Site Saturation of the Histidine-46 Position in *Pseudomonas aeruginosa* Azurin: Characterization of the His46Asp Copper and Cobalt Proteins†

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ABSTRACT: Cassette mutagenesis has been used to replace the copper ligand His46 of *Pseudomonas aeruginosa* azurin with 19 other amino acids and a stop codon. Several mutant proteins were expressed in *Escherichia coli* and isolated; however, only the variant in which His was replaced by Asp exhibited the spectral characteristics of a blue (type 1) center. The spectroscopic and electrochemical properties of this mutant protein show that the copper site is perturbed relative to wild-type azurin. The absorption spectrum of Cu(II)(His46Asp) azurin exhibits a S(Cys)→Cu(II) band at 612 nm, as well as weaker features at ~300, 454, and ~850 nm; its EPR spectrum is rhombic ($g_\parallel = 2.327(1)$, $g_x \approx 2.03$, and $g_y \approx 2.07$; $A_\parallel = 22(2) \times 10^{-4}$, $A_x \approx 46 \times 10^{-4}$, and $A_y \approx 22 \times 10^{-4}$ cm^{-1}). The reduction potential of the mutant (260 mV *vs* NHE at pH 8.5; 297 mV at pH 5.0) is lower than that of wild-type azurin (288 mV at pH 8.5; 349 mV at pH 5.0). The S(Cys)→Co(II) absorption bands (~300 and 362 nm) in Co(II)(His46Asp) azurin are strongly blue-shifted relative to those (330 and 375 nm) in the spectrum of the Co(II)(His46) protein, whereas the intensities of the ligand-field bands in the 500–650-nm region ($\epsilon \approx 100$ M^{-1} cm^{-1}) indicate a five-coordinate Co(II) environment.

Blue (or type 1) copper proteins are versatile electron-transfer systems (Solomon et al., 1992; Adman, 1985; Farver & Pecht, 1984). The blue copper center in these molecules possesses several unusual properties, including an intense visible absorption centered around 600 nm, a small parallel EPR hyperfine splitting, and a relatively high reduction potential (Solomon & Lowery, 1993; Adman, 1985; Gray & Malmström, 1983; Gray & Solomon, 1981). Crystal structure analyses of blue copper proteins (Adman, 1991) have revealed that the copper is bound tightly to the polypeptide framework by two histidines and one cysteine, thereby forming an approximately trigonal CuN_2S core coordination unit. Other residues with donor atoms within ~3 Å of the Cu in azurin are Met121 and Gly45. Although the $His_2CysMet$ copper environment is highly conserved in most blue proteins (Rydén, 1984), some variations are known: stellacyanin, for example, does not contain methionine and its place may be taken by glutamine (Fields et al., 1991).

Mutagenesis of the metal-binding amino acids of blue proteins has provided insight into the minimal ligand requirements of the copper site as well as information about the functional roles of the residues. It has been demonstrated that Met121 of *Pseudomonas aeruginosa*[1] azurin can be substituted by many other amino acids with retention of type 1 properties (Karlsson et al., 1991; Chang et al., 1991), and His117 of azurin has been replaced by glycine to produce a type 2 copper protein that converts to type 1 upon the addition of external ligands (den Blaauwen et al., 1991). Not surprisingly, site-directed mutagenesis of Cys112 in *P. aeruginosa* azurin has shown that this residue must be present to obtain a type 1 site (Mizoguchi et al., 1992).

Very little is known about the position 46 ligand interactions in azurin. Since Cu–N(His46) is one of the strong metal–ligand bonds (Adman, 1991; Nar et al., 1991), it could play an important role in determining many of the spectroscopic and functional features of the protein. In the course of our investigations of position 46 azurin mutants, we have found that replacement of His46 with Asp does not destroy the blue copper center; indeed, the spectroscopic and electrochemical properties of Cu(II)(His46Asp) azurin are those of a blue copper protein with modest perturbations attributable to carboxylate interaction at the binding site. Additional information about the ligand field of the His46Asp protein has been obtained from spectroscopic studies of the cobalt(II) derivative.

MATERIALS AND METHODS

Enzymes and Chemicals. All enzymes were obtained from Boeringer Mannheim Biochemicals or New England Biolabs. Amplitaq Taq DNA polymerase (Perkin-Elmer Cetus), antibiotics (Sigma), radioactive materials (Amersham), isopropyl β-thiogalactoside (IBI), Nusieve GTG agarose (FMC), and all other molecular biology grade reagents were used as supplied.

