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Nitrosocyanin, a Red Cupredoxin-like Protein from *Nitrosomonas europaea*†

David M. Arciero,‡ Brad S. Pierce,§ Michael P. Hendrich,§ and Alan B. Hooper*,‡

Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, Minnesota 55108, and Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

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ABSTRACT: Nitrosocyanin (NC), a soluble, red Cu protein isolated from the ammonia-oxidizing autotrophic bacterium *Nitrosomonas europaea*, is shown to be a homo-oligomer of 12 kDa Cu-containing monomers. Oligonucleotides based on the amino acid sequence of the N-terminus and of the C-terminal tryptic peptide were used to sequence the gene by PCR. The translated protein sequence was significantly homologous with the mononuclear cupredoxins such as plastocyanin, azurin, or rusticyanin, the type 1 copper-binding region of nitrite reductase, and the binuclear CuA binding region of N_2O reductase or cytochrome oxidase. The gene for NC contains a leader sequence indicating a periplasmic location. Optical bands for the red Cu center at 280, 390, 500, and 720 nm have extinction coefficients of 13.9, 7.0, 2.2, and 0.9 mM^{-1}, respectively. The reduction potential of NC (85 mV vs SHE) is much lower than those for known cupredoxins. Sequence alignments with homologous blue copper proteins suggested copper ligation by Cys95, His98, His103, and Glu60. Ligation by these residues (and a water), a trimeric protein structure, and a cupredoxin β-barrel fold have been established by X-ray crystallography of the protein [Lieberman, R. L., Arciero, D. M., Hooper, A. B., and Rosenzweig, A. C. (2001) *Biochemistry 40*, 5674−5681]. EPR spectra of the red copper center indicated a Cu(II) species with a $g_{||}$ of 2.25 and an $A_{||}$ of 13.8 mT (144 $\times$ 10^{-4} cm^{-1}), typical of Cu in a type 2 copper environment. NC is the first example of a type 2 copper center in a cupredoxin fold. The open coordination site and type 2 copper suggest a possible catalytic rather than electron transfer function.

The autotrophic, nitrifying bacterium *Nitrosomonas europaea* derives energy from the oxidation of ammonia to nitrite (*1*). Ammonia is oxidized to hydroxylamine ($NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$) by ammonia monooxygenase (AMO)[1] and hydroxylamine to nitrite ($NH_2OH + H_2O \rightarrow NO_2^- + 4e^- + 5H^+$) by hydroxylamine oxidoreductase (HAO). Of the four electrons abstracted by HAO from NH_2OH and transferred to the tetraheme cytochrome c_{554}, two electrons are subsequently directed to AMO to regenerate NH_2OH and the remaining two pass through ubiquinone and a cytochrome bc_1 complex to terminate in the product of one of several enzymes: a cytochrome aa_3 terminal oxidase, a copper-containing nitrite reductase, a nitric oxide reductase,

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* To whom correspondence should be addressed. Telephone: (612) 624-4930. Fax: (612) 625-5780. E-mail: hooper@cbs.umn.edu.

‡ University of Minnesota.

§ Carnegie Mellon University.

[1] Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NC, nitrosocyanin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance spectroscopy; PCR, polymerase chain reaction.

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an ATP-dependent NAD^+ reductase, or a cytochrome *c* peroxidase (*2*). Aside from the monoheme cytochrome c_{552} (*3*), a membrane tetraheme cytochrome c_{M552} (*2*), and the terminal oxidase (*4*), many components of the paths of electron transfer from cytochrome c_{554} to the terminal electron-accepting enzymes have yet to be described.

The broad cupredoxin evolutionary family of copper proteins includes the classic small, mononuclear blue copper cupredoxins (e.g., plastocyanin, azurin, pseudoazurin, and rusticyanin), the mononuclear type 1 copper-binding region of nitrite reductase, and the binuclear type 3 CuA binding region of N_2O reductase or cytochrome oxidase. All these copper centers function in electron transfer. Although the small mononuclear cupredoxins have been shown to carry out electron transport in a wide variety of bacterial aerobic or anaerobic energy-generating pathways and in photosynthesis, an example has yet to be reported in the autotrophic nitrifying bacteria.

