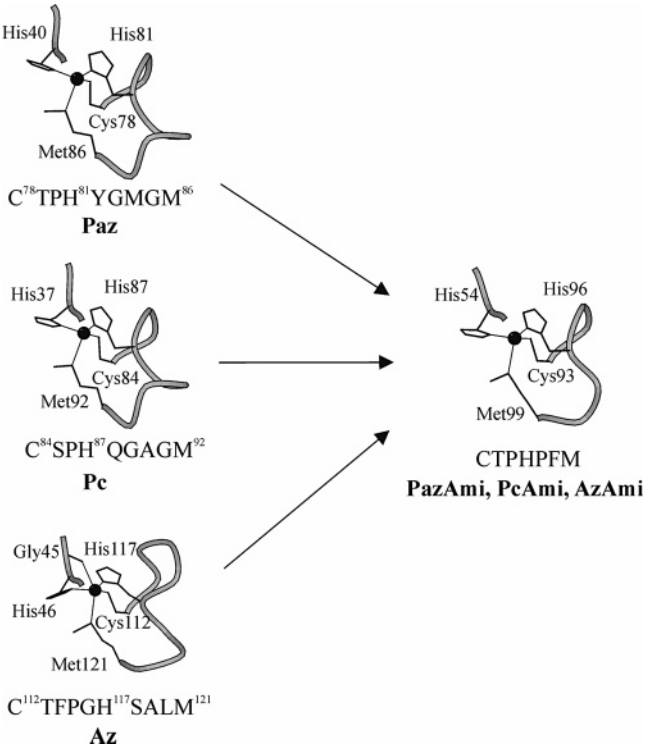


***Figure 1.*** The active site structures of *A. cycloclastes* pseudoazurin (Paz),[22] spinach plastocyanin (Pc),[21] *P. aeruginosa* azurin (Az),[23,24] and *P. versutus* amicyanin[25] drawn with MOLSCRIPT.[28] The sequences of the C-terminal active site loops are shown and indicate the loop-contraction mutations that have been made to Paz, Pc, and Az to produce PazAmi, PcAmi, and AzAmi, respectively.

residue C-terminal extension consisting of two α-helices that pack onto the surface of the $\beta$-barrel. The structure of Az is different with more residues within the $\beta$-barrel structure mainly accommodated in the loops joining strands 3 and 4 and strands 4 and 5. The additional residues in the former loop contribute to a more buried active site in Az, and the extension of the loop linking strands 4 and 5 results in a α-helical flap which sits on the surface of the barrel in a location analogous to the α-helical regions of Paz. All of these proteins have type 1 copper sites situated in similar positions within their cupredoxin folds involving strong coordination of the metal by the thiolate sulfur of a Cys and the imidazole nitrogens of two histidines (see Figure 1). In Pc,[20,21] Paz,[22] and Ami,[25] the coordination environment is completed by a weak axial Met ligand. In Az, the metal ion has two weak axial interactions provided by the thioether sulfur of a Met and the backbone carbonyl oxygen of a Gly residue.[23,24] Thus, the active site geometry of Az is trigonal bipyramidal, while in the other three proteins a distorted tetrahedral arrangement is found. The active site structures of type 1 copper sites give rise to distinct spectroscopic properties including a S(Cys) → Cu(II) ligand to metal charge-transfer (LMCT) transition at around 600 nm.[29,30] Additionally, type 1 copper sites possess an unusually small hyperfine coupling constant in their electron paramagnetic resonance (EPR) spectra due to the highly covalent Cu−S(Cys) bond.[29,30] Type 1 copper sites exhibit a large range of reduction potentials (from ~0.2 to 1.0 V) which are higher than that of the Cu(II)/Cu(I) aqua couple.[29,30]

The architecture of type 1 copper sites involves three of the four canonical ligands being situated on a single loop which links $\beta$-strands 7 and 8. The shortest loop is found in Ami, and previous loop-elongation mutagenesis experiments introduced larger cupredoxin loops into this protein.[14−16] Herein, we replace the loops of Paz (we have previously reported an initial characterization of this variant[19]), Pc, and Az with the short sequence from Ami (see Figure 1). Thus, the same sequence has been introduced into three different cupredoxins and the properties of these loop-contraction variants, along with those of Ami, Paz, Pc, and Az, provide an intriguing insight into the role of the ligand loop and the $\beta$-barrel scaffold in controlling the properties of type 1 copper sites.

## Materials and Methods

**Loop-Contraction Mutagenesis.** Loop-contraction mutagenesis was carried out using the QuickChange (Stratagene) site-directed mutagenesis kit. The genes encoding Paz, Pc, and Az were sub-cloned from pTrcsPAz [a pTrc99A (Pharmacia) derivative harboring the gene for *Achromobacter cycloclastes* Paz including the transit peptide],[31] pTrcPc (a pTrc99A derivative harboring the gene for spinach Pc behind the *Pseudomonas aeruginosa* Az transit peptide), and pTrcAz (a pTrc99A derivative harboring the gene for *Pseudomonas aeruginosa* Az including the transit peptide), respectively, into pGEM-T (Promega) and were used as templates for mutagenesis. The following primers were used to bring about the mutations: gcgtaaagtgcacgccgcacccgttcatggtcggcgtcgttcagg (forward primer) and cctgaacgacgccgaccatgaacgggtgcggcgtgcactttacgc (reverse primer) for PazAmi [Paz loop-contraction mutant in which the C-terminal loop is replaced with that of Ami], cttacaagttctactgcacgccgcacccgttcatggtgggaaaagtaac (forward) and gttacttttcccaccatgaacgggtgcggcgtgcagtagaacttgtaag (reverse) for PcAmi (Pc loop-contraction mutant in which the C-terminal loop is replaced with that of Ami), and catgttcttctgcaccccgcacccgttcatgaagggcaccctg (forward) and cagggtgccccttcatgaacgggtgcggggtgcagaagaacatg (reverse) for AzAmi (Az loop-contraction mutant in which the C-terminal loop is replaced with that of Ami). DNA sequencing was used to verify the mutations in all cases. pTrc99A derivatives harboring the PazAmi, PcAmi, and AzAmi genes (pTrcPazAmi, pTrcPcAmi, and pTrcAzAmi, respectively) were used as expression vectors.

**Cell Growth and Initial Protein Isolation.** *Escherichia coli* BL21, TG1, and JM101 were transformed with pTrcPazAmi, pTrcPcAmi, and pTrcAzAmi, respectively. Cells were grown (cultures were grown for typically 3−5 h after induction) and harvested as described previously.[31] The periplasmic proteins were obtained as described previously, and isolation and purification of the loop-contraction mutants was carried out using the procedures described below.