Bacterial Strains. Escherichia coli strains XL-1 Blue (Bullock et al., 1987), JM101 (Yanisch-Perron et al., 1985), HMS174(DE3) (Studier et al., 1990), and TG 1 (Carter, 1986) were used in experiments as described. The culture medium was L-broth unless otherwise indicated.

DNA. Oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry

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[1] Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. coli*, *Escherichia coli*; LMCT, ligand-to-metal charge transfer; LF, ligand field; EPR; electron paramagnetic resonance; NHE, normal hydrogen electrode; CD, circular dichroism; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; FPLC, fast protein liquid chromatography.

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(Beaucage & Caruthers, 1981) on an Applied Biosystems Model 380A automated DNA synthesizer. Degenerate oligonucleotides were made equimolar in A, C, G, and T at positions 1 and 2 of the codon and in G and C at position 3. They were then purified by preparative gel electrophoresis on a 15% acrylamide gel. Plasmid pET3a was obtained from Novagen, and pBluescriptII was from Stratagene. Mutant plasmids were purified from *E. coli* by the alkaline lysis method (Sambrook et al., 1989).

Restriction digests typically used 0.5 μg of plasmid DNA, 2–5 units of restriction enzyme, and 2 mL of 10× digest buffer in 20 μL at the suggested temperature for 2–3 h. DNA restriction fragments were run on 1.2% agarose gels, visualized with ethidium bromide, and isolated with a Schleicher and Schuell Elutrap apparatus.

The polymerase chain reaction (PCR) was carried out as described (Saiki et al., 1988). Each reaction contained 1 ng of template DNA, 50 pmol of the two primers, 200 μM dNTPs in 50 mM KCl, 10 mM Tris (pH 8.3), and 1.5% $MgCl_2$ in 50 μL. The reactions were overlayered with 50 μL of mineral oil and subjected to 25 cycles of the following program: 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min. PCR products were subcloned by ethanol precipitation, treatment with T4 DNA polymerase to remove ragged ends, ligation into *Eco*RV-treated pBluescriptII, and restriction enzyme digestion. This procedure proved to be far more efficient than direct treatment of PCR products with restriction enzymes, as restriction sites at the termini of DNA molecules are very poorly digested (Kaufman & Evans, 1990).

Cassette Mutagenesis. The construction of the plasmid pUCASA containing the synthetic gene coding for proazurin has been described (Chang et al., 1991). Complementary oligonucleotides spanning the region of pUCASA between the *Sac*II and *Bst*EII sites were synthesized with a degenerate codon (NNG/C) for position 46. For cassette mutagenesis, 50 pmol of each of the oligonucleotides were first treated with ATP and polynucleotide kinase, combined in 100 μL of 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl, heated to 95 °C, and then slowly cooled to room temperature. A three-fragment ligation was then carried out as follows: 1 pmol of the annealed oligonucleotides was combined with approximately 0.12 pmol each of the *Eco*RI/*Sac*II fragment and the *Eco*RI/*Bst*EII fragment in a 20-μL volume. This was incubated in a mixture of 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 1 unit of T4 DNA ligase for 12–14 h at 16 °C. Ten microliters of the ligation mixture were directly transformed into competent *E. coli* strain TG 1 and plated out onto LB–ampicillin.

Transformant Sequencing. Sixty-four transformants from the ligation reaction were randomly picked by the toothpick method and grown in 2-mL cultures of L-broth containing 0.05 mg/mL ampicillin. Plasmid DNA from these cultures was isolated following the alkaline lysis protocol and sequenced by a variation of the Sanger dideoxy method for double-stranded DNA (Manfioletti & Schneider, 1988). DNA sequencing reagents including Sequenase version 2.0 were obtained from United States Biochemicals.

Western Blots. Colonies harboring mutants were grown to the late log phase; a 1.5-mL sample of each was pelleted by centrifugation, resuspended in 100 μL of protein sample buffer, and heated to 95 °C for 10 min to lyse the cells. Aliquots of 20 μL each were loaded onto a 15-cm, 15% polyacrylamide gel with a 6% stack and run at a constant current of 5 mA for 12–16 h. Protein was then transferred from the gel onto DEAE nitrocellulose using a Bio-Rad Transblot cell for 1 h at 100 mA. Azurin was visualized following the binding of rabbit anti-azurin antibody using the Vectastain ABC immunoperoxidase system.

