An Engineered Azurin Variant Containing a Selenocysteine Copper Ligand

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · APRIL 2002

Impact Factor: 12.11 · DOI: 10.1021/ja0169163 · Source: PubMed

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Published on Web 02/16/2002

An Engineered Azurin Variant Containing a Selenocysteine Copper Ligand

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Received August 22, 2001

Modulating the properties of proteins through de novo design or redesign of existing proteins has been a longstanding goal in protein chemistry. Over the past two decades, site-directed mutagenesis has been a powerful tool to probe the role of certain residues and to fine-tune the activity of proteins. A limitation of this approach has been the accessibility of only a restricted number of functional groups through the 20 amino acids in the genetic code. The recent technique of expressed protein ligation (EPL) provides an alternative route that allows efficient incorporation of unnatural residues into proteins.[1] We report here the preparation and characterization of an azurin variant in which a cysteine copper ligand has been replaced with selenocysteine (Sec) by EPL.

Pseudomonas aeruginosa azurin is a member of the family of type 1 blue copper proteins involved in biological electron transfer (ET).[2] Its metal center is coordinated in a pseudotrigonal bipyrimidal geometry by two histidines and a cysteine (Cys112) as equatorial ligands and a methionine and main chain carbonyl in the axial positions (Figure 1).[3] It possesses all the features unique to type 1 blue copper proteins, such as an intense blue color ($\epsilon_{625\ nm} = 5000\ M^{-1}\ cm^{-1}$), and an unusually small parallel EPR hyperfine splitting ($A_{||} \approx 55$ G).[4] The protein has been the subject of extensive investigation by site-directed mutagenesis, spectroscopy, electronic structure calculation, and X-ray crystallography.[4,5] These studies point to the cysteine, specifically the strong covalency of the Cu−Cys bond, as essential in defining the properties of the protein.[6] For example, mutations of the histidines and methionine resulted mostly in variant azurins with spectra still dominated by the strong blue color.[5,7] In contrast, the C112D mutation lacked the spectral features of type 1 copper centers and resulted in a type 2 copper center.[8] Furthermore, this mutation resulted in a dramatically decreased covalency at the active site and reduced ET rates.[8d] In general, only a limited number of site-directed mutagenesis studies have been reported because of the large perturbations caused by substituting Cys112 with one of the other 19 natural amino acids. On the other hand, replacement with Sec may allow minimal structural changes and thereby fine-tuning of its spectroscopic and redox properties. Furthermore, such replacement would provide an opportunity to characterize a mononuclear complex with a single Cu^{2+}−Se bond of which there are few examples in the inorganic literature.[9]

Sec has been incorporated previously into a small number of proteins using methods that are very specific for the proteins involved.[10] We and others have recently reported on the use of native chemical ligation between peptides with a C-terminal thioester and an N-terminal Sec.[11,12] This methodology is expanded here to EPL.[12] A peptide corresponding to the C-terminal 17 residues of azurin was prepared using automated solid-phase peptide synthesis (Scheme 1). The N-terminus of this peptide, corresponding to Cys112 in WT azurin, contained *Se-p*-methoxybenzyl (PMB) selenocysteine and was prepared as reported previously.[11a] A small amount of deselenation occurred during cleavage from the resin, consistent with previous reports.[13] Removal of the PMB protecting group with I_2 under acidic conditions provided a product that showed molecular ions for both the symmetrical diselenide and selenol monomer.[14a] Truncated azurin (minus the 17 C-terminal residues) was expressed as a fusion to the N-terminus of the *Mxe* intein domain.[14b] The expressed protein ligation between the fusion protein and the synthetic peptide was carried out in the presence of mercaptoethane sulfonic acid (MESNA) as mediator.[15] The products of the ligation reaction were analyzed by matrix-assisted laser desorption ionization mass spectrometry (azurin 1−111-MESNA: calcd M_w, 12282; obsd 12281 ± 6; Sec ligation product, Sec112 azurin hereafter: calcd M_w, 13993; obsd 13991 ± 7).

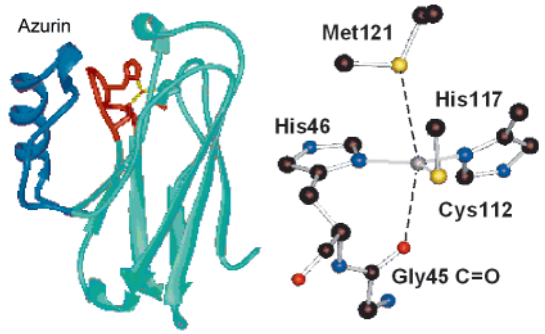
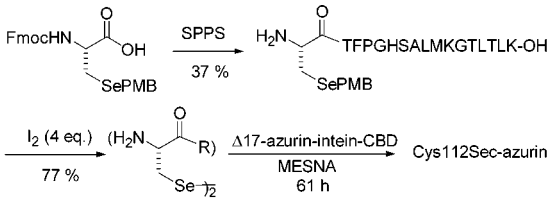


Figure 1. Greek-key β-barrel fold of *P. aeruginosa* azurin (left) and the active site residues (right). (PDB ID: 4Azu).[3a]