**Isolation and Purification of PazAmi.** The PazAmi-containing solution was adjusted to pH 6.5 and incubated with carboxymethyl (CM) sepharose (Pharmacia) for 60 min at 4 °C with stirring. The bound proteins were eluted with 10 mM phosphate pH 7.0 containing 250 mM NaCl. The protein was exchanged into 10 mM phosphate pH 6.0 by ultrafiltration (Amicon stirred cell, 5 kDa membrane) and loaded onto a CM-sepharose column. The bound PazAmi was eluted with a gradient of 0−250 mM NaCl in the same buffer. The PazAmi-containing fractions were exchanged into 20 mM phosphate pH 6.0 by ultrafiltration and loaded onto a CM-sephadex (SIGMA) column. Pure PazAmi was eluted with a gradient of 0−150 mM NaCl in the same buffer. Fully oxidized PazAmi with an $A_{278}/A_{595}$ ratio of ≤1.3 gave a single band on a 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The final yield of PazAmi was ~4

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mg/L of cell culture. Wild-type (WT) Paz was isolated and purified using the same procedure.

**Isolation and Purification of PcAmi.** The PcAmi-containing solution was incubated with (diethylamino)ethyl (DEAE) sepharose (Pharmacia), for 60 min at 4 °C with stirring. The bound proteins were eluted with 10 mM tris(hydroxymethyl)aminomethane (Tris) pH 8.0 containing 300 mM NaCl. The protein mixture was exchanged into 10 mM Tris pH 8.0 and loaded onto a DEAE-sepharose column and eluted with a gradient of 0−250 mM NaCl in the same buffer. The PcAmi fractions were oxidized using a sufficient volume of a 20 mM solution of $K_3[Fe(CN)_6]$ and exchanged into 10 mM 2-(*N*-Morpholino)-ethanesulfonic acid (Mes) pH 5.6 and loaded onto a DEAE-sepharose column. The protein was eluted with a gradient of 0−250 mM NaCl in 10 mM Mes pH 5.6. The PcAmi-containing fractions were exchanged into 20 mM Tris pH 7.0 containing 200 mM NaCl and concentrated by ultrafiltration. The solution was applied onto a Superdex 75 (Pharmacia) gel-filtration column in the same buffer. Pure oxidized PcAmi has an $A_{278}/A_{596}$ ratio of ≤1.3 corresponding to a single band on a 14% SDS-PAGE gel. The final yield of PcAmi was ∼4 mg/L of cell culture. WT Pc was isolated and purified using the same procedure.

**Isolation and Purification of AzAmi.** The AzAmi-containing solution was diluted to give a Tris concentration of 1 mM, and the pH was adjusted to 9.0. The protein mixture was incubated with DEAE-sepharose, for 2 h at 4 °C with stirring. The bound proteins were eluted with 1 mM Tris pH 9.0 containing 200 mM NaCl, exchanged into 1 mM Tris pH 9.0, and loaded onto a DEAE-sepharose column. The bound proteins were eluted with a gradient of 0−100 mM NaCl. The AzAmi fractions were exchanged into 1 mM Tris pH 9.0 containing 100 $\mu$M sodium ascorbate, to maintain AzAmi in the Cu(I) state, and were loaded onto a DEAE-sepharose column. The bound protein was eluted with a gradient of 0−50 mM NaCl. Pure oxidized AzAmi with an $A_{278}/A_{609}$ ratio of ≤2.0 gave a single band on a 14% SDS-PAGE gel. The final yield of AzAmi was ∼26 mg/L of cell culture. WT Az was isolated and purified using the same procedure.

**Preparation of the Co(II)-Substituted Loop Variants.** Apo-proteins were prepared by an unfolding/refolding procedure described previously.[32] Refolded PazAmi was exchanged into 10 mM phosphate pH 6.0 containing 2 mM ethylenediaminetetraacetic acid (EDTA), purified on a CM sepharose column at pH 6.0, and exchanged into 10 mM 4-(2-hydroxyethyl)piperadine-1-ethanesulfonic acid (Hepes) pH 8.0. A 20-fold excess of Co(II) ($CoCl_2 \cdot 6H_2O$) was added, and after incubation at room temperature (RT) overnight the excess metal was removed by ultrafiltration. Refolded PcAmi was exchanged into 10 mM Hepes pH 8.0, and a 20-fold excess of Co(II) was added, and the mixture was left overnight at RT. The Co(II) protein was purified on a DEAE sepharose column at pH 8.0. Refolded AzAmi was exchanged into 10 mM Hepes pH 8.0 and was left for 3 days in the presence of a 20-fold excess of Co(II) at RT. The Co(II) protein was purified on a DEAE sepharose column at pH 9.0. In all cases, some (≤5%) Cu protein remained in the Co(II) samples. For the characterization studies, this was maintained in the spectroscopically silent Cu(I) form by the addition of a small amount of sodium ascorbate.

**UV/Vis Spectrophotometry.** UV/Vis spectra were measured at 25 °C on either a Perkin-Elmer $\lambda$ 35 or a Shimadzu UV-2101PC spectrophotometer in 10 mM phosphate buffer pH 8.0.

**Determination of Molar Absorption Coefficients.** The molar absorption coefficients of the Cu(II) loop-contraction mutants were determined using inductively coupled plasma-atomic emission (ICP-AE) spectroscopy. The proteins were fully oxidized using a sufficient volume of a 20 mM solution of $K_3[Fe(CN)_6]$ and washed with 0.5 mM EDTA to remove adventitiously bound metal. After the excess oxidant and EDTA were removed using ultrafiltration, a UV/vis spectrum was acquired and the copper concentration was determined with a Unicam 701 ICP-AE spectrometer. The molar absorption coefficients of the Co(II) loop variants were determined in the same way but without oxidizing the samples.

**EPR Spectroscopy.** X-band EPR spectra were recorded at −196 °C on a Bruker EMX spectrometer. The proteins were fully oxidized using $K_3[Fe(CN)_6]$ and washed with 0.5 mM EDTA as above. The protein samples (2 mM) were in 25 mM Hepes pH 7.6 plus 40% glycerol. DPPH (diphenylpicrylhydrazyl) was used as an external reference, and the program SIMFONIA (Bruker) was used for spectral simulations.

**Reduced Protein Samples for $^1$H NMR Spectroscopy.** The proteins were fully reduced by the addition of sodium ascorbate. The excess reductant was removed, and the protein was exchanged into 10 or 100 mM phosphate, typically at pH* 8.0 (99.9% $D_2O$), using ultrafiltration (pH values quoted for deuterated solutions are uncorrected for the deuterium isotope effect and thus are indicated by pH*). A small amount of sodium ascorbate (100−200 $\mu$M) was added to the sample to maintain the protein in the reduced form. The sample (usually 1−2 mM) was transferred to an NMR tube, flushed with argon, and sealed.

