The Copper Chaperone NosL Forms a Heterometal Site for Cu Delivery to Nitrous Oxide Reductase

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Abstract: The final step of denitrification is the reduction of nitrous oxide (N\textsubscript{2}O) to N\textsubscript{2}, mediated by Cu-dependent nitrous oxide reductase (N\textsubscript{2}OR). Its metal centers, Cu\textsubscript{A} and Cu\textsubscript{B}, are assembled through sequential provision of twelve Cu\textsuperscript{2+} ions by a metallochaperone that forms part of a nos gene cluster encoding the enzyme and its accessory factors. The chaperone is the nosL gene product, an 18 kDa lipoprotein predicted to reside in the outer membrane of Gram-negative bacteria. In order to better understand the assembly of N\textsubscript{2}OR, we have produced NosL from Shewanella denitrificans and determined the structure of the metal-loaded chaperone by X-ray crystallography. The protein assembled a heterodinuclear metal site consisting of Zn\textsuperscript{II} and Cu\textsuperscript{II}, as evidenced by anomalous X-ray scattering. While only Cu\textsuperscript{2+} is delivered to the enzyme, the stabilizing presence of Zn\textsuperscript{II} is essential for the functionality and structural integrity of the chaperone.

Introduction

Nitrous oxide reductase (N\textsubscript{2}OR) is the terminal oxidoreductase in microbial denitrification, where nitrogen derived from biomass is recycled back into the atmosphere\textsuperscript{[1]} It mediates the reduction of nitrous oxide (N\textsubscript{2}O) to dinitrogen gas (N\textsubscript{2}) and water.\textsuperscript{[1]} Although highly exergonic at \(\Delta G^\circ = -339.5 \text{kJmol}^{-1}\), this 2-electron reduction is challenging due to the chemical inertness of nitrous oxide that manifests in an activation energy barrier of 250 kJmol\textsuperscript{-1}. N\textsubscript{2}OR is a two-domain protein that utilizes two multinuclear copper centers (Figure 1A).\textsuperscript{[2] In a C-terminal cupredoxin-type domain the enzyme binds the binuclear copper center Cu\textsubscript{A}, that is very similar to the site of the same name in cytochrome \(c\) oxidase.\textsuperscript{[3]} Its oxidized state is a mixed-valent [Cu\textsuperscript{I+}Cu\textsuperscript{II}], and it is liganded by two histidine residues, two cysteines, one methionine and a backbone carbonyl.\textsuperscript{[4]} Cu\textsubscript{A} accepts and transfers a single electron, with a midpoint potential of +260 mV for the enzyme from \textit{Pseudomonas stutzeri}.\textsuperscript{[5]} The second metal site, Cu\textsubscript{B}, is a unique [4Cu:2S] cluster coordinated by 7 histidine residues in the hub of a seven-bladed \(\beta\)-propeller domain.\textsuperscript{[6]} Cu\textsubscript{B} binds and activates N\textsubscript{2}O for reduction during catalysis (Figure 1A).\textsuperscript{[7]} In the 130 kDa periplasmatic metalloprotein N\textsubscript{2}OR, two chains form a tight head-to-tail homodimer that closely aligns the Cu\textsubscript{A} site of one monomer to the Cu\textsubscript{B} cluster of the other, creating the complex active site of the enzyme at and around the dimer interface (Figure 1A).\textsuperscript{[8]} Although apo-N\textsubscript{2}OR is exported from the cytoplasm in a folded state via the Tat system, the assembly of both metal sites occurs exclusively in the periplasm. It requires an intricate machinery of accessory transporters, reductases and chaperones around the cytoplasmic membrane and in the periplasmic space that afford a comprehensive assembly line, preventing undesired side reactions and assuring the specific delivery of all required components to their appropriate physiological targets.\textsuperscript{[9]}
In the denitrifying Gammaproteobacterium *Shewanella denitrificans* N,OR is encoded by the nosZ gene that forms part of a nos gene cluster with the canonical structure nosRZDFYL (Figure 1B).[^9] NosR, a polytopic transmembrane iron-sulfur flavoprotein, is the physiological electron donor for N₂O reduction.[^8] The open reading frames nosDFY, located downstream of nosZ, the structural gene for N,OR, encode a multi-subunit ABC-type transporter that is strictly required for the formation of Cux₂.[[^9] Herein, NosF is the cytoplasmic ATPase, NosY forms the integral membrane domain of the transporter with six transmembrane helices and NosD was suggested to be an associated carrier protein located in the periplasm.[[^19,10] NosL, the subsequent gene in the cluster, is present in most genomes of denitrifying organisms, including those in which the relative order of the nosDFY genes is not conserved, suggesting an important role in N,OR assembly.[[^19,90] NosL is a lipoprotein of 18 kDa that was proposed to be anchored to the inner leaflet of the outer membrane of the Gram-negative bacterium via a conserved N-terminal cysteine that gets lipidated after translocation through the inner membrane.[[^11] As NosD is presumably anchored in the cytoplasmic membrane via the ABC transporter NosFY, this raises the question how copper-bound to NosL crosses the periplasm to reach NosD.

