Preparation and Characterization of Mercury and Silver Derivatives of an Engineered Purple Copper Center in Azurin

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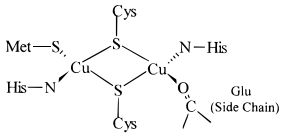
Understanding the structure and function of purple copper centers in proteins have been the focus of recent studies.[1,2] Purple copper centers, found in cytochrome *c* oxidase (COX)[3] and nitrous oxide reductase,[4] are a new class of copper centers in biology. They all display an intense purple color due to strong absorptions around 480, 530, and 800 nm,[2] a typical EPR spectrum indicative of a mixed-valence binuclear copper center,[5] and a characteristic resonance Raman Cu−S stretching frequency around 340 cm^{-1}.[6] Recent X-ray structural studies of two COX[7] and one engineered cytochrome *o* quinol oxidase[8] have shown that the purple copper center is a dithiolate-bridged binuclear copper center where both coppers are in distorted tetrahedral sites.



Cu_A

While the structure of the purple copper center in COX is now known, many questions related to the structure and function of the center, such as metal-binding properties, rigidity, and electronic structure of the center, still remain to be understood. Metal ion substitution has contributed greatly to the understanding of the structural properties of metalloproteins,[9] particularly copper thiolate proteins such as blue copper proteins,[10] ACE1 protein,[11] and metallothionein.[12] Studies by UV electronic absorption,[13] X-ray absorption,[14] ^{199}Hg NMR spectroscopy,[15] and X-ray crystallography[16] have shown that Hg(II) is an effective probe of Cu(II) in copper thiolate centers such as blue copper proteins and metallothioneins. Ag(I) has also been used to mimic Cu(I) in proteins such as superoxide dismutase[17] and metallothionein.[18] Therefore, we prepared mercury and silver derivatives of a purple copper center that had been engineered in *Pseudomonas aeruginosa* azurin by our group.[2g] Electronic absorption (UV-vis) and electrospray mass spectrometry (ES-MS) characterizations show that a Hg(II)Ag(I) derivative of the purple copper center can be prepared by a sequential addition of Hg^{2+} and Ag^+ ions to either holo or apo proteins.

The holoprotein of the engineered Cu_A azurin construct was prepared and purified as previously reported,[2g] with a few modifications.[19] Titration of $HgCl_2$ into holoprotein of the purple copper construct[20] resulted in a quench of the visible spectrum, at approximately 1 equiv of Hg^{2+} (Figure 1, insert). At the same time, increases in absorptions around 250 and 290 nm, typical of S-to-Hg(II) charge transfer,[13] were observed (Figure 1Ab). The ES-MS spectrum shows that Hg(II) has replaced one of the coppers in the binuclear center (Figure 1Bb).[21] The strong absorptions around 485 and 530 nm have been assigned as S-to-Cu(II) charge transfers.[6] The complete quench of these absorptions by addition of Hg(II) indicates that

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(19) $CuSO_4$ was titrated into the apoprotein slowly until saturation at 485 nm occurs. The protein was then purified through a Resource Q column (Pharmacia, NJ) on a BioCad Sprint system (PerSeptive Biosystem, MA).

(20) The metal stock solution was freshly prepared in 50 mM ammonium acetate buffer (pH 5.1). Metal ions were slowly titrated into the protein solution. When $AgNO_3$ was used, caution was taken to avoid exposure to light. Once the reaction is complete, excess metal ions were removed by passage through a PD-10 gel filtration column (Pharmacia, NJ). The fractions containing protein, determined by UV, were concentrated in a Centricon-10 (Amicon, MA) and subjected to ES-MS study. The protein concentration was estimated based on $\epsilon_{480} = 4100\ M^{-1}\ cm^{-1}$ for holoproteins and $\epsilon_{280} = 8440\ M^{-1}\ cm^{-1}$ for apoproteins.

(21) The ES-MS data were acquired in the continuum mode using a Quattro mass spectrometer capable of unit mass resolution to 4000 molecular weight (50% valley definition). The mass scale was calibrated with CsI. The electrospray ionization was performed with a 100% water flow system at 15 μL/min into the ion source, and the sample was in ammonium acetate (pH 5.1) buffer. Multiple charged ions (typically 6, 7, and 8 positive-charged species) were observed under the conditions employed. The multiple charged data were then transformed into the singly charged spectra presented in the paper.