Protein Expression and Purification. Azurin mutants in pUCASA were expressed in moderate yields in *E. coli* strain TG1 as described previously (Chang et al., 1991). Higher yields of azurin could be obtained by the use of the T7 RNA polymerase expression system of the pET3a vector (Studier et al., 1990), provided by Novagen. The expression vector pET3aAz was constructed by transferring the azurin gene into pET3a by simultaneously amplifying the gene from pUCASA and introducing *Nde*I and *Bgl*II restriction sites at the 5′ and 3′ ends, respectively, of the coding strand by PCR with the oligonucleotide primers **1** and **2**:

M1Az

1: 5′-CGCGCCATATGCTGCGTAAGCTGGCTGCAGTGTCT-3′

NdeI

Term

2: 5′-GCGCAGATCTCTATTTCAGAGTCAGGGTACCTTTCAT-3′

BglII

Following subcloning of the PCR product into *Eco*RV-treated pBluescriptII as above and sequencing of the entire coding region, the azurin gene was excised from the shuttle vector with *Nde*I and *Bgl*II, isolated from a 1.5% low-melting agarose gel, and cloned into the *Nde*I and *Bam*HI sites of pET3a. Following transformation and isolation of pET3aAz in XL-1 Blue, the plasmid was transformed into the strain HMS174(DE3), and the protein was expressed and isolated from the periplasmic fraction of the cells as described previously (Chang et al., 1991). The Cu(II)(His46Asp) protein was purified to homogeneity on an FPLC system using 10 mM Hepes (pH 8.7) and eluting with a NaCl gradient. A purity ratio $A_{612}/A_{280} \geq 0.30$ was obtained. The Co(II) derivative of the His46Asp protein was prepared either by demetalation of Cu(I)(His46Asp) azurin and subsequent reconstitution with Co(II) (Di Bilio et al., 1992) or by addition of Co(II) [as cobalt(II) acetate in 10 mM Hepes (pH 7.5)] to 1 mM to the periplasmic fraction containing azurin. Co(II)(His46Asp) azurin has a faint green color and is stable to metal loss at pH >7, but it loses Co(II) quantitatively and reversibly at pH 4.5. The Co(II)(His46Asp) protein appears to be more stable to metal loss at high pH than Cu(II)(His46Asp) azurin. This property was used to advantage in the isolation and purification of the protein.

Isoelectric Focusing. Isoelectric points were determined by isoelectric focusing using Pharmacia Ampholine PAGplates (pH 4.0–6.5) on a Multiphor apparatus and were referenced to standards supplied by Bio-Rad. Protein bands were visualized by staining with Coomassie Blue.

Spectroscopic Measurements. CD spectra of 0.1 mM protein solutions were taken on a JASCO Model J600 spectropolarimeter using 1.0-cm cells. X-band EPR spectra were taken on a Bruker ESP 300 spectrometer. Absorption spectra were recorded using a modified Cary 14 spectrophotometer. Hepes was chosen as buffer for the spectroscopic measurements due to its low affinity for metal ions (Perrin & Boyd, 1968) and relatively low pH variation upon freezing (Williams-Smith et al., 1977). Glycerol was added to samples for low-temperature EPR experiments in order to increase the quality of the glassy frozen solutions. The spin-Hamiltonian parameters were estimated by simulation of the EPR spectra.

Electrochemistry. Differential pulse polarography was carried out with a Princeton Applied Research polarographic analyzer (Model 174A) equipped with a microvolt digital multimeter (Keithley 177). Reduction potentials were determined by placing argon-deaerated solutions (0.7 mL, 1 mM in 50 mM NH_4OAc, 100 mM NaCl) into a mini stirred cell equipped with a platinum counter electrode, glassy carbon working electrode, and a calomel reference electrode and subjecting them to voltammetric scans between −200 and 500 mV at scan rates of 2 mV/s and modulation amplitudes of 25 mV. Potentials were calculated from the numerical mean of the reduction and oxidation waves.

Computer Modeling. Computer modeling of the coordination site of Cu(II)(His46Asp) azurin employed Quanta (V. 3.2) and the 2.7-Å resolution structure of the *P. aeruginosa* protein (Adman & Jensen, 1981).