NosL from *Achromobacter cycloclastes* specifically and stochiometrically binds Cux, supporting its involvement in copper delivery to N,OR as a metallochaperone.[[^12] The NMR solution structure of an apo form of NosL from A. *cycloclastes* heterologously produced in *E. coli* revealed a close structural homology to the organomercury lyase MerB and implicated one of the conserved residues, Met109, in metal binding. However, the entire N-terminal region of apo-NosL was disordered in the NMR structure, and while this was in line with NosL acting in copper delivery during N,OR maturation,[[^14] little was learned about the site and mode of metal binding. In *P. stutzeri*, the Tns5-mediated disruption of the C-terminus of NosL had no effect on N,OR synthesis.[[^13] Furthermore, the expression of the nosZDFY genes from *P. stutzeri* in *Pseudomonas putida*, a related but non-denitrifying organism, was sufficient for the production of catalytically competent N,OR, confirming that NosL is not indispensable for Cux₂ or Cux₄ biosynthesis or—more likely—that there is a functional backup system present in these particular hosts.[[^9,14] A further caveat may be that copper was added to the growth media in these studies, so that a dedicated chaperone may still be essential under conditions of metal limitation.

The complex role of NosL was further highlighted in recent studies on different denitrifiers. NosL of *Paracoccus denitrificans*, for instance, binds a single Cux₁ per protein with attomolar affinity and is specifically required for Cux₂ center assembly in N,OR.[[^14] More recently, we reported the recombinant production of active *P. stutzeri* holo-N,OR in *Escherichia coli*, where we found the ABC transporter NosFY and the accessory NosD protein to be required for the formation of the catalytic Cux₂ site, but not Cux₄.[[^25] In this study, however, the enzyme from *Marinobacter hydrocarbonoclasticus* could not be matured at all, independent of nosL coexpression, while the Cux₄ site of *S. denitrificans* was correctly assembled only if *S. denitrificans* NosL was present.[[^25] We therefore chose *S. denitrificans* as a source organism for the present study, so as to assure that the Cux₄ site in NosZ can only be formed if a functional NosL protein is coproduced.

### Results and Discussion

To facilitate the isolation and crystallization of NosL we generated a soluble version with a cleavable leader peptide and a C-terminal Streptag(II) that lacked the lipidation site, residue Cys38, of the wild type protein. *S. denitrificans* NosL (SdNosL) was produced recombinantly in *E. coli* and was isolated as a monomer (Figure S1). The pure protein showed no UV/vis absorption features beyond 280 nm, but although the medium was not further supplemented with metals, an ICP-MS analysis revealed 0.56 Zn²⁺ ions per NosL monomer, but only 0.02 Cux. From here, the holoprotein could be reconstituted in vitro by providing Cux under anoxic conditions.

Cux-reconstituted SdNosL crystallized in the cubic space group F432 with one monomer per asymmetric unit, allowing for the determination of its structure to 1.85 Å resolution by the single-wavelength anomalous dispersion method (Figure 2A, Table S2). In good agreement with the apo-NosL
structure of *A. cycloclastes* that aligned to our model with a root-mean-squared deviation of 2.3 Å for all atoms, the overall fold of *SdNosL* consists of two structurally similar, independent domains of four and three antiparallel β-sheets and a mixed β/α topology with a characteristic ββαβ motif in each domain (Figure 2B).[11a] In contrast to the NMR solution structure, however, the N-terminal part of *SdNosL* was well-ordered, likely due to the coordination of two metal ions close to the surface of the protein.