"Classic" blue copper proteins have been extensively characterized with respect to the structural basis of their spectral and potentiometric properties (*5*−*9*). They are most readily identified by the presence of an intense electronic absorption band near 600 nm and a much higher reduction potential and a different EPR spectrum compared to those of inorganic copper derivatives. The EPR spectra of blue copper proteins (type 1) have an $A_{||}$ of <6.7 mT, whereas "normal" tetragonal copper sites have an $A_{||}$ between 11.9 and 21.6 mT (*26*). The distinguishing absorption band apparently is derived from the Cu being in a distorted tetrahedral ligand environment created by two normal Cu−N bonds from His residues, a short Cu−S(Cys) bond, and a long Cu−S(Met) bond in which Cu−S covalency is a strong contributing factor (*10*). In several examples of "perturbed" blue copper sites in which ligand field transitions are shifted to higher energies, the perturbation is attributed to distortion to either tetragonal or tetrahedral geometries. To date, the most strongly perturbed blue copper site in nature is the type 1 Cu site of nitrite reductase from *Alcaligenes cycloclastes* (*11*).

We report the isolation, optical and EPR spectra, metal composition, monomer molecular mass, and amino acid sequence of nitrosocyanin (NC), a novel red copper protein from *N. europaea*. Preliminary description of this protein has appeared (*2*, *12*). On the basis of its primary sequence and β-barrel "cupredoxin" tertiary fold, nitrosocyanin appears to belong to the broad cupredoxin evolutionary family. However, NC is strikingly novel in having a red color, an electronic absorption band at 390 nm, and a relatively low midpoint reduction potential. The EPR characteristics of NC are also not typical of type 1 copper but resemble those of type 2 tetragonal copper centers. The latter are often found to have catalytic functions. The 1.65 Å crystal structure (*13*) shows the protein to be a trimer of mononuclear cupredoxin monomers each having a copper within a cupredoxin β-fold and coordinated by His, His, Cys, the side chain O of Glu, and a solvent molecule. NC represents the first example in nature of a type 2 copper ligation in a cupredoxin β-barrel fold.

EXPERIMENTAL PROCEDURES

Growth of Cells and Purification of the Protein. Growth of *N. europaea* in minimal salts medium in a laboratory-designed continuous culture fermentor (*14*, *15*) and disruption of cell membranes and fractionation of the soluble fraction with ammonium sulfate (*16*) have been described. Subsequent fractionation of the individual resolubilized ammonium sulfate pellets was carried out by gel filtration chromatography on a Sephadex G-100 column equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.2 M KCl. Nitrosocyanin was found primarily in the Sephadex G-100 eluate following chromatography of the 60−80% ammonium sulfate pellet and nearly coeluted with cytochrome P460 (*17*). The two proteins were subsequently separated on an octyl-Sepharose CL-4B column (4.0 cm × 16.0 cm) using a 2.0 to 0.0 M ammonium sulfate gradient in a 25 mM potassium phosphate solution (pH 7.5, 500 × 500 mL). Although nitrosocyanin was almost pure at this point, the remaining contaminant protein (predominantly a *c*-type cytochrome accounting for <2% of the protein) was removed by chromatography on either a Sephadex G-75 column [50 mM potassium phosphate solution (pH 7.0)] or a DEAE-Sepharose column [5 mM potassium phosphate solution (pH 7.0), 0 to 0.25 M KCl gradient].

Sequencing of the Protein. Analytical separation of the protein and peptides by HPLC (Hewlett-Packard 1090 M system) was carried out on a Vydac C8 column (4.6 mm × 250 mm; pore size of 300 Å) using the standard trifluoroacetic acid/acetonitrile buffer system. For proteolysis, 20 μM nitrosocyanin was first denatured by incubation at pH 2.0 for 60 min at room temperature in the dark. Digestion by 2% (w/w) TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) was carried out in a 0.1 M Tris/acetate solution (pH 7.6) in the dark at room temperature. Aliquots were removed at various times, acidified, and analyzed by reverse-phase HPLC. Before sequencing had been carried out, nitrosocyanin or the proteolytic peptides were collected and analyzed with a Bruker MALDI-TOF mass spectrometer in the Mass Spectrometry Consortium for the Life Sciences facility at the University of Minnesota. Automated Edman degradation of HPLC-purified nitrosocyanin (N-terminal analysis) or proteolytic peptides was performed on an Applied Biosystems 470A gas-phase sequentiator by the Microchemical Facility of the Institute of Human Genetics at the University of Minnesota. Phenyl thioisocyanate-derivatized amino acids were analyzed directly on an automated on-line ABI model 120 HPLC system containing a Brownlee C18 column (4.6 mm × 250 mm). Analysis by the Microchemical Facility of the amino acid composition was carried out on samples of nitrosocyanin, which had been hydrolyzed for 24 h. The C-terminal tryptic peptide was identified on the basis of (a) the amino acid composition of the protein (which indicated the number of trypsin sites) and (b) the precise mass values of the intact protein and the peptides as determined by MALDI; i.e., only one combination of amino acids which did not contain a trypsin-recognizable amino acid residue could have given the observed molecular mass. The putative C-terminal peptide and one other purified tryptic peptide were sequenced. Both the N-terminal region and the putative C-terminal peptide were long enough and contained enough sequence information for the design of oligonucleotide primers for PCR.

