# Paramagnetic NMR Spectroscopy of Cobalt(II) and Copper(II) Derivatives of *Pseudomonas aeruginosa* His46Asp Azurin

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NMR spectra of paramagnetic Co(II) and Cu(II) derivatives of *Pseudomonas aeruginosa* His46Asp azurin have been investigated. In each derivative, assignment of hyperfine-shifted resonances outside the diamagnetic envelope of spectra recorded at 200 and 500 MHz confirms that the Asp carboxylate is coordinated to the paramagnetic metal center. The reduced paramagnetic shifts of the Cys112 proton resonances in Cu(II) and Co(II) His46Asp azurins compared to those of the corresponding wild type proteins indicate that metal−S(Cys) bonding is weakened in this mutant. The downfield shifts of the $\gamma$-$CH_2$ of Met121 suggest a stronger interaction between the metal and the Met thioether group than is present in the wild type protein. Molecular modeling of the metal site structure indicates a distorted tetrahedral geometry with Asp46 (monodentate carboxylate), Cys112, and His117 equatorial ligands. In this structure, the metal ion is shifted 0.3 Å out of the O(Asp)S(Cys)N(His) trigonal plane toward Met121.

## Introduction

Site-directed mutagenesis has assisted in identifying the essential features of the unique coordination environment of blue copper proteins.[1−4] Of the three equatorial ligands in *Pseudomonas aeruginosa* azurin, only Cys112 is absolutely required for a blue site;[4] the two histidines, 46 and 117, are less critical since the characteristic blue spectroscopic signature is observed in derivatives with mutations at these positions.[2,3] The engineered His46Asp azurin is of special interest because it exhibits a perturbed blue copper center.[3] Since an Asp carboxylate side chain is readily accommodated at position 46, the change from an N to an O donor should affect only the ligand field at the copper[5] and not the overall structure (cupredoxin fold) of the protein.[6]

Employing paramagnetic NMR spectroscopy,[7] we have investigated the coordination environment in the Cu(II) and Co(II) derivatives of *Pseudomonas aeruginosa* His46Asp azurin. Previous work has shown that paramagnetic NMR spectra of oxidized blue copper proteins can be obtained,[8] although the peaks are not as readily assigned as those of analogous Co(II) and Ni(II) species.[13−18] By studying the paramagnetic NMR spectra of Cu(II) and Co(II) derivatives of His46Asp azurin, we have elucidated the main features of the coordination environment of this engineered protein.

## Experimental Section

**Protein Preparation.** His46Asp azurin was prepared as described previously.[3,16] Samples for NMR experiments were prepared by first concentrating the His46Asp metal derivatives in Centricon-10 concentrator units (Amicon) to a final concentration of 3−4 mM. The $D_2O$ solutions were prepared by exchanging in Centricon-10 units. The reported pH values measured in $D_2O$ solutions are not corrected for the isotopic effect.

**Spectroscopic Measurements.** Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

NMR spectra were recorded on Bruker ACE 200 and AMX 500 spectrometers operating at proton frequencies of 200.13 and 500.13 MHz, respectively. All chemical shifts were referenced to the chemical shift of water at the appropriate temperature, which in turn was calibrated against internal DSS. 1D experiments were performed using the superWEFT pulse sequence (180°−$\tau$−90°)[9] or by presaturating the

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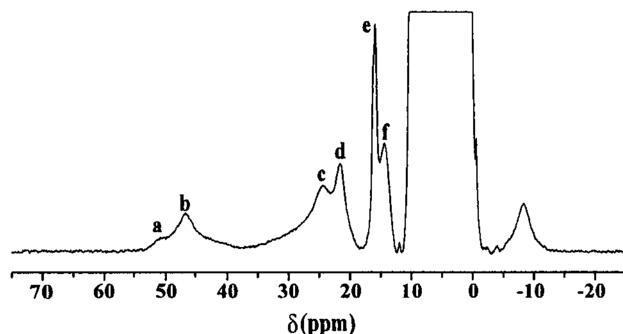


**Figure 1.** 500 MHz $^1$H NMR spectrum of Cu(II) His46Asp *P. aeruginosa* azurin recorded at 305 K in 50 mM sodium acetate buffer in $H_2O$ (pH 5.5). Signal a is absent in spectra recorded at higher pH values or in $D_2O$ solutions.

