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Metalloproteins Diversified: The Auracyanins Are a Family of Cupredoxins That Stretch the Spectral and Redox Limits of Blue Copper Proteins

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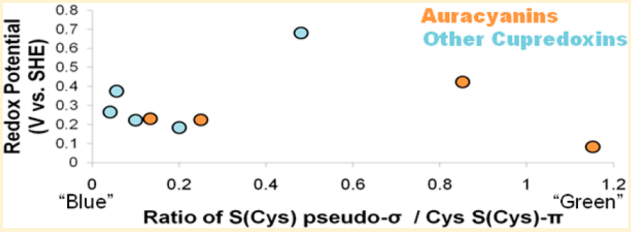
Supporting Information

ABSTRACT: The metal sites of electron transfer proteins are tuned for function. The type 1 copper site is one of the most utilized metal sites in electron transfer reactions. This site can be tuned by the protein environment from +80 mV to +680 mV in typical type 1 sites. Accompanying this huge variation in midpoint potentials are large changes in electronic structure, resulting in proteins that are blue, green, or even red. Here, we report a family of blue copper proteins, the auracyanins, from the filamentous anoxygenic phototroph *Chloroflexus aurantiacus* that display the entire known spectral and redox variations known in the type 1 copper site. *C. aurantiacus* encodes four auracyanins, labeled A–D. The midpoint potentials vary from +83 mV (auracyanin D) to +423 mV (auracyanin C). The electronic structures vary from classical blue copper UV–vis absorption spectra (auracyanin B) to highly perturbed spectra (auracyanins C and D). The spectrum of auracyanin C is temperature-dependent. The expansion and divergent nature of the auracyanins is a previously unseen phenomenon.



Metalloproteins direct electron transfer in biological systems.[1,2] The natural range of biological redox reactions extends from −500 mV to +800 mV vs NHE. Four families of proteins are responsible for most of this range: heme-iron proteins, non-heme iron proteins, blue copper proteins, and flavodoxins. Efficient electron transfer using only these cofactors across such a broad range requires significant protein tuning.[3] Great interest exists in understanding the protein factors that tune electron transfer proteins,[4,5] particularly in blue copper sites.[6] Here, we report a family of blue copper proteins called auracyanins from a single organism *Chloroflexus aurantiacus* that span the entire redox range of known blue copper proteins.

C. aurantiacus employs unique electron transport chains to perform aerobic respiration and anoxygenic photosynthesis.[7] Unlike other phototrophs, it lacks a cytochrome bc_1 or the related cytochrome b_6f complex normally responsible for establishing the proton motive force.[8] Instead, *C. aurantiacus* utilizes a structurally and evolutionarily unrelated protein complex called alternative complex III.[9] *C. aurantiacus* also lacks soluble cytochromes as electron carriers.[7] The mobile electron carriers in both photosynthesis and respiration appear to be blue copper proteins called auracyanins.[10] *C. aurantiacus* encodes four auracyanins, named A–D.[11,12] Auracyanins A and B are predicted to be involved in respiration and photosynthesis, respectively.[13] Auracyanins A and B are well characterized.[10] Auracyanins C and D are first reported here.

The auracyanins are members of the Type 1 (T1) blue copper protein (BCP) family, also referred to as cupredoxins. Typical features of this family are (1) a midpoint potential between +250 mV and +400 mV; (2) a deep blue color; and (3) four ligands that coordinate the copper ion (cysteine thiolate, two histidine imidazoles, and methionine thioether).[14] A detailed understanding of the T1 copper site has been elucidated from natural variation and mutational studies. Natural variation is observed in the extremely high potential BCP rusticyanin ($E_{m,3.2}$ = +680 mV),[15] and the extremely low potential BCP nitrosocyanin ($E_{m,7}$ = +86 mV).[16] Type 1 copper sites can range from blue (azurin, plastocyanin) to green (nitrite reductase) to red (nitrosocyanin) in color.[17] The axial ligand of most blue copper proteins is methionine, but glutamine has been observed in stellacyanin.[18] Mutational studies have addressed much of this variation.[6,19,20]

Ideal target proteins for understanding the variation observed in Type 1 copper sites have yet to be found. The high potential rusticyanin is stable only at low pH.[21] The low potential red

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Table 1

creation of pET21aSig by performing PCR on pET21 with primers containing NheI, digesting the PCR product with NheI, followed by ligation, and transformation		
	forward	ATTAGCTAGCCCTGCTCAGTGCGCCGCTGCTGGCTGCTAGTATGACTGGTGGACAG
	reverse	TATAGCTAGCAGGGATACCGCAGCGAGTTTACGTAGCATATGTATATCTCCTTCTTAAAG
gene cloning into pRSETa and pET21aSig at BamHI and EcoRI		
	forward - BamHI	reverse - EcoRI
auracyanin A	GATCGGATCCGGTGGTGGCGGGAGTAGC	CCGGGCTGCAGGAATTCGCCAACAATAGA
auracyanin B	GATCGGATCCACGCAGCCCCCGGCG	GATCGAATTCCTACGGCGTTACCGTTAATG
auracyanin C	CGGGATCCGGACGCAGGAACAGTTATTC	CGGAATTCCTATCGTTGCTTCGCATTCAG
auracyanin D	CGGGATCCGGTGAACCGCTGCGTGAAC	CGGAATTCCTCGTAATGACGGTCATCATG

copper protein nitrosocyanin has abnormal ligands and copper loops.[22] Green copper nitrite reductase contains a Type 2 copper site in addition to the Type 1 copper site.[23] Here, we report a family of blue copper proteins that show nearly the full variation observed in Type 1 copper sites in a single organism. Auracyanins A and B have normal redox potentials of +238 mV and +230 mV, whereas auracyanin C has a high potential of +423 mV and auracyanin D has a low potential of +83 mV. The potential of auracyanin D is the lowest measured for a BCP. The color of the proteins ranges from deep blue (auracyanins A and B) to light green (auracyanin C) to gray (auracyanin D). The spectrum of auracyanin C is temperature-dependent like green nitrite reductase, whereas auracyanin D is not. Finally, auracyanin D has a glutamine predicted as an axial ligand, similar to stellacyanin. In summary, the auracyanins contain the majority of known variation in Type 1 copper sites in a single family of proteins isolated from one organism.