In the dinuclear metal site of *NosL*, both metal ions adopted a distorted tetrahedral geometry (Figure 2C). With five sulfur-containing residues in a total of seven amino acids ligands, the site appeared quite unlike any other known biological metal center, and the identification of the nature of the metal cations was of obvious importance (Figure 3A). We collected three diffraction data sets around the X-ray absorption K-edges of Cu and Zn (Figure 3B), at energies of (i) 8958 eV, (ii) 8998 eV and (iii) 9678 eV. From these, anomalous double difference (∆∆\textsubscript{anom}) electron density maps were calculated for Zn ((iii)–(ii)) and Cu ((ii)–(i)). These maps allowed for the unambiguous identification of the more buried metal as zinc (Figure 3C), while the one at the surface of *NosL* was identified as copper (Figure 3D). Importantly, this analysis also revealed that the population of both sides is unique, with each species fully restricted to its designated position.

Despite the presence of molecular oxygen during crystallization the crystals remained colorless (Figure S1), indicating a reduced state of the copper atom and a resistance to oxidation upon exposure to air. The Cu-Zn distance was 3.4 Å, and most of the coordinating ligands belonged to the N-terminal domain that was disordered in the apo-*NosL* structure: Cys59 is a ligand to Zn\textsuperscript{II}, Met64 coordinates Cu\textsuperscript{I} and Cys62 is μ\textsubscript{2}-bridging both metals (Figure 2C). Cys88 and Asp92, also part of the N-terminal region, coordinate zinc, and the hard carboxylate of Asp92 as a ligand strongly disfavors the coordination of a second, soft Cu\textsuperscript{II} ion at this position. The environment of copper, however, consists exclusively of soft sulfur ligands that will favor Cu\textsuperscript{II} over the harder Cu\textsuperscript{II}, and in addition the more flexible methionine sidechains of residues M64 and M145 will facilitate uptake and release of copper, while zinc is held tightly in place.

The only ligand originating from the C-terminal part of *NosL* is Met145, and the C-terminal loop where it is located seems to undergo a major conformational rearrangement upon Cu binding, as indicated by a superposition of the crystal structure of *S. denitrificans* holo-*NosL* with an ensemble of the 20 lowest energy NMR structures of *A. cycloclastes* apo-*NosL* (Figure 2B).[11a] Only in one of these 20 states the loop in question pointed towards the Zn,Cu binding site, revealing a possible transition state on the way from the apo- to the holo-form, where Zn\textsuperscript{II} is already bound and the repositioning of Met109 readies the system for Cu\textsuperscript{II} capture (Figure S2). In addition, the heterobimetallic site in holo-*NosL* was coordinated by a seventh ligand, but this His104 derived from another *NosL* monomer within the crystal, leading to the formation of a homotrimer in *crystallo* (Figure 2C, Figure S3). This histidine ligand shows a limited degree of conservation among *NosL* proteins of other *N*\textsubscript{2}\textsubscript{O}-reducing organisms (Figure S4), while the other Cu-binding residues, Cys62, Met64 and Met145, are strictly conserved among fifteen analyzed *NosL* homologs. Note that not all of these form part of a canonical *nos* cluster, indicating the protein to be a more widely used type of metallochaperone. It cannot be ruled out that the observed coordination of His104 to the Cu\textsuperscript{II} ion is only induced by packing interactions within the crystal lattice, and we therefore conducted a geometry optimization of the heterobimetallic binding site of *NosL* in the absence of the external ligand. Interestingly, this led to an inversion of the tetrahedral geometry of the Cu ion and its retraction into the chaperone, creating a more symmetric dinuclear site with both Cys62 and Cys88 serving as bridging ligands (Figure 4A). Although only a model, this structure suggests a chemically reasonable metal-loaded resting state for a monomeric NosL-type Cu chaperone. Five of the analyzed organisms retain Cys59, Cys88 and Asp(Glu)92. Among these exclusively Zn-binding ligands, Cys59 is the most highly conserved, while the positions of Cys88 and Asp92 are swapped in the
NosL proteins from three of the analyzed N₂O-reducers that belong to clade II (Figure S4).

