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Creation of a Binuclear Purple Copper Site within a *de Novo* Coiled-Coil Protein

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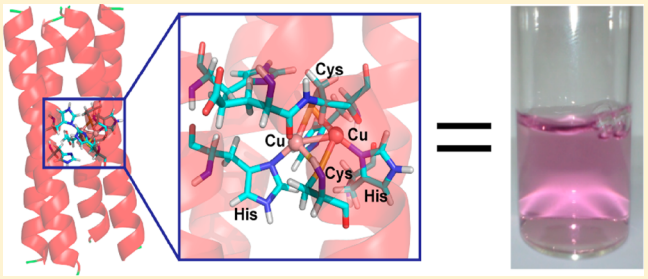
Supporting Information

ABSTRACT: Although various kinds of metal binding proteins have been constructed by *de novo* design, the creation of a binuclear metal binding site remains especially challenging. The purple copper site in subunit II of COX, referred to as the Cu_A site, has two copper ions bridged by two Cys residues. We constructed the Cu_A site consisting of two Cys and two His residues in a *de novo* designed four-helical coiled-coil protein. The protein bound two copper ions and exhibited a purple color, with relatively intense absorption bands at 488 and 530 nm in the UV−vis spectrum. The EPR spectrum displayed unresolved hyperfine splittings in the $g_{\|}$ region, which was similar to the native or engineered Cu_A site with an $A_{\sim 480}/A_{\sim 530} > 1$. The extended X-ray absorption structure analyses of the protein revealed the presence of the Cu_2S_2 core structure, with two typical N(His)−Cu bonds per core at 1.90 Å, two S (Cys)−Cu bonds at 2.21 Å, and the Cu−Cu bond at 2.51 Å, which are also characteristic structures of a purple copper site.



Cytochrome *c* oxidase (COX) and nitrous oxide reductase (N_2OR) belong to purple copper proteins and possess electron transfer functions. Both proteins receive the electron from cytochrome *c* at an unusual copper electron transfer center called CuA. In COX, the electron is then transferred to heme *c* to catalyze dioxygen to water, while in the case of N_2OR, the electron is transferred to the Cu_Z site, where the reduction of N_2O to N_2 takes place.[1] The Cu_A site contains a binuclear copper center, which is composed of two bridging Cys residues forming a Cu_2S_2 core. The Cu_A site has two additional His residues interacting equatorially and several weakly interacting axial groups, such as Met, His, and the main chain carbonyl of Glu.[2−4] These sites have been characterized as the binuclear copper center with mixed valence [Cu(1.5)−Cu(1.5)] species in various spectroscopic studies.[5−10] The typical Cu_A sites show relatively intense absorption bands at around 480 and 530 nm, which are attributed to ligand-to-metal charge transfer (LMCT), and exhibit a purple color.[11−13] Hence, the proteins with the Cu_A site are referred to as purple copper proteins. Weaker bands are also observed at ∼350 nm and the near-infrared region.[11,14] These copper sites have also been characterized by their electron paramagnetic resonance (EPR) spectra, which show seven-line hyperfine splittings in the $g_{\|}$ region.[13] The Cu_Z site in N_2OR contains four copper ions bound by seven His residues.[15] Recent X-ray structural analysis revealed that the Cu_Z site has a 4Cu:2S composition and showed a UV−vis maxima at 562 and 650 nm, which is different from those of the Cu_A site.[1]

The creation of a metal binding site bearing unusual characteristic features within a scaffold protein would improve our understanding of the correlation between the coordination structures and the spectral features and facilitate design strategies for metal binding sites.[16,17] The Cu_A site of purple copper proteins have a conserved CXXXC motif in a loop.[18] Thus far, creation of purple copper proteins has been accomplished by loop-directed mutagenesis methods, in which the loop of the Cu_A site was successfully transferred to

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the copper binding loop of the blue copper proteins, azurin and amicyanin. The resultant proteins showed purple copper properties.[19,20] Previously, we successfully created a type 1 blue copper site, within a *de novo* designed four-helical coiled-coil protein.[21] In this report, we provide evidence that the purple copper site can also be introduced into a *de novo* designed four-helical coiled-coil protein.

MATERIALS AND METHODS

Construction of the Gene Encoding the Designed Protein, bi-AM2C. To obtain the expression plasmid, pET32-S(−)-biAM2C, containing the gene encoding bi-AM2C, we mutated the AM2C gene[21] by PCR reactions, using the following primers: 5′-GTAAGCATTATGCT**CAC**G-AAAACGAGTTAGC-3′, 5′-GCACTCGAAAA-TGAG**TGC**GCTTATTGCAAAAG-3′, and 5′-CAAAG-CACTACGCT**CAT**GAGAATGAACTTGC-3′. Only the forward primers are indicated. Bold letters indicate the newly residues, His at 16, Cys at 47, and His at 84, respectively. The entire DNA and amino acid sequences as well as the restriction enzyme sites are shown in the Supporting Information. The *Nco*I and *Hin*dIII DNA fragment was ligated to the large *Nco*I-*Hin*dIII fragment of pET 32-S(−) with T4 DNA ligase to obtain pET32-S(−)-bi-AM2C. Expression of bi-AM2C using the plasmid in *Escherichia coli* BL21 (DE3) and purification of the protein were described before.[21]

Measurements of UV−vis Absorption, Circular Dichroism, and Mass Spectrometries. The lyophilized purified protein was dissolved in 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)−NaOH buffer (pH 7.5), containing 500 mM NaCl and 200 μM dithiothreitol (DTT) (0.7 mg/mL). The solution was centrifuged, and the supernatant was concentrated by ultrafiltration with an Amicon Ultra-15 MWCO 3000 filter (Millipore), to a protein concentration of 1 mM. This stock solution was diluted with appropriate buffers without DTT for each measurement.

The measurements of UV−vis absorption, circular dichroism (CD), and mass spectrometries were described before.[21] Used buffers were 50 mM PIPES-NaOH, pH 7.5, containing 500 mM NaCl for UV−vis absorption spectroscopy and 10 mM phosphate buffer, pH 7.5, containing 140 mM NaCl for CD spectroscopy.

