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Spectroscopic Characterizations of Bridging Cysteine Ligand Variants of an Engineered $Cu_2(S_{Cys})_2$ Cu_A Azurin

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Bridging cysteine ligands of the Cu_A center in an engineered Cu_A azurin were replaced with serine, and the variants (Cys116Ser and Cys112Ser Cu_A azurin) were characterized by mass spectrometry, as well as UV–vis and electron paramagnetic resonance (EPR) spectroscopic techniques. The replacements resulted in dramatically perturbed spectroscopic properties, indicating that the cysteines play a critical role in maintaining the structural integrity of the Cu center. The replacements at different cysteine residues resulted in different perturbations, even though the two cysteines are geometrically symmetrical in the primary coordination sphere with respect to the two copper ions. The Cys112Ser variant contains two distinct type 2 copper centers, while the Cys116Ser variant has one type 1 copper center with slight tetragonal distortion. Both the UV–vis and EPR spectra of the Cys116Ser variant change with pH, and the pK_a of the transition is 6.0. A type 1 copper EPR spectrum with $A_{||} = 26$ G was obtained at pH 7.0, while a type 2 copper EPR spectrum with $A_{||} = 140$ G was found at pH 5.0. Interestingly, lowering the temperature from 290 to 85 K resulted in conversion of the Cys116Ser variant from a type 1 copper center to a type 2 copper center, suggesting rearrangement of the ligand around the copper or binding of an exogenous ligand at low temperature. This difference in mutation effects at different cysteines may be due to different constraints exerted on the two cysteines by hydrogen-bonding patterns in the ligand loop.

Introduction

Copper thiolate centers, found in redox-active copper proteins commonly called cupredoxins, have been of great interest to both inorganic chemists and biochemists.[1,2] Two such copper thiolate centers, the mononuclear blue (type 1) copper and the dinuclear purple Cu_A, are unique in inorganic coordination chemistry because few examples existed before the discovery of these centers in nature. The type 1 blue copper center contains a $CuS_{Cys}(N_{His})_2$ in a distorted tetrahedral geometry, while the purple Cu_A center contains a mixed-valence $Cu_2(S_{Cys})_2$ core with two sulfurs bridging the two coppers (Figure 1). Both copper centers function as electron-transfer (ET) centers in proteins such as plastocyanin and azurin (in the case of the blue copper center) and cytochrome *c* oxidase (C*c*O) and nitrous oxide reductase (N_2OR) (in the case of the purple Cu_A center).[1−9] A detailed understanding of these unique copper thiolate centers can enrich our knowledge of inorganic coordination chemistry while enabling the design of ET centers with tunable redox properties for biological functions. Toward this goal, biochemical and biophysical studies of the Cu_A center

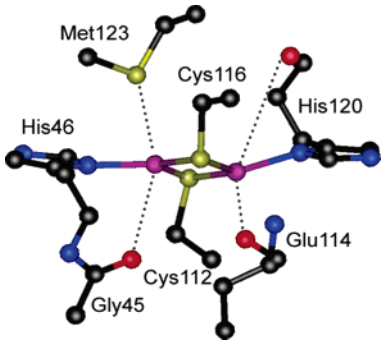

Figure 1. Active site structure of the Cu_A site in Cu_A azurin.

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in both native enzymes,[10−22] soluble fragments,[23−36] and engineered proteins[37−45] have been carried out.

The bridging cysteine residues in the purple Cu_A center play crucial roles in defining its structure and function. They are important for the spectroscopic characteristics of the dinuclear copper center. The optical spectra of the Cu_A center are characterized by two intense $S_{Cys} \rightarrow Cu$ charge-transfer (CT) bands around 480 and 530 nm and a near-IR band around 800 nm, originating from a class III mixed-valence $\Psi \rightarrow \Psi^*$ transition. A small hyperfine splitting constant in the $g_{||}$ region of the electron paramagnetic resonance (EPR) spectra of purple copper proteins is the result of high-degree delocalization of the unpaired electron onto the bridging cysteine ligands.[46] Furthermore, sulfur K-edge X-ray absorption spectroscopy indicated a relatively high $Cu-S_{Cys}$ covalency in the Cu_A center (∼23% per sulfur) when compared to a normal (type 2) copper thiolate center (∼15%).[47−49] This large copper thiolate covalency is responsible for both the mixed-valence character and the efficient ET properties of the Cu_A center.