MATERIALS AND METHODS

Cloning and Expression. All four auracyanins were cloned into two plasmids for overexpression in *Escherichia coli*: pRSETa and a modified pET21a containing the azurin signal peptide,[24,25] pET21aSig. Auracyanins A, C, and D were cloned directly after the cysteine involved in lipidation to avoid processing complications in *E. coli* (see Results). Recombinant auracyanin A and B genes were previously described.[26] The pRSETa vector introduces an N-terminal 6× histidine tag followed by an enterokinase cleavage site. All four genes expressed suitably in pRSETa producing 2−10 μg per liter of media. The pET21aSig vector includes an N-terminal T7 tag used for protein identification when Western blotting. Auracyanins A and B produced 50−100 μg of periplasmic protein per liter in the pET21aSig vector. The recovered proteins were identical to the native auracyanins A and B from *C. aurantiacus* when expressed in pRSETa or pET21aSig in *E. coli*. Auracyanins C and D were highly toxic when expressed in the periplasm using pET21aSig and produced little detectable protein. As a result, auracyanins C and D were expressed using pRSETa, and the 6× histidine tag was removed by enterokinase treatment.

Protein Purification. Auracyanins A and B were purified using a modified azurin preparation method.[27] Periplasmic fractions were applied to the CM Sepharose (Sigma-Aldrich Company, MO, USA) at pH 3.5, instead of pH 4.5. This lower pH denatured more contaminating proteins without affecting the stability of auracyanins A and B. Gel filtration was not required to obtain pure samples.

Auracyanins C and D were purified using nickel affinity chromatography. Cells were lysed by sonication in the presence of lysozyme and DNase (1 mg/mL) in 20 mM phosphate buffer supplemented with 500 mM sodium chloride and 1 mM imidazole. Samples were applied to a 5 mL HisTrap HP (GE Healthcare). The column was washed with buffer supplemented with 20 mM imidazole and eluted with 500 mM imidazole. The eluted fraction was dialyzed against 20 mM tris buffer pH 7.5 three times and treated with enterokinase (P8070, NEB). The samples were run through the nickel column again to remove undigested protein and contaminating proteins. To remove enterokinase, the samples were applied to a 5 mL Q HP column (GE Healthcare).

Spectroscopy. Room temperature and low temperature UV−vis spectra were measured on a Shimadzu UV-1800 spectrophotometer. Temperature was set to 77 K using a Janis VNF-100 liquid nitrogen cryostat (Janis Research Company, MA, USA). Temperature gradient spectra were recorded on Perkin-Elmer Lambda 950 UV−vis spectrophotometer with a peltier attachment.

Circular dichroism spectra were measured using a Jasco J-815 spectropolarimeter controlled at 25 °C. Continuous scanning was done from 900 to 300 nm at a scanning speed of 50 nm/min. Additional settings were a data pitch of 0.5, a response of 0.5 s, and a bandwidth of 1 nm. Four accumulations were carried out and averaged for each sample.

Continuous wave electron paramagnetic resonance (CW-EPR) spectra were acquired using an X-band Bruker ESP-300E spectrometer equipped with an Oxford Instruments ESR-9 helium cryostat with liquid nitrogen to maintain temperature of 80 K. The modulation frequency was 100 kHz. Spectra were processed in the Matlab environment (The Mathworks, Natick, NJ) using the easyspin toolbox.[28]

Redox Measurements. Spectroelectrochemistry was performed using a CHI620C CH Instruments potentiostat to vary the potential and a Shimadzu UV-1800 spectrophotometer to record spectra. Measurements were carried out in 100 μM potassium phosphate buffer (pH 7.0) and 200 mM potassium chloride. The concentration of each auracyanin was adjusted so that ODε_{max} was approximately 0.2 in the 1.7 mM path length of the honeycomb spectroelectrochemical cell (Pine Instruments). Twenty micromolar phenazine ethosulfate (+60 mV), 40 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (+260 mV), and 20 μM ferricyanide (+425 mV) mediated electron transfer between the honeycomb and protein. All measurements were carried out in an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2). Solutions were allowed to equilibrate anaerobically for several hours prior to measurement. Titrations were performed in the both the oxidative and reductive directions for each auracyanin.

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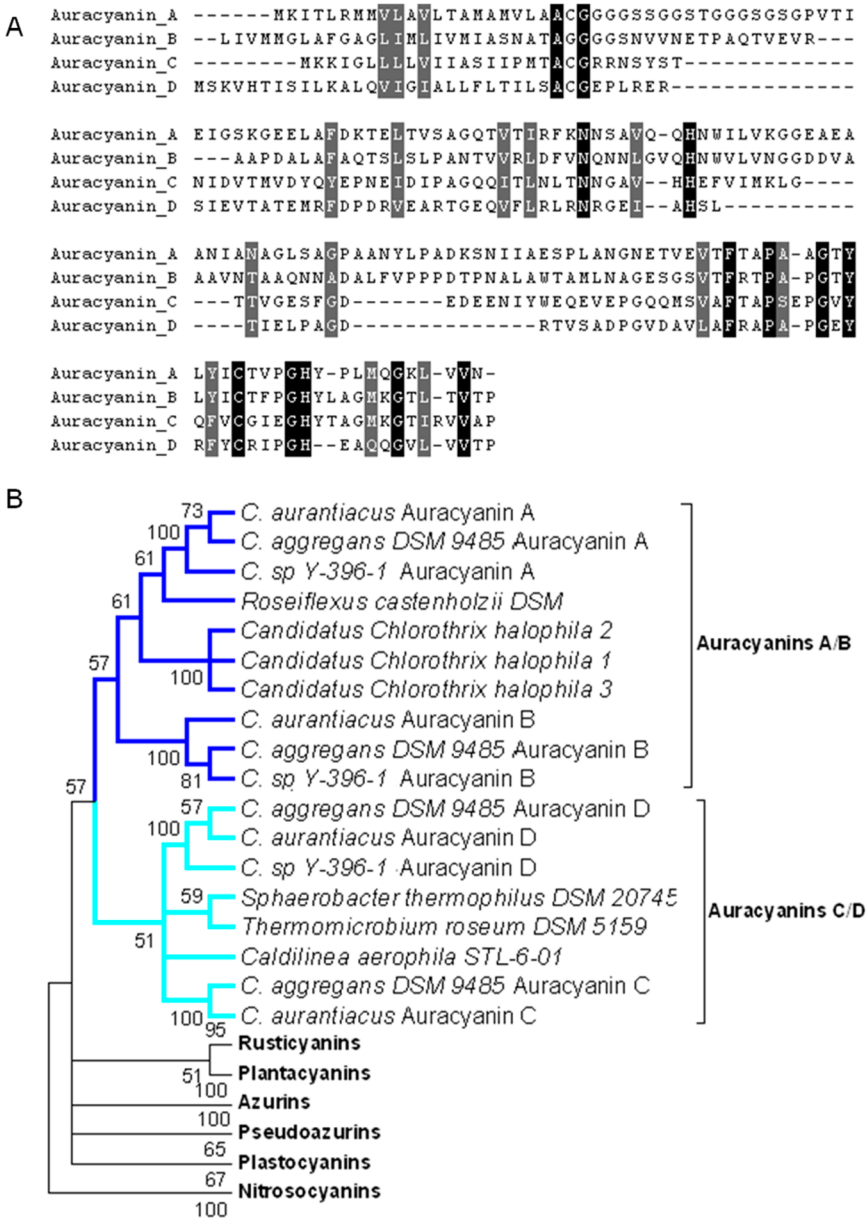

Figure 1. (A) Sequence alignment of the four auracyanins. Sequence alignment was performed with tcoffee[31] using auracyanin A (2AAN) and auracyanin B (1QHQ) crystal structures as guides and displayed with MultiAlignShow (http://www.bioinformatics.org/sms/multi_align.html). Black and gray shading represent conserved and similar residues, respectively. (B) Phylogenetic tree of the four auracyanins with other BCPs. The branches of the other BCPs were condensed for space (see Supplementary Figure 1 for full tree).