Electron Paramagnetic Resonance (EPR) Spectroscopy. The EPR spectra of the copper complexes were observed with an X-band (9.4 GHz) EPR spectrometer (JEOL, TE-300), operating with 100 kHz field modulation of ca. 0.63 mT. The microwave frequency for EPR excitation was monitored by an internal digital frequency counter. The magnetic field strength was calibrated by the hyperfine coupling constant of the Mn(II) ion (8.69 mT) doped in MgO powder. The *g*-values of the paramagnetic species were estimated on the basis of the *g*-value (2.0025) of a radical salt (lithium salt of tetracyanoquinodimethane). The *g*-values of the copper complexes were obtained as the mean value of three independent EPR measurements. A modified cryostat system (JANIS, ST-170) was used for temperature control of the frozen sample from 4.2 to 100 K.

X-ray Absorption Spectroscopy (XAS). Cu K-edge XAS data were obtained on beamline BL01B1 at SPring-8 under ring conditions of 8 GeV and ca. 100 mA (Proposal No. 2011B1238). The lyophilized sample of the protein was pressed into pellets, and the resulting purple pellets were maintained at 30 K during the measurement. The dicopper complexes, $Cu_2(CH_3COO)_4$ and $[Cu_2(S_2)(Bn_3TACH)](SbF_6)$, were obtained from Wako and prepared as described previously,[22] respectively. The data collections were performed in the fluorescence mode with a 19-element solid state detector for the protein sample and in the transmittance mode for the dicopper compounds, using a Si(111) double crystal monochromator. Cu foil was utilized to calibrate the energy. For the analysis of the protein sample, 20 scans were measured and then averaged to give the reported spectra. The XAS data were processed in the standard manner by the WinXAS software (version 3.1)[23] and Fourier transformed over the range of $2 < k < 12$ Å^{-1}. For the EXAFS analysis, the Cu_A center was modeled using bond lengths derived from the crystallographically characterized Cu_A center of COX (PDB 2ZXW). One His directly coordinated to the Cu atom was included in the model, but the more distant ligation atoms, such as the main-chain carbonyl atoms, were excluded due to their lower contributions to the EXAFS data of the Cu_A sites in *T. thermophilus* and *B. subtilis*.[24] The FEFF 8.4 code was used for the calculation of the theoretical EXAFS signals.[25] The parameters in the curve-fitting analysis were as follows: *R*, the distance from Cu; σ^2, the Debye−Waller factor; and ΔE_0, the photoelectron energy shift. Coordination numbers were fixed, as shown in Table 1.

Model Setting and Minimization. The model of the bi-AM2C−$(Cu)_2$ complex was constructed with Swiss-PDBViewer.[26] The modeling of bi-AM2C was based on the crystal structure of a parallel, four-stranded coiled-coil, GCN4-pLI (PDB ID: 1GCL).[27,28] However, bi-AM2C consists of antiparallel coiled coils because it is a single chain peptide. The crystal structure of the retro GCN4 leucine zipper (PDB ID: 1C94)[29] was used to build the second and fourth α-helical domains. The side chains of the model were replaced according to the sequence of AM2C, using SCWRL 3.0.[30] Hydrogen atoms were added using the tplgene module of myPresto.[31] All ionizable side chains were configured in their characteristic ionized states at pH 7.0. We placed two Cu^{2+} ions at the putative metal binding site of bi-AM2C.

The starting structure was solvated in a 35 Å sphere of 5006 TIP3P water[32] molecules from the center of mass of the model. A harmonic potential of 100 kcal mol^{-1} Å^{-2} was applied to any water molecules that moved outside the sphere. The AMBER96 force field parameters[33,34] were used for the amino acids of the model of the bi-AM2C−Cu^{2+} complex in the simulations. The atomic charges of the amino acids were also obtained from the AMBER96 database.[33,34]

Conjugate gradient minimization was performed on the starting structure, using the cosgene module of myPresto.[30] The long-range nonbonded interactions were truncated with a 12 Å cutoff distance. The nonbonded interactions were updated every 10 steps. The root-mean-square force criterion to terminate the minimizations was 0.01 kcal mol^{-1} Å^{-2}. In the minimization, the Cu−N(His), Cu−Cu, and Cu−S(Cys) atom−atom distances were restrained within 0.1 Å deviation from the EXAFS data by a harmonic potential.

RESULTS AND DISCUSSION

Amino Acid Sequence and Structure of the Protein. To create the purple copper site, we used a tetrameric α-helical coiled coil as the *de novo* scaffold protein,[35] in which we previously successfully created a blue copper site.[21] The blue copper site was placed within the hydrophobic core of the coiled coil by the introduction of two His residues and one Cys residue, to form a trigonal plane geometry (Figure 1). The axial

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position of this site provided the binding site for various anionic ligands, such as chloride or phosphate ions.

N-term. Q IEDKLEE ILSK**H**YA **X**ENELAR IKKLLG E G
S G G Q IEDKLEE ILSK**C**YA **Y**ENELAR IKKLLG G T
G G K Q IEDKLEE ILSK**H**YA **X**ENELAR IKKLLG E G
C-term. Q IEDKLEE ILSKAYA AENELAR IKKLLG G L

Figure 1. Amino acid sequences of AM2C (X, Y = A) for the blue copper protein and bi-AM2C (X = H, Y = C) for the purple copper protein. The proteins were prepared by genetic engineering in *E. coli* and hence contain the extra G-S-A-M-A-K and R-S sequence at the N- and C-termini, respectively. The amino acid numbering is only for the core sequence. The protein has four α-helical portions forming the α-helical coiled-coil structure, and each heptad position (a to g) is indicated above the amino acids.