water resonance. Different delays ($\tau$) were used in the superWEFT pulse sequence to optimize the detection of the fastest relaxing signals. The chemical shift values reported in the text were taken at 313 K, except when specified. Non-selective $T_1$ values were determined by means of an inversion-recovery experiment. The $T_1$ values were calculated from the initial slope of the semilogarithmic plots of the fractional deviation of the $z$-magnetization from the equilibrium *vs* the intermediate delay $\tau$.[10]

The difference steady state and truncated 1D NOE spectra were recorded using the methodology reported by Banci *et al.*[11] Recycle times spanning between 16 and 400 ms were used in most of the cases, with irradiation times ranging from 10 to 70 ms. All the NOE and 2D experiments were performed at least at two different temperatures in order to discard accidental signal degeneracies. 2D NOESY spectra were recorded in the phase-sensitive TPPI mode.[12] In order to detect dipole-dipole connectivities corresponding to the fastest relaxing signals, recycle times of 120 ms and mixing times of 6−10 ms were used. The NMR data sets were processed using the Win-NMR and UXNMR software packages (Bruker). Phase-sensitive 2D NMR data were phase-corrected and baseline-leveled in both dimensions.

**Molecular Modeling.** All molecular modeling utilized Biograf v3.21 incorporating the DreidingII force field. This force field was tested previously in modeling the coordination site of the Cys112Asp mutant.[4] All modeling was done on the apoprotein. The His46 side chain of *P. aeruginosa* azurin (4azu)[19] was replaced with an Asp side chain, having similar main chain and side chain dihedral angles as the native His46 residue. The ligand protonation state corresponded to pH 7. All hydrogens were added to the structure explicitly and charges assigned from the AminoLib of POLARIS (overall charge of zero). Energy minimization was done with all atoms movable. An initial energy calculation gave an overall rms force ∼25 (kcal/mol)/Å, and the structure minimized to an overall rms force of ∼0.3 (kcal/mol)/Å after 200 steps of conjugate gradient minimization. A copper ion, having the coordinates of the ion as in the 4azu azurin crystal structure, was added to the structure. The copper ion was translated 0.4 Å toward Met121 based upon shifts observed in the 1D paramagnetic NMR spectra.

## Results

**Cu(II) His46Asp Azurin.** The 500 MHz paramagnetic $^1$H NMR spectrum of Cu(II) His46Asp azurin is shown in Figure 1. Several broad resonances outside the diamagnetic envelope arise from nuclei >4.5 Å from the Cu(II) center.[8] The resonances of nuclei closer to the paramagnetic Cu(II) are broadened beyond detection. The observed resonances can be assigned by reference to the spectrum of the wild type protein.[8] The broadest signals (a, b) are associated with the coordinated His117 ligand;[8] indeed, one of these resonances (a) is absent in spectra recorded at high pH or in $D_2O$ solutions. The resonance

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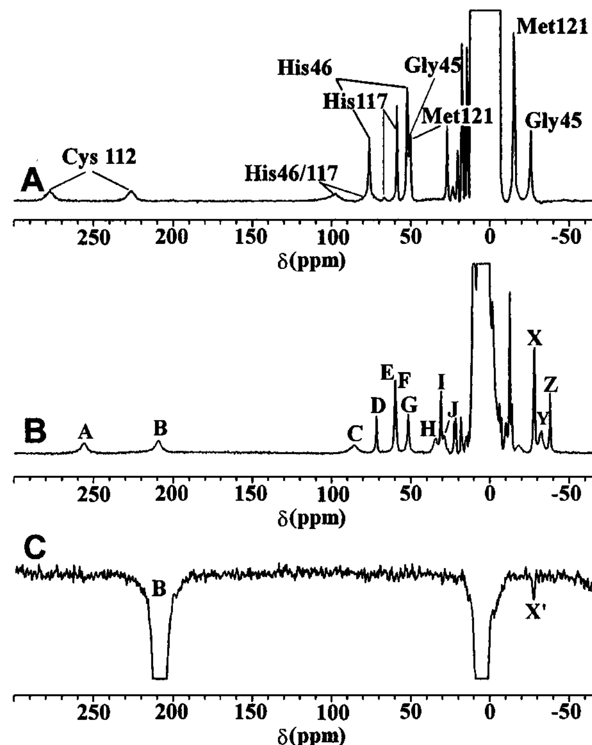