Phylogenetic Tree. ClustalW was used to align the auracyanin and BCP sequences. The tree was created in MEGA4[29] using the neighbor-joining method with a bootstrap of 500 replicates. Branches with bootstrap values lower than 50% were condensed. Other BCP family branches were compressed (see Supplementary Figure 1 for noncompressed tree). Supplementary Table 1 contains accessions for each BCP used.

RESULTS

Identification and Cloning of Auracyanins C and D. The sequencing of the *C. aurantiacus* genome led to the discovery of the genes that code for auracyanins C and D.[11] Auracyanin C is also present in the sequenced genome of *Chloroflexus aggregans*, but not *Chloroflexus sp. Y-396-1*. Auracyanin D is found in both *Chloroflexus sp. Y-396-1* and *Chloroflexus aggregans*. Sequence analysis suggests that a single auracyanin gene duplicated giving rise to the auracyanin A/B lineage and the auracyanin C/D lineage (data not shown).

The sequences of auracyanins C and D contain a signal peptide and a traditional BCP domain. The signal peptide contains a consensus sequence for bacterial lipid modification (ProSiteProfiles PS51257). Auracyanin A has been shown to have lipid modification in *C. aurantiacus*.[30] A conserved ACG motif is present in the terminal portion of the signal peptide in auracyanins A, C, and D. Auracyanin B contains a transmembrane domain for membrane anchoring. This suggests the auracyanins were originally attached to the membrane through lipidation, and auracyanin B's transmembrane domain is a recent adaptation. This is also confirmed by examining the auracyanins of other non-photosynthetic members of the Chloroflexi bacteria, which also contain the same conserved

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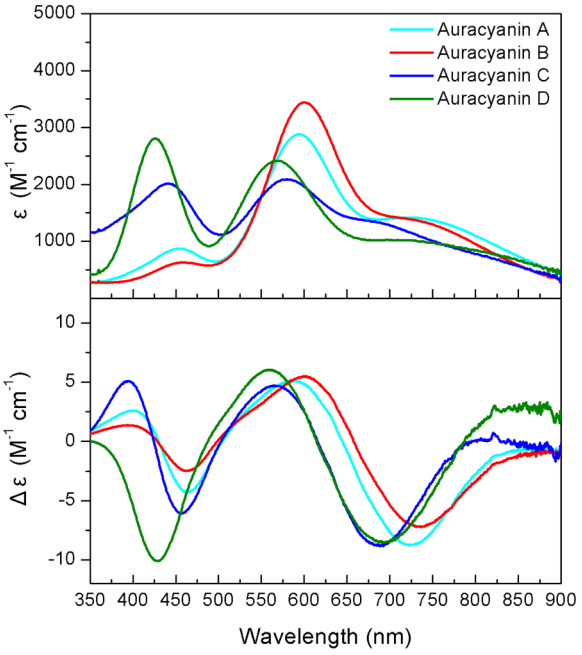

Figure 2. (A) UV−vis of all four auracyanins. (B) Circular dichroism of all four auracyanins.

ACG motif. Putative ligands can be identified by sequence alignment with auracyanins A and B. Auracyanin C is predicted to have a canonical ligand coordination with a cysteine, two histidines, and a methionine. Glutamine is predicted to replace methionine in auracyanin D, similar to the replacement seen in stellacyanin. Examination of the variable regions of the cupredoxin fold, thought to be involved in protein−protein interaction, suggest auracyanins C and D have different redox partners than auracyanins A and B (Figure 1a).

UV−vis and CD Spectroscopy of Auracyanins C and D. The spectral origins of blue copper proteins are well-described.[17,32,33] Solomon ascribes eight transitions to fit the three blue copper peaks.[34] The highest energy peak (425−460 nm) consists of three transitions arising from S(Met), His π, and S(Cys) σ to the Cu^{2+} $3dx^2-y^2$ charge transfers. The dominant peak around 570−625 nm consists of the S(Cys) π to the Cu^{2+} $3dx^2-y^2$ charge transfer. The lower energy peak from 650 to 1100 nm consists of the four ligand field transitions. The ratio of S(Cys) σ/ S(Cys) π varies between blue copper proteins. "Classical" blue copper proteins have a ratio under 0.15. As the ratio increases, the blue copper site is described as being "perturbed".

The UV−vis spectra of all four auracyanins were compared. Auracyanins C and D show highly perturbed T1 Cu sites, and the charge-transfer transitions are blue-shifted compared with other blue copper proteins. For auracyanin C, the S(Cys) → Cu π CT transition occurs at 581 nm and the S(Cys) → Cu σ CT transition occurs at 442 nm with an $\varepsilon_{442}/\varepsilon_{581}$ ratio of 0.89. In auracyanin D, these transitions are even further blue-shifted to 569 and 426 nm, and the $\varepsilon_{426}/\varepsilon_{569}$ ratio increases to 1.15. Auracyanin A shows a less perturbed copper site with $\varepsilon_{450}/\varepsilon_{593}$ = 0.32. Auracyanin B has a more classical spectrum with $\varepsilon_{458}/\varepsilon_{600}$ = 0.16.