The Cu_A sites are composed of the Cu_2S_2 core, with one strongly interactive His residue for each copper ion and additional weakly interactive axial ligands.[2−4] The two His and two Cys ligands are well preserved in the reported Cu_A domains. On the other hand, the axial interactions of the Cu_A sites involved several species of amino acids and other ligands, with various lengths and strengths of the interactions. This indicates that the positions of the two Cys and two His residues are essential for forming the Cu_A site, and the axial interactions would allow some flexibility. The distance between the two Cys residues is ∼3.6 Å and should be important for the formation of the thiolate bridging complex.[3] When the two Cys residues are present at the i and i + 3 positions on the α-helix, the distance between the two Cys residues is about ∼4 Å, which is close to the distance of the Cu_A site. At first, we placed the two Cys residues at the i and i + 3 positions of the second α-helix. The two His residues that interact with the two copper ions must exist near them. Therefore, we placed four His residues on neighboring α-helices. We intended two His residues to interact with copper, while the other two His residues might function as axial interacting groups. We also placed two Ala residues on the fourth α-helix to provide space for these ligands. This protein is referred to as bi-AM2C (Figure 1).

The protein bi-AM2C was expressed in *E. coli* BL21 (DE3) and purified, as described in the Materials and Methods section. The CD spectrum of bi-AM2C revealed minima at 208 and 222 nm (Figure 2). A typical coiled-coil structure has $[\theta]_{222}/[\theta]_{208}$ > 1.[36] Like AM2C, bi-AM2C has three loops and flexible structures at the N- and C-termini. Considering these amino acid sequences, bi-AM2C is considered to adopt the coiled-coil structure.

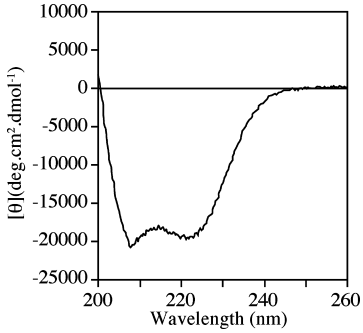

Figure 2. Circular dichroism spectrum of bi-AM2C. The measurement was performed in PBS, pH 7.5, with a 10 μM protein concentration.

The association state and the size of bi-AM2C were analyzed by analytical ultracentrifugation[21,35] under reducing conditions, with 10 mM tris[2-carboxyethyl]phosphine hydrochloride (TCEP). The sedimentation coefficient ($C(s)$) distributions from the sedimentation velocity (SV) experiments with 50 μM bi-AM2C indicated that the majority of bi-AM2C had an $s_{20,w}$ of 1.3 S (Figure 3), and the estimated molecular mass of this

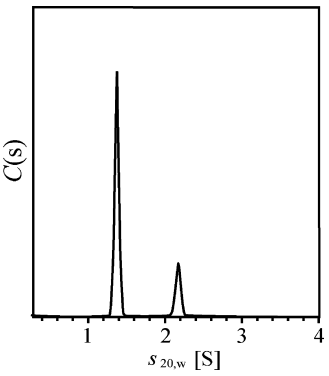

Figure 3. Analytical ultracentrifugation of bi-AM2C. The sedimentation coefficient distribution of 50 μM bi-AM2C from the SV experimental analysis is indicated.

species was 13 000 Da. This value is quite consistent with the molecular mass calculated from the amino acid sequence of bi-AM2C (15 250 Da), clearly indicating that the majority of bi-AM2C is monomeric under these conditions. The observed $s_{20,w}$ of bi-AM-2C was comparable to that (1.62 S) calculated using the 3D structure of GCN4-pLI (PDB ID: 1GCL),[27] supporting the formation of a similar four α-helical stranded coiled-coil structure in solution. The presence of a small amount of the dimer was also evident ($s_{20,w}$ = 2.5), as shown in Figure 3. Since the dimer was detected under highly reducing conditions, noncovalent intermolecular interactions mediate dimer formation.

UV−vis Spectroscopy. The Cu_A site reportedly has relatively intense absorption bands at around 480 and 530 nm, arising from LMCT, and hence exhibits the purple color.[14] Weaker bands were also observed at around 350 nm and in the near-infrared region. The bands around 480 and 530 nm are attributed to the thiolate to copper charge transfer, while the bands in the near-infrared region are due to the Cu−Cu ψ−ψ* transition. When we added excess Cu^{2+} to the bi-AM2C solution, the mixture immediately exhibited the purple color (Figure 4a), indicating the presence of the purple copper site. To further verify the purple copper site, we measured the UV−vis spectra of bi-AM2C in the presence of Cu^{2+}. At first, we measured the UV−vis spectrum of bi-AM2C in the presence of 2 equiv of Cu^{2+} (Figure 4b). The mixture showed an intense band at 488 nm with a shoulder at around 530 nm, which is identical to the characteristic absorption band of the purple copper sites. Additional weaker bands were present at around 350 and 850 nm, which were also observed in the purple copper sites. These spectral features (ε_{488} = 1350, ε_{530} = 1085, ε_{849} = 695, ε_{350} = 420) are very similar to those of the engineered purple copper site in azurin (ε_{488} = 1550, ε_{530} = 1380, ε_{765} = 770, ε_{350} = 380).[19] These results suggested that bi-

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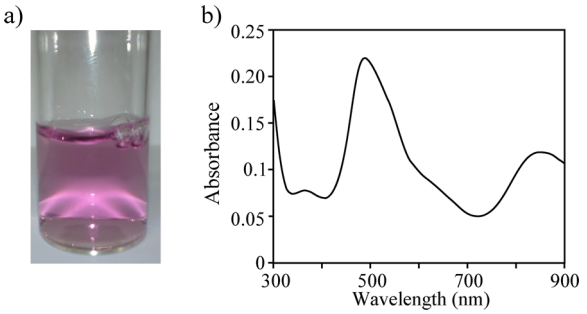

Figure 4. (a) Purple color of the solution of bi-AM2C and Cu^{2+} in 50 mM PIPES-NaOH, pH 7.5, containing 500 mM NaCl. [bi-AM2C] = 200 μM, [Cu^{2+}] = 400 μM. (b) UV−vis spectrum of bi-AM2C and Cu^{2+} in the same buffer. [bi-AM2C] = 160 μM, [Cu^{2+}] = 320 μM.