**Figure 2.** 200 MHz $^1$H NMR spectra: (A) *P. aeruginosa* Co(II) azurin recorded at 313 K in 50 mM sodium acetate buffer (pH 5.5) (assignments from refs 13 and 16); (B) Co(II) His46Asp azurin recorded at pH 5.5 in 50 mM sodium acetate buffer in $H_2O$; (C) NOE difference spectrum of Co(II) His46Asp azurin obtained by 30 ms saturation of signal B. The NOE corresponds to the one proton signal X′, which overlaps resonance X at this pH.

at 19 ppm in wild type azurin, previously assigned to the $H_\alpha$ of Cys112, is upfield shifted (f),[8] and a new isotropically shifted signal is observed at 16 ppm (e). We assign the latter peak to the resonance of a Met121 $\gamma$-$CH_2$ proton. Similar signals corresponding to the Met ligands have been observed in the spectra of wild type and mutant amicyanins.[8] Finally, we assign the additional broad resonances at 21.5 and 24.3 ppm (c, d) to protons of the newly introduced ligand, Asp 46. All the isotropic signals are slightly shifted in the spectra recorded at alkaline pH.

**Co(II) His46Asp Azurin.** The 200 MHz $^1$H NMR spectrum of Co(II) His46Asp azurin (pH 5.8, 313 K) is shown in Figure 2. Almost 20 well-resolved hyperfine-shifted signals, with shifts ranging from 260 to −40 ppm, are observed outside the diamagnetic envelope. The chemical shifts, line widths, and $T_1$ values are characteristic of Co(II)-substituted blue copper proteins; the lines are sharper than those of the analogous Cu(II) proteins because the Co(II) ion exhibits higher electron relaxation rates ($10^{11}-10^{12}$ s$^{-1}$). The hyperfine-shifted resonances correspond to protons of the five potential ligands: Cys112, His117, Asp46, Met121, and Gly45.

The line widths of the broad resonances A−C, H, and J are characteristic of protons 3−4 Å from the metal center. The $\beta$-$CH_2$ of Cys112, the $H_\epsilon1$ proton of His117, the $\beta$-$CH_2$ of His117, and one of the $\beta$ protons of Asp46 (if bound) are good candidates for these signals. The downfield-shifted signals A and B are assigned to the $\beta$-$CH_2$ protons of Cys112; this assignment is supported by their large chemical shifts, line widths, and short $T_1$ values (Figure 2A), as found in wild type Co(II) azurin[13−16] and Co(II) stellacyanin.[17,18] We assign the third broad signal C to the $H_\epsilon1$ resonance of His117. Signal D is absent when the spectra are recorded in $D_2O$ solution or at