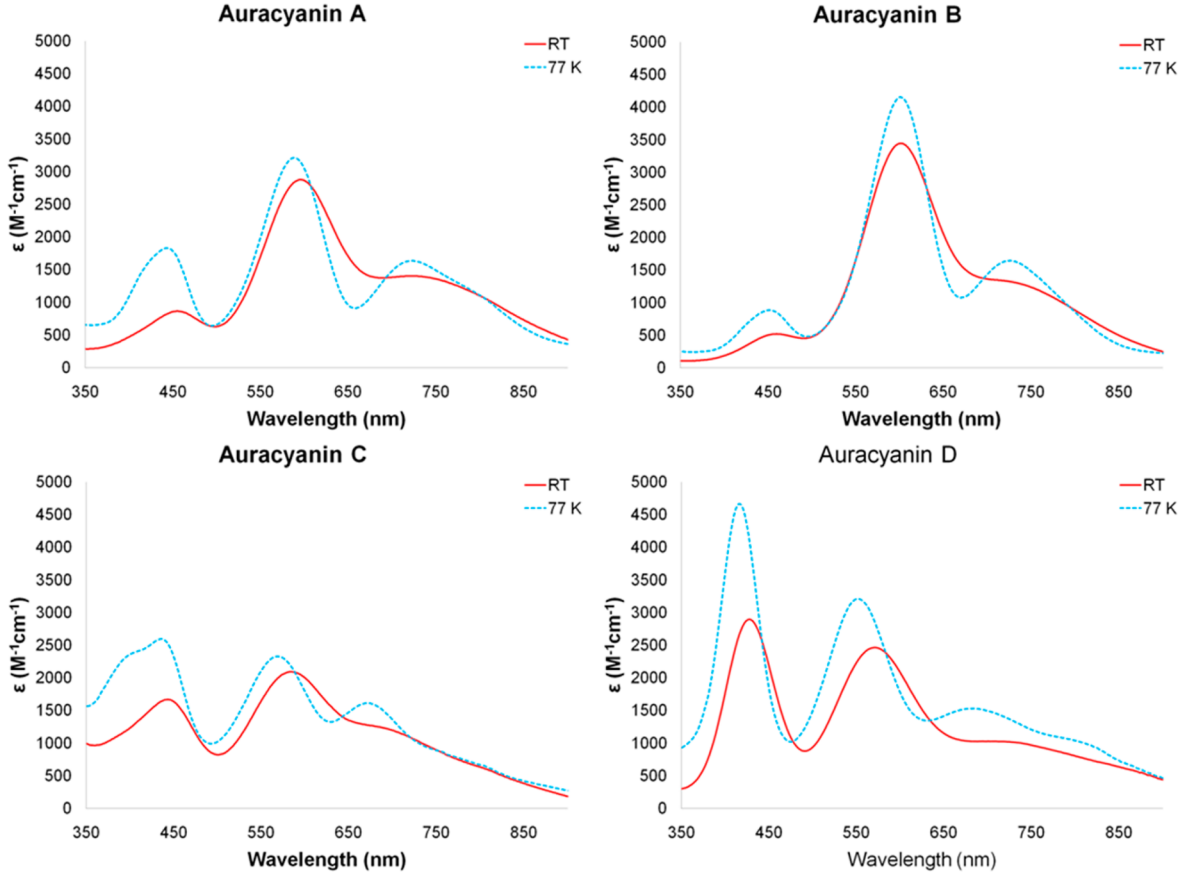

Figure 3. Comparison of room temperature (RT, red) and 77 K (blue) spectra.

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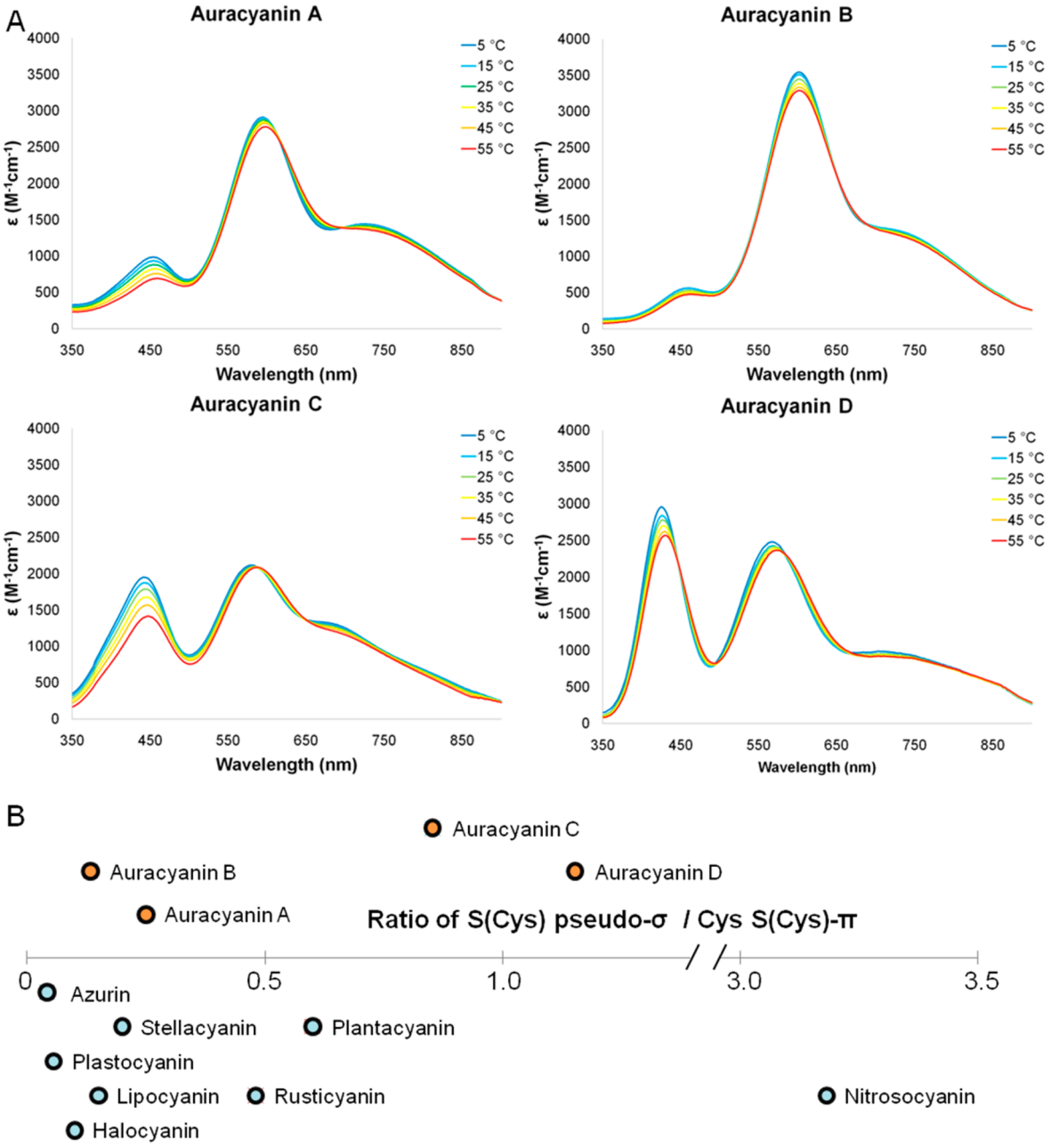

Figure 4. Further analysis of spectral features of auracyanins. (A) The effect of temperature on UV−vis transitions of the auracyanins. (B) A dot-plot comparison of the auracyanins' spectra with other blue copper proteins. Graphed is the ratio of pseudo-σ to π transitions. The pseudo-σ value is taken to be the maximum of the three high energy transitions (S(Met), His π, and S(Cys) σ to the Cu^{2+} $3dx^2-y^2$ charge transfers).