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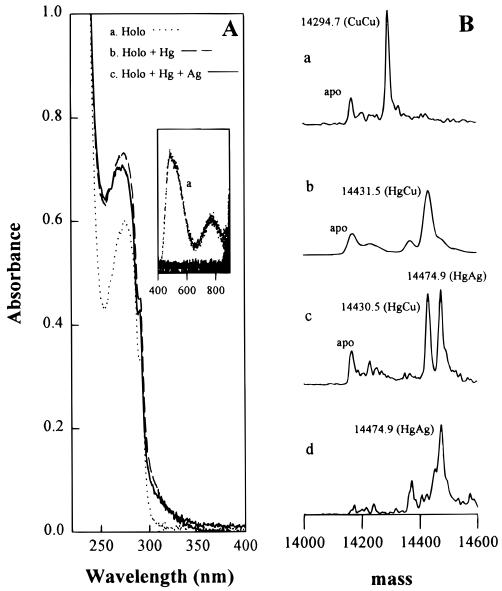

Figure 1. Addition of metal ions to holo-Cu_A azurin construct. (A) Electronic absorption spectra: (a) holo-Cu_A azurin construct (···), (b) holo-Cu_A + 1 equiv of Hg^{2+} (- - -), (c) holo-Cu_A + 1 equiv of Hg^{2+} + 1 equiv of Ag^+ (—). Insert shows spectra in the visible region. (B) ES-MS spectra: (a) holo-Cu_A azurin construct, (b) holo-Cu_A + 1 equiv of Hg^{2+}, (c) holo-Cu_A + 1 equiv of Hg^{2+} + 1 equiv of Ag^+, (d) holo-Cu_A + 1 equiv of Hg^{2+} + 10 equiv of Ag^+.

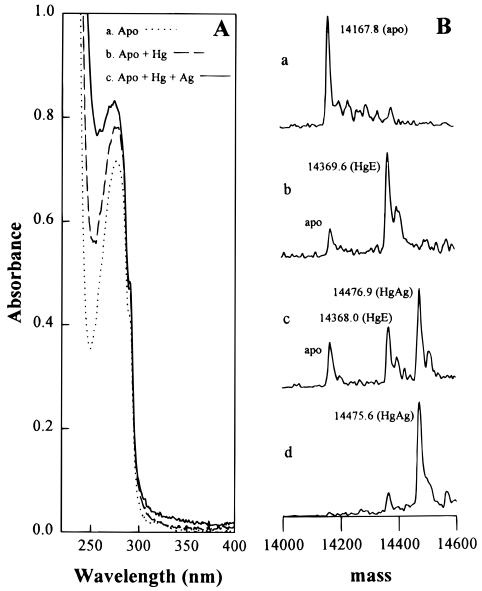

Figure 2. Addition of metal ions to apo-Cu_A azurin construct. (A) Electronic absorption spectra: (a) apo-Cu_A azurin construct (···), (b) apo-Cu_A + 1 equiv of Hg^{2+} (- - -), (c) apo-Cu_A + 1 equiv of Hg^{2+} + 1 equiv of Ag^+ (—). (B) ES-MS spectra: (a) apo-Cu_A azurin construct, (b) apo-Cu_A + 1 equiv of Hg^{2+}, (c) apo-Cu_A + 1 equiv of Hg^{2+} + 1 equiv of Ag^+, (d) apo-Cu_A + 10 equiv of Hg^{2+} + ~1 equiv of Ag^+.

the Cu(II) was replaced first, rather than the Cu(I). Therefore, we assign the product to be the Hg(II)Cu(I) derivative.[22] When this derivative is further treated with 1 equiv of $AgNO_3$, a new species with a molecular weight consistent with a Hg(II)Ag(I) derivative was observed by ES-MS, in addition to the Hg(II)Cu(I) derivative (Figure 1Bc), indicating that Ag(I) is capable of replacing the Cu(I) in the Hg(II)Cu(I) derivative. Addition of 10 equiv of $AgNO_3$ can replace all the Cu(I) in the Hg(II)Cu(I) derivative (Figure 1Bd).

