A [3Cu:2S] cluster provides insight into the assembly and function of the CuZ site of nitrous oxide reductase†

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Nitrous oxide reductase (N₂OR) is the only known enzyme reducing environmentally critical nitrous oxide (N₂O) to dinitrogen (N₂) as the final step of bacterial denitrification. The assembly process of its unique catalytic [4Cu:2S] cluster CuZ remains scarcely understood. Here we report on a mutagenesis study of all seven histidine ligands coordinating this copper center, followed by spectroscopic and structural characterization and based on an established, functional expression system for Pseudomonas stutzeri N₂OR in Escherichia coli. While no copper ion was found in the CuZ binding site of variants H129A, H130A, H178A, H326A, H433A and H494A, the H382A variant carried a catalytically inactive [3Cu:2S] center, in which one sulfur ligand, S22, had relocated to form a weak hydrogen bond to the sidechain of the nearby lysine residue K454. This link provides sufficient stability to avoid the loss of the sulfide anion. The UV-vis spectra of this cluster are strikingly similar to those of the active enzyme, implying that the flexibility of S22 may have been observed before, but not recognized. The sulfide shift changes the metal coordination in CuZ and is thus of high mechanistic interest.

Introduction

Nitrous oxide (N₂O) is an inert, odorless and non-toxic gas that nevertheless acts as a greenhouse agent with a global warming potential exceeding that of carbon dioxide (CO₂) by a factor of 290.

Nitrous oxide reductase (N₂OR) is the only known enzyme reducing environmentally critical nitrous oxide (N₂O) to dinitrogen (N₂) as the final step of bacterial denitrification. The assembly process of its unique catalytic [4Cu:2S] cluster CuZ remains scarcely understood. Here we report on a mutagenesis study of all seven histidine ligands coordinating this copper center, followed by spectroscopic and structural characterization and based on an established, functional expression system for Pseudomonas stutzeri N₂OR in Escherichia coli. While no copper ion was found in the CuZ binding site of variants H129A, H130A, H178A, H326A, H433A and H494A, the H382A variant carried a catalytically inactive [3Cu:2S] center, in which one sulfur ligand, S22, had relocated to form a weak hydrogen bond to the sidechain of the nearby lysine residue K454. This link provides sufficient stability to avoid the loss of the sulfide anion. The UV-vis spectra of this cluster are strikingly similar to those of the active enzyme, implying that the flexibility of S22 may have been observed before, but not recognized. The sulfide shift changes the metal coordination in CuZ and is thus of high mechanistic interest.

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binds Cu⁺ and delivers it to apo-N₂OR,²² and a recent study has suggested its role to be in particular for Cu₂ site assembly.²³

In addition, the ABC transporter NosFY in conjunction with the periplasmic NosD protein is required to provide a sulfur species to the periplasm in order to complete Cu₂ maturation.¹⁷ We recently established a recombinant system that included all essential genes nosRZDFYDX for N₂OR production, able to generate functional enzyme containing both Cu₆ and Cu₂ sites in E. coli,²⁴ facilitating mutagenesis studies of the key residues coordinating the copper sites.

Results and discussion

Properties of the seven histidine variants of Cu₂

In the present study we focus on understanding the role of the seven ligands of the Cu₂ site, namely H129, H130, H178, H326, H382, H433 and H494 of P. stutzeri N₂OR (PsN₂OR). All histidine residues were individually mutated to alanine. The corresponding variants were purified (Fig. S1†), followed by spectroscopic characterization and the determination of three-dimensional structures by X-ray crystallography (Table S1†).

Not unexpectedly, six of the seven variants of the coordinating histidine residues, namely H129A, H130A, H178A, H326A, H433A and H494A only showed spectral properties confirming the presence of the Cu₄ site (Fig. S2†), with absorption maxima at 485 nm, 525 nm and 795 nm, although the relative occupancy of Cu₄ was found to differ (Table S2†). However, the H382A variant showed UV-vis spectra that were nearly identical to those of wild-type N₂OR (Fig. 1). The oxically isolated H382A was of blue colour, with partially oxidized copper sites as indicated by comparing the spectra of sample as isolated (Fig. 1D, blue) and ascorbate-reduced (Fig. 1E, blue).