In addition to biochemical and biophysical studies of the $Cu_2(S_{Cys})_2$ center, replacement of the residues in the coordination sphere can be a powerful tool in elucidating the role of each residue around the metal-binding site. For example, in contrast to small perturbations of the spectroscopic properties when one of the active site histidines or the axial ligand methionine was replaced with other residues,[22,34,36,43,45,50,51] replacing one of the two cysteine residues has resulted in greatly perturbed structural, functional, and spectroscopic characteristics[52−55] or even in loss of copper-binding capability.[52,56] To provide further insight into the role of cysteines in the purple Cu_A center, we report here

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the preparation and spectroscopic characterization of two variants where the bridging cysteines in an engineered Cu_A center in azurin are replaced by a serine (Cys112Ser and Cys116Ser). In addition to confirming the importance of both cysteines in defining the spectroscopic signatures of the Cu_A center, we have observed different effects of Cys to Ser mutation at the two locations and interesting pH- and temperature-dependent conversions between different types of copper thiolate centers in one of the variants (Cys116Ser).

Materials and Methods

Protein Preparation. Construction, expression, and purification of the Cys112Ser and Cys116Ser variants of Cu_A azurin were carried out using procedures reported previously.[39,41]

Spectroscopic Measurements. UV−vis absorption spectra were obtained at 4 °C on a Varian Cary 3E spectrophotometer. X-band EPR spectra were collected at 35 K on a Varian-122 X-band spectrometer operating at 9.05 GHz and employing 100-kHz field modulation. For the Cys112Ser variant, a buffer of 50 mM ammonium acetate (pH 5.1) was used for both UV−vis and EPR measurements, with 50% glycerol added as a glassing agent in EPR studies. For the Cys116Ser variant, pH titrations were first carried out using a mixed buffer system [50 mM sodium acetate, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 50 mM 3-(*N*-morpholino)propanesulfonic acid, and 50 mM Tris]. Once the pH range where the variant was stable was determined, a mixed buffer of 50 mM sodium acetate and 50 mM MES was used for EPR and low-temperature studies, with 25% glycerol added as a glassing agent. Electrospray ionization mass spectrometry (ESI-MS) data were acquired using a Quattro mass spectrometer (Micromass, Manchester, U.K.). The ESI was performed with a 100% water flow system with an applied cone voltage of 30 V. The mass scale was calibrated with CsI. Typical protein concentrations were 0.1 mM in a 50 mM pH 5.1 ammonim acetate buffer.

Low-Temperature UV−Vis Measurements. Optical spectra at low temperature were obtained using a Cary 3E UV−vis spectrophotometer and a homemade cryostat with liquid nitrogen as a cooling agent. A sample in 75% glycerol was placed in a disposable UV-enhanced methacrylate semi-microspectroscopic cell (Fisher Scientific, Pittsburgh, PA) with a 4.3-mm path length, and the cell was mounted on the cuvette holder of the cryostat. The protein solution was cooled without direct contact with liquid nitrogen to prevent cracking and to maintain the high optical quality of the frozen glassy solid solutions.

Results and Discussion

The two bridging cysteine variants of Cu_A azurin, Cys112Ser and Cys116Ser, were constructed, expressed, and purified using a protocol previously reported.[39,41] The mutations were confirmed by sequencing of the variant DNA (data not shown) and ESI-MS of the purified proteins. (Cys112Ser variant: calcd, 14154.99 Da; obsd, 14153.0 Da. Cys116Ser variant: calcd, 14154.99 Da, obsd, 14155.1 Da). The addition of $CuSO_4$ to the apo Cys112Ser variant at pH 5.1 resulted in the appearance of an intense yellow color with a strong absorption band at 390 nm and a broad band at 713 nm (Figure 2Aa). In contrast, the addition of $CuSO_4$ to the apo Cys116Ser variant resulted in an intense blue color with a strong absorption band at 623 nm and a weaker band at 424 nm (Figure 2Ab). ESI-MS of the Cys112Ser variant shows

