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Palladium(II) and Platinum(II) Bind Strongly to an Engineered Blue Copper Protein

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Supporting Information

ABSTRACT: Studies of palladium(II) and platinum(II) binding to well-characterized proteins contribute to understanding the influence of these metals in the environment and body. The well-characterized apoprotein of azurin has a soft-metal binding site that may be exposed to solvent by mutation of a coordinating His-117 residue to glycine (H117G). Palladium(II) and platinum(II) form strong 1:1 adducts with the apo form of H117G azurin. A combination of UV−vis, circular dichroism, and inductively coupled plasma mass spectrometry techniques suggests that the metal binds specifically at His-46 and Cys-112 of the protein.

Platinum-group elements (PGEs) play an integral role in modern chemistry, catalysis, and chemotherapeutic treatments. Because of their heavy use, they have entered our environment, particularly from leaching of catalyst from catalytic converters in automobiles.[1,2] Catalytic converters emit PGEs in inhalable and commutable particle sizes (0.1−20 nm) that are rapidly complexed into soluble and mobile species after deposition.[3−5] As a result, PGEs are highly bioavailable and known to bioaccumulate in plant and aquatic life.[6,7] Human exposure to PGEs is an increasing concern, especially following treatment with platinum for battling cancer.[8−10] Exposure to cisplatin and its derivatives leads to highly elevated and persistent *in vivo* exposure to bioreactive platinum.[11] Thus, it is important to understand the chemistry underlying PGE interactions with biomolecules.

Though there has been progress on understanding the interactions of these metals with nucleic acids,[12] there remains a need for information on the binding of Pd^{2+} and Pt^{2+} to proteins. Interactions of Pd^{2+} and Pt^{2+} with proteins have been studied, but the precise location of metal binding is rarely known.[13−22] There is also interest in Pd^{2+}−protein adducts as potential scaffolds for catalysis[23−25] including hydrolytic cleavage of peptides.[26−31] Pd^{2+} salts also influence amyloid fibril formation.[32,33]

This contribution describes studies utilizing the apoprotein of azurin, a type 1 copper metalloprotein with a characteristic deep-blue color. The canonical copper binding site of *Pseudomonas aeruginosa* (*Pa*) azurin consists of four amino acid residues: His-46, Cys-112, His-117, and Met-121 (Figure 1a).[34] *Pa* azurin has been used extensively in the study of protein−metal interactions, where it consistently provides a mononuclear binding site for Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Au^{+}, Ag^{+}, and Hg^{2+}.[35−43] Another advantage of azurin is that, as a small protein that is amenable to expression on a large scale from recombinant *Escherischia coli*, it may easily be mutated to modify the coordination environment of copper[44−47] or other metals[48,49] in its metal binding site. Among mutations to the primary coordination sphere of the copper site, the H117G (His-117 residue to glycine) mutation is special because it is a small change that allows significant solvent access to the metal, enabling exogenous ligands to bind to copper(II) in place of the missing His-117 residue.[46,50,51] For example, the addition of copper(II) and an excess of *N*-methylimidazole to the apo form of H117G *apo*-azurin results in a characteristic 630 nm absorbance band and a coordination environment similar to that of the wild-type protein.[50] Figure 1b illustrates the binding of *N*-methylimidazole to the H117G copper(II) azurin. We anticipated that the compiled knowledge of H117G azurin would offer a way to unambiguously characterize Pd^{2+} and Pt^{2+} binding to native protein residues.

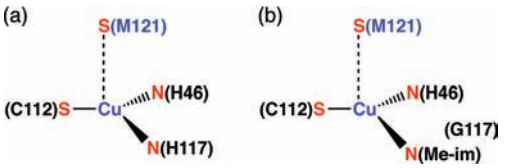

Figure 1. Copper(II) sites of (a) azurin and (b) H117G azurin with added *N*-methylimidazole (Me-im).

Upon the addition of 1 equiv of MCl_4^{2-} to a 0.48 mM solution of the apo form of H117G azurin [buffered at pH 7.0 with 5 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS)], a new broad absorbance developed in the UV−vis spectrum for M = Pd (ε = 2.6 mM^{-1} cm^{-1} at 324 nm) and M = Pt (ε = 0.54 mM^{-1} cm^{-1} at 330 nm). The spectra are shown in the Supporting Information. The addition of these metal salts to wild-type protein does not yield any significant new absorbance, showing that the mutation is essential for PGE binding. The new absorptions in the Pd^{2+} and Pt^{2+} adducts of the H117G *apo*-azurin are reminiscent of those seen in Pd^{2+} and Pt^{2+} complexes with anionic sulfur donors, suggesting coordination to cysteinate.[52,53] The dependence of the absorbance on the added metal concentration (Figure 2) fits

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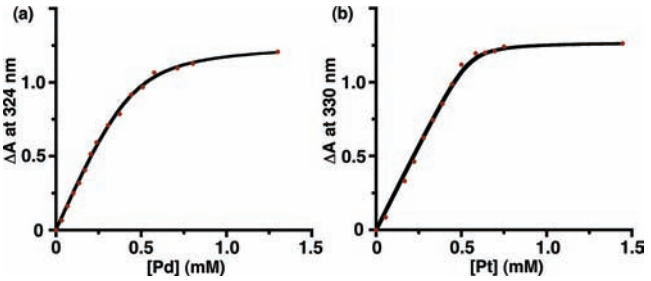


Figure 2. Development of the UV−vis intensity upon the addition of (a) Pd^{2+} and (b) Pt^{2+} to 0.48 mM H117G *apo*-azurin in a 5 mM MOPS buffer (pH = 7.0). The lines represent fits to the binding curves using eq 1.