RESULTS

Mutagenesis and Protein Expression. In the site-saturation experiment, the codon for His46 of azurin was replaced by cassette mutagenesis with a degenerate codon containing a mixture of all four nucleotides at the first two positions and a mixture of G and C at the third position. This mixture codes for all 20 amino acids as well as a stop codon. After ligation of the mutagenesis cassette into the previously described vector containing the preazurin gene in pUC18 (pUCASA) (Chang et al., 1991) and transformation into *E. coli*, 64 of the resulting transformants were grown to saturation in small cultures, their plasmid DNA was isolated, and the identity of the codon at position 46 was determined by double-stranded plasmid DNA sequencing (Manfioletti & Schneider, 1987). From this pool all 20 possible amino acid variants were identified.

To determine whether the mutant proteins were expressed under the control of the lac promoter and correctly processed in the *E. coli* host, a Western blot analysis of the mutants was carried out (Chang et al., 1991). All proteins showed bands at the expected molecular mass for azurin (14.6 kDa), except for the stop-codon mutant, which displayed a very faint band at this position, most likely due to a small amount of suppression of the mutation by the supE phenotype of strain TG 1 (Carter, 1986). The blots confirmed that the mutant proteins had been properly secreted into the periplasmic space of *E. coli* with concomitant cleavage of the azurin signal sequence.

Since the desired proteins could be isolated only in moderate yield under the control of the lac promoter in the pUC18 vector, alternative expression systems were explored. High yields of azurin were obtained from the commercially available pET3a vector (Novagen), in which expression of the azurin gene is under the control of the T7 RNA polymerase promoter (Studier et al., 1990). Expression of the wild-type as well as mutant azurins in *E. coli* strain HMS174(DE3) with this system yielded 10–12 mg of protein/L of culture. Rather than undertaking the tedious task of expressing and isolating all 19 mutant azurins on a large scale, we selected a subset for investigation. This subset included a number of mutants with side chains capable of metal coordination (Asp, Glu, Ser, Asn, and Lys), as well as one with a noncoordinating side chain (Ala). These mutant azurins were isolated, treated with $CuSO_4$ to 1 mM, and analyzed by absorption spectroscopy. Of the above variants, only the one with aspartate at position 46 displayed the strong 600-nm absorption of a blue protein.

Spectroscopic and Electrochemical Properties. Some differences between the spectra of the Cu(II)(His46Asp) mutant and the wild-type proteins are apparent (Figure 1).

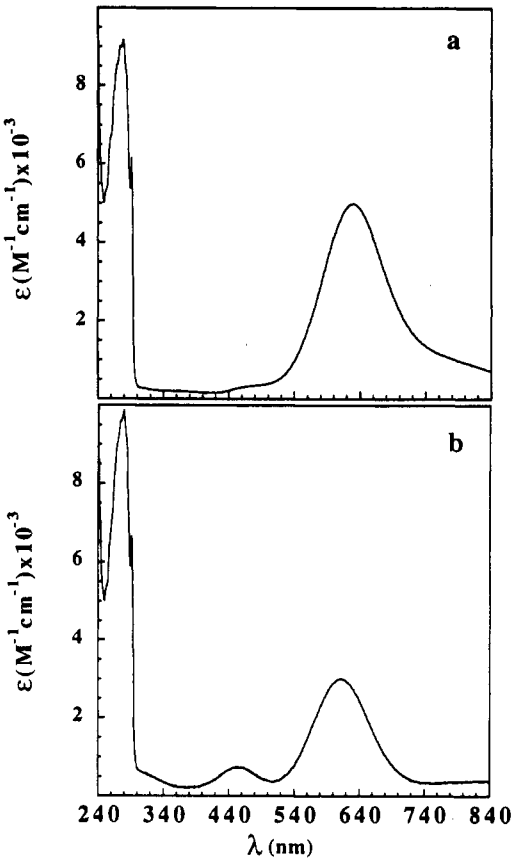

FIGURE 1: UV–visible absorption spectra of wild-type azurin (a) and Cu(II)(His46Asp) azurin (b) in 10 mM Hepes/36 mM NaCl buffer (pH 8.7) at room temperature.

The S(Cys)→Cu(II) charge-transfer absorption in the spectrum of the mutant is centered at 612 nm, and the band at 454 nm is more intense ($A_{454}/A_{612} = 0.25$) than the analogous band (481 nm) in the spectrum of the wild-type protein. Moreover, a band at ~300 nm is partially resolved in the spectrum of Cu(II)(His46Asp) azurin.