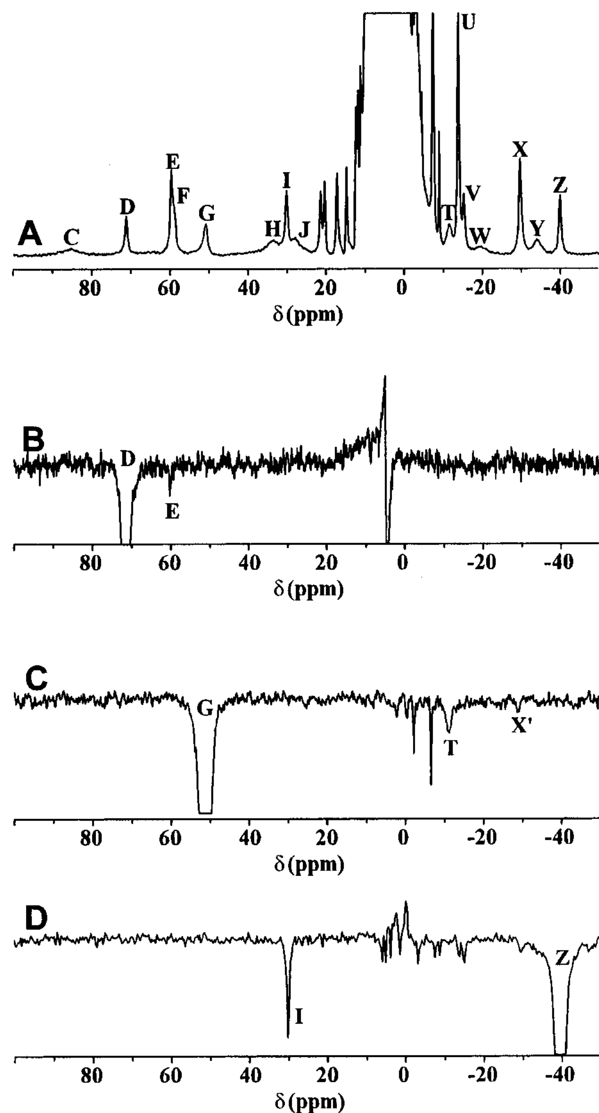


**Figure 3.** $^1$H NMR spectra of Co(II) His46Asp azurin at 313 K in $H_2O$ (pH 5.5): (A) 200 MHz 1D reference spectrum. Traces B−D correspond to the NOE difference obtained by irradiation of signals D, G, and Z, respectively.

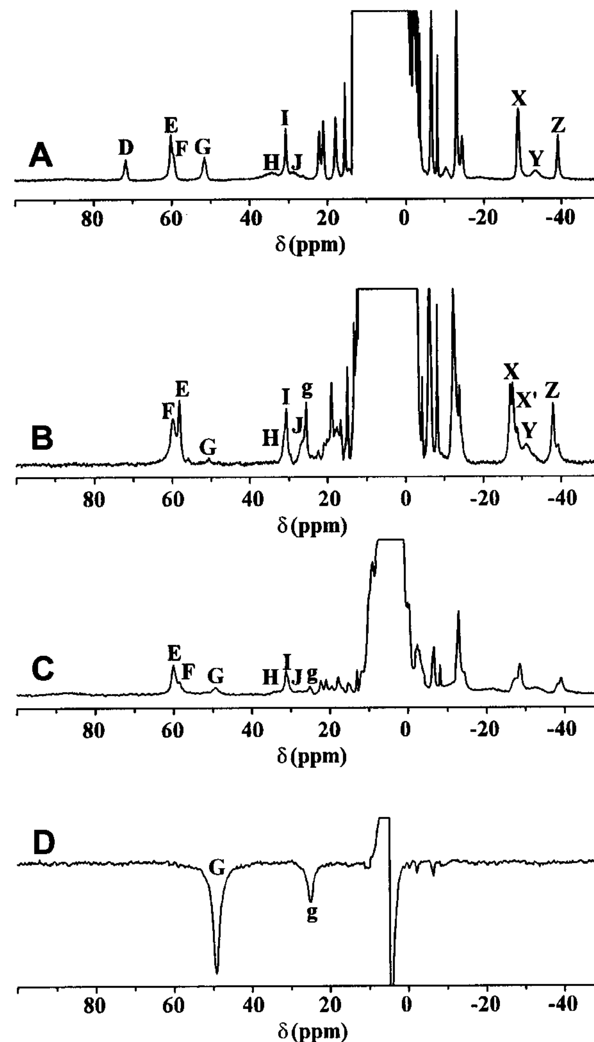


**Figure 4.** $^1$H NMR spectra of Co(II) His46Asp azurin at 313 K in $H_2O$: (A) pH 5.5 (500 MHz); (B) pH 9.0 (500MHz); (C) pH 7.4 (200 MHz); (D) trace obtained in saturation transfer experiment by irradiation of signal G, pH 7.4 (200 MHz).

pH values above 7.0; therefore, it is likely associated with the exchangeable $H_\epsilon 2$ proton of His117, which is solvent-exposed. Signal E gives an NOE upon irradiation of signal D; thus, the latter resonance is attributable to the $H_\delta 2$ proton of the same His residue. The shift pattern of signals C, D, and E resembles closely the one observed for the His ligands in Co(II) derivatives of azurin and stellacyanin.[13,14,18] A pattern corresponding to a second His ligand is clearly absent from this spectrum.

Resonance G is dipole-coupled to the upfield signals T and X (Figure 3). This latter signal is seen as a composite peak (X), partially overlapping with other resonances (see below). The strong coupling between the (G,T) pair of signals suggests that these features are a geminal proton couple. Irradiation of signal B ($H_\beta 1$ Cys112) gives an NOE with signal X′. Since the $\beta$-$CH_2$ protons of Cys112 are oriented toward the $\beta$-$CH_2$ of Met121 in the wild type protein,[13,19] we associate resonance X′ with a $\beta$ proton of Met121. Consequently, the (G,T) pair is due to the $\gamma$-$CH_2$ protons of Met121.