AM2C bound two copper ions, and they were organized with a similar coordination structure to those of the purple copper sites.

As in the case of the blue copper protein of AM2C, the purple color decayed in a single-exponential process, with a half-life of 2.4 h (Figure S4 in the Supporting Information).[21] The color-faded protein was insensitive to the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), indicating that the Cys residue of bi-AM2C was oxidized. Electron spray ionization time-of-flight mass spectrometry (ESI-TOF MS) of the denatured protein provided molecular masses of 15 313 and 15 329 Da in an equimolar ratio. The molecular masses were increased by 63 and 79, respectively, as compared to that of bi-AM2C (calculated value: 15 250 Da). This result suggested that the sulfurs of the two Cys residues were oxidized to SO_2 in the former case and to SO_2 and SO_3 in the latter case.

The UV−vis spectrum of the mixture of the protein and Cu^{2+} at a 1:1 molar ratio exhibited an intense band at 488 nm, with shoulders at around 530 and 600 nm (Figure 5a). In contrast,

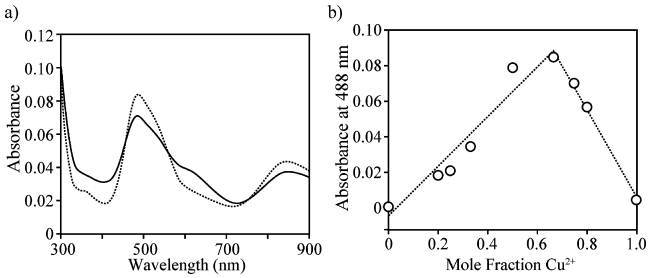

Figure 5. (a) UV−vis spectra of mixtures of bi-AM2C and Cu^{2+} at 1:1 (solid line) and 1:2 (dotted line) molar ratios. (b) Job's plot analysis. The total concentration ([bi-AM2C] + [Cu^{2+}]) was fixed at 200 μM.

the shoulder at around 600 nm disappeared at the 1:2 molar ratio of the protein and Cu^{2+}. On the other hand, the blue copper protein, AM2C with one Cu^{2+}, exhibited an intense band at around 600 nm ($\varepsilon \sim 4100$) and a weaker band at 476 nm.[21] Accordingly, these results suggested that the absorptions around 600 and 488 nm correspond to the mononuclear and binuclear Cu^{2+} sites, respectively. The UV−vis spectrum of the 1:1 mixture of biAM2C and Cu^{2+} corresponded to that of 20:1 mixture of the binuclear site and mononuclear site, indicating that more than 90% of the protein was present as the binuclear type (Supporting Information Figure S3). To determine the stoichiometry of bi-AM2C and copper, we measured the UV−vis spectra by mixing different molar ratios of bi-AM2C and Cu^{2+} at a fixed total concentration ([bi-AM2C] + [Cu^{2+}]) of 200 μM. The absorption intensity at 488 nm was plotted as a function of the molar ratio of Cu^{2+} (Figure 5b). The Job's plot analysis revealed a maximum at an approximate 1:2 molar ratio, indicating that the protein bound two copper ions.

Electron Paramagnetic Resonance (EPR) Spectroscopy. An EPR measurement was performed for a frozen solution of the bi-AM2C-$(Cu)_2$ complex. The frozen solution of the complex exhibited a characteristic purple color, indicating the retention of copper ions by bi-AM2C in the frozen matrix. As shown in Figure 6a, the EPR spectrum exhibited a rhombic

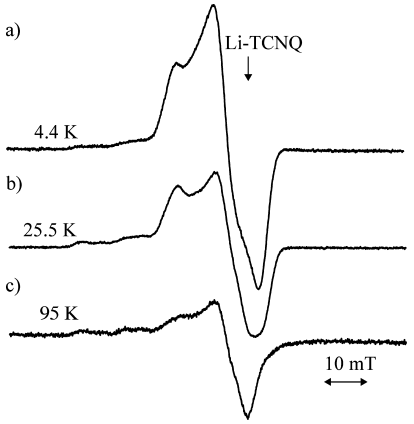

Figure 6. EPR spectra recorded for the frozen solutions of the bi-AM2C−$(Cu)_2$ complex at (a) 4.4, (b) 25.5, and (c) 95 K. The final concentrations of bi-AM2C and Cu^{2+} were 1 and 2 mM, respectively, in 50 mM PIPES−NaOH, pH 7.5, containing 500 mM NaCl and 200 μM DTT. EPR spectra of the complex at 4.4, 25.5, and 95 K were recorded by adjusting the receiver amplitude of EPR apparatus at 500, 500, and 1500, respectively. The lithium salt of tetracyanoquinodimethane, Li-TCQN, was used as an external standard (g = 2.0025).

symmetric line shape (g_1 = 2.19, g_2 = 2.04, and g_3 = 1.98), and the hyperfine splitting due to the copper nuclei was unresolved in the g_1 region. The EPR spectrum of the sample was recorded in the second-derivative EPR mode, but the hyperfine splitting remained unresolved. This indicated that the hyperfine splitting could be hidden in the line width of the g_1 component. The EPR spectra of the purple copper proteins showed seven-line hyperfine splittings in the $g_{\|}$ region. On the other hand, the Cu_A site of COX lacks the detectable hyperfine splitting.[11,13,37] These different spectral behaviors are attributed to the correlation of the $A_{\sim480}/A_{\sim530}$ ratio of the UV−vis spectrum.[19] At a ratio greater than 1, no detectable hyperfine splitting is observed in the $g_{\|}$ region. On the other hand, seven hyperfine splittings were observed at a ratio lower than 1. The $A_{\sim480}/A_{\sim530}$ ratio of the bi-AM2C−$(Cu)_2$ complex was 1.23, which is similar to that of the purple copper site of COX. This suggested that the complex would not show hyperfine splitting, which was quite consistent with the measured results. The obtained parameters were similar to those of the Cu_A site of COX ($g_{\|}$ = 2.18, g_{xy} = 2.00−2.03) and the engineered Cu_A site within the azurin scaffold ($g_{\|}$ = 2.18, g_{xy} = 2.06).[19,38] Since binuclear copper sites show short relaxation times arising from the spin exchange interaction between the two copper nuclei, the EPR signal intensity would be markedly diminished above 77 K. As depicted in Figure 6b,c, the signal intensity of the complex became weaker with increasing temperature, and the weak EPR