The absorption spectrum of Co(II)(His46Asp) azurin is shown in Figure 2. The LMCT absorptions at ~300 and 362 nm are blue-shifted with respect to the bands in the Co(II)-(His46) protein. A system of relatively weak ($\epsilon \approx 100$ M^{-1} cm^{-1}) but well-resolved LF bands is present in the 500–650-nm region. The EPR spectrum of a frozen solution of isotopically enriched (^{63}Cu) Cu(II)(His46Asp) azurin exhibits the features of a type 1 copper center, with $A_\parallel = 22(2) \times 10^{-4}$ cm^{-1} and $g_\parallel = 2.327(1)$; moreover, the spectrum is rhombic ($g_x \approx 2.03$ and $g_y \approx 2.07$; $A_x \approx 46 \times 10^{-4}$ and $A_y \approx 22 \times 10^{-4}$ cm^{-1}; Figure 3). These properties are similar to those exhibited by stellacyanin (Gray & Solomon, 1981). Of particular interest is the observation that the frozen solution EPR spectrum in NH_4OAc buffer at pH >7 is attributable to a tetragonal Cu(II) site ($g_\parallel = 2.18$, $A_\parallel = 165 \times 10^{-4}$ cm^{-1}; spectrum not shown). When glycerol was added to this solution, however, both type 1 and type 2 EPR signals were observed. When Tris or phosphate buffer at basic pH was used, only the type 1 EPR spectrum was obtained.

Reduction potentials for Cu(II)(His46Asp) azurin were obtained from differential pulse polarographic measurements at room temperature referenced to wild-type azurin as a standard (St. Clair Strong et al., 1992). The values are $E°$ = 260 mV *vs* NHE at pH 8.5 and 297 mV at pH 5.0 (wild-type azurin: $E°$ = 288 mV at pH 8.5 and 349 mV at pH 5.0).

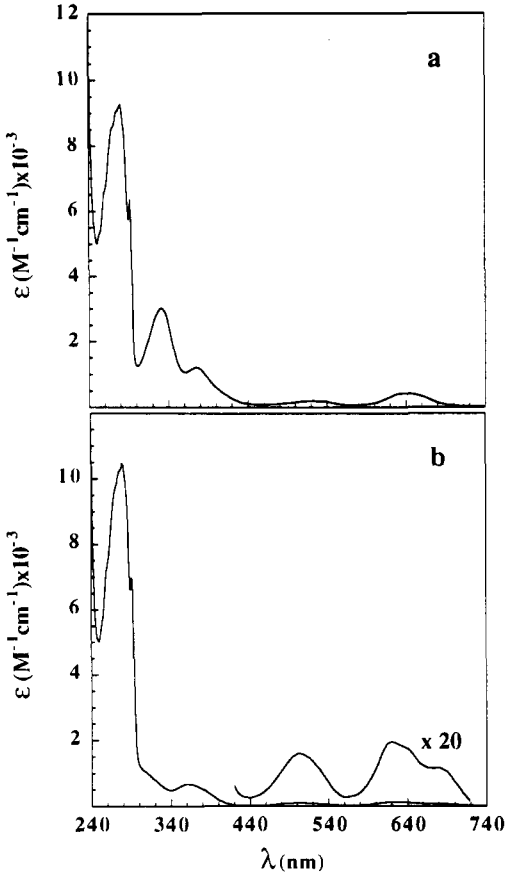

FIGURE 2: UV–visible absorption spectra of the Co(II) derivative of wild-type azurin (a) and Co(II)(His46Asp) azurin (b) in 10 mM DEA buffer (pH 9.0) at room temperature.

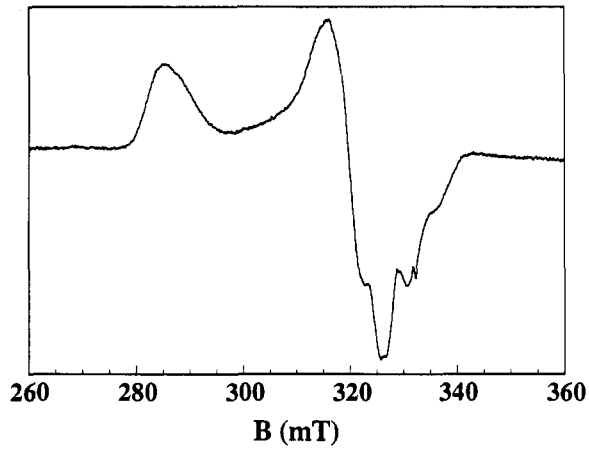

FIGURE 3: X-band (9.314 GHz) EPR spectrum of ^{63}Cu(II)-(His46Asp) azurin in a frozen solution (10 mM Hepes/36 mM NaCl–glycerol (1:1) mixture) at 77 K. The sharp feature at 332 mT is an instrumental artifact.