Amplification of the Gene for Nitrosocyanin by PCR. Genomic DNA was isolated as described previously (*18*). Degenerate oligonucleotide primers with the sequences

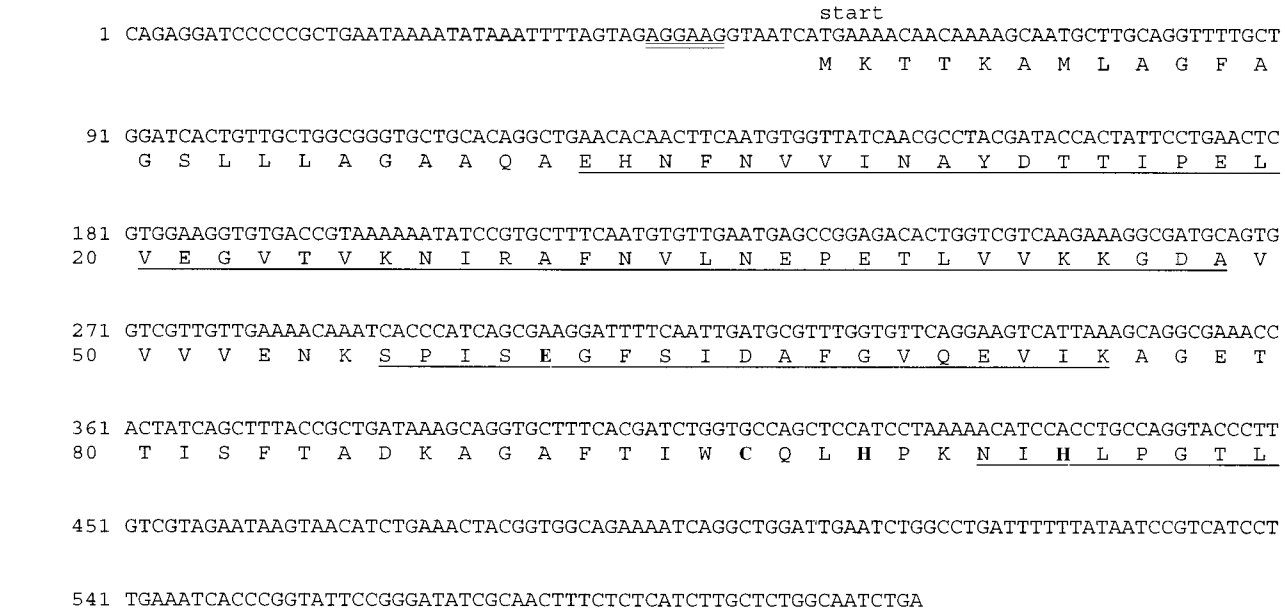

FIGURE 1: Nucleotide sequence of the gene encoding nitrosocyanin (top) and amino acid sequence for NC (bottom). The gene is tentatively assigned the name ncy. Amino acid numbers are for the mature protein. Single underlines denote the amino acid sequence of N-terminus or tryptic peptides. Double underlines denote the probable ribosomal binding site. Bold denotes the copper binding residues.

5′-ACITAYGAYACIACIATHCC-3′ and 3′-GGICCITGI-RAITTRCAICA-5′ based on the N-terminal and putative C-terminal amino acid sequences AYDTTIP and PGTLNVV, respectively, were prepared (by Life Technologies, Gaithersburg, MD). PCRs were carried out in 100 μL volumes in a Perkin-Elmer GeneAmp 2400 thermal cycler. The first amplification reaction employing 35 cycles (consisting of denaturation at 94 °C for 10 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1 min) resulted in a product seen as a faint band of 300 base pairs as analyzed on agarose gels. The second amplification reaction, which used the product of the first reaction as the template, consisted of 40 cycles (denaturation at 94 °C for 10 min, annealing at 39 °C for 1 min, and extension at 72 °C for 1 min). After confirmation of the success of the second reaction by gel electrophoresis, five additional "second" reactions were carried out simultaneously with the product of the first reaction as the primer. The PCR-generated product was purified by gel electrophoresis, the relevant section cut from the gel, and the PCR product isolated using a QIAquick gel extraction kit (Qiagen). The combined PCR product was sequenced directly by the Advanced Genetic Analysis Center in the Department of Veterinary Medicine, University of Minnesota, using the forward and reverse primers previously employed for amplification.