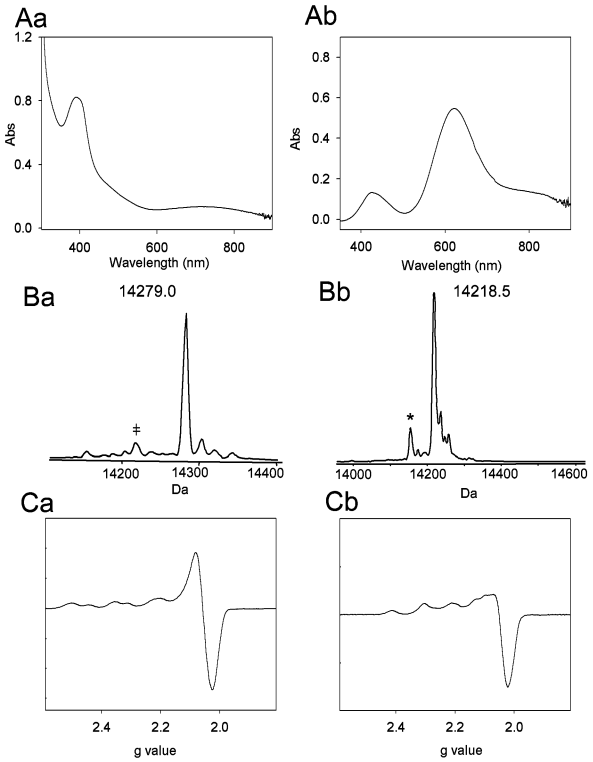

Figure 2. (A) UV−vis spectrum of Cys112Ser (Aa) and Cys116Ser (Ab). (B) ESI-MS of Cys112Ser (Ba) and Cys116Ser (Bb) variants of Cu_A azurin. The double dagger indicates a peak from a species with one copper, and the asterisk indicates a peak from the apo protein. (C) X-band EPR spectrum of Cys112Ser (Ca) and Cys116Ser (Cb). UV−vis and EPR spectra were recorded in a pH 5.0 ammonium acetate buffer. EPR parameters: microwave power 0.2 mW, modulation amplitude 4 G, and temperature 35 K.

a peak corresponding to a species with two copper ions (obsd, 14279.0 Da; calcd, 14281.09 Da; Figure 2Ba), while a single major peak corresponding to a species with one copper ion was observed in ESI-MS of the Cys116Ser variant (obsd, 14218.5 Da; calcd, 14217.54 Da; Figure 2Bb).

It has now been established that the copper(II) thiolate in a type 2 normal copper center often displays a strong yellow or red color because of an intense ligand-to-metal CT band around 400 nm. The typical tetragonal geometry and normal copper−thiolate bond of the type 2 copper center orients one lobe of the $d_{x^2-y^2}$ orbital along the ligand−metal bond, producing an intense, high-energy S_{Cys} pseudo-σ to Cu(II) CT transition and a weak, low-energy S_{Cys} π to Cu(II) CT transition.[6,9] Therefore, from optical spectra, it can be concluded that the Cys112Ser variant contains a type 2 copper center.

In contrast to the type 2 copper center, a type 1 copper center has a distorted tetrahedral geometry. Because of the distorted tetrahedral geometry, the short copper−thiolate bond rotates the Cu $d_{x^2-y^2}$ orbital such that its lobes are bisected by the Cu−S_{Cys} bond rather than being aligned with the bond, resulting in better overlap with the S_{Cys} π orbital than with the pseudo-σ orbital.[6,9] As a result, the lower energy band (i.e., S_{Cys} π orbital to Cu CT, around 600 nm) is much more intense than its higher energy band (i.e., S_{Cys} pseudo-σ orbital to Cu CT, around 400 nm). Therefore, the ratio, R_ϵ [$=\epsilon$(higher energy band)/ϵ(lower energy band)], is a good measure of different types of copper centers, with a low R_ϵ

indicative of a type 1 blue copper center and a high R_ϵ indicative of a type 2 copper center.[2,6,9,57]

Green copper proteins with intermediate R_ϵ exist between the above two extremes, and they are due to tetragonal distortion of the type 1 blue copper center and elongation of the copper−thiolate bond. With a $R_\epsilon \sim 0.23$, the copper center in the Cys116Ser variant can be described as a type 1 blue copper center with some degree of tetragonal distortion.