**Sample Preparation for Electron Self-Exchange Rate Constant Measurements.** For electron self-exchange (ESE) rate constant measurements, the proteins were exchanged into 99.9% deuterated phosphate buffer (usually at $I = 0.10$ M) and prepared as described previously.[31] Measurements on AzAmi were carried out in 20 mM phosphate at pH* 8.2 ($I = 0.06$ M). To maintain the ionic strength, as the pH* values were altered for the other two loop-contraction variants, the phosphate concentration was modified in the following manner. For PazAmi, experiments were carried out in 37 mM phosphate at pH* 7.6 ($I = 0.10$ M) and 60 mM phosphate at pH* 6.5 ($I = 0.10$ M). For PcAmi, measurements were made in 37 mM phosphate at pH* 7.5 ($I = 0.10$ M) and 73 mM phosphate at pH* 6.2 ($I = 0.10$ M). The concentration of Cu(II) protein in the sample was determined by measuring the absorbance at 595 nm ($\epsilon = 4000\ M^{-1}\ cm^{-1}$) for PazAmi, 596 nm ($\epsilon = 5100\ M^{-1}\ cm^{-1}$) for PcAmi, and 609 nm ($\epsilon = 5600\ M^{-1}\ cm^{-1}$) for AzAmi.

**Samples for Paramagnetic $^1$H NMR Studies.** Paramagnetic $^1$H NMR spectra of Cu(II) and Co(II) proteins were obtained in 10 mM phosphate in 99.9% $D_2O$ typically at pH* 8.0. Spectra were also obtained of Cu(II) PcAmi in 37 mM phosphate buffer pH 7.5 in 90% $H_2O$/10% $D_2O$. Saturation-transfer experiments were carried out on a 1:1 mixture of Cu(I) and Cu(II) PcAmi (1.6 mM of each) in 37 mM phosphate pH* 7.5 ($I = 0.10$ M, 99.9% $D_2O$). Spectral were acquired of the Co(II) proteins in 10 mM phosphate in 90% $H_2O$/10% $D_2O$ at pH 8.1.

**NMR Spectroscopy.** $^1$H NMR spectra were acquired on either a JEOL Lambda 500 or a Bruker Avance 300 spectrometer as described previously.[33,34] All of the ESE rate constant determinations were at 25 °C. Paramagnetic $^1$H NMR spectra were acquired using the super-WEFT[35] pulse sequence (d−180°−$\tau_D$−90°−acq). Saturation-transfer experiments on 1:1 mixtures of oxidized and reduced PcAmi were acquired as described previously,[33] as were 1D NOE difference experiments on the Co(II) loop variants.[34] When measuring ESE rate constants, spectra of AzAmi were obtained using the super-WEFT sequence. This approach was used to observe resonances from reduced AzAmi in the presence of small amounts of the oxidized protein (2−20%) which experience paramagnetic relaxation enhancement as a consequence of the ESE reaction (and due to them being situated close to the copper site).[33] The spectra were acquired as described previously,[33] and spin−spin ($T_2$) relaxation times were derived from peak widths at half-height using the relation $\nu_{1/2} = (\pi T_2)^{-1}$.

**Electrochemistry of Proteins.** The direct measurement of the reduction potential of proteins was carried out at ambient temperature

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***Table 1.*** Properties of the Loop-Contraction Mutants (PazAmi, PcAmi, AzAmi) and Those of the Corresponding Wild-Type Cupredoxins

| parameter | Ami | Paz | PazAmi | Pc | PcAmi | Az | AzAmi |
|---|---|---|---|---|---|---|---|
| UV/vis[a] | | | | | | | |
| $\lambda_{max1}$[nm] | 460 | 454 | 463 | 460 | 473 | 460(sh)[b] | 470(sh)[b] |
| $\lambda_{max2}$[nm] | 596 | 594 | 595 | 597 | 596 | 628 | 609 |
| $\epsilon_{\sim 600}$ [$mM^{-1}$ $cm^{-1}$] | 3.9 | 3.7 | 4.0 | 4.7 | 5.1 | 5.7 | 5.6 |
| $A_{\sim 460}/A_{\sim 600}$ | 0.11 | 0.45 | 0.54 | 0.13 | 0.12 | 0.07 | 0.07 |
| EPR[c,d] | ax | rh | rh | ax | ax | ax | ax |
| $g_x$ | 2.032 | 2.015 | 2.020 | 2.035 | 2.035 | 2.035 | 2.033 |
| $g_y$ | 2.047 | 2.053 | 2.058 | 2.044 | 2.043 | 2.054 | 2.048 |
| $g_z$ | 2.235 | 2.213 | 2.205 | 2.233 | 2.227 | 2.261 | 2.225 |
| $A_x$ [mT] | 0.6 | 7.3 | 6.0 | 0.5 | 0.5 | 1.4 | 1.1 |
| $A_y$ [mT] | 0.8 | 1.8 | 1.0 | 0.8 | 0.5 | 1.4 | 1.0 |
| $A_z$ [mT] | 5.4 | 3.5 | 4.5 | 6.0 | 5.6 | 5.3 | 6.9 |
| $pK_a^{red}$ [e] (CV) | 6.6[f] | 4.9[g] | 6.6 | | 6.8 | <2[h] | 5.5 |
| $pK_a^{red}$ [e] (NMR) | 6.7[i] | | 6.7 | 4.9 | 6.7 | | |
| $E_m$[j] [mV] (CV) | 238[f] | 266 | 215 | 378 | 315 | 295[k] | 261[k] |
| $k_{ESE}$ [$M^{-1}$ $s^{-1}$][l] | $1.3 \times 10^5$ [m] | $3.7 \times 10^3$ [n] | $4.6 \times 10^2$ [o] | $2.5 \times 10^3$ [p] | $1.3 \times 10^3$ [q] | $7.0 \times 10^5$ [r] | $6.9 \times 10^5$ [s] |

[a] Measured in 10 mM phosphate at pH 8.0 (25 °C). [b] Shoulder. [c] Recorded at −196 °C in 25 mM Hepes at pH 7.6 plus 40% glycerol. All of the EPR parameters were derived from simulations using the program SIMFONIA (Bruker). [d] ax = axial, rh = rhombic. [e] For the C-terminal His ligand in the Cu(I) proteins. [f] Taken from ref 14. [g] From kinetic studies, see ref 31. [h] Calculated value for Cu(I) Az, see ref 38. [i] Taken from ref 39. [j] Measured at pH 8.0. [k] Measured at pH 7.5. [l] ESE rate constant. [m] Measured at pH* 8.2.[39] [n] Measured at pH* 7.6.[40] [o] Measured at pH* 7.6. [p] Measured at pH* 8.0.[33] [q] Measured at pH* 7.5. [r] Measured at pH 9.0.[41] [s] Measured at pH* 8.2.