Spectroscopy. Optical spectra of a sample in cuvette with a path length of 1 cm were recorded with a Hewlett-Packard 8453 diode array spectrometer equipped with a constant-temperature cuvette holder. X-band EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 cryostat for low-temperature measurement. The microwave frequency and magnetic field were calibrated with a frequency counter and a NMR gaussmeter, respectively. Spin quantitation was determined relative to a CuEDTA standard. The Oxford thermocouple temperature was calibrated using a carbon glass resistor temperature probe (CGR-1-1000 Lake Shore Cryotronics). All experimental data were recorded with a magnetic field modulation of 100 kHz and were collected under nonsaturating conditions. Simulations of the EPR spectra use software written by the authors (*19*).

Assay Procedures. Subunit molecular mass determination was carried out by SDS−PAGE on mini-slab gels (*20*) using the buffer system of Laemmli (*21*) and by MALDI-MS. Samples were analyzed for metal content by inductively coupled plasma emission spectroscopy at the University of Minnesota Research Analytical Laboratory, in the Department of Soil, Water, and Climate.

Electrochemistry. Spectroelectrochemistry of nitrosocyanin was carried out in a 50 mM potassium phosphate solution (pH 7.0) containing 0.1 M KCl using an optically transparent thin layer electrode cell described previously (*22*). The redox mediator dyes that were used were 2,6-dichloroindolephenol (BDH Chemicals Ltd.; $E^{\circ\prime} = 215$ mV), 2,6-dibromo-3′-methoxyindophenol (TCI America, Inc.; $E^{\circ\prime} = 161$ mV), phenazine methosulfate (Sigma Chemical; $E^{\circ\prime} = 80$ mV), gallocyanine (Sigma Chemical; $E^{\circ\prime} = 20$ mV), and indigo tetrasulfonate (AMRESCO; $E^{\circ\prime} = -46$ mV).

RESULTS AND DISCUSSION

Molecular Mass, Oligomeric Structure, and Metal Content. Analysis by SDS−PAGE indicated a single subunit with a molecular mass of approximately 12 kDa. MALDI-MS revealed a subunit molecular mass (M_{av}) of 12 218 Da. On a Sephadex G-100 gel filtration column, nitrosocyanin nearly coeluted with cytochrome P460, molecular mass 52 kDa (*23*). Thus, nitrosocyanin, which had been seen previously as a trimer in the crystal structure (*13*), was shown to be an oligomer in solution. The metal composition varied between preparations, but all contained copper. Some preparations contained Cu and Ca, but no other metals were detected.

Sequence of the Gene. Figure 1 shows the amino acid sequence of nitrosocyanin and the complete nucleotide sequence of its gene. The amino acid sequence was determined directly for the first 48 residues (N-terminal analysis

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A.    ALIGNMENT WITH PLASTOCYANIN
                                10        20        30        40        50
NC        EHN------------FNVV--INAYDTTIPELNVEGVTVKNIRAFNVLN-EPETLVVKKGDAVKVVV
           .              : .:  .... ...    .:  :::     ..:. :: ...:. ::.:: :
PLAS_P    MKLIAQISRSLSLALFALVLMVGSFVAVMSPAAAETFTVKMGADSGLLQFEPANVTVHPGDTVKWV-
                 10        20        30        40        50        60

               60                      70        80         90       100       110
NC        ENKSPISE---------GFS---IDAFG-VQEVIKAGETKTISFTAD-KAGAFTIWCQLHPKNIHLPGTLNVVE
          .:: :  .         : :    : ..  : ... ::.  :.:..:  ::..: .:  :    .. : ..:
PLAS_P    NNKLPPHNILFDDKQVPGASKELADKLSHSQLMFSPGESYEITFSSDFPAGTYTYYCAPHRGAGMV-GKITVEG
            70        80        90       100       110       120       130