Further information on the geometry of the active site can be obtained from EPR spectra. The X-band EPR spectrum of the Cys112Ser variant was found to be axial with a large hyperfine coupling constant in the $g_{||}$ region (Figure 2Ca). Two sets of hyperfine structures were observed with $A_{||}^1 =$ 155 G and $A_{||}^2 =$ 145 G, respectively. The observation of two sets of hyperfine structures is consistent with the presence of two coppers in this variant, as confirmed by MS. The large hyperfine constants are typical of type 2, normal copper centers where the geometry around the copper center is tetragonal. Therefore, the EPR spectrum of the Cys112Ser can be best interpreted as the presence of two distinct type 2 copper centers. This EPR result is consistent with the UV−vis spectral studies of the same variant described above. In contrast, the X-band EPR spectrum of the Cys116Ser variant at pH 5.0 (Figure 2Cb) exhibited axial character with a large parallel hyperfine splitting constant (∼140 G), suggesting the presence of a type 2 copper center at this pH. This result is inconsistent with the UV−vis spectral studies described above because blue copper proteins normally display smaller parallel hyperfine splitting constants.

To provide further insight into the structure of the Cys116Ser variant, the pH dependence of its UV−vis spectral changes was examined (Figure 3A). As the pH increased from 5.0 to 7.0, the band at 623 nm was blue shifted to 603 nm with a concomitant increase in intensity, while the higher energy band around 424 nm was shifted to slightly lower energy. This transition is reversible, and a single protonation/deprotonation equilibrium model gives a good fit with the experimental data and a pK_a value of 6.0 can be obtained (Figure 3A, inset). Furthermore, EPR spectra of Cys116Ser at various pHs were also collected (Figure 3B). As the pH increased from 5.0 to 7.0, the type 2 copper features disappeared and a new feature with a small hyperfine splitting constant in the $g_{||}$ region (26 G) emerged. This small hyperfine constant is characteristic of type 1 blue copper centers, originating from a highly covalent S_{Cys}−Cu bond. Therefore, pH-dependent UV−vis and EPR spectra of the Cys116Ser variant could be interpreted as a change in the geometry around the copper ion, thus affecting the Cu−S_{cys} interaction upon protonation/deprotonation of a neighboring residue.

To reconcile the inconsistency between UV−vis and EPR studies of the Cys116Ser variant at the same pH, temperature effects on its UV−vis spectra were investigated because

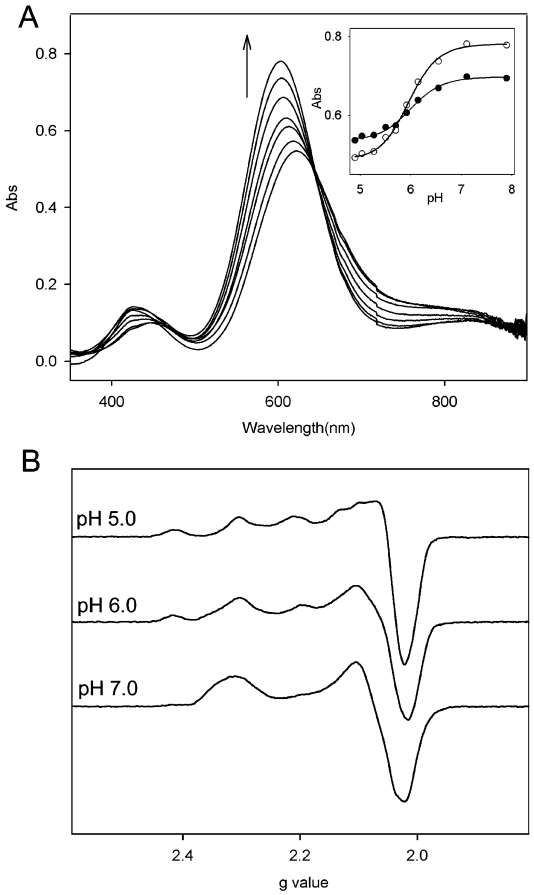

Figure 3. (A) pH dependence of the UV−vis spectrum of the Cys116Ser variant in the pH range of 5.0−7.0. The arrow indicates the direction of increasing pH. Inset: changes in the peak intensities at 623 nm (●) and 603 nm (○) as a function of pH. The solid lines represent the best fit of the experimental data. (B) X-band EPR spectrum of Cys116Ser at various pHs.