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signal obtained at 95 K was characteristic of a monomeric copper complex, due to the free copper ion present in the reaction solution. The mononuclear copper sites usually have long relaxation times, which allow EPR signal detection even at room temperature. This result strongly indicated that the major paramagnetic species (g_1 = 2.19, g_2 = 2.04, and g_3 = 1.98) detected at 4.4 K was a binuclear copper site involved in a purple copper protein.

X-ray Absorption Spectroscopy. The Cu_A site forms the Cu_2S_2 core with a mixed-valence Cu(1.5)−Cu(1.5) center, and so far, the Cu^{2+}−Cu^{2+} state in the Cu_A site has not been reported for either a native or engineered system.[39] To examine the oxidization state of the copper ions in the bi-AM2C−copper complex, we compared the XANES spectra of the bi-AM2C−copper complex and authentic Cu^{2+}−Cu^{2+} compounds, such as $Cu_2(CH_3COO)_4$ (Cu−Cu distance: 2.64 Å) and a sulfur-bridged dimer, $[Cu_2(S_2)(Bn_3TACH)](SbF_6)$ (Cu−Cu distance: 3.92 Å).[22] The peaks of the bi-AM2C−copper complex in the first-derivative spectra appeared at a lower energy than those of the authentic Cu^{2+}−Cu^{2+} compounds (Figure 7a, inset). This result indicated that the copper ions in bi-AM2C were in more reduced states than those in the oxygen- and sulfur-bridged copper, where the copper ions were present as Cu^{2+}. The Cu(1)Cu(1) center is colorless, and therefore, considering the appearance of the purple color and the results from the EPR spectral analyses, it is reasonable to assume the formation of the mixed-valence Cu(1.5)−Cu(1.5) center in the Cu_2S_2 core of bi-AM2C, although excess Cu^{2+} was present in the protein solution. In another example of purple copper site creation, the addition of Cu^{2+} ion to the protein solution resulted in the formation of blue copper; however, after a prolonged incubation, the color of the solution turned from blue to purple.[40] In our cases, this phenomenon might happen immediately, and would be caused by the reduction of Cu^{2+} to Cu^{+}.

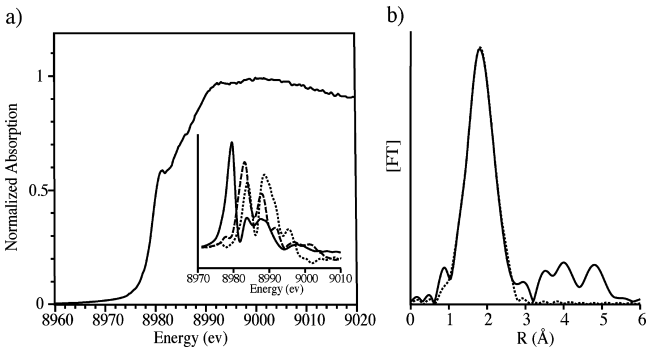

Figure 7. (a) The normalized Cu K-edge XANES spectra of the bi-AM2C−$(Cu)_2$ complex. Inset: the first derivatives of Cu K-edge absorption spectra of the bi-AM2C−$(Cu)_2$ complex (solid line), $Cu_2(OAc)_4$ (dotted line), and $[Cu_2(S_2)BnTACH]^+$ (broken line). (b) FT of EXAFS of the bi-AM2C−$(Cu)_2$ complex. The experimental and best-fitting curves are indicated by the black and dotted lines, respectively.

The Cu_A site forms the Cu_2S_2 core, with Cu−S distances of 2.2−2.3 Å and a Cu−Cu distance of 2.4−2.6 Å.[4] These distances within the core are one of the structural characteristics of the site. The Fourier transformed EXAFS of the bi-AM2C−$(Cu)_2$ complex in Figure 7b shows a single peak at a phase-shifted distance $R' \sim 1.8$ Å. The peak is produced by scattering from the shell of one of the N(His) atoms at 1.90 Å, the two S(Cys) atoms at 2.21 Å, and one Cu atom at 2.51 Å (Table 1), in excellent agreement with reported Cu_A site structures. These results, together with the other spectroscopic data presented in this paper, provide evidence that the purple copper site in bi-AM2C is electronically and geometrically identical to those in native or engineered proteins.

Table 1. EXAFS Analysis of the bi-AM2C−$(Cu)_2$ Complex[a]

	N^b	r (Å)	σ^2 (Å^2)	ΔE_0 (eV)
Cu−N(His)	1	1.90	0.0021	7.8
Cu−S(Cys)	2	2.21	0.0035	9.2
Cu−Cu	1	2.51	0.0039	0.2

[a]The coordination numbers (N), distances (r), Debye−Waller factors (σ^2), and energy shifts (ΔE_0) are shown. The residuals of the fit for the first peak, which was defined as $\sum\{\varphi_{exp} - \varphi_{theo}\}/\sum\{\varphi_{exp}\} \times 100$, were 4.3. [b]Fixed parameters for the curve-fitting procedure.

Modeling the Structure of the bi-AM2C−$(Cu)_2$ Complex. We investigated the coordination geometry of the active site in the model of the bi-AM2C−$(Cu)_2$ complex, using molecular mechanics simulations. There are four His residues present in bi-AM2C. We performed the mutagenesis studies, and it revealed the importance of the two His residues at 13 and 81 (Supporting Information Figure S5). Since no crystal structure of bi-AM2C has been reported, the model of the bi-AM2C−$(Cu)_2$ complex was constructed and minimized by the conjugate gradient method. Overall, in spite of the distance restraints using the EXAFS data, there were few changes in the backbone structure between the minimized structures and the starting structure because the backbone root-mean-square deviation (RMSDs) was 1.15 Å.