B.    ALIGNMENT WITH N2O REDUCTASE
               50        60        70        80        90        100       110
NC      VKKGDAVKVVVENKSPI---SEGFSIDAFGVQEVIKAGETKTISFTADKAGAFTIWCQL--HPKNIHLPGTLNVVE
        :::.:. : ::. : . :   :.:: .   ::.  :.  .:..:.: ::: :. . .:..  :   ... : ..:
NOSZ    VKQGNEVTVVITNMDQIEDVSHGFVMVNHGVSMEISPQQTSSITFIADKPGLHWYYCSWFCHALHMEMVGRMMVEPA
       550       560       570       580       590       600       610
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FIGURE 2: Alignment of the amino acid sequence of nitrosocyanin with (A) plastocyanin from the cyanobacterium *P. laminosum* (*23*) and (B) the C-terminal Cu-binding region of nitrous oxide reductase, NOSZ, of *Ps. aeruginosa* (*24*).

of the protein) and for two tryptic peptides, one of which was the C-terminus. The base sequence of the product of the PCR using oligonucleotides to the N- and C-terminal ends gave the coding region of the gene. From blast searches of sequence databases, nitrosocyanin was significantly homologous to mononuclear blue copper proteins, "cupredoxins" such as plastocyanin (Figure 2A; *24*), azurin, pseudoazurin, amicyanin, and rusticyanin, to a region encompassing one of the ligands to the type 1 copper center of nitrite reductase (data not shown), and to a region binding the binuclear CuA of N_2O reductase (Figure 2B; *25*) or cytochrome oxidase. This evolutionary relationship was also reflected in the shared cupredoxin β-barrel fold found in the copper binding regions of NC (*13*). The sequence of the 69 or 71 C-terminal amino acids of NC (gaps excluded) was 36 or 35% identical and 65 or 66% homologous to the corresponding region of plastocyanin from the cyanobacterium *Phormidium laminosum* or N_2O reductase from *Pseudomonas aeruginosa*, respectively. The copper ligand set of plastocyanin (His73, Cys120, His123, Met130) was recognizable as Glu60, Cys95, His98, and His103 in NC. This was an early indication that nitrosocyanin has Glu60 and His103 where His and Met, respectively, are found in classic blue copper centers. Copper ligation by Glu60, Cys95, His98, His103, and a solvent molecule was established by the X-ray structure (*13*). The complete sequence of the gene, from the *Nitrosomonas* genome database (*26*), contained a leader sequence. Thus, the mature protein is periplasmic.

Optical Spectra. The optical spectrum of oxidized nitrosocyanin (Figure 3) shows absorption bands at 280, 390, 496, and 720 nm. Reduction of nitrosocyanin by dithionite resulted in complete bleaching of the optical spectrum above 280 nm. The three bands disappeared simultaneously (data not shown). Bleaching of the optical spectrum also occurred in the presence of phenylhydrazine but not other aryl- or alkylhydrazines. All three bands also disappeared completely and simultaneously when nitrosocyanin was denatured at pH <2.0 or >11.0 or in the presence of guanidine hydrochloride (pH 7.0).

Reduction Potential. A plot of optical absorption at 390 nm as a function of reduction potential and the corresponding

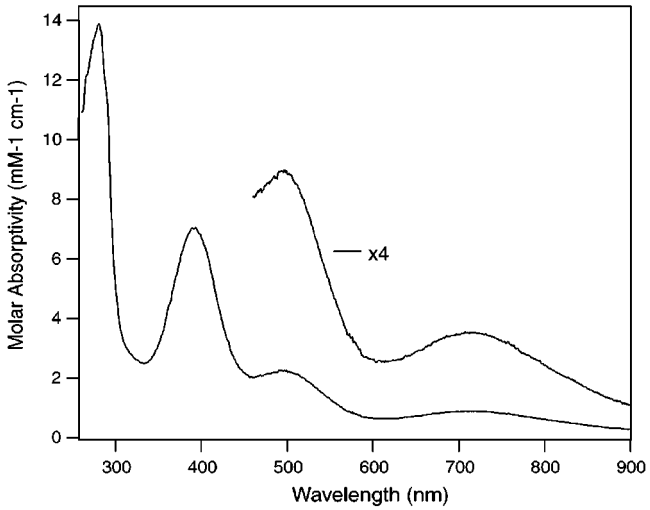

FIGURE 3: Absorption spectrum of nitrosocyanin (the protein sample used in Figure 5B) diluted in a 50 mM potassium phosphate solution and 200 mM KCl (pH 7.0).