UV−vis spectra were obtained at room temperature while EPR spectra were taken at 35 K. As the temperature decreased from 290 to 85 K, the intensity of the band around 430 nm increased and was blue shifted, while the intensity and the position of the band around 625 nm changed only moderately at pH 5.0 (Figure 4A). R_ϵ ($\epsilon_{432}/\epsilon_{626}$) changed from 0.26 at 290 K to a $\epsilon_{417}/\epsilon_{626}$ ratio of 0.62, suggesting an increased interaction between the Cu $d_{x^2-y^2}$ and S_{Cys} pseudo-σ orbital at lower temperatures. This result explains the large $A_{||}$ value in EPR spectra at pH 5.0 obtained at cryogenic temperatures because lowering the temperature resulted in an increase in R_ϵ toward a more tetragonally distorted green copper center. The temperature dependence of the UV−vis spectrum is more drastic at pH 6.0 (Figure 4B). The $\epsilon_{419}/\epsilon_{623}$ ratio increases from 0.42 to a $\epsilon_{411}/\epsilon_{611}$ ratio of 1.8 as the temperature decreases from 290 to 85 K. At pH 7.0, both bands around 420 and 620 nm increase in intensity and blue shift as the temperature decreases. The $\epsilon_{421}/\epsilon_{617}$ ratio of 0.41 slightly increases to a $\epsilon_{408}/\epsilon_{597}$ ratio of 0.52 in the same temperature range (Figure 4C).

Potential reasons for the spectral changes observed at low temperatures arise either from the rearrangement of the ligands to give a different geometry around the metal site or from the binding of an exogenous ligand. At all pHs, an increase in R_ϵ was observed, suggesting a tetragonal distor-

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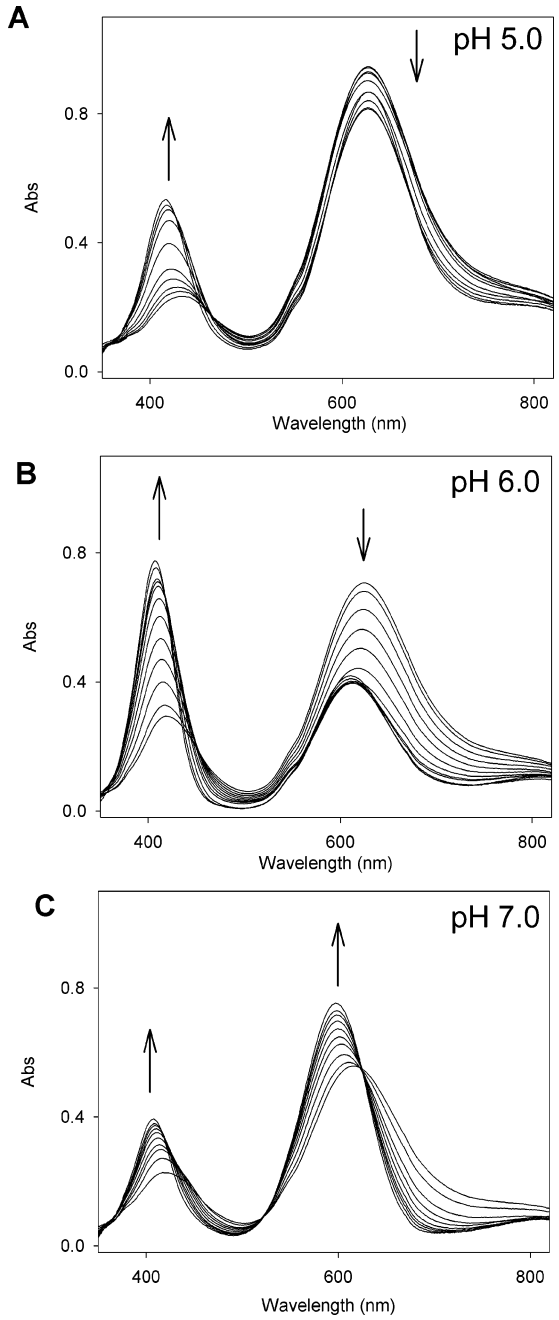

Figure 4. Temperature dependence of UV−vis spectra of the Cys116Ser variant (A) at pH 5.0, (B) at pH 6.0, and (C) at pH 7.0. Arrows indicate the direction of decreasing temperature from 290 to 85 K. Glycerol (75%, v/v) was used as a glassing agent.