When the apoprotein of the purple copper construct is reacted with 1 equiv of $HgCl_2$, strong absorptions in the UV region were also observed (Figure 2Ab).[23] The ES-MS spectrum (Figure 2Bb) shows that this species is the single mercury adduct, resulting in a Hg(II)E derivative. Addition of one equiv of $AgNO_3$ to Hg(II)E resulted in an ES-MS spectrum (Figure 2Bc) consisting of both Hg(II)E and Hg(II)Ag(I) derivatives. In a separate experiment, 10 equiv of $HgCl_2$ were added to the apoprotein.[20] Both Hg(II)E and Hg(II)Hg(II) were observed by ES-MS and both can be converted to Hg(II)Ag(I) by addition of $AgNO_3$ (Figure 2Bd). These results suggest that, although Hg(II) is capable of binding to both sites in the purple copper center, it binds to one site more strongly, and the Hg(II) in the weak-binding site can be replaced by Ag(I). This finding is consistent with the results presented in Figures 1 and 2: Hg(II) binds to the Cu(II) site more strongly than the Cu(I) site and Ag(I) can replace both Hg(II) and Cu(I) in the Cu(I) site when Hg(II) is present in the Cu(II) site.[24]

In conclusion, we have demonstrated that Hg(II) can replace the Cu(II) of the purple copper center preferentially and the Hg(II)Ag(I) derivative of the purple copper center can be prepared by a sequential addition of Hg^{2+} and Ag^+ ions to either holo- or apoproteins. The observation of the different preference of Hg(II) and Ag(I) suggests that the two copper sites in this purple copper protein may not be equivalent with respect to metal substitution, even though it has been shown that both sites are spectroscopically equivalent. This is not surprising because no spectral evidence attributable to Cu(II)Cu(II) derivative has been observed after addition of Cu^{2+}.[2] The mixed-valent Cu(II)Cu(I) center can be obtained only after Cu^+ is formed during the titration, presumably because of reduction of Cu^{2+} either by externally-added reductants such as ascorbate or by the cysteines in the apoproteins, some of which serve as a sacrificial reductant.[2g] The different preference of metal ion substitution of the two sites could be due to their different ligand environments. The site containing Met could be the putative binding site for Hg(II) because the thioether is softer than the carbonyl oxygen of Gly in the other site.[25] It is not known if our Hg(II)Ag(I) derivative mimics the intermediate or the final state of Cu(II)/Cu(I) binding to the purple copper center. After Cu(II) and Cu(I) bind to their respective sites, similar to our Hg(II)Ag(I) derivative, a local structural change may or may not be necessary to achieve the spectroscopic equivalency. Further characterization of the above derivatives by ^{199}Hg NMR spectroscopy, X-ray absorption, and X-ray crystallography are in progress. Comparison of these results with those of the Cu(II)Cu(I) derivative will contribute to our understanding of the metal-binding affinity, rigidity, and other structural properties of this new class of copper centers.

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(22) In this paper, we use MM′ to represent different metal derivatives in this binuclear copper center, where M is in the putative Cu(II) site, M′ is in the putative Cu(I) site, and an E represents an empty site.

(23) The absorption bands are similar to those observed in the Hg(II) titration into the holoprotein (Figure 1Ab), but the peaks are less well-defined as revealed by difference spectra (data not shown).

(24) Preliminary results show that the Hg(II)Ag(I) derivative can also be obtained by addition of Ag^+ first, followed by Hg^{2+} to either the holo- or apoproteins. However, Ag^+ of more than 3 equiv has to be used in these experiments. If only 1 equiv Ag^+ is titrated into the holoprotein, (2Ag(I))Cu(I) was detected by ES-MS and UV-vis, indicating two Ag(I) have replaced one Cu(II) in the purple copper center. The two Ag(I) in the Cu(II) site can be replaced by Hg(I) by addition of 1 equiv of Hg^{2+}, resulting in a Hg(II)Cu(I) derivative. Addition of 1 equiv of Ag^+ to apoprotein resulted in mainly a (2Ag(I))Ag(I) derivative. The Hg(II)Ag(I) derivative can be obtained after further addition of 1 equiv of Hg^{2+}. Therefore, the metal affinity for the putative Cu(II) site is in the order of Hg(II) > 2Ag(I) > Cu(II) and the metal affinity for the putative Cu(I) site is Ag(I) > Cu(I) > Hg(II).

(25) An alternative explanation can rationalize the observation that Hg(II) replaces Cu(II) preferentially and Hg(II)Ag(I) is the most stable derivative; the purple copper center is built so that a net +4-charged M(II)M(II) in the binding site is less favored than the +3-charged M(II)M(I) derivative.