Nernst plot is shown in Figure 4. The reductive titration of nitrosocyanin followed Nernstian behavior for a single one-electron center with a midpoint potential of 85 mV (vs NHE). Identical results were obtained for the absorption band at 500 nm (data not shown). The change in the intensity of the absorption band at 720 nm as a function of reduction potential was similar, although the data were not analyzed directly by the Nernst equation. The midpoint reduction potential of nitrosocyanin (85 mV) is $\sim$100 mV below the lowest value reported for blue copper proteins (*10*, *27*). Given the 2N2OS ligation of the NC red copper site (*13*), it is reasonable that the additional oxygen ligand from Glu60 is at least partly responsible for the unusually low redox potential of this enzyme. This downward trend in redox potential upon replacement of a sulfur by an oxygen donor has also been observed for M121X mutants of azurin (where X is the end, Glu, or Asp) (*28*).

EPR Spectroscopy. Figure 5A shows a frozen solution EPR spectrum of NC as isolated. The simulation (dashed line) overlaid on the data indicates the presence of two Cu(II) species at approximately equal concentrations. The *g* values

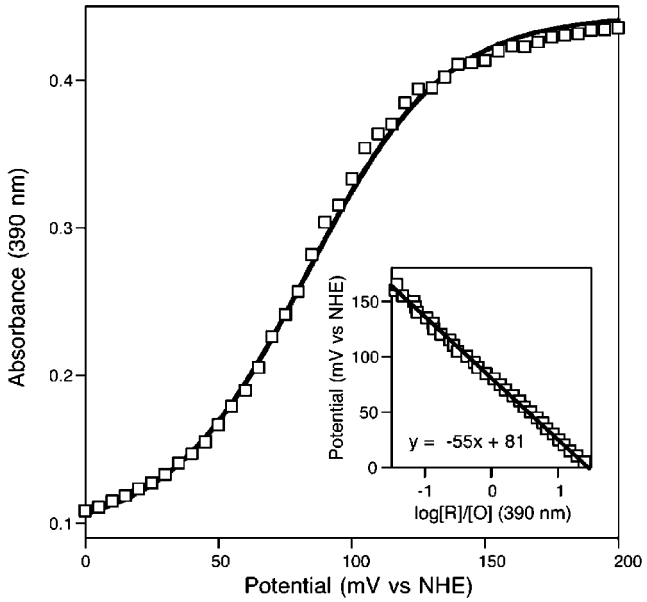

FIGURE 4: Reduction of nitrosocyanin as a function of the reduction potential of the media. Absorption at 392 nm was collected at 5 mV increments in a spectropotentiometric titration proceeding from fully oxidized to fully reduced nitrosocyanin. The line through the data points is a simulated curve for a one-electron Nernstian system with a midpoint potential of 80 mV. The inset is a Nernst plot of the data.

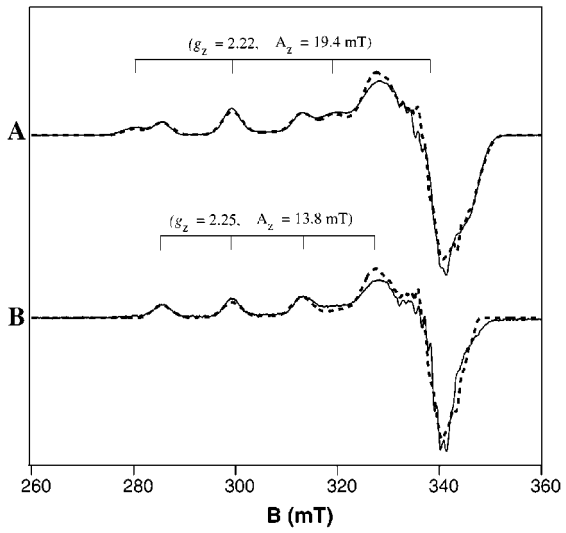

FIGURE 5: X-Band EPR data (—) and simulations (— — —) of the nitrosocyanin monomer in 50 mM potassium phosphate and 200 mM KCl (pH 7). (A) EPR signals of nitrosocyanin as isolated. Least-squares fitting of each distinct Cu(II) species indicates a sum of 36% species **I** and 64% species **II**. (B) EPR signal of a sample of NC after EDTA cleanup and gel filtration. $[Cu^{2+}] = 0.43$ mM. Parameters used for simulation of each Cu(II) species are given in Table 1. The instrumental conditions were as follows: 15 K, frequency of 9.62 GHz, modulation amplitude of 0.3 mT, and microwave power of 0.002 mW.