After oxidation with ferricyanide the protein underwent a colour change to purple, with absorption maxima at 533 nm and 780 nm and two prominent shoulders at 485 nm and 625 nm (Fig. 1D, purple). These features do not merely reflect a mixed-valent Cu₄ site, but correspond to the properties of a wild-type form I N₂OR (Fig. 1A)²⁶,²⁶ except for the more pronounced shoulder peak at 625 nm. The selective reduction of Cu₄ by ascorbate resulted in two distinct bands at 535 nm and 625 nm (Fig. 1E, blue). We have earlier described this two-peak spectrum of Cu₄ as a signature of the [4Cu₂:2S] form I, with the 625 nm maximum originating from S₂₁ and the second peak, found at 562 nm in wild-type N₂OR, as originating from S₂₂.²⁶ Although a difference assignment attributing this transition to also originate from S₂₂ was made by other.²₆ The spectrum of H382A N₂OR thus indicated that – unlike in Cu₂⁻ sulfur S₂₂ was still present, but the shifted peak indicated a change in its chemical environment. Also, the intensity ratio of the two Cu₂ bands indicated an incomplete occupancy for S₂₂. Intriguingly, further reduction with dithionite did not lead to a loss of either band (Fig. 1F, cyan), indicating the “Cu₂” site in H382A was redox-inert. In wild-type N₂OR, dithionite reduction leads to a single charge transfer peak at 650 nm (Fig. 1C), which was assigned to the reduction of Cu₂ from a [2Cu⁺:2Cu²⁺] to a [3Cu⁺:1Cu²⁺] form.²⁴,²⁷ We then determined the N₂O-reducing activity using reduced benzyl viologen as electron donor. H382A was not active in N₂O reduction (Fig. S3F†). We also determined the specific activities for the other six variants, and while H129A, H130A, H326A, H433A and H494A were completely inactive as expected (Fig. S3†), H178A showed low activity, with a decrease in vₘₐₓ to 0.03 ± 0.01 μmol N₂O per min per mg, approximately 50-fold lower than that of wild-type enzyme,²⁴ and Kₘ (N₂O) of 268 ± 24 μM (Fig. S3D†). This residual activity (validated by 3 replicates) might be originated from a small portion of H178A containing a functional Cu₄ site, but the overall occupancy of possibly only 2% given the 50-fold lower activity was too low to be observed in the UV-vis spectra and crystal structure (vide infra).

We further proceeded to crystallize all variants and determined their three-dimensional structures to resolutions ranging from 1.67 Å to 1.49 Å (Table S3 and Fig. S4–S11†). As expected, the overall fold and dimeric structure of all variants remained unchanged,²⁴ with root-mean-squared deviations for all atoms from wild-type N₂OR at 0.34 Å (H129A), 0.28 Å (H130A), 0.14 Å (H178A), 0.48 Å (H326A), 0.11 Å (H382A), 0.32 Å (H433A), and 0.23 Å (H494A). Overall, the major structural difference was that the Ca²⁺-binding loop (N257–D273) was disordered in some of variants. This disorder seemed to correlate with the occupancy of the Cu₄ site (Tables 1 and S2†), in line with an early report that the presence of Ca²⁺ ions was required for a stable insertion of the center.¹¹

Although at different occupancies, the Cu₄ site was present in all seven-variants (Table S2 and Fig. S4–S11†). The Cu₄ ligands C618, W620, C622, H626, and M629 were in place to coordinate two copper ions, but the remaining ligand, H583, was in one of two possible conformations (e.g. Fig. S4†). In a ‘bound’ conformation, the N₆ atom of the imidazole moiety coordinated Cu₄ at a distance of 2.5 Å, and the N₆ atom formed