Scheme 1



Addition of $CuSO_4$ to ∼0.3 mM Sec112-azurin resulted in a blue solution. The protein was purified by anion-exchange chromatography, and electrospray ionization mass spectrometry indicated a single bound copper (calcd M_w, 14055; obsd, 14053 ± 7). The yield of Sec112-azurin was estimated to be ∼0.4 mg/L of culture. The electronic absorption spectra of Cu(II)-containing wild type (WT) and Sec112 azurin are shown in Figure 2. As reported previously, the visible spectrum of WT azurin is dominated by a strong absorption at ∼628 nm, due to a S(Cys112) 3pπ → Cu(II) $3d_{x^2-y^2}$ charge-transfer transition.[4,6] In addition, weaker absorption bands at 366, 466, and ∼800 nm are observed and have been assigned to Met a_1 and His π_1 to Cu(II) $3d_{x^2-y^2}$ charge-transfer (CT) transitions and Cu(II) d→d transitions, respectively.[4,6] Interestingly, the visible absorption spectrum of the mutant is similar to that of WT azurin, with the absorption bands red-shifted. The most intense transition

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10.1021/ja0169163 CCC: $22.00

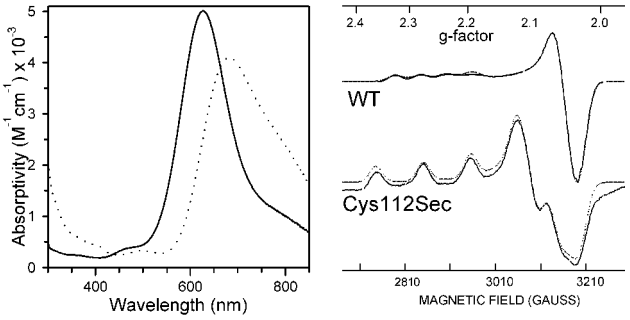

Figure 2. (Left) UV-visible absorption spectra of WT (solid) and Sec112 azurin (dotted) in 50 mM NH_4OAc pH 5.1. (Right) X-band EPR spectra of WT and Sec112 azurin (solid: exptl, dashed: simulated). Instrument settings: frequency 9.08 GHz; power 0.2 mW; modulation amplitude 5 G; temp 20 K.

at 677 nm is likely the Se→Cu(II) CT band, shifted from the S→Cu(II) band at 628 nm. The direction of this shift is consistent with the lower ionization energy of selenium. Other peaks located at 408 and 498 nm are possibly red-shifted Met and His→Cu CT bands, respectively.

The X-band EPR spectrum of Sec112 azurin displays a much larger parallel hyperfine splitting (104 G) than WT (56 G), suggesting a reduced covalency in the Sec112 protein (Figure 2). In addition to the larger $A_{\|}$, simulation[16] of the EPR spectrum gave g-values of 2.234, 2.109, and 2.047 (compared with 2.262, 2.056, and 2.039 for WT azurin). Thus, the site has more rhombic character than WT, emphasizing the difference in ligand field strengths between Se and S. The large increase in g_y, with modest decrease in g_z, is consistent with either an increased interaction with an axial ligand, or a decreased Cu−Se bond strength, because selenolate, being softer than thiolate, does not interact as favorably with Cu(II), a borderline metal ion in terms of hardness.

The potentials of WT and Sec112 azurin are surprisingly similar (328 ± 3 mV and 316 ± 2 mV vs NHE, respectively)[17] given the large change in $A_{\|}$, the position of the CT-band, and the expected stabilization of Cu(I) by a selenolate. This modulation of the effect of Se-for-S substitution suggests a compensating change in geometry, consistent with the observed increase in g-anisotropy.

In summary, an azurin variant containing selenocysteine in place of cysteine at the blue copper center has been obtained through expressed protein ligation. This report marks the first time that selenocysteine is artificially incorporated into the active site of a metalloprotein. The variant displays a significantly increased $A_{\|}$ and red-shifted CT band, while maintaining the general type 1 copper characteristics, including similarity in reduction potentials. This study illustrates that isostructural substitution using EPL can fine-tune the structural and functional properties of a metal-binding site without loss of its characteristics. At the same time, even an apparently subtle change like a Cys-to-Sec mutation may induce compensating changes due to other factors at the metal binding site. Further spectroscopic and X-ray crystallographic studies are under way to fully elucidate the effect of Sec on the structure and function of the blue copper center.

Acknowledgment. This work was supported by grants from the Beckman Foundation (Young Investigator Award to W.A.V.) and the National Science Foundation (to Y.L.). S.M.B. thanks the NIH for biophysics training grant support. The University of Illinois EPR Research center is supported by the NIH. Y.L. and W.A.V. are Cottrell Scholars of the Research Corporation and Alfred P. Sloan Fellows, and Y.L. is a Camille Dreyfus Teacher-Scholar of the Camille and Henry Dreyfus Foundation.

Supporting Information Available: Procedure for the preparation of the Sec-containing peptide, a Table of EPR simulation parameters, and details of the electrochemical experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) (a) The product elutes as a single peak by HPLC, and in the presence of I_2 one would expect oxidation of the selenol monomer to the corresponding diselenide. Nevertheless, the monomer molecular ion is observed by mass spectrometry. (b) The truncated azurin gene from *P. aeruginosa* was ligated into the pTXB1 vector from New England Biolabs (Beverly, MA).

(15) Typical EPL reaction conditions: 6 mL of chitin beads containing the bound fusion protein in 20 mM Tris buffer, pH 8, 500 mM NaCl, 1 mM EDTA, 50 mM MESNA, and 1 mM peptide. The mixture was agitated for 61 h at 4 °C.

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JA0169163