(22 ± 1 °C) using an electrochemical setup described previously.[36] Measurements were carried out at scan rates of typically 20 mV/s. All reduction potentials were referenced to the NHE, and voltammograms were calibrated using the $[Co(phen)_3]^{3+/2+}$ couple (370 mV vs NHE).[37] The gold working electrode was modified using either a 1 M solution of 2-diethylaminoethanethiol (DEAE-SH) for the measurements of Pc and PcAmi, or a saturated solution of 4,4-dithiodipyridine (Aldrithiol-4) for all other proteins, as described previously.[36] A pH jump method was used by diluting a stock protein solution (∼1−2 mM in 1 mM buffer at $I = 0.10$ M, NaCl) 10−20-fold with 20 mM buffer ($I = 0.10$ M, NaCl). The studies in the pH range 3.8−5.3 were carried out in acetate, Mes was used in the pH range 5.5−6.7, and Tris was used in the pH range 7.0−8.1.

## Results

**The UV/Vis Spectra.** The UV/vis spectra of Cu(II) PazAmi, PcAmi, and AzAmi are shown in Figures S1A−C, respectively, along with those of the WT proteins, and peak positions and intensities are listed in Table 1. In the case of PazAmi, the spectrum is more similar to that of Paz (same scaffold) than Ami (whose loop has been introduced). PcAmi possesses a UV/vis spectrum similar to those of both Pc and Ami (which are alike). In AzAmi, there is a considerable shift of the main LMCT band to 609 nm as compared to 628 nm in Az, which is more similar to the position in the Ami spectrum where the band is at 596 nm. The intensity of the band is not altered significantly by the AzAmi loop contraction, and thus this feature is more like Az than Ami.

The UV/vis spectra of the Co(II) loop variants are shown in Figure S2, and the peak positions are listed in Table S1. The spectra of the Co(II) loop variants are alike, with those for PazAmi and PcAmi being most homologous. The spectra of the loop variants are also similar to those of the WT proteins except in the case of AzAmi (see Table S1). In this variant, the LMCT bands are shifted by ca. 10 nm to higher wavelength as compared to Co(II) Az, and thus are in similar positions to those of WT Co(II) Ami. The d−d transitions in the spectrum of Co(II) AzAmi are more similar to those for Co(II) Az rather than Co(II) Ami.

**EPR Spectra.** The EPR spectra of the loop-contraction variants are shown in Figures S3A−C along with those of the WT proteins. In all cases, loop contraction has very little influence, which is highlighted by the *g* and *A* values which have been determined from simulations (see Figures S4A−C) and are listed in Table 1. The most significant effect is in AzAmi where $g_z$ is considerably smaller than in Az and is accompanied by a sizable increase in $A_z$.

**Paramagnetic $^1$H NMR Spectra of the Cu(II) Proteins.** The paramagnetic $^1$H NMR spectra of the Cu(II) loop-contraction variants are compared with those of Az, Pc, and Paz in Figure 2 (the spectrum of Ami is not included but is very similar to that of Paz[42]). The spectra of Paz[40] and PazAmi[19] have been assigned previously, as have those of spinach Pc[43] and *P. aeruginosa* Az.[44] The paramagnetic $^1$H NMR spectrum of PcAmi was assigned utilizing saturation-transfer difference experiments on 1:1 mixtures of oxidized and reduced protein. The experiments in which signals a, b, f, and g were irradiated are shown in Figure S5, and the assignments made are listed in Table S2 and Table 2. We have not been able to observe saturation transfer from the isotropically shifted resonances of Cu(II) AzAmi to their diamagnetic counterparts (unsuccessful attempts were made at a variety of conditions at 500 MHz). This is probably due to the ESE reaction being too fast in this protein (vide infra).[42] However, a comparison to the assigned spectra of Az and the other Cu(II) cupredoxins included in Figure 2 allows tentative assignments to be made for AzAmi (see Table 2).

**Paramagnetic $^1$H NMR Spectra of the Co(II) Proteins.** The paramagnetic $^1$H NMR spectra of the Co(II) loop-contraction variants are shown in Figure S6. Detailed information about

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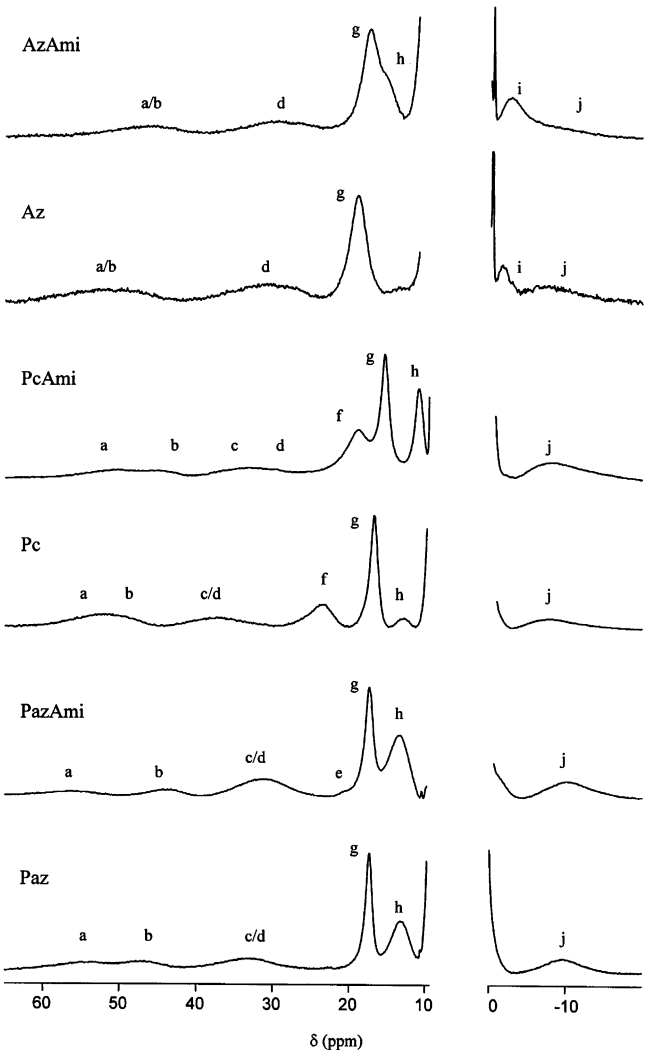


***Figure 2.*** $^1$H NMR spectra (500 MHz) of the Cu(II) loop-contraction variants and the WT proteins (25 °C) in 99.9% deuterated 10 mM phosphate buffer. The spectra of AzAmi, Az, PcAmi, and Pc were acquired at pH* 8.0, while those of PazAmi and Paz were measured at pH* 7.6.

peak positions and assignments can be found in Tables S3−S5, and the data for all three loop variants are compared to those for the Co(II) WT proteins in Table S6.[32,34,45−48] The assignment of the $^1$H NMR spectra of Co(II) PazAmi and PcAmi is straightforward from a comparison to the WT data (see Table S6) along with the observation of selective dipolar connectivities (see Tables S3 and S4, respectively). The analysis of the spectrum of Co(II) AzAmi is more complicated, and thus detailed studies have been performed (see Tables S5 and S6).