The active site of the bi-AM2C−$(Cu)_2$ complex is located in the vicinity of the second α-helix of the coiled coil, as shown in Figure 8a. The active site contains copper ions bridged with the deprotonated cysteinyl groups of Cys47 and Cys50. The copper ions and cysteinyl sulfur atoms form an almost planar Cu_2S_2 core with a dihedral angle of 163.2°, as shown in Figure 8b. The dihedral angle of the Cu_2S_2 core of the bi-AM2C−Cu^{2+} complex is close to those of the nitrous oxide reductase from *Paracoccus denitrificans* (164.0°−165.9°). The copper ions are strongly and equatorially coordinated with two His residues (His13 and His81) (Figure 8c). The peptide carbonyl groups of Glu46 and Cys47 reside within 2.7 Å from the copper ion and might axially interact with it. His16 and His84 are located over the Cu_2S_2 core, at distances of 4.07 and 2.70 Å from the copper ions, respectively, implying that these His residues might also form axial interactions with the copper ions. However, an X-ray crystallographic study is required to determine the precise interactions. The two His residues, two Cys residues and the axial Met residue are reportedly the major ligands for the two copper ions in the Cu_A site of COX. On the other hand, the axial ligands of the Cu_A protein do not significantly affect the physical properties.[41] Our work suggested that the Met residue is not essential to form the purple copper Cu_A site.

CONCLUSION

On the basis of the blue copper site described previously, we successfully created the purple copper Cu_A site within a *de novo* scaffold coiled-coil protein, where the positioning of the Cys residues was the key for Cu_A site formation. We expect that bi-AM2C will be a fascinating target for studying the correlations between the coordination structure, the spectra, and the redox

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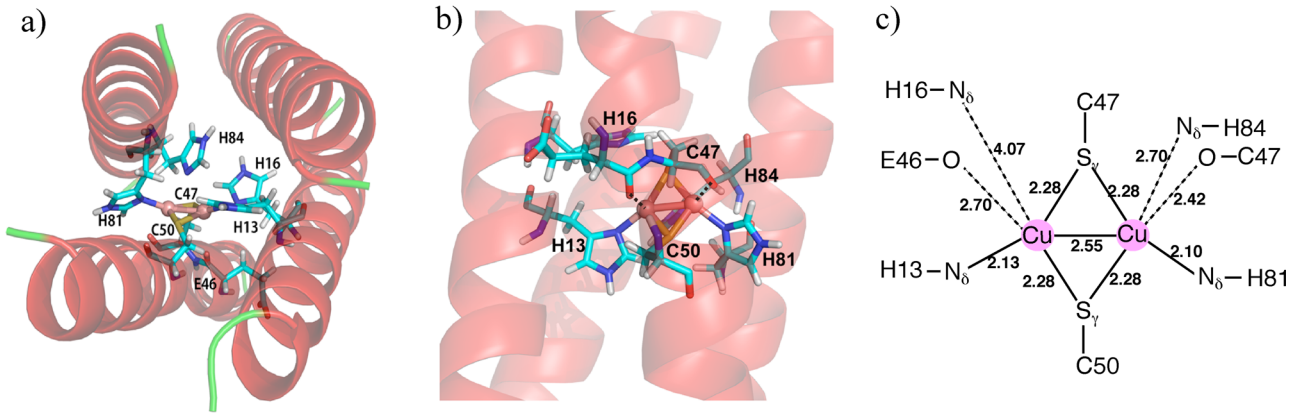

Figure 8. (a) Bottom view of the minimized model structure of the bi-AM2C−$(Cu)_2$ complex. The pink sphere indicates the copper ion. (b) Side view of the active site of the minimized model structure of the bi-AM2C−$(Cu)_2$ complex. (c) Schematic view of the active site of the minimized model structure of the bi-AM2C−$(Cu)_2$ complex. The putative axial interactions are indicated by the dotted lines. Bond lengths are shown in angstroms.

behavior, through mutagenesis analyses of bi-AM2C. In COX, the electron is first trapped in subunit II, containing the purple copper Cu_A site, and then transferred to subunit I containing the heme Fe_B−Cu_B through heme−Fe_A. The tetrameric coiled-coil domain is a suitable scaffold for the introduction of heme compounds in the hydrophobic core.[42,43] In the future, it might be possible to construct a model of subunit I with the heme−Fe_B−Cu_B site in the coiled-coil scaffold. The protein is stable and structurally simple, and both the original version and its mutants can be obtained by genetic approaches; hence, it is suitable for such studies. Our ultimate target is to create the minimal soluble model for COX and to elucidate the reduction mechanism of the four electrons of oxygen to water.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

DNA and amino acid sequences of bi-AM2C, ESI-TOF-MS, UV−vis spectral analysis, time course of the color decay, and mutagenesis studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

C(*s*), sedimentation coefficient; COX, cytochrome *c* oxidase; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HPLC, high-performance liquid chromatography; LMCT, metal charge transfer; N2OR, nitrous oxide reductase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SV, sedimentation velocity; TCEP (tris[2-carboxyethyl]-phosphine hydrochloride); Tris, tris(hydroxymethyl)-aminomethane; XANES, X-ray absorption near-edge structure.

■ REFERENCES

(1) Pomowski, A., Zumft, W. G., Kroneck, P. M. H., and Einsle, O. (2011) N_2O binding at a [4Cu:2S] copper-sulphur cluster in nitrous oxide reductase. *Nature 477*, 234−237.

(2) Tsukihara, T., Aoyama, H., Yamashita, E., Tomigaki, T., Yamaguchi, H., Sinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å. *Science 269*, 1069−1074.