The visible circular dichroism spectra varied significantly between the auracyanins as well. Most notable is the loss of the positive extreme in auracyanin D that is present in auracyanins A, B, and C at ~400−420 nm (Figure 2b). This transition is normally assigned to the S(met) → Cu CT.[35] A similar phenomenon is observed in mavicyanin, which also has a glutamine as an axial ligand. Site-directed mutagenesis to swap the glutamine for a methionine in mavicyanin results in the appearance of this extreme.[36,37] This suggests that the glutamine found in the auracyanin D sequence behaves similarly to its role in stellacyanin and mavicyanin.

Low Temperature and Temperature-Dependent Spectra of the Auracyanins. In order to get better resolution of the perturbed copper sites in auracyanins C and D, low temperature (77 K) spectra were recorded (Figure 3). At low temperature, multiple transitions become obvious for the higher energy peak in auracyanin C. This is similar to what is observed in nitrite reductase at lower temperatures.[17] The intensity of the charge-transfer transitions increases in auracyanin D upon freezing. Auracyanin D's color changes from gray to purple upon freezing. Auracyanin A becomes significantly more perturbed upon freezing.

The spectral changes associated with freezing led us to measure the UV−vis spectra at various temperatures (Figure 4a). Perturbed blue copper sites can have temperature dependence, as is the case of green copper nitrite reductase.[38,39] Spectra were taken from 5 to 55 °C for each auracyanin. Auracyanin C showed dramatic spectral changes over this range. Visually, auracyanin C appeared light green at 5 °C and blue at 55 °C. It is interesting that auracyanin C appears blue at

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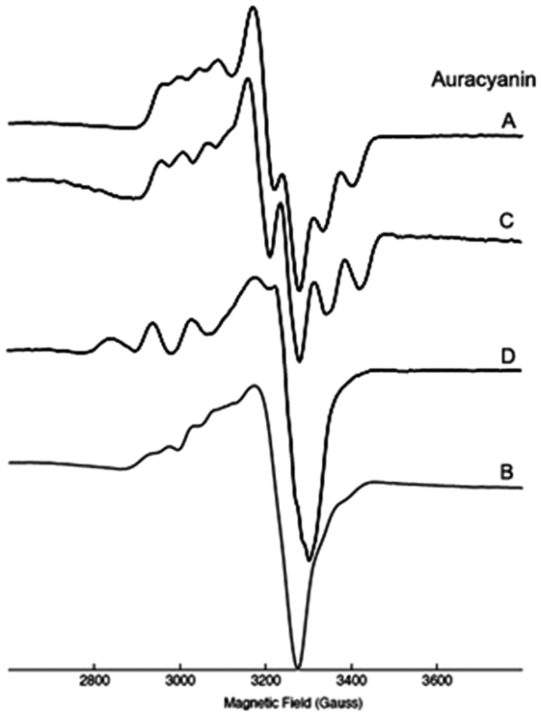

Figure 5. EPR of the auracyanins at 80 K.

the physiological temperature of 55 °C. Auracyanin A shows a similar but smaller change over this range, whereas the spectra of auracyanins B and D undergo negligible changes.

The visible spectral features of the four auracyanins span the entire range of known BCP spectra (Figure 4b).[16,21,35,40−43] Auracyanin B is a classical BCP, like azurin or plastocyanin. Auracyanin A is a slightly perturbed BCP, like pseudoazurin. Auracyanin C is a highly perturbed BCP with temperature-dependent spectra similar to green nitrite reductase. Auracyanin D is a highly perturbed BCP with temperature-independent spectra.

EPR Spectroscopy of Auracyanins C and D. Experimental EPR spectra for auracyanins A and B are in good agreement with previous X-band characterization.[10] The auracyanin C spectrum is very similar to that of auracyanin A, while auracyanin D is different from A, B, or C (Figure 5). Simulated fits to the auracyanin A spectrum were in agreement with a rhombic copper center (g_x = 2.026, g_y = 2.053, g_z = 2.220), but hyperfine coupling from nearby ligands could not be distinguished. Coupling constants are also similar to previous characterization by McManus et al.[10] in that $A_x > A_z > A_y$. A simulated fit to the auracyanin C spectrum was consistent with a rhombic copper center with down-shifted g values (g_x = 2.023, g_y = 2.048, g_z = 2.201) compared to auracyanin A. Auracyanins B and D could have an axial geometry or at least do not have a significant contribution from g_x if rhombic.

Spectroelectrochemical Redox Titration. Spectroelectrochemistry was performed to determine the potential of the four auracyanins (Figure 6). The midpoint potentials (vs NHE) of auracyanins A (+238 mV) and B (+230 mV) match well with those previously found (auracyanin A +205−240 mV and auracyanin B +215−240 mV). Auracyanin C has a midpoint potential of +423 mV, giving it one of the higher known potentials of a BCP. The midpoint potential of auracyanin D is +83 mV. This is approximately the value of nitrosocyanin (+86 mV),[16] the lowest known potential of a blue copper protein. Thus, the auracyanins also span the entire redox range of blue copper proteins.[6,16,42−44]

DISCUSSION

Existence of Four Auracyanins. The auracyanins are a unique family of BCPs. Despite sequence similarity and evidence of gene duplication, each auracyanin appears to have an independent function. Gene duplications in other BCPs, such as plastocyanin and pseudoazurin, appear to produce fairly similar proteins. The two plastocyanins from *Halothece sp. PCC 7418* (PCC7418_1913 and PCC7418_1914) are 80% identical. The two pseudoazurins from *Sinorhizobium meliloti* are 52% identical.[45] Interestingly, the second pseudoazurin has a potential 30 mV lower than those of other pseudoazurins and is on an operon with sulfite dehydrogenase, not nitrite reductase like other pseudoazurins.[46] The auracyanins have much lower sequence identity than other duplicated blue copper proteins with auracyanins B, C, and D having 57%, 48%, and 37% similarity to auracyanin A, respectively (Figure 1a). Additionally, they have very different redox potentials and electronic structures indicating more divergent functions.