**$^1$H NMR Spectra of the Reduced Loop-Contraction Variants.** In all cases, the $^1$H NMR spectra of the Cu(I) loop-contraction variants show only localized changes as compared to those of the WT proteins. In PazAmi, the resonances arising from the two His ligands are found at different chemical shifts than in Paz. These variations are greater for the His81 signals (see Figure S9) and indicate a structural alteration at the Cu(I) site which has a larger influence on this His ligand. In Cu(I) PcAmi, the His ligand resonances are found at positions similar to those in Pc (see Figure S10), and thus loop contraction has a more limited influence on the structure of the Cu(I) site of this protein. This is also supported by the fact that the $C^{\epsilon}H_3$ resonance of Met90 is found at 0.64 ppm in PcAmi as compared to 0.56 ppm (Met92) in the spectrum of Cu(I) Pc (data not shown). In Cu(I) AzAmi, His ligand resonances are not resolved in the $^1$H NMR spectrum. Active site resonances are observed in WEFT spectra of partially oxidized samples of AzAmi, but we have not been able to unambiguously assign these signals to the imidazole ring protons from particular His ligands. The resonances observed in the WEFT spectra of AzAmi are found in positions similar to those in the WT protein [for example, the axial Met $C^{\epsilon}H_3$ resonance occurs at 0.10 ppm in Cu(I) AzAmi as compared to −0.05 ppm in the reduced WT protein].

The influence of pH* on the chemical shift of the His ligand resonances in the $^1$H NMR spectrum of PazAmi has been reported previously and yields p$K_a$* values of 6.7−6.8 (see Figure S11). The behavior of these resonances is consistent with the protonation of His81 (the chemical shift of the His81 $C^{\epsilon 1}H$ resonance is most affected by changing pH*) which dissociates from the Cu(I) ion leading to a three-coordinate site,[49] and with fast exchange on the NMR time scale between the deprotonated and protonated forms. In PcAmi, the dependence on pH* of the position of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of both His37 and His87 can be followed (see Figure S12). The fits of the data, to the equation given in ref 40, for all four resonances yield p$K_a$* values of 6.7. The chemical shifts of a number of other active site resonances of PcAmi are also influenced by altering the pH, including the Met90 $C^{\epsilon}H_3$ signal, and a fit of the data yields a p$K_a$* of 6.6 (data not shown). This is consistent with the protonation of the C-terminal His87 ligand and dissociation leading to a trigonal Cu(I) site.[50] The influence of pH* on the position of the His ligand resonances in the spectrum of Cu(I) Pc has also been investigated, and the data for the His87 $C^{\epsilon 1}H$ signal in Pc and PcAmi are compared in Figure 3. The dramatic difference between the behavior of this resonance as a function of pH* in the two proteins is immediately apparent, and the data for Pc yield p$K_a$* values of 4.9 (from fits of the data for the His87 $C^{\epsilon 1}H$ and His37 $C^{\delta 2}H$ resonances). In AzAmi, altering pH* has very little influence on the WEFT spectrum of a partially oxidized sample down to pH 6.0. Below this pH value, resonances are no longer observed and thus the ESE rate constant of AzAmi must be smaller preventing selection of resonances arising from the Cu(I) form in the WEFT spectra (vide infra).[33]

**The Reduction Potentials of the Loop-Contraction Variants and Their Dependence on pH.** The loop-contraction variants yield good quasi-reversible responses on modified gold electrodes in the pH range ∼8−4. In all cases, the anodic and cathodic peaks are of equal intensity and their separation is typically ∼60 mV at a scan rate of ∼20 mV/s (at neutral pH). As the pH is decreased, the peak separation increases slightly, except in the case of PcAmi. The peak currents are proportional to the (scan rate)$^{1/2}$ in the range ∼5−200 mV/s. The reduction

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***Table 2.*** Assigned Hyperfine Shifted Resonances in the $^1$H NMR Spectra of the Cu(II) Loop-Contraction Mutants (PazAmi, PcAmi, and AzAmi) and Those of the Corresponding Wild-Type Cu(II) Cupredoxins

| | | $\delta_{obs}$ (ppm)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| peak | assignment[b] | Ami[c] | Paz[d] | PazAmi | Pc[e] | PcAmi | Az[f] | AzAmi |
| a | HisB $C^{\delta 2}H$ | 50 | 54 | 56 | 53 | 50 | ∼52 | ∼46 |
| b | HisA $C^{\delta 2}H$ | 43 | 46 | 44 | 50 | 45 | | |
| c/d | HisA/B $C^{\epsilon 1}H$ | ∼35 | 33 | 31 | 37 | 34/30[g] | ∼30[h] | ∼30 |
| e | HisA $N^{\epsilon 2}H$ | 27.5 | 22.8[i] | 21.0 | 31.4[j] | 29.2[k] | 26.9[l] | |
| f | Met $C^{\gamma 2}H$ | 12/11.1 | | | 23.7 | 18.9 | | |
| g | Asn $C^{\alpha}H$ | 14.1 | 17.3 | 17.5 | 17.0 | 15.4 | 19.0 | 17.5 |
| h | Met $C^{\gamma 1}H$ | 12/11.1 | 13.0 | 13.6 | 13.1 | 10.8 | | ∼15 |
| i | HisA $C^{\beta 1}H$ | −2.5 | | | *m* | | −2 | −3 |
| j | Cys $C^{\alpha}H$ | −9.5 | −9.7 | −10.5 | −8.0 | −7.7 | −7.8 | ∼ −10 |