(3) Wilmanns, M., Lappalaomem, P., Kelly, M., Sauer-Eriksson, E., and Saraste, M. (1995) Crystal structure of the membrane-exposed domain from a respiratory quinol oxidase complex with an engineered dinuclear copper center. *Proc. Natl. Acad. Sci. U. S. A. 92*, 11955−11959.

(4) Robinson, H., Ang, M. C., Gao, Y.-G., Hay, M. T., Lu, Y., and Wang, A. H.-J. (1999) Structual Basis of Electron transfer Modulation in the Purple Cu_A Center. *Biochemistry 38*, 5677−5683.

(5) Neese, F., Zumft, W. G., Antoholine, W. E., and Kroneck, P. M. H. (1996) The Purple Mixed-Valence Cu_A Center in Nitrous-oxide reductase: EPR of the Copper-63-, Copper-65-, and Both Copper-65- and [^{15}N]Histidine-Enriched Enzyme and a Molecular Orbital Interpretation. *J. Am. Chem. Soc. 118*, 8692−8699.

(6) Fee, J. A., Sanders, D., Slutter, C. E., Doan, P. E., Aasa, R., Karpefors, M., and Vanngard, T. (1995) Multi-frequency EPR Evidence for a Binuclear Cu_A Center in Cytochrome c Oxidase: Studies with a ^{63}Cu- and ^{65}Cu-Enriched, Soluble Domain Subunit II from *Thermus thermophilus*. *Biochem. Biophys. Res. Commun. 212*, 77−83.

(7) Farrar, J. A., Neese, F., Lappalainen, P., Kroneck, P. M. H., Saraste, M., Zumft, W. G., and Thomson, A. J. (1996) The Electronic Structure of Cu_A: A Novel Mixed-Valance Dinuclear Copper Electron-Transfer Center. *J. Am. Chem. Soc. 118*, 11501−11514.

(8) Blackburn, N. J., Barr, M. E., Woodfuff, W. H., van der Oost, J., and de Vries, S. (1994) Metal-Metal Bonding in Biology: EXAFS Evidence for a 2.5 Å. Copper-Copper Bond in the Cu_A Center of Cytochrome Oxidase. *Biochemistry 33*, 10401−10407.

(9) Williams, K. R., Gamelin, D. R., LaCroix, L. B., Houser, R. P., Tolman, W. B., Mulder, T. C., de Vries, S., Hedman, B., Hodgson, K., O, and Solomon, E. I. (1997) Influence of Copper-Sulfur Covalency and Copper-Copper Bonding on Valance Delocalization and Electron Transfer in the Cu_A site of Cytochrome *c* Oxidase. *J. Am. Chem. Soc. 119*, 613−614.