Fig. 1  UV-vis spectra of WT PsN₂OR (A–C) and variant H382A (D–F). As isolated, H382A (D) showed two absorption peaks at 535 nm and 625 nm (blue); upon oxidation with ferricyanide the spectra (purple) were similar to the wild type form I N₂OR (A). (B and E) Selective reduction of Cu₄ with ascorbate yielded a two-peak spectrum indicative of a [4Cu₂:2S] Cu₂ site (blue). Upon extended reduction with dithionite, the typical loss of the 535 nm band seen for WT PsN₂OR (C) was not observed in the H382A (F) variant (cyan).
a short hydrogen bond (2.7 Å) to residue D576 (Fig. S4A†). This is a state for Cu4,28 that is most commonly observed and was also found in N2OR of *M. hydrocarbonoclasticus,*27 *P. denitrificans,*29 and *A. cyclolastes,*29 as well as in cytochrome c oxidases (PDB ID: 2CUA).29 In the second, ‘unbound’ state, however, the imidazole group of H583 was rotated away from CuA by approximately 135°, so that the N3 atom now formed a hydrogen bond to residue S550, while the H-bond between N1 and D576 remained unchanged (Fig. S4B†). This histidine flip at CuA was previously reported for *P. stutzeri* N2OR,29 and we proposed a role in gating electron transfer from an external redox partner to CuA.29 In our previous study, H583 showed partial ligation of CuA in either form I and II of recombinant *PsN2OR.*24 However, the conformational switch of H583 was randomly distributed among the variants (Table 1). Geometry changes of CuA were also observed between the two conformations of H583. The major difference was that the bond lengths of CuA to the sulfur atoms of C618 and C622 were about 0.1 Å longer when H583 was not a ligand (Table S4†).

The UV-vis spectra of the six variants H129A, H130A, H178A, H326A, H433A and H494A lacked the S → Cu charge transfer bands typically associated with the Cu2 center (Fig. S2†). This is primarily indicative of an absence of sulfide, but the presence of histidine-coordinated Cu alone should also lead to similar N → Cu CT bands, albeit with lower intensity. Only the H326A variant might show such a peak at 630 nm (Fig. S2D†), while the other five gave no indication for the presence of Cu. Structural analysis revealed that in the variants H130A, H178A and H433A the Cu2 site was only occupied by water molecules (Fig. S5, S6 and S10†). In the H129A, H326A and H494A variants, one or two zinc ions were instead found at the binding site for Cu2 (Fig. S4, S7 and S11†), as confirmed by anomalous scattering data collected at the X-ray absorption K-edge of Zn (9700 eV). The presence of Zn2+ in the Cu2 site can be rationalized by non-specific incorporation via a periplasmic zinc chaperone such as ZinT30 or ZraP,31,32 and the presence of multiple histidines, which are suitable ligands for zinc.33 The metal might occupy this site if the regular Cu2 maturation pathway is dysfunctional. Note that in no case either a single or two Cu ions were observed. Since the maturation factors NosDFYL were present for the production of all N2OR variants in *E. coli,* copper (and sulfide) delivery should have been possible. The complete lack of Cu thus either means that each of the six histidines mutated here is essential for assembly, or that any site assembled without the full complement of ligands is unstable and the metal is quickly lost.

### Table 1 Structural information on WT *PsN2OR* and the seven histidine variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Side</th>
<th>Ca2⁺-binding loop</th>
<th>Cu2</th>
<th>CuA-His583</th>
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<tr>
<td>WT</td>
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<td>[4Cu:2S]</td>
<td>Unbound 3.74 Å</td>
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<td>Unbound 3.44 Å</td>
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<td>A</td>
<td>Ordered</td>
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<td>Bound 2.51 Å</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Disordered</td>
<td>1 Zn²⁺</td>
<td>Unbound 3.46 Å</td>
</tr>
<tr>
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<td>Empty</td>
<td>Unbound 3.49 Å</td>
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<tr>
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</tr>
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<td>A</td>
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</tr>
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<tr>
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<td>Bound 3.85 Å</td>
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<tr>
<td></td>
<td>B</td>
<td>Disordered</td>
<td>2 Zn²⁺</td>
<td>Bound 2.82 Å</td>
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</table>

* Side A shows CuA of chain A, Ca²⁺-binding loop and Cu2 of chain B, which together form one active site; side B means the other way around. CuA is present in all seven variants. ‘Bound’ indicates H583 is a ligand of CuA, while ‘unbound’ means it is turned away. The given distances are between CuA and S550; see Table S4 for more details. PDB accession code 6RL0. Chain A of H382A shows weak density for Cu1 in the Cu2 site. See Fig. S9B for more details.

The unexpected UV-vis spectra (Fig. 1D) were reflected in an unprecedented, only partially assembled [3Cu:3-S:3-S] cluster at the Cu2 site (Fig. 2B). In this cluster, Cu1 was absent, leaving S2‡ as a μ2-bridging sulfide ligating the remaining three copper ions. The histidine coordination of these was identical to native Cu2, in that Cu2 was coordinated by H129 and H178, Cu1 by H130 and H433, and Cu4 by H494. Furthermore, the second sulfide, S2, was still present as a ligand to Cu4, in spite of the absence of