[a] Data recorded at 25 °C in 10 mM phosphate (99.9% $D_2O$) pH* 7.6 (Paz, PazAmi), pH* 8.0 (Pc, PcAmi), pH* 8.0 (Az, AzAmi). The observed shifts ($\delta_{obs}$) arise from the three contributing factors $\delta_{dia}$, $\delta_{pc}$, and $\delta_{Fc}$ [$\delta_{dia}$ is the shift in an analogous diamagnetic system, $\delta_{pc}$ is the pseudo-contact (through-space) contribution, and $\delta_{Fc}$ is the Fermi contact (through-bond) contribution]. The $\delta_{pc}$ values are small, due to the small anisotropy of the g tensor, and therefore $\delta_{obs}$ minus $\delta_{dia}$ for a particular proton provides a good estimate of $\delta_{Fc}$, which is a measure of the spin density. [b] HisA is the N-terminal His ligand, and HisB is the C-terminal His ligand. The Asn residue is adjacent to the N-terminal His ligand. [c] For *P. versutus* Ami in 50 mM phosphate at pH 7.0 and 32 °C.[42] [d] Assignment as in ref 40. [e] Assignment as in ref 43. [f] Assignment as in ref 44. [g] Peaks c and d were assigned as $C^{\epsilon 1}H$ resonances of His87 and His37, respectively, from saturation-transfer experiments. [h] The second His $C^{\epsilon 1}H$ resonance is found at 46.7 ppm at 800 MHz (5 °C) in 10 mM phosphate pH 8.0 but was not observed in this study. [i] Data recorded at 25 °C in 10 mM phosphate (90% $H_2O$/10% $D_2O$) at pH 8.1. [j] Recorded at 800 MHz (25 °C) in 50 mM phosphate (90% $H_2O$/10% $D_2O$) pH 7.5. [k] Data recorded at 40 °C in 37 mM phosphate (90% $H_2O$/10% $D_2O$) pH 7.5. [l] Recorded at 800 MHz (5 °C) in 10 mM phosphate (90% $H_2O$/10% $D_2O$) at pH 8.0. [m] Found[43] at −1.5 ppm at 800 MHz (25 °C) in 50 mM phosphate at pH 7.5 but was not observed in the present study.

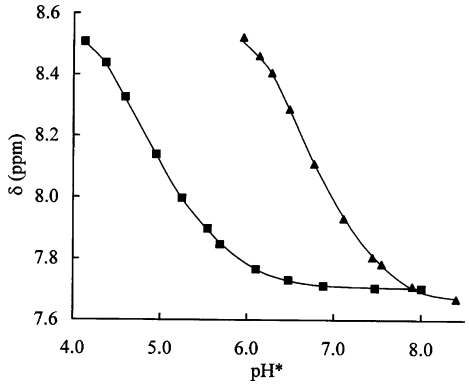


***Figure 3.*** Dependence on pH* of the chemical shift of the His87 $C^{\epsilon 1}H$ resonance in the $^1$H NMR spectrum of Pc (■) and PcAmi (▲) in 100 mM phosphate (25 °C). The solid lines are fits of the data to an equation given in ref 40 and result in a p$K_a$ of 4.9 for Pc and 6.7 for PcAmi.

potentials ($E_m$) obtained are 215 mV for PazAmi at pH 8.0, 315 mV for PcAmi at pH 8.0, and 261 mV for AzAmi at pH 7.5. The $E_m$ of Paz is 266 mV (at pH 8.0), that of Pc is 378 mV (at pH 8.0), while Az has an $E_m$ of 295 mV (at pH 7.5) and Ami 238 mV (at pH 8.0).[14] From these values, it can be seen that loop-contraction mutagenesis leads to an increase in the $E_m$ by ∼30−60 mV with the influence largest in PcAmi and smallest for AzAmi (see Table 1).

The influence of pH on the $E_m$ of PazAmi (Figure 4A), PcAmi (Figure 4B), and AzAmi (Figure 4C) has been studied along with those of the corresponding WT proteins. In all cases, loop contraction has a marked effect on the pH dependence of the $E_m$, and dramatic increases are observed upon lowering the pH in the range 7−4, which have slopes of −60 mV/pH. This confirms that the reduction of the loop variants is accompanied by the uptake of a proton at the active site (protonation of the C-terminal His ligand in all cases) in this pH range. A similar behavior has previously[51,52] been observed for Pc at pH values below 5.5, which we have not reproduced here (a similar behavior is expected for Paz but cannot be observed as the electrochemical response of this protein deteriorates dramatically at pH < 5.5[40]). In PazAmi and AzAmi, there are smaller changes

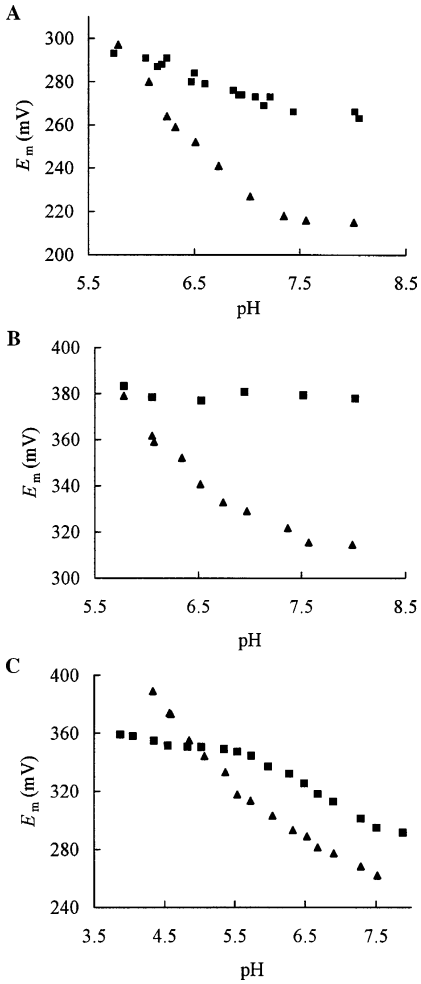


***Figure 4.*** Dependence on pH ($I$ = 0.10 M, NaCl) of the reduction potential ($E_m$) of (A) Paz (■) and PazAmi (▲), (B) Pc (■) and PcAmi (▲), and (C) Az (■) and AzAmi (▲). All of the values are referenced to the NHE at 22 °C.

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in the $E_m$ values at higher pH that are also observed in the WT proteins and which can be assigned to noncoordinated His residues (His6 in Paz[40] and PazAmi and His35 and His83 in Az[53] and AzAmi). The influence of the protonation of these residues on $E_m$ is almost identical in the loop-contraction variants and the WT proteins. From the pH dependence of $E_m$ (at lower pH), p$K_a$'s of 6.6 for PazAmi (obtained by fitting the difference in $E_m$ for PazAmi and Paz in the pH range 7.6−5.8 using an equation given in ref 33), 6.8 for PcAmi, and 5.5 for AzAmi (obtained by fitting the difference in $E_m$ for AzAmi and Az in the pH range 7.0−4.0) are obtained.