The functional roles of the auracyanins still need to be clarified. Auracyanins A and B are likely involved in respiration and photosynthesis, respectively. The variable domains of auracyanins A and B are similar, indicating similar redox partners. This is logical as respiration and photosynthesis use different ACIII complexes as proton pumps.[12] Auracyanin C's variable region is shorter, and contains an entirely different sequence than auracyanins A, B, or D. Interestingly, *Chloroflexus sp. Y-396-1* lacks an auracyanin C gene entirely (Figure 1b). Phylogenetic profiling for genes common to *C. aurantiacus* and *Chloroflexus aggregans*, but not present in *C. sp. Y-396-1*, revealed 14 periplasmically expressed genes including auracyanin C (Supplementary Table 2). The most interesting of these genes is nitrite reductase, which would require a periplasmic electron transfer partner. The nitrite reductase from *Alcaligenes faecalis* uses the blue copper protein pseudoazurin during denitrification.[47] The remaining genes are unidentified or solute binding proteins, making them unlikely interaction partners. This suggests auracyanin C is possibly involved in denitrification. Auracyanin D has the smallest variable region, and no obvious redox partner can be elucidated by genomic surveying.

Perturbed Copper Sites. The highly perturbed nature of auracyanins C and D is of great interest to the BCP field.[17] Their spectra are the most perturbed of any single copper T1 site using standard ligands (Figure 4b). The red copper nitrosocyanin has a more perturbed spectrum, but it contains a different ligand set.[22] The spectra of auracyanins C and D are difficult to explain at the sequence level. The sequence of the copper binding loops is dissimilar between auracyanins C and D. In fact, auracyanins C's sequence is very close to that of auracyanin B in the copper loop (Figure 1a). Mutational studies of the auracyanins are underway in order to understand the sequence origin of "classical" and "perturbed" spectra.

It is noteworthy that auracyanin C is temperature-sensitive and auracyanin D is not. A possible explanation for auracyanin C's temperature dependence is the existence of two adjacent histidine residues at the first coordinating ligand. It is possible that depending on the temperature one histidine may be favored over the other as a ligand. It is not surprising that auracyanin D is temperature-independent. According to

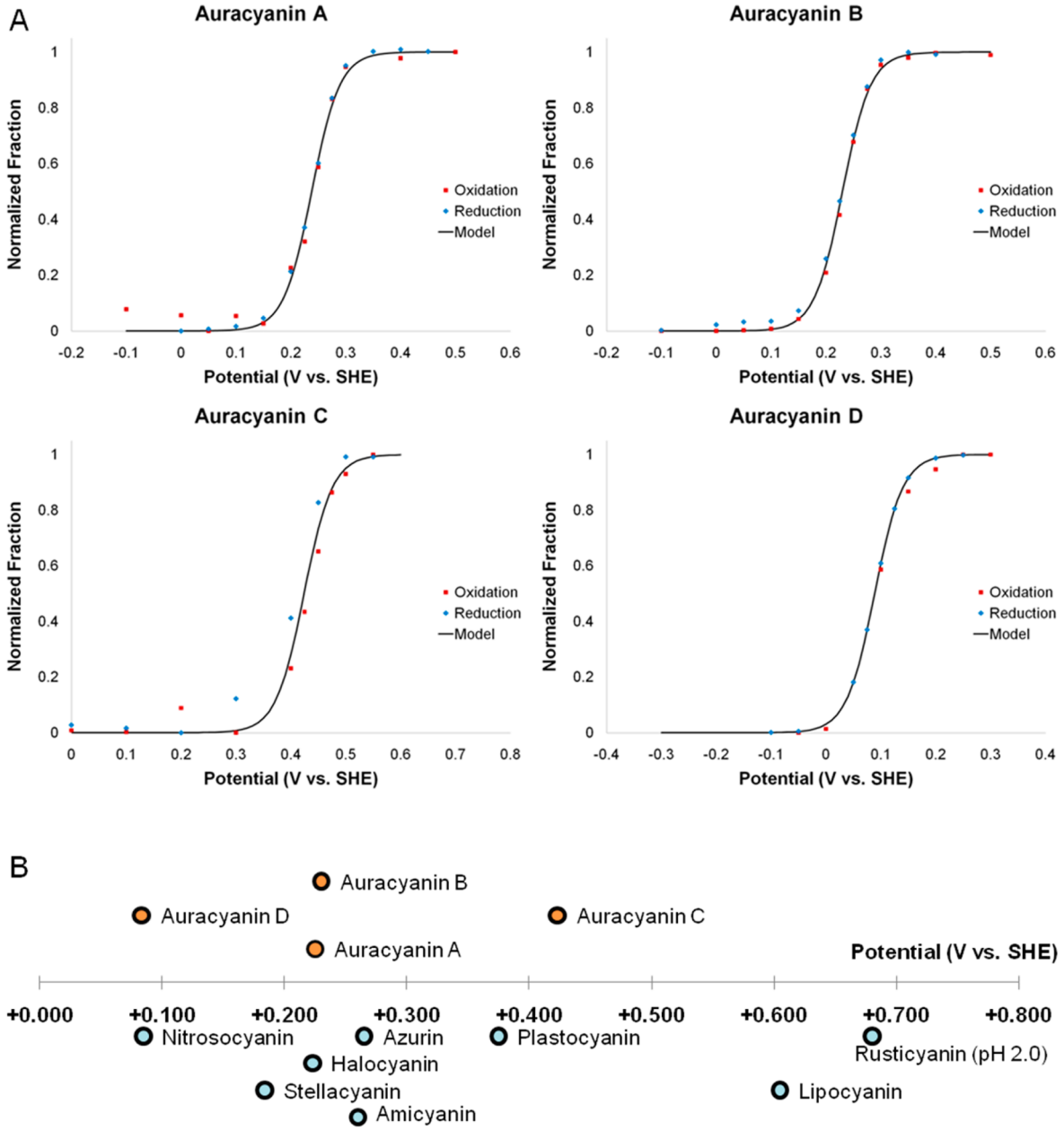

Figure 6. (A) Spectroelectric redox titrations of the four auracyanins. Each titration was carried out in the oxidative (red) and the reductive (blue) directions. (B) Dot plot showing the potentials of each auracyanin relative to other known blue copper proteins.

Solomon's perturbation theory, temperature dependence depends on the axial methionine distance shortening. Since auracyanin D lacks an axial methionine, it would not be expected to have temperature dependence.