and hyperfine constants are listed in Table 1. Copper species **I** has a relatively large $A_{||}$ value of 19.4 mT (201×10^{-4} cm^{-1}) and a $g_{||}$ value of 2.22, consistent with a type 2 copper site (*29*, *30*). Figure 5B shows the EPR spectrum of NC after treatment with a 20-fold excess of EDTA followed by removal of EDTA by Sephadex G-25 gel filtration. This spectrum shows >90% copper species **II** with an $A_{||}$ of 13.8 mT (144×10^{-4} cm^{-1}). Titration of NC with up to 20 equiv of EDTA did not decrease the absorbance at 390 nm, indicating that the chromophore of NC is not susceptible to EDTA chelation. After the removal of EDTA-bound copper by gel filtration, EPR quantitation of samples of NC confirmed the loss of 43% of the Cu(II). The loss of EPR intensity originated primarily from the copper species **I** signal, while the intensity of the signal from copper species **II** was essentially unchanged; hence, the red chromophore can be assigned to EPR copper species **II**. Importantly, the crystals used for three-dimensional structure determinations were also red (*13*).

Table 1: EPR Parameters for Each Cu(II) Species in Nitrosocyanin[a]

species	g_x, g_y, g_z	A_x, A_y, A_z (Cu) ($\times 10^{-4}$ cm^{-1})	$A_o^{(N)}$ ($\times 10^{-4}$ cm^{-1})
I	2.025, 2.072, 2.224	−, −, 201	−
II	2.023, 2.067, 2.245	−, 20, 144	13.3

[a] The nitrogen hyperfine band of species II was fit with two equivalent isotropic A values (A_o).

Although the $g_{||}$ and $A_{||}$ values observed for copper species **II** can be categorized as those of a type 2 copper site, these values are not typical of square planar Cu(II) complexes with similar ligation (*31*−*34*). Type 2 model complexes with five-coordinate N2O2S ligation have observed $g_{||}$ values similar to those of the red Cu of NC, ranging from 2.20 to 2.27, but with larger $A_{||}$ values ($167-187 \times 10^{-4}$ cm^{-1}) (*33*, *34*). However, as copper complexes become tetrahedrally distorted away from square planar, the $g_{||}$ increases concomitant with a decrease in the observed $A_{||}$ value (*28*, *32*, *35*−*37*), as is observed for NC copper species **II**. Given the distorted square pyramidal geometry of the NC active site (*13*), as well as the heterogeneous nature of the ligands, the observed deviation of $g_{||}$ and $A_{||}$ values from those typical of square planar geometry is reasonable.

Copper species **I** might be considered adventitious, as it is removed by EDTA treatment and not responsible for the characteristic red color of NC. The $g_{||}$ and $A_{||}$ values of copper species **I** are similar to those observed for Cu(II) bound to four N donors. Previously, it was demonstrated that the N-terminal region of bovine serum albumin (BSA) binds Cu to four N donors, one of which is histidine (*38*, *39*). Nitrosocyanin has three His residues per monomer: two bind to species **II** Cu and one is at the N-terminus. We suggest that the N-terminal His is a candidate for the binding site of species **I** Cu. The possibility of a second metal binding site is also consistent with the observation of equal amounts of Ca and Cu observed in the metal analysis of one preparation of NC as well as the equimolar concentrations of copper EPR species **I** and **II** prior to EDTA chelation in two preparations.

Additional hyperfine peaks are observed near a g value of 2 in Figure 5A (NC as isolated), and these peaks are retained in Figure 5B (EDTA-treated), indicating that the hyperfine pattern is associated with copper species **II**. The simulation (dashed line) overlaid on the data is from a least-squares fit for a Cu(II) species ($I = {}^3/_2$) and two equivalent nitrogen atoms ($I = 1$). The nitrogen hyperfine pattern near a g value of 2 could be reasonably fit assuming two equivalent isotropic A values of 1.3 mT (13.3×10^{-4} cm^{-1}). This value is typical of nitrogen ligands coordinated to Cu, and the number of nitrogens is consistent with two His ligands to Cu as shown in the crystal structure (*13*).

Spin quantitation of species **II** in the EDTA-treated sample gave 0.43 mM Cu(II). On the basis of this value, the subunit molar absorptivities for the 280, 390, 500, and 720 nm optical bands of the red copper center were adjusted from their previously reported values (*12*) to 13.9, 7.0, 2.2, and 0.9 mM^{-1} cm^{-1}, respectively.