tion around the metal center at low temperatures. Interestingly, a temperature-induced type 1 to type 2 transition was also observed for His46Asp and His117Gly variants of blue copper azurin.[58,59] In both cases, lowering the temperature favors type 2 copper centers. These results, combined with the observations described in this paper, indicate that the highly conserved ligands to either the blue copper (e.g., His46 and His 117 in azurin) or the purple Cu_A center (e.g., Cys116) play a critical role in keeping the integrity of the centers at all temperatures; mutations of these ligands can result in changes of both copper centers into a type 2 copper center. Furthermore, binding of an exogenous ligand, CH_3OH, to a Met121Ala azurin variant was found to be temperature-dependent.[60] Binding of an alcohol to Met121Ala was observed at temperatures below freezing. On the basis of these observations, it is quite possible that the hydroxyl group of Ser116 coordinates to copper at low temperatures, causing rearrangement of the ligand set to give a tetragonal geometry as found in type 2 copper centers. Alternatively, an exogenous ligand, such as water or hydroxide may contribute to the ligation at low temperatures.

Given that the two cysteines in the Cu_A center are geometrically symmetrical in the primary coordination sphere with respect to the two copper ions, it is rather surprising that Cys112Ser and Cys116Ser mutation resulted in entirely different species. This difference might be explained by the different constraints exerted on the two cysteines by the ligand loop. Inspection of the X-ray crystal structure of Cu_A azurin[61] revealed that the position of Cys112 is restrained by a series of hydrogen-bonding interactions (Figure 5). Strong interactions are present between Ser113 hydroxyl group and Asn47 side-chain amide nitrogen and Ser113 amide nitrogen and Asn47 side-chain amide oxygen (denoted as A in Figure 5A). These interactions have been recognized previously in *Alcaligenes denitrificans* azurin.[62] Additional interactions between the Cys112 thiolate and Ser113 and Glu114 amide nitrogens (denoted as B), Ser113 hydroxyl group and Asp71 backbone carbonyl (denoted as C), as well as Cys112 amide nitrogen and Met123 carbonyl oxygen (denoted as D) further constrain the position of Cys112 in the ligand loop. Restricted movement of Cys112 and His46 could force tetrahedral geometry around the copper, thus creating a type 1 like site in the Cys116Ser variant. Cys116 is, however, likely to be more flexible with fewer such hydrogen-bonding interactions, with interactions between Cys116 amide nitrogen and Glu114 carbonyl oxygen, Asn119 amide nitrogen and Gly117 carbonyl oxygen, denoted as E and F, respectively. As a result, rearrangement of the ligand set (including Cys116 and His120) could happen to yield a tetragonal environment around the copper, yielding a type 2 like center in the Cys112Ser variant. Additional ligands from backbone carbonyl or exogenous ligands such as water or buffer molecules can also coordinate to the copper ion through the open binding site.

It is interesting to compare our results with the bridging cysteine mutagenesis studies on the Cu_A center from *Paracoccus denitrificans*. Both Cys224Ser (corresponding to Cys112Ser in Cu_A azurin) and Cys228Ser (corresponding to Cys116Ser in Cu_A azurin) variants in the soluble Cu_A binding domain exhibited very weak transitions in the visible region, with $\lambda_{max} = 400-420$ nm.[53] Metal analyses revealed the copper content of 1 Cu per protein for Cys224Ser and 2