For zinc-containing proteins, the first coordination sphere of the metal ion typically reflects its function as either a structural, catalytic or regulatory moiety. Accordingly, the cysteine-rich coordination sphere of Zn\textsuperscript{II} and its buried position in holo-NosL suggest a predominantly structural role, possibly by organizing and stabilizing the N-terminus of the protein, providing a stable yet flexible environment for the reversible uptake of Cu\textsuperscript{I} mediated by the strictly conserved Cys62 and Met64 located in this region (Figure S4). Hereby, zinc can stabilize the protein structure and prevent intramolecular disulfide conformation, as was suggested for the Zn-dependent sulfurylase SulU from Bacillus subtilis that exhibits the same 3Cys:1Asp Zn\textsuperscript{II} coordination as found for holo-NosL\textsuperscript{[17]}. An additional role may be in preventing the unspecific binding of zinc to N-OR or other proteins by scavenging the metal ions. As recombinant *S. denitrificans* N₂OR produced in *E. coli* only contained copper in its Cu\textsubscript{A} site when SmNosL was coproduced,\textsuperscript{[15]} we proceeded to generate the Zn\textsuperscript{II} ligand variants C59G\textsuperscript{A}, C88A\textsuperscript{L} and D92A\textsuperscript{L}, as well as a C88D/D92C\textsuperscript{L} swap to mimic the situation in clade II N₂OR, in order to assess the relevance of Zn\textsuperscript{II} for the copper-donating role of NosL. Indeed, the coproduction of each of these mutants with *S. denitrificans* N₂OR resulted in a decreased Cu content of the enzyme as determined by ICP-MS (Figure S5J). In addition, the correct assembly of the Cu\textsubscript{A} site of N₂OR was impaired, as only very little Cu\textsubscript{A} signal was observed after oxidation with ferricyanide (Figure S5A–D), indicating that both residues—and hence the ability to bind Zn\textsuperscript{II}—are essential for copper trafficking by NosL. Individual replacement of the conserved, sulfur-containing Cu ligands of NosL (C62A\textsuperscript{L}, M64A\textsuperscript{A}), M145A\textsuperscript{L}) consistently resulted in apo-N₂OR when coproduced and isolated (Figure S5E–G). This confirmed the importance of the three strictly conserved ligands to copper and their role in copper transfer from NosL to N₂OR. In contrast, the swap of C88G and D92L did not yield a functional NosL, suggesting that the properties of clade II NosL proteins also must differ in other aspects (Figure S5I). The coproduction of H104A\textsuperscript{L}, on the other hand, resulted in a correctly assembled Cu\textsubscript{A} site in N₂OR, albeit with a further decreased copper content (Figure S5H), indicating that His104, which is responsible for the trimer formation observed in the crystals of *S. denitrificans* NosL (see Supplementary Discussion), is not essential for the uptake and transfer of copper. This is underlined by the lack of conservation of His 104 among NosL orthologs. It may, however, replace a physiological histidine ligand supplied by the interaction partner of NosL, likely apo-N₂OR (NosZ) and/or the NosDFY system that is required for Cu\textsubscript{Z} formation. In this way, the coordination of Cu by His104 (Figure 2A), together with the modeled structure of a putative metal-loaded state (Figure 4A) outline the first two steps of the mobilization of Cu from the chaperone when the metal ion is eventually transferred to the apo-enzyme (Figure 4B).

**Conclusion**

Our analysis of the metal-replete state of the copper chaperone NosL revealed the unexpected integration of a Cu\textsuperscript{I} ion into a heterobimetallic binding site together with Zn\textsuperscript{II} that presumably plays a predominantly structural role. In its apo form, the N-terminus of NosL is disordered, and the association of a zinc ion that is bound with high affinity is required to pre-form a reversible binding site for copper that must be of lower affinity to facilitate the transition of the metal from and to its interaction partners. According to its character, Cu\textsuperscript{I} is bound via soft sulfur ligands, including cysteines that may be prone to forming disulfide bonds upon oxidation. Avoiding this may well be a secondary function of the coordinated zinc. The serendipitous interaction of the heterobimetallic site with a non-conserved histidine residue from a neighboring monomer within the crystal lattice is highly suggestive of a possible pathway for copper delivery to an interaction partner, and we propose that in the absence of a suitable ligand the Zn,Cu site is likely to attain a more symmetric conformation that represents a metal-loaded resting state. It remains to be determined whether NosL will functionally interact with NosZ or NosDFY or even both. As NosL is predicted to be a lipoprotein residing in the inner leaflet of the outer membrane, an interaction with the soluble NosZ protein is well conceivable, while NosD seems to be permanently anchored to the cytoplasmic membrane. It remains to be clarified if and how an interaction of NosL and NosD can be achieved or whether the assembly steps for Cu\textsubscript{A} (via NosL) and Cu\textsubscript{Z} (via NosD) are spatially separated.
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Conflict of Interest

The authors declare no conflict of interest.