**ESE Reactivity.** The ESE rate constants of the loop-contraction variants have been determined using $^1$H NMR spectroscopy. For PazAmi and PcAmi, the influence of oxidized protein concentration on the $T_1^{-1}$ values of His ligand imidazole ring resonances of the Cu(I) proteins was measured. Using eq 1,

$$T_i^{-1} = T_{i,red}^{-1} + k\,[\mathrm{Cu(II)}] \qquad (1)$$

where $T_i^{-1}$ is the observed relaxation rate of a resonance [either longitudinal ($T_1$) or transverse ($T_2$)], $T_{i,red}^{-1}$ is the observed relaxation rate in the fully reduced protein, and [Cu(II)] is the concentration of oxidized protein, which is valid for protons belonging to the slow exchange regime, the rate constant ($k$) is determined.[41,54] For both proteins, only $T_1^{-1}$ values were used as the ESE rate constants ($k_{ESE}$) are small making the measurement of $T_2^{-1}$ (peak widths) imprecise. Plots of $T_1^{-1}$ against [Cu(II)] are shown in Figures S13 and S14 for His ligand resonances in PcAmi and PazAmi, respectively. The slopes of these plots yield $k_{ESE}$ values (25 °C) of $1.3 \times 10^3$ M$^{-1}$ s$^{-1}$ for PcAmi at pH* 7.5 ($I = 0.10$ M) and $4.6 \times 10^2$ M$^{-1}$ s$^{-1}$ for PazAmi at pH* 7.6 ($I = 0.10$ M). These data and the ESE rate constants for the WT proteins under identical conditions are listed in Table 1. Determinations (25 °C) were also carried out at pH* 6.2 ($I = 0.10$ M) for PcAmi where an ESE rate constant of $5.1 \times 10^2$ M$^{-1}$ s$^{-1}$ was obtained and at pH* 6.5 ($I = 0.10$ M) for PazAmi where $k_{ESE}$ is $1.6 \times 10^2$ M$^{-1}$ s$^{-1}$.

In AzAmi, the His ligand imidazole ring resonances are not sufficiently well resolved to be useful for ESE rate constant determinations. We have therefore utilized the super-WEFT pulse sequence to select fast relaxing resonances in the Cu(I) protein in the presence of small amounts of the Cu(II) form. This approach is only useful if the ESE rate constant is approximately $\geq 10^5$ M$^{-1}$ s$^{-1}$.[33] The resonances which are selected are those which are close to the active site and are likely to belong to the slow-exchange regime. Plots of $T_2^{-1}$ against [Cu(II)] for a number of active site resonances are shown in Figure S15, yielding slopes ranging from $6.2 \times 10^5$ to $7.8 \times 10^5$ M$^{-1}$ s$^{-1}$. Thus, an ESE rate constant (25 °C) of $6.9 \times 10^5$ M$^{-1}$ s$^{-1}$ (average) is obtained for AzAmi at pH* 8.2 (20 mM phosphate). The value for the WT protein also determined in 20 mM phosphate (25 °C) but at pH* 9.0 is $7.0 \times 10^5$ M$^{-1}$ s$^{-1}$ (see Table 1).[41] At lower pH* values (<6.0), application of the WEFT sequence to partially oxidized samples of AzAmi does not result in the selection of resonances from the Cu(I) protein. Thus, as the His115 ligand protonates, ESE slows and prevents the determination of the rate constant.[33]

## Discussion

The introduction of the five-residue active site loop of Ami into cupredoxins, which possess a corresponding sequence ranging from seven to eight residues, has a limited effect on their active site structures. In all cases, stable type 1 copper centers are produced which are redox active and are able to undergo intermolecular ET. Thus, the $\beta$-barrel scaffolds of Paz, Pc, and Az can all accommodate shorter C-terminal loops giving authentic cupredoxin active sites. The spectroscopic properties of the loop-contraction variants indicate that the presence of a classic or distorted type 1 center[30] seems to be controlled more by the scaffold than the loop (see Table 1). However, loop elongation of Ami usually resulted in a slight distortion of its classic site.[14−16] The UV/vis and EPR spectra of PazAmi are remarkably similar to those of Paz, indicating that the Cu(II) site is almost unaffected by loop contraction, which is confirmed by paramagnetic $^1$H NMR studies. The comparison of the data for the Co(II)-substituted proteins indicates a slightly enhanced M(II)−S(Cys) interaction in PazAmi as compared to Paz. In Pc, the introduction of the Ami loop has almost no effect on the UV/vis and EPR spectra of the protein, and thus the active site structures of the WT protein and PcAmi are very similar. The paramagnetic $^1$H NMR spectra indicate a small increase in the spin density on the Cys ligand and a decreased M(II)−S(Met) interaction in PcAmi as compared to Pc.

In the case of AzAmi, there are significant changes in the UV/vis and EPR spectra of the Cu(II) protein as compared to Az, which make this variant more like Ami. This is also apparent from the paramagnetic $^1$H NMR studies of the Cu(II) protein with the appearance of an extra hyperfine shifted signal, presumably from the C$^\gamma$H proton of the axial Met118, indicating increased spin density on this ligand. The UV/vis spectrum of Co(II) AzAmi shows that the interaction with the Cys ligand is altered as compared to Co(II) Az, but that the overall geometry is similar. The NMR spectrum of Co(II) AzAmi demonstrates a significant increase in the M(II)−S(Cys) bond strength and a decreased M(II)−S(Met) interaction as compared to Co(II) Az, with the M(II)−O(Gly45) interaction similar in both. The discrepancy between the influence of loop contraction on the M(II)−S(Met) interaction in the Cu(II) and Co(II) proteins is due to the preference of Co(II) for the harder axial oxygen ligand (from Gly45), which is also the case in Az.[55] In Az, the thiolate sulfur of the Cys112 ligand accepts two hydrogen bonds from the backbone NH's of Asn47 and Phe114. In Ami (and Paz and Pc), the second hydrogen bond is absent due to a Pro residue being found in the position corresponding to Phe114. In AzAmi, the Phe114 residue has been replaced by Pro114, and thus the second hydrogen bond donor is missing. The presence of only a single hydrogen bond to the Cys ligand enhances the electron density on the thiolate sulfur, which leads to the increased $\delta_{obs}$ values for the C$^\beta$H resonances of this residue in the NMR spectrum of the Co(II) protein. Furthermore, the removal of this hydrogen bond to the Cys ligand results in increased spin density on the axial Met ligand. The alterations in the UV/vis and EPR spectra of Cu(II) AzAmi as compared

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to Az can also be assigned to the different hydrogen-bonding pattern which is consistent with previous suggestions.[26] The loss of an aromatic residue at position 114 could also contribute to the altered spectral properties of AzAmi as the Phe114Ala mutation in Az results in the main LMCT band shifting to 617 nm.[56]