Low and High Redox Potentials of Auracyanins C and D. Auracyanin D and nitrosocyanin have the lowest known potentials of any known blue copper proteins. The origin of auracyanin D's low potential is likely the glutamine axial ligand. An axial glutamine ligand results in a 100 mV decrease in potential relative to a methionine for a given BCP.[6] Subtracting 100 mV from the slightly low potentials of auracyanins A and B gives a potential around +130−140 mV. Small hydrogen bonding changes near the copper site can additionally alter the redox potential by 40−60 mV.[6] The combination of the glutamine ligand and changes to the hydrogen bonding network would give a potential close to auracyanin D's potential of +83 mV. Such mutations have produced an azurin variant with a potential of +90 mV.[6]

The reason for the high potential of auracyanin C is not clear from sequence information. The copper-binding loop of auracyanin C is nearly identical to the copper-binding loop of auracyanin B. This makes it unlikely to be responsible for the high potential. It is more likely that the residue following the first coordinating histidine is responsible for the high potential. In most blue copper proteins, this residue is an asparagine and participates in the hydrogen bonding network of the secondary coordination sphere.[21,48] Mutation of the asparagine to serine in azurin raises its potential by 130 mV.[6] It is not clear from the primary sequence if the first histidine (His 64) or the second histidine (His 65) is the coordinating ligand. Regardless, auracyanin C lacks a conserved asparagine at this position, which likely contributes to the high redox potential.

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CONCLUDING REMARKS

The auracyanins are an interesting family of BCPs. They coexist within the same organism and share a common ancestor, but have entirely different spectral and redox properties. Due to the extreme nature of auracyanins C and D compared with auracyanins A and B, it will be possible to use the auracyanin family as a mutational platform for understanding how copper sites are tuned.

ASSOCIATED CONTENT

Supporting Information

Full, uncompressed phylogenetic tree of blue copper proteins; table of blue copper proteins used in phylogenetic tree with species and accession number; table of phylogenetic profiling of sequenced *Chloroflexus* genomes. 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.

REFERENCES

(1) Gray, H. B., and Winkler, J. R. (2010) Electron flow through metalloproteins. *Biochim. Biophys. Acta 1797*, 1563−1572.

(2) Holm, R. H., Kennepohl, P., and Solomon, E. I. (1996) Structural and functional aspects of metal sites in biology. *Chem. Rev. 96*, 2239−2314.

(3) Banci, L., Bertini, I. , Luchinat, C., and Turano, P. (2006) Electron-Transfer Proteins, in *Biological Inorganic Chemistry: Structure and Reactivity* (Gray, H. B., Stiefel, E, I., Valentine, J. S., and Bertini, I., Eds.) 1st ed., pp 229−261, University Science Books, Herendon, VA.

(4) Lin, I.-J., Gebel, E. B., Machonkin, T. E., Westler, W. M., and Markley, J. L. (2005) Changes in hydrogen-bond strengths explain reduction potentials in 10 rubredoxin variants. *Proc. Natl. Acad. Sci. U.S.A. 102*, 14581−14586.

(5) Stephens, P. J., Jollie, D. R., and Warshel, a. (1996) Protein control of redox potentials of iron−sulfur proteins. *Chem. Rev. 96*, 2491−2514.

(6) Marshall, N. M., Garner, D. K., Wilson, T. D., Gao, Y.-G., Robinson, H., Nilges, M. J., and Lu, Y. (2009) Rationally tuning the reduction potential of a single cupredoxin beyond the natural range. *Nature 462*, 113−116.

(7) Pierson, B. (1985) Cytochromes in *Chloroflexus aurantiacus* grown with and without oxygen. *Arch. Microbiol. 143*, 260−265.

(8) Yanyushin, M. F., Del Rosario, M. C., Brune, D. C., and Blankenship, R. E. (2005) New class of bacterial membrane oxidoreductases. *Biochemistry 44*, 10037−10045.

(9) Gao, X., Xin, Y., Bell, P. D., Wen, J., and Blankenship, R. E. (2010) Structural analysis of alternative complex III in the photosynthetic electron transfer chain of *Chloroflexus aurantiacus*. *Biochemistry 49*, 6670−6679.

(10) McManus, J. D., Brune, D. C., Han, J., Sanders-Loehr, J., Meyer, T. E., Cusanovich, M. a, Tollin, G., and Blankenship, R. E. (1992) Isolation, characterization, and amino acid sequences of auracyanins, blue copper proteins from the green photosynthetic bacterium *Chloroflexus aurantiacus*. *J. Biol. Chem. 267*, 6531−6540.

(11) Tang, K.-H., Barry, K., Chertkov, O., Dalin, E., Han, C. S., Hauser, L. J., Honchak, B. M., Karbach, L. E., Land, M. L., Lapidus, A., Larimer, F. W., Mikhailova, N., Pitluck, S., Pierson, B. K., and Blankenship, R. E. (2011) Complete genome sequence of the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus*. *BMC Genomics 12*, 334.

(12) Majumder, E. L. W., King, J. D., and Blankenship, R. E. (2013) Alternative Complex III from phototrophic bacteria and its electron acceptor auracyanin. *Biochim. Biophys. Acta 1827*, 1383−1391.

(13) Cao, L., Bryant, D. a, Schepmoes, A. a, Vogl, K., Smith, R. D., Lipton, M. S., and Callister, S. J. (2012) Comparison of *Chloroflexus aurantiacus strain J-10-fl* proteomes of cells grown chemoheterotrophically and photoheterotrophically. *Photosynth. Res. 110*, 153−168.

(14) Gray, H. B., Malmström, B. G., and Williams, R. J. P. (2000) Copper coordination in blue proteins. *J. Biol. Inorg. Chem. 5*, 551−559.

(15) Ingledew, J W, and C., J. G. (1980) A potentiometric and kinetic study on the respiratory chain of ferrous-iron-grown *Thiobacillus ferrooxidans*. *Biochim. Biophys. Acta 590*, 141−158.

(16) Arciero, D., Pierce, B., Hendrich, M., and Hooper, A. (2002) Nitrosocyanin, a red cupredoxin-like protein from *Nitrosomonas europaea*. *Biochemistry 41*, 1703−1709.

(17) Solomon, E. I. (2006) Spectroscopic methods in bioinorganic chemistry: blue to green to red copper sites. *Inorg. Chem. 45*, 8012−8025.

(18) Hart, P. J., Eisenberg, D., Nersissian, A. M., Valentine, J. S., Herrmann, R. G., and Nalbandyan, R. M. (1996) A missing link in cupredoxins: Crystal structure of cucumber stellacyanin at 1.6 Å resolution. *Protein Sci. 5*, 2175−2183.

(19) Berry, S. M., Bladholm, E. L., Mostad, E. J., and Schenewerk, A. R. (2011) Incorporation of the red copper nitrosocyanin binding loop into blue copper azurin. *J. Biol. Inorg. Chem. 16*, 473−480.

(20) Li, C., Banfield, M. J., and Dennison, C. (2007) Engineering copper sites in proteins: loops confer native structures and properties to chimeric cupredoxins. *J. Am. Chem. Soc. 129*, 709−718.