The naming of nitrosocyanin was based its homologous amino acid sequence and same β-barrel cupredoxin fold (*13*) as compared with those of the small mononuclear copper protein, plastocyanin. In contrast, the spectroscopic, redox properties, and oligomeric state of NC are very different from those of known cupredoxins and suggest that NC may represent a new class of copper proteins. Classic blue copper proteins such as azurin and plastocyanin typically have copper ion bound in a distorted trigonal plane comprised of two histidine residues and a cysteinyl thiolate. In addition, a methionyl sulfur atom is weakly bound in an axial position. This ligand environment is characterized by a prominent absorption band near 600 nm which is not observed for NC. While the red color of NC is fairly unique among copper proteins, its absorption bands and molar absorptivities are in line with other five-coordinate copper model complexes (*33*, *40*). The EPR spectra of blue copper proteins are characterized by $A_{||}$ values of $<70 \times 10^{-4}$ cm^{-1} (*30*), in contrast to the $A_{||}$ value of 144×10^{-4} cm^{-1} for NC. Interestingly, there are optical and EPR spectral similarities between NC and mutants of azurin and rusticyanin in which a Glu has replaced one of the ligands to the copper (*41*−*43*). However, comparison of X-ray structures indicates that the differences in the electronic structure between the red copper protein, NC, and the classic blue copper proteins result from several structural differences (*13*).

Possible Metabolic Role. Nitrosocyanin is present in *Nitrosomonas* at molar levels similar to those of proteins involved in nitrification and central electron transport pathways (AMO, HAO, cyt c_{554}, cyt c_{552}, cyt bc_1, and cyt aa_3) and thus may play a central metabolic role (*2*). The genome (*26*) does not provide a clue as to its function; the adjacent genes, a nucleoside diphosphate kinase and probable aminotransferase, neither are in the same operon nor represent likely functional partners. The small, monomeric, mononuclear blue copper proteins such as plastocyanin, azurin, pseudoazurin, and rusticyanin, which are homologous to nitrosocyanin, mediate electron transfer. Significantly, a member of this family is not seen in the genome of *Nitrosomonas.* In theory, there are many potential partners for NC in electron transfer. Possible electron donors include HAO, cytochrome c_{554}, cytochrome c_{552}, and cytochrome c_{m552}, and possible electron acceptors include cytochrome c_{554}, cytochrome c_{552}, and the enzymes ammonia monooxygenase, nitrite reductase, nitric oxide reductase, and cytochrome oxidase (*2*).

Given that the redox potential of NC is considerably lower than those of other cupredoxins, that the NC copper-binding site is open to solvent (*13*), and that type 2 copper sites are often involved in catalysis (*44*), one must consider the possibility that NC has a catalytic function. This possibility is also supported by the presence of a water coordinated to the Cu and the ability of oxidized NC to accommodate a large molecule, 1,6-hexanediol, near the water (*13*). Given the reactions which are unique or predominant in *Nitrosomonas*, nitrosocyanin could, in theory, have a role in a novel nitric oxide dehydrogenase (carrying out the last step in nitrite synthesis), a nitric oxide reductase, or the oxidation of ammonia. Reversible sequestration of a potentially toxic compound such as NO is also a possible function.

Evolutionary Relationship to Other Copper Proteins. A diverse group of proteins or protein domains share homologous amino acid sequences which form a common β-barrel cupredoxin fold and provide scaffolding for ligands to a mono- or binuclear copper center with an electron transfer function. The members of this cupredoxin evolutionary family include (a) monomeric monodomain proteins such as plastocyanin which have mononuclear blue, type 1 copper centers, (b) monomeric monodomain proteins such as stellacyanin which have perturbed type 1 mononuclear blue copper centers, (c) a domain within a trimeric protein such as nitrite reductase which has a perturbed type 1 copper center, and (d) a domain within a multidomain protein such as N_2O reductase which has a binuclear purple, type 3, CuA center. Categories a and b are the classic cupredoxins. On the basis of primary and tertiary structure, nitrosocyanin is clearly a member of the general cupredoxin evolutionary family. Interestingly, for a sequence region of ~70 amino acids, NC is ~66% homologous with the Cu-binding regions of either plastocyanin or N_2O reductase (Figure 2). Nevertheless, NC is remarkably different from all known members of this cupredoxin evolutionary family. It is a trimeric monodomain protein. Most dramatically, the mononuclear copper center has a unique ligand set (Glu, His, Cys, and H_2O) and geometry, resulting in a type 2 copper center with a relatively low redox potential. Thus, it may have a catalytic rather than electron transport function. Enzymes such as nitrite reductase (*45*) or ceruloplasmin (*46*) have an electron transport type 1 copper center in a cupredoxin-like fold and a type 2 copper site involved in catalysis in a separate region of the protein. NC represents the first example of a cupredoxin fold in which the copper ligands generate a type 2 copper center.

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