Signals I and Z also show strong dipole−dipole coupling, as seen from 1D NOE (Figure 3) and NOESY experiments (not shown). This strong coupling between upfield- and downfield-shifted resonances resembles the one observed for the Met121 $\gamma$-$CH_2$ pair. As demonstrated for wild type[13] and mutant[14,16] azurins, as well as for Co(II) stellacyanin,[18] these patterns are the geminal proton couples of the axial ligands. Since the Met couple has been accounted for, this couple must be due to the α-$CH_2$ protons of Gly45. The chemical shifts and relaxation properties (short $T_1$ values and large line widths) of signals F, H, and J indicate that they also belong to a metal ligand. The resonances can be reasonably attributed to the $\beta$-$CH_2$ protons of Asp46 and to a $\beta$-$CH_2$ proton of His117.

Our finding that some of the hyperfine-shifted signals split at higher pH values (Figure 4) suggests that the protein displays a pH-dependent equilibrium, which is slow on the NMR time scale (Figure 4C). Similar equilibria observed for wild type Co(II) azurin,[13] as well as the Cys112Asp mutant,[16] have been accounted for by His35 deprotonation. However, the metal site in the His46Asp mutant seems to be affected more than in wild type azurin. Since the p$K_a$ of this transition is 8.2, assignment to His35 would require alterations in the environment around this residue. It appears there is a structural change in the region around Met121, since the Met121 $H\gamma 1$ proton resonance (G) is dramatically shifted in the spectrum of the high pH species. A saturation transfer experiment performed by irradiating signal G at pH 7.4 indicates that a new resonance at 25 ppm (g) corresponds to the $H\gamma 1$ proton (Figure 4D). From this experiment, we calculate an exchange rate of 550 $s^{-1}$ (pH 7.5, 313 K) for $H\gamma 1$.

## Discussion

Assignment of the hyperfine-shifted signals in the NMR spectra of Cu(II) and Co(II) derivatives of His46Asp azurin confirms that Asp is bound to the paramagnetic center. Notably, the shift patterns for the conserved ligands are similar to those in wild type azurin, and the shift differences are attributable to the structural changes arising from the introduction of the Asp carboxylate group. A similar conclusion was reached from an examination of the electronic spectrum of the Cu(II) mutant, which, although slightly altered, is still characteristic of a blue copper protein.[4]

The reduced paramagnetic shifts and line widths of the $\beta$-$CH_2$ protons of Cys112 in the Co(II) protein reflect a weakened metal−S(Cys) interaction with respect to wild type azurin. The shifts of the Cys $\beta$-$CH_2$ protons in Co(II)-substituted blue copper proteins depend on the extent of d-electron delocalization into Cys sulfur p orbitals.[14,18] In the His46Asp protein, the presence of a carboxylate ligand in the metal coordination sphere introduces an additional negative charge that repels the Cys thiolate, weakening the Co(II)−S(Cys) bond. In Co(II) Met121Gln azurin[14] and Co(II) stellacyanin,[18] similarly reduced metal−S(Cys) interactions are likely due to the influence of Co(II)−O bonding from an axial glutamine ligand. The $\gamma$-$CH_2$ resonances of Met121 (G,T) are downfield-shifted with respect to those in wild type azurin, indicating stronger coordination in both the Co(II) and Cu(II) derivatives. The upfield-shifted signals of Gly45 are presumably due to smaller contact shifts.

The qualitative features of the structure near the paramagnetic center of Co(II) His46Asp are fixed by NMR constraints. In an effort to refine this structural picture, we have done molecular modeling experiments to evaluate possible modes of binding of Asp46 to the metal ion (at first the model was built independent of the constraints inferred from paramagnetic NMR spectroscopy). The result of the modeling experiments is an energy-minimized distorted tetrahedral coordination geometry with equatorial ligands Cys112, His117, and Asp46; in this model, the metal ion is shifted 0.4 Å toward the thioether with respect to its position in wild type azurin (Figure 5). The model predicts that a single oxygen of the Asp46 carboxylate is bound to the metal in an *anti* fashion; this mode closely resembles the atomic arrangement of the native His46 ligation.[19] Although binding of metals by carboxylate in an *anti* fashion is not commonly observed,[20] it may be enforced by the overall fold of the protein.[21] Furthermore, the other oxygen of the carboxylate is oriented such that it can possibly hydrogen bond to His35. Alternate modes of binding were explored but were rejected due to the large deviation from known coordination geometries. Our observation that the paramagnetic shift of the Cys $H_\alpha$ in the Cu(II) protein also is smaller than in the wild type protein is consistent with this model. The downfield shift of the Met121 $\gamma$-$CH_2$ and the upfield shift of the Gly45 resonances with respect to the wild type protein are accounted for by a longer Co-O(Gly45) bond. These data fit with a general picture in which the Co(II) ion is displaced from the equatorial plane in the direction of the Met residue.