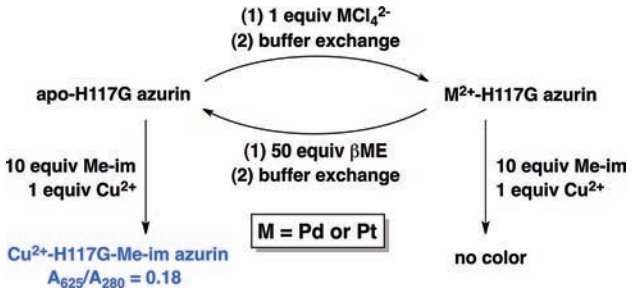
to a standard binding curve (eq 1), yielding association constants of $2.1(5) \times 10^4\ M^{-1}$ for Pd^{2+} and $1.2(7) \times 10^5\ M^{-1}$ for Pt^{2+}. Thus, both PGEs bind strongly, and Pt^{2+} binds roughly 6 times more strongly than Pd^{2+}.

$$[ML] = \left\{\left([M]_0 + [L]_0 + \frac{1}{K_{eq}}\right) - \left\{\left([M]_0 + [L]_0 + \frac{1}{K_{eq}}\right)^2 - 4[L]_0[M]_0\right\}^{1/2}\right\}/2 \quad (1)$$

The development of the new absorbance saturated in each case at approximately 1 equiv of Pd^{2+} or Pt^{2+} relative to protein (Figure 2). Exchanging the buffer after the addition of the metal salt gave no significant change to the UV−vis spectra, indicating low k_{off} rates. After buffer exchange and dilution, the PGE-loaded proteins were digested with acid followed by analysis with inductively coupled plasma mass spectrometry (ICP-MS). The metal analysis indicated that the proteins incorporated 95% Pd^{2+} and 98% Pt^{2+} into the protein. Because there are no alternative metal binding sites known in azurin,[46] and the new UV absorbance is consistent with a thiolate-to-metal charge-transfer transition,[52−54] these results suggest that the heavy metals bind at the copper site with Cys and His donors.

In order to further support the location of metal binding, we took advantage of the fact that Cu^{2+}-bound H117G azurin develops a intense blue color upon binding of *N*-methylimidazole to regenerate the N_2S_2 coordination environment of the wild-type copper(II) protein.[50] This property enabled us to query the status of the copper binding site, as shown in Scheme 1.

Scheme 1. Using the Cu^{2+} Binding Ability of H117G Azurin To Query M^{2+} Binding at the Copper Site



The addition of copper(II) and excess *N*-methylimidazole to the apo form of H117G azurin resulted in the expected copper-based ligand-to-metal charge-transfer absorbance at λ_{max} = 630 nm, with a ratio of absorbances A_{630}/A_{280} = 0.48. In constrast, the addition of Cu^{2+} and *N*-methylimidazole to the Pd^{2+}- or Pt^{2+}-substituted protein did not result in the appearance of the 630 nm absorbance band. The inability of the Pd^{2+}- and Pt^{2+}-substituted proteins to bind copper suggests that the heavy metals bind at the copper site, but does not preclude the possibility that the protein is somehow damaged by the heavy metals in a manner that prevents copper binding. We tested for protein damage by incubating the Pd^{2+}- and Pt^{2+}-loaded proteins in an excess of the soft ligand 2-mercaptoethanol for 12 h to chelate the PGE salt, followed by buffer exchange. After this treatment to remove the heavy metal, the copper binding ability of the protein was completely restored (A_{630}/A_{280} = 0.48). These results indicate that the protein had undergone no irreversible change and provide additional support for Pd^{2+} and Pt^{2+} binding specifically at the His-46/Cys-112 site of the engineered azurin.

To evaluate whether the addition of Pd^{2+} and Pt^{2+} changed the secondary structure of H117G azurin, circular dichroism (CD) experiments were performed after the addition of either Pd^{2+} or Pt^{2+} to the apo form of H117G azurin. The CD spectrum, which matches the literature spectrum for the azurin protein,[55] was unchanged by H117G mutation or by the addition of either metal (Figure 3). These results suggest that

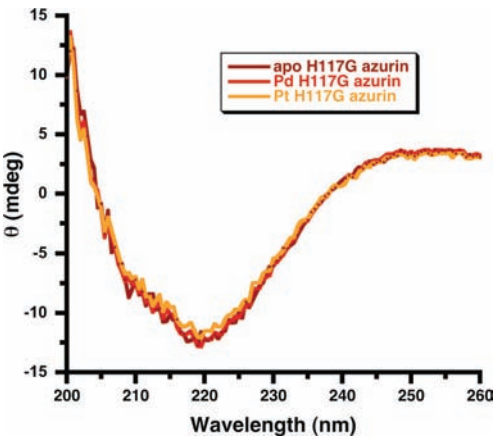


Figure 3. CD spectra of H117G azurin in the apo form and after the addition of Pd^{2+} and Pt^{2+}. Samples have 0.22 mM protein in a 5 mM MOPS buffer (pH = 7.0).

Pd^{2+} and Pt^{2+} binding to the protein does not induce any significant conformational changes. Thus, our experiments clearly point toward PGE binding at the vacant copper binding site of H117G *apo*-azurin.

In conclusion, the copper-binding apoprotein of azurin can be engineered so that it coordinates a single Pd^{2+} or Pt^{2+} ion. Each ion binds with $K_{assoc} > 20\,000\ M^{-1}$, and binding to Pt^{2+} is stronger than that to Pd^{2+}. Our evidence indicates that the metal binds to the cysteine at the vacant copper site, and thus we suggest that the metals have square-planar $NSCl_2$ coordination that includes His-46 and Cys-112 of the protein.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, working conditions, and additional spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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