(10) Blackburn, N. J., de Vries, S., Barr, M. E., Houser, R. P., Tolman, W. B., Sanders, D., and Fee, J. (1997) X-ray Absorption Studies on the Mixed-Valence and Fully Reduced Forms of the Soluble Cu_A Domains of Cytochrome *c* Oxidase. *J. Am. Chem. Soc. 119*, 6135−6143.
(11) Slutter, C. E., Sanders, D., Wittung, P., Malmström, B. G., Aasa, R., Richards, J. H., Gray, H. B., and Fee, J. A. (1996) Water-Soluble, Recombinant Cu_A-Domain of the Cytochrome ba_3 Subunit II from *Thermus thermophilus*. *Biochemistry 35*, 3387−3395.
(12) Farrar, J. A., Lappalainen, P., Zumft, W. G., Saraste, M., and Thomson, A. J. (1995) Magnetic and optical properties of copper-substituted alcohol dehydrogenase: a bisthiolate copper (II) complex. *Eur. J. Biochem. 232*, 294−303.
(13) Riester, J., Zumft, W. G., and Kroneck, P. M. H. (1989) Nitrous oxide reductase from *Pseudomonas stutzeri*. Redox properties and spectroscopic charactrization of different forms of the multicopper enzyme. *Eur. J. Biochem. 178*, 751−762.
(14) Gamelin, D. R., Randall, D. W., Hay, M. T., Houser, R. P., Mulder, T. C., Canters, G. W., de Vries, S., Tolman, W. B., Lu, Y., and Solomon, E. I. (1998) Spectroscopy of Mixed-Valence Cu_A-Type Centers: Ligand-Field Control of Ground-State Properties Related to Electron Transfer. *J. Am. Chem. Soc. 120*, 5246−5263.
(15) Rasmussen, T., Berks, B. C., Sanders-Loehr, J., Dooley, D. M., Zumft, W. G., and Thomson, A. J. (2000) The Catalytic Center in Nitrous reductase, CuZ, Is a Copper-Sulfide Cluster. *Biochemistry 39*, 12753−12756.
(16) Lu, Y., Berry, S. M., and Pfister, T. D. (2001) Engineering Novel Metalloproteins: Design of Metal-Binding Sites into Native Protein Scaffolds. *Chem. Rev. 101*, 3047−3080.
(17) DeGrado, W. F., and Summa, C. M. (1999) de Novo Design and Structural Characterization of Proteins and Metalloproteins. *Annu. Rev. Biochem. 68*, 779−819.
(18) van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R. B., Aasa, R., Pascher, T., and Malmström, B. G. (1992) Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the E. coli cytochrome o quinol oxidase complex. *EMBO J. 11*, 3209−3217.
(19) Hay, M., Richards, J. H., and Lu, Y. (1996) Construction and characterization of an azurin analog for the purple copper site in cytochrome *c* oxidase. *Proc. Natl. Acad. Sci. U. S. A. 93*, 461−464.
(20) Dennison, C., Vijgenboom, E., de Vries, S., van der Oost, J., and Canters, G. W. (1995) Introduction of a Cu_A site into the blue copper protein amicyanin from *thiobacillus versutus*. *FEBS Lett. 365*, 92−94.
(21) Shiga, D., Nakane, D., Inomata, T., Funahashi, Y., Masuda, H., Kikuchi, A., Oda, M., Noda, M., Uchiyama, S., Fukui, K., Kanaori, K., Tajima, K., Takano, Y., Nakamura, H., and Tanaka, T. (2010) Creation of a Type 1 Blue Copper Site within a de Novo Coiled-Coil Protein Scaffold. *J. Am. Chem. Soc. 132*, 18191−18198.
(22) Kajita, Y., Matasumoto, J., Takahashi, I., Hirota, S., Funahashi, Y., Ozawa, T., and Masuda, H. (2008) Syntheses, Characterization, and Reactivities of (m-h2:h2-Disulfido)dicopper(II) Complexes with *N*-Alkylated *cis*,*cis*-1,3,5-Triaminocyclohexane Derivatives. *Eur. J. Inorg. Chem.*, 3977−3986.
(23) Ressler, T. (1998) WinXAS: A XAS Data Analysis Program under MS Windows. *J. Synchrotron Radiat. 5*, 118−122.
(24) Blackburn, N. J., de Vries, S., Barr, M. E., Houser, R. P., Tolman, W. B., Sanders, D., and Fee, J. A. (1997) X-ray Absorption Studies on the Mixed-Valence and Fully Reduced Forms of the Soluble Cu_A Domains of Cytochrome *c* Oxidase. *J. Am. Chem. Soc. 119*, 6135−6143.
(25) Ankudinov, A. L., Ravel, B., Rehr, J. J., and Conradson, S. D. (1998) Real-space multiple-scattering calculation and interpretation of x-ray-absorption near-edge structure. *Phys. Rev. B 58*, 7565−7576.
(26) Guex, N., and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis 18*, 2714−2723.
(27) Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993) A Switch between Two-, Three-, and Four-Stranded Coiled Coils in GCN4 Leucine Zipper Mutants. *Science 262*, 1401−1407.
(28) Harbury, P. B., Kim, P. S., and Alber, T. (1994) Crystal structure of an isoleucine-zipper trimer. *Nature 371*, 80−83.
(29) Mittl, P. R. E., Deillon, C., Sargent, D., Liu, N., Klauser, S., Thomas, R. M., Gutte, B., and Grütter, M. G. (2000) The *retro*-GCN4 leucine zipper sequence forms a stable three-dimension structure. *Proc. Natl. Acad. Sci. U. S. A. 97*, 2562−2566.
(30) Canutescu, A. A., Shelenkov, A. A., and Dunbrack, R. L., Jr. (2003) A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci. 12*, 2001−2014.
(31) Fukunishi, Y., Mikami, Y., and Nakamura, H. (2003) The filling potential method: A method for estimating the free energy surface for protein-ligand docking. *J. Phys. Chem. B 107*, 13201−13210.
(32) Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for modelling water. *J. Chem. Phys. 79*, 926−935.
(33) Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Jr., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J. Am. Chem. Soc. 117*, 5179−5197.
(34) Kollman, P., Dixon, R., Cornell, W., Fox, T., Chipot, C., and Pohorille, A. (1997) The development/application of a 'minimalist' organic/biochemical molecular mechanic force field using a combination of ab initio calculations and experimental data, in *Computer Simulation of Biomolecular Systems* (van Gunsteren, W. F., Weiner, P. K., and Wilkinson, A. J., Eds.) Vol. 3, pp 83−96, Kluwer Academic Publishers, Dordrecht.
(35) Shiga, D., Nakane, D., Inomata, T., Masuda, H., Oda, M., Noda, M., Uchiyama, S., Fukui, K., Takano, Y., Nakamura, H., Mizuno, T., and Tanaka, T. (2009) The Effect of the Side Chain length of asp and Glu on Coordination Structure of Cu^{2+} in a *de novo* Designed Protein. *Biopolymers 91*, 907−916.
(36) Sakurai, Y., Mizuno, T., Hiroaki, H., Oku, J., and Tanaka, T. (2005) Optimization of aromatic side chain size complementarity in the hydrophobic core of a designed coiled-coil. *J. Peptide Res. 66*, 387−394.
(37) Malmström, B. G., and Aasa, R. (1993) The nature of the Cu_A center in cytochrome *c* oxidase. *FEBS Lett. 325*, 49−52.
(38) Antholine, W. E., Kastrau, D. H. K., Steffens, G. C. M., Buse, G., Zumft, W. G., and Kroneck, P. M. H. (1992) A comparative EPR investigation of the multicopper proteins nitrous oxide reductase and cytochrome c oxidase. *Eur. J. Biochem. 209*, 875−881.
(39) Savelieff, M. G., and Lu, Y. (2010) Cu_A centers and their biosynthetic models in azurin. *J. Biol. Inorg. Chem. 15*, 461−483.
(40) Savelieff, M. G., Wilson, T. D., Elias, Y., Nilges, M., Garner, D. K., and Lu, Y. (2008) Experimental evidence for a link among cupredoxins: Red, blue, and purple copper transformations in nitrous oxide reductase. *Proc. Natl. Acad. Sci. U. S. A. 105*, 7919−7924.
(41) Hwang, H. J., Berry, S. M., Nilges, M. J., and Lu, Y. (2005) Axial Methionine Has Much Less Influence on Reduction Potentials in a Cu_A Center than in a Blue Copper Center. *J. Am. Chem. Soc. 127*, 7274−7275.
(42) Rabanal, F., DeGrado, W. F., and Dutton, P. L. (1996) Toward the Synthesis of a Photosynthetic Reaction Center Maquette: A Cofacial Porphyrin Pair Assembled between Two Subunits of a Synthetic Four-Helix Bundle Multiheme Protein. *J. Am. Chem. Soc. 118*, 473−474.
(43) Cochran, F. V., Wu, S. P., Wang, W., Nanda, V., Saven, J. G., Therien, M. J., and DeGrado, W. F. (2005) Computational De Novo Design and Characterization of a Four-Helix Bundle Protein that Selectively Binds a Nonbiological Cofactor. *J. Am. Chem. Soc. 127*, 1346−1347.