The C-terminal His ligand of Ami,[57] Paz,[49] and Pc[50] protonates and dissociates from the Cu(I) sites with p$K_a$ values of 6.7,[39] 4.9,[31] and 4.9[58] respectively. In all cases, the three-coordinate centers formed favor Cu(I) and lead to an increase in $E_m$.[14,51,52] Furthermore, protonation of this ligand increases the reorganization energy for the site, which results in decreased ET reactivity,[33,59] and thus this property of cupredoxins may play an important physiological role.[50,60] Az is unusual in that the C-terminal His ligand does not protonate in the accessible pH range and the p$K_a$ has been calculated to be less than 2.[38] The factors which control this feature of cupredoxins are not well understood,[52,61] and an interesting outcome of the present study is the influence loop contraction has on the p$K_a$ for this residue. In PazAmi and PcAmi, shortening the His to Met loop by two residues increases the p$K_a$ of the C-terminal His from 4.9 to 6.7. This elevated p$K_a$ is identical to that observed for His96 in Cu(I) Ami, the protein whose loop has been introduced. This demonstrates that in these three cupredoxins (Ami, Paz, and Pc) the length of the loop between the His and Met ligands (the loop length between the Cys and the His ligands is identical) regulates the p$K_a$, perhaps by altering the strength of the Cu−N(His) bond.[52] The introduction of the Ami loop into Az results in the C-terminal His ligand protonating, albeit with a p$K_a$ of 5.5. The AzAmi variant not only has a shorter loop than Az, but it is also missing the second hydrogen bond to the thiolate sulfur of the Cys ligand (vide supra). It has been suggested that this hydrogen bond, absent in Ami, Paz, and Pc, provides additional stabilization at the active site of Az which may prevent His ligand protonation and dissociation.[62] This is supported by the observation that mutation of the corresponding Pro residue to a Phe in Ami results in a large decrease in the p$K_a$ of the C-terminal His ligand to below 5.[63] However, the introduction of a Ala at the same position does not have such a dramatic effect on the p$K_a$ of the His ligand.[63] It could be that the presence of an aromatic amino acid in this position is important and in Az the $\pi-\pi$ interaction of Phe114 with the His ligand stabilizes the Cu(I)−N(His) bond.[31,56,61] Thus, within the Az scaffold the loop length also influences this effect although other structural factors contribute to the p$K_a$ value. Loop elongation in Ami[14−16] always resulted in a decrease in the p$K_a$ of His96 and in the case of the variant possessing the rusticyanin loop, the value is $<4.5$ and is not observed.[16] The spectroscopic properties of the variant in which the Az loop was introduced indicate that the second hydrogen bond is not present in this protein[15] and could explain why the p$K_a$ of the His ligand is only lowered to 5.6. The second hydrogen bond to the Cys ligand is present in the phytocyanins, and some of these have a C-terminal His ligand which protonates.[64−66] Thus, there appears to be a number of factors in the cupredoxins which are important for controlling the p$K_a$ of the C-terminal His ligand. Our studies demonstrate that the length and structure of the ligand-containing loop is one of these with a shorter loop destabilizing the Cu(I) center.

All of the loop-contraction variants have $E_m$ values that are well within the range normally observed for cupredoxins. Loop contraction always results in a decrease in $E_m$ (at ∼neutral pH), and the magnitude of this effect ranges from ∼30 mV in AzAmi to ∼60 mV in PcAmi. Ami has the lowest $E_m$ of all of the WT cupredoxins used in this study. Thus, the introduction of its C-terminal active site loop into Paz, Pc, and Az results in a $E_m$ which is closer to that of Ami. Similarly, the loop elongation experiments on Ami, in which the active site loops of Paz, Pc, and Az were introduced, all result in an increase in $E_m$ to a value closer to that of the protein whose loop was introduced.[14,15] The decrease in $E_m$ in all of the loop-contraction variants indicates that the shortening of this region provides an active site environment preferable for the cupric ion, whereas loop elongation always leads to an active site which favors Cu(I). Whether these variations are due to subtle geometrical modifications, altered solvent exposure, or a combination of these and other factors must await detailed structural studies on the loop-contraction variants. However, it is apparent that the C-terminal loop does tune the $E_m$ of a type 1 copper site. When considering the effect of the loop contractions on $E_m$, it is surprising that the mutation in Az has the smallest influence. This is especially so considering that the replacement of the Pro at the position corresponding to Phe114 of Az in Paz and Ami, with residues which form the second hydrogen bond to the thiolate sulfur of the Cys ligand, resulted in up to 180[67] and 150 mV[63] increases in $E_m$, respectively. Thus, the removal of this hydrogen bond in AzAmi would be expected to cause a much larger decrease in $E_m$. The Phe114Ala mutation in Az results[68] in a 50 mV increase in $E_m$, and thus there are probably a number of contributing factors to the observed influence of loop contraction on the $E_m$ of this protein.

The ET reactivity of the loop-contraction variants has been assessed by determining their ESE rate constants, which are not influenced by alterations in $E_m$, as this reaction has no driving force. The largest effect is seen in Paz where an 8-fold decrease in $k_{ESE}$ is observed upon loop contraction. PcAmi has a $k_{ESE}$ which is only half that of Pc, whereas in Az loop contraction has almost no influence on ET reactivity. Loop elongation in Ami usually resulted in a greater decrease in $k_{ESE}$ ranging from ∼6 to >12-fold.[14−16] The proteins studied in this work, in particular Pc and Az, have scaffolds that can accommodate the shorter loop of Ami without significantly influencing

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ET reactivity. In PazAmi and PcAmi, decreases in $k_{ESE}$ were observed at lower pH* consistent with the protonation of the C-terminal His ligand having a detrimental effect on ET due to an increased reorganization energy.[33,59] In AzAmi, a similar decrease in $k_{ESE}$, although not quantified, is indicated by the lack of selection of active site resonances from the Cu(I) protein in the WEFT spectra of partially oxidized samples at lower pH* values.

This study has identified that a shorter ligand-containing loop at a type 1 copper center results in an environment which stabilizes Cu(II) over Cu(I). Previous loop elongation studies, carried out on a single cupredoxin, showed that in Ami a longer loop leads to a site which has a preference for Cu(I).[14−16] For many years, it has been suggested that the active site structure of a cupredoxin is more suited to Cu(I) rather than oxidized copper, thus explaining the elevated $E_m$ values for these proteins as compared to the Cu(II)/Cu(I) aqua couple.[69−71] Recent studies on denatured Az have demonstrated that the folded cupredoxin domain of this protein actually stabilizes Cu(II).[72−74] The studies that we have carried out show that the preference for oxidized or reduced copper is not only controlled by the $\beta$-barrel scaffold of a cupredoxin but is also dependent on the C-terminal ligand-containing loop. The loop-contraction variants possess active sites which demonstrate an enhanced preference for Cu(II), yet they are still entatic in that they support fast intermolecular ET.

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**Supporting Information Available:** Tables and figures of experimental data and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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