DISCUSSION

Replacement of His46 in azurin with other amino acids resulted in a pool of mutant proteins, all of which were expressed and properly processed into the periplasmic space of *E. coli*. Selected mutants whose side chains contain heteroatoms capable of metal coordination can be prepared on a large scale, isolated, and treated with Cu(II). Only the mutant with aspartate at position 46 displays the intense absorption in the visible spectrum characteristic of a type 1 center. The possibility exists, however, that some of the non-type 1 position 46 mutants that were prepared do in fact bind copper; it has been established, for example, that a position 117 mutant (His117Gly) forms an intensely green Cu(II) protein (den Blaauwen et al., 1991). The close similarity of the CD spectra of Cu(II)(His46Asp) azurin and the wild-type protein (data not shown) eliminates the possibility of substantial structural rearrangement to allow one of the noncoordinating histidine residues (35 or 83) to bind to copper. Inspection of the side chains of histidine and aspartate reveals that they can be superimposed such that Asp O_δ coincides with His N_δ. We believe that the structure of Cu(II)-(His46Asp) azurin, therefore, includes coordination of copper to the carboxylate side chain of aspartate. Since the side chains of the other amino acids cannot easily be accommodated at position 46 in a conformation that places a strongly coordinating heteroatom near the copper, it is likely that only Asp substitution can preserve the blue center.

The reduction potential of Cu(II)(His46Asp) azurin is lower than that of the wild-type protein, owing to the stabilization of Cu(II) by the negatively charged aspartate ligand. The spectroscopic properties of Co(II)(His46Asp) azurin also reflect the substitution of a neutral His imidazole by a carboxylate in the coordination sphere. The blue shifts of the LMCT bands in Co(II)(His46Asp) azurin relative to the Co-(II)(His46) protein are roughly the same as those for the Co(II) derivatives of Met121Asp and Met121Glu azurins (Di Bilio et al., 1992). Although less intense, the LF bands of Co(II)(His46Asp) are similar to those observed for the approximately trigonal sites in Co(II) derivatives of wild-type azurin and plastocyanin (Gray & Solomon, 1981). Comparison of the LF spectra of Co(II)(His46Asp) and Co-(II)(Met121Gly) azurins is particularly interesting, since weaker LF bands are also observed for the latter protein (Di Bilio et al., 1992). Such a decrease in band intensity can be rationalized in terms of a change of the Co(II) coordination geometry from a trigonal pyramidal to a distorted trigonal bipyramidal structure (Bertini & Luchinat, 1984). There is little doubt that the three strong Co(II) ligands in Co(II)-(His46Asp) azurin are Cys112, His117, and Asp46, but the assignment of axial coordination is less certain; possible ligands include Met121, Gly45, and water. Water coordination is likely for Co(II)(Met121Gly) azurin because of the space created by placing Gly at position 121.

A frozen sample of the Cu(II)(His46Asp) protein in ammonia-containing buffer at pH >7 (which no longer has a blue color) exhibits an EPR spectrum characteristic of a type 2 copper site. Such behavior can be explained by a structural rearrangement to a tetragonal copper geometry possibly accompanied by coordination of a molecule of ammonia. Inspection of a computer model of azurin reveals that replacement of His46 with Asp results in the creation of a small cavity in the region previously occupied by atoms of the imidazole ring, along with the loss of a hydrogen bond between the N_ϵ of His46 and the peptide carbonyl of Asn10. Rearrangement of the side-chain conformations in the vicinity of Asp46 could result in an increased accessibility of the copper center to solvent and other exogenous ligands.

We have shown that a histidine at position 46 is not required for a blue copper center. In our view, His46 plays an important role in these proteins by fine tuning the reduction potential of the copper site, maintaining the stability of the protein, and limiting access to the copper center by solvent and other potential exogenous ligands.

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SUPPLEMENTARY MATERIAL AVAILABLE

CD spectra [native and Cu(II)(His46Asp) azurins] and tables of absorption spectral data [Cu(II)(His46Asp) azurin] (23 pages). Ordering information is given on any current masthead page.

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