(21) Hall, J. F., Kanbi, L. D., Harvey, I., Murphy, L. M., and Hasnain, S. S. (1998) Modulating the redox potential and acid stability of rusticyanin by site-directed mutagenesis of Ser86. *Biochemistry 37*, 11451−11458.

(22) Lieberman, R., and Arciero, D. (2001) Crystal structure of a novel red copper protein from *Nitrosomonas europaea*. *Biochemistry*, 5674−5681.

(23) Godden, A. J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., and Legall, J. (1991) The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycoclastes*. *Science 253*, 438−442.

(24) Canters, G. W. (1987) The azurin gene from *Pseudomonas aeruginosa* codes for a pre-protein with a signal peptide. Cloning and sequencing of the azurin gene. *FEBS Lett. 212*, 168−172.

(25) Arvidsson, R. H. A., Nordling, M., and Lundberg, L. G. (1989) The azurin gene from *Pseudomonas aeruginosa*. *Eur. J. Biochem. 179*, 195−200.

(26) Lee, M., Del Rosario, M. C., Harris, H. H., Blankenship, R. E., Guss, J. M., and Freeman, H. C. (2009) The crystal structure of auracyanin A at 1.85 A resolution: the structures and functions of auracyanins A and B, two almost identical "blue" copper proteins, in the photosynthetic bacterium *Chloroflexus aurantiacus*. *J. Biol. Inorg. Chem. 14*, 329−345.

(27) Manetto, G. D., Grasso, D. M., Milardi, D., Pappalardo, M., Guzzi, R., Sportelli, L., Verbeet, M. P., Canters, G. W., and La Rosa, C. (2007) The role played by the alpha-helix in the unfolding pathway and stability of azurin: switching between hierarchic and nonhierarchic folding. *ChemBioChem 8*, 1941−1949.

(28) Stoll, S., and Schweiger, A. (2006) EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson. 178*, 42−55.

(29) Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol. 24*, 1596−1599.

(30) Van Driessche, G., Hu, W., Van de Werken, G., Selvaraj, F., McManus, J. D., Blankenship, R. E., and Van Beeumen, J. J. (1999) Auracyanin A from the thermophilic green gliding photosynthetic

bacterium *Chloroflexus aurantiacus* represents an unusual class of small blue copper proteins. *Protein Sci. 8*, 947−957.
(31) Notredame, C., Higgins, D. G., and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol. 302*, 205−217.
(32) Solomon, E. I., Szilagyi, R. K., DeBeer George, S., and Basumallick, L. (2004) Electronic structures of metal sites in proteins and models: contributions to function in blue copper proteins. *Chem. Rev. 104*, 419−458.
(33) Solomon, E. I., and Hadt, R. G. (2011) Recent advances in understanding blue copper proteins. *Coord. Chem. Rev. 255*, 774−789.
(34) Gewirth, A., and Solomon, E. (1988) Electronic structure of plastocyanin: excited state spectral features. *J. Am. Chem. Soc. 110*, 3811−3819.
(35) LaCroix, L., and Randall, D. (1998) Spectroscopic and geometric variations in perturbed blue copper centers: electronic structures of stellacyanin and cucumber basic protein. *J. Am. Chem. Soc. 7863*, 9621−9631.
(36) Kataoka, K., Nakai, M., Yamaguchi, K., and Suzuki, S. (1998) Gene synthesis, expression, and mutagenesis of zucchini mavicyanin: the fourth ligand of blue copper center is Gln. *Biochem. Biophys. Res. Commun. 250*, 409−413.
(37) Kataoka, K., Kondo, a, Yamaguchi, K., and Suzuki, S. (2000) Spectroscopic and electrochemical properties of the Met86Gln mutant of *Achromobacter cycloclastes* pseudoazurin. *J. Inorg. Biochem. 82*, 79−84.
(38) Buning, C., Canters, G. W., Comba, P., Dennison, C., Jeuken, L., Melter, M., and Sanders-Loehr, J. (2000) Loop-directed mutagenesis of the blue copper protein amicyanin from *Paracoccus versutus* and its effect on the structure and the activity of the type-1 copper site. *J. Am. Chem. Soc. 122*, 204−211.
(39) Ghosh, S., Xie, X., Dey, A., Sun, Y., Scholes, C. P., and Solomon, E. I. (2009) Thermodynamic equilibrium between blue and green copper sites and the role of the protein in controlling function. *Proc. Natl. Acad. Sci. U.S.A. 106*, 4969−4974.
(40) Husain, M., and Davidsons, V. L. (1985) An inducible periplasmic blue copper protein from. *J. Biol. Chem. 260*, 14626−14629.
(41) Hadt, R. G., Sun, N., Marshall, N. M., Hodgson, K. O., Hedman, B., Lu, Y., and Solomon, E. I. (2012) Spectroscopic and DFT studies of second-sphere variants of the type 1 copper site in azurin: covalent and nonlocal electrostatic contributions to reduction potentials. *J. Am. Chem. Soc. 134*, 16701−16716.
(42) Worrall, J. a R., Machczynski, M. C., Keijser, B. J. F., Di Rocco, G., Ceola, S., Ubbink, M., Vijgenboom, E., and Canters, G. W. (2006) Spectroscopic characterization of a high-potential lipo-cupredoxin found in *Streptomyces coelicolor*. *J. Am. Chem. Soc. 128*, 14579−14589.
(43) Scharf, B., and Engelhard, M. (1993) Halocyanin, an archaebacterial blue copper protein (type I) from *Natronobacterium pharaonis*. *Biochemistry 32*, 12894−12900.
(44) Gray, K., Knaff, D., Husain, M., and Davidson, V. (1986) Measurement of the oxidation-reduction potentials of amicyanin and c-type cytochromes from *Paracoccus denitrificans*. *FEBS Lett. 207*, 2−5.
(45) Laming, E. M., McGrath, A. P., Guss, J. M., Kappler, U., and Maher, M. J. (2012) The X-ray crystal structure of a pseudoazurin from *Sinorhizobium meliloti*. *J. Inorg. Biochem. 115*, 148−154.
(46) Low, L., Ryan Kilmartin, J., Paul, V, B., and Ulrike, K. (2011) How are "atypical" sulfite dehydrogenases linked to cell metabolism? Interactions between the SorT sulfite dehydrogenase and small redox proteins. *Front. Microbiol. 2*, 58.
(47) Zumft, W. G. (1997) Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev. 61*, 533−616.
(48) Hoitink, C. W., and Canters, G. W. (1992) The importance of Asn47 for structure and reactivity of azurin from *Alcaligenes denitrificans* as studied by site-directed mutagenesis and spectroscopy. *J. Biol. Chem. 267*, 13836−13842.