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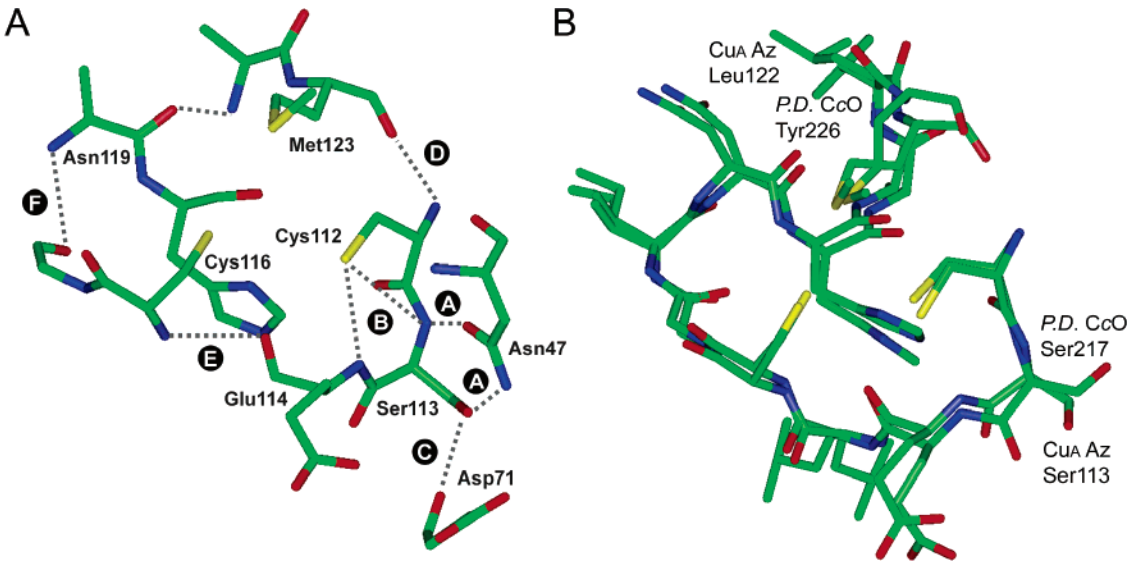

Figure 5. (A) Cu_A ligand loop in Cu_A azurin with hydrogen-bonding interactions shown as dotted lines. See the text for details. (B) Superimposed structure of the Cu_A ligand loop in *P. dentrificans* C*c*O (Cys216−Met227) and the Cu_A ligand loop in Cu_A azurin (Cys112−Met123). The structure of *P. dentrificans* C*c*O was generated from a PDB file (1AR1) using Insight II (Accelrys, San Diego, CA) software.

Cu per protein for Cys228Ser. EPR spectra consisting of a mixture of two signals with large hyperfine coupling constant, typical of a type 2 copper center, were detected for both variants.[53] The Cys216Ser variant in a deletion strain of *P. denitrificans* was also studied, and the loss of the near-IR peak in the optical spectrum and the four-line EPR spectrum was reported.[54] It is very intriguing that the same mutation in Cu_A azurin yielded different species because the Cu_A azurin ligand loop was based on the Cu_A ligand loop in *P. denitrificans* C*c*O.[39] The sequence of the ligand loop is the same as those for Cu_A azurin and *P. denitrificans* C*c*O except one amino acid (Leu122 in Cu_A azurin in place of Tyr226 in *P. denitrificans* C*c*O), and two loop structures overlay very well (Figure 5B).[61,63] A noticeable difference was detected in the position of Ser113 (Cu_A azurin numbering). The hydroxyl group in Ser113 orients in different directions in the two loop structures, and hydrogen-bonding interaction present in the Cu_A azurin (with Asn47, see Figure 5B) is absent in the Cu_A from *P. denitrificans*. These subtle differences might render more flexibility in the ligand loop around Cys216 (corresponding to Cys112) in the Cu_A from *P. denitrificans*. Therefore, formation of the type 1 copper center in the Cys116Ser Cu_A azurin is probably due to the residual structural features of the scaffold protein, azurin, that hold the Cys112 in position via strong hydrogen-bonding interactions as described above.

In conclusion, spectroscopic properties of the purple Cu_A azurin were completely lost upon replacement of either of the two bridging cysteines, Cys112 and Cys116, with serine, demonstrating the critical roles played by these two residues. Substitution of Cys112 with Ser produced a dinuclear copper species with a type 2 like optical spectrum. The existence of two separate type 2 copper centers was deduced from EPR spectra. Replacement of Cys116 with Ser resulted in a mononuclear copper species. On the basis of spectroscopic data, the geometry around the copper is close to distorted tetrahedral and it maintains one of the strong $Cu-S_{Cys}$ interactions. It also exhibits interesting pH and temperature dependence, possibly because of rearrangement of the ligand set around the copper or binding of an exogenous ligand. Even though the two cysteines are geometrically symmetrical in the primary coordination sphere with respect to the two copper ions, the Cys112Ser and Cys116Ser mutations exert different effects on their spectroscopic properties. This difference may be due to different constraints exerted on the two cysteines by hydrogen-bonding patterns in the ligand loop.

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