The alkaline transition ($pK_a \sim 8.2$) most likely involves the deprotonation of a residue that alters the hydrogen-bond network in the site, inducing a slight conformational rearrangement. The observed drastic shift of the Met121 $H\gamma 1$ resonance (49 to 25 ppm) indicates that this rearrangement occurs near the methionine.[22] We suggest that the conformational change could be triggered by deprotonation of His35, because the introduction of a carboxylate oxygen in place of the His46 $C_\delta$−$H_\delta$ could shift the His35 $pK_a$ from 5.7 to 8.2. In wild type azurin, the His35 $N_\epsilon$ hydrogen bonds to the main chain oxygen of Met44; however, in the His46Asp protein this nitrogen is equidistant (3.0 Å) from the Asp46 $O_\delta$ and the main chain oxygen of Met44. Thus, it is possible that the His35 $N_\epsilon H^+$ hydrogen bonds to both oxygen-atom acceptors (the Asp46 $O_\delta$ and the Met44 carbonyl) in the mutant; alternatively, the $N_\epsilon H^+$ bonding interaction could be shifted entirely to the Asp46 oxygen. In the ideal orientation for hydrogen bonding, the Asp carboxylate group and His imidazole ring are coplanar. This arrangement can be achieved by a slight rotation (≤20°) of the His35 and Asp46 side chains. Upon deprotonation of His35, the Asp carboxylate is free to rotate around the $C_\beta$−$C_\gamma$ bond. Such a rotation would shift the O(Asp)S(Cys)N(His) trigonal plane, altering the position of the metal ion. As a result, the metal−S(Met) bond length would be perturbed in the alkaline conformer, thereby accounting for the shift in the Met121 $H\gamma 1$ resonance.

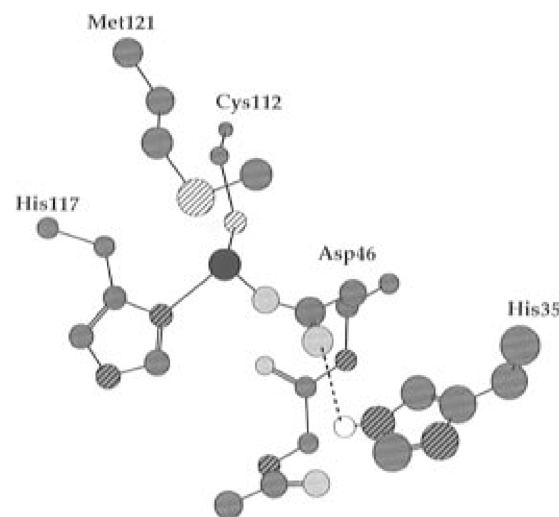


**Figure 5.** Structural model of the Co(II) His46Asp azurin active site. The His46Asp mutation was modeled by replacing the His46 imidazole side chain of *P. aeruginosa* azurin with the carboxylate side chain of Asp. The positions of common atoms (i.e., $C_\beta$ and $C_\gamma$) were maintained. After energy minimization of the apoprotein, a Co(II) ion, having the coordinates of the Cu(II) ion in the azurin X-ray structure (4azu),[19] was added to the site. The Co(II) was displaced 0.4 Å toward Met121 based on the NMR data. Bidentate and semidentate coordination modes of the Asp side chain were examined, but due to large deviations from known geometries, they were rejected as possibilities. In the monodentate model shown, one oxygen of the carboxylate group binds to Co(II). The other oxygen is within range to form a hydrogen bond to the His35 side chain, an interaction that is not present in the native protein.

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**Supporting Information Available:** A figure showing the absorption spectra of Co(II) His46Asp at pH 5.5 and 9.0 (1 page). Ordering information is given on any current masthead page.

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(22) This distortion around Met121 does not strongly affect the electronic structure of the blue copper site, as judged by the mildly perturbed absorption spectrum of the His46Asp protein in alkaline (pH 9.0) solutions (see Supporting Information).