Keywords: copper chaperone · denitrification · metalloenzyme biogenesis · nitrous oxide reductase · X-ray crystallography

[1] a) S. R. Pauleta, S. Dell’Acqua, I. Moura, Coord. Chem. Rev. 2013, 257, 332–349; b) W. G. Zumft, P. M. H. Kroneck, Adv. Microb. Physiol. 2006, 52, 107–225.
[2] A. Pomowski, W. G. Zumft, P. M. H. Kroneck, O. Einsle, Nature 2011, 477, 234–237.
[3] a) P. M. H. Kroneck, W. A. Antholine, J. Riester, W. G. Zumft, FEBS Lett. 1989, 248, 212–213; b) P. M. H. Kroneck, W. E. Antholine, D. H. W. Kastrau, G. Buse, G. C. M. Steffens, W. G. Zumft, FEBS Lett. 1990, 268, 274–276.
[4] J. A. Farrar, F. Neese, P. Lappalainen, P. M. H. Kroneck, M. Saraste, W. G. Zumft, A. J. Thompson, J. Am. Chem. Soc. 1996, 118, 11501–11514.
[5] a) J. Riester, W. G. Zumft, P. M. H. Kroneck, Eur. J. Biochem. 1989, 178, 751–762; b) C. L. Coyle, W. G. Zumft, P. M. H. Kroneck, H. Körner, W. Jakob, Eur. J. Biochem. 1985, 153, 459–467.
[6] A. Wüst, L. Schneider, A. Pomowski, W. G. Zumft, P. M. H. Kroneck, O. Einsle, Biol. Chem. 2012, 393, 1067–1077.
[7] L. Zhang, C. Trnecík, S. L. Andrade, O. Einsle, Biochim Biophys Acta 2017, 1858, 95–102.
[8] P. Wunsch, W. G. Zumft, J. Bacteriol. 2005, 187, 1992–2001.
[9] a) E. I. Solomon, D. E. Heppner, E. M. Johnston, J. W. Ginsbach, J. Cirera, M. Qayyum, M. T. Kieber-Emmons, C. H. Kjaergaard, R. G. Hadt, L. Tian, Chem. Rev. 2014, 114, 3659–3853; b) P. Wunsch, M. Herb, H. Wieland, U. M. Schick, W. G. Zumft, J. Bacteriol. 2003, 185, 887–896.
[10] U. Honisch, W. G. Zumft, J. Bacteriol. 2003, 185, 1895–1902.
[11] a) L. M. Taubner, M. A. McGuirl, D. M. Dooley, V. Copie, Biochemistry 2006, 45, 12240–12252; b) S. Narita, S. Matsuyama, H. Tokuda, Arch. Microbiol. 2004, 182, 1–6.
[12] M. A. McGuirl, J. A. Bollinger, N. Cospic, R. A. Scott, D. M. Dooley, J. Biol. Inorg. Chem. 2001, 6, 189–195.
[13] A. Dreusch, J. Riester, P. M. H. Kroneck, W. G. Zumft, Eur. J. Biochem. 1996, 237, 447–453.
[14] S. P. Bennett, M. J. Soriano-Laguna, J. Bradley, D. A. Svistunenko, D. J. Richardson, A. J. Gates, N. E. Le Brun, Chem. Sci. 2019, 10, 4985–4993.
[15] L. Zhang, A. Wust, B. Prasser, C. Müller, O. Einsle, Proc. Natl. Acad. Sci. USA 2019, 116, 12822–12827.
[16] a) I. L. Alberts, K. Nadassy, S. J. Wodak, Protein Sci. 1998, 7, 1700–1716; b) W. Marek, Adv. Nutr. 2013, 4, 82–91.
[17] B. P. Selbach, A. H. Chung, A. D. Scott, S. J. George, S. P. Cramer, P. C. Dos Santos, Biochemistry 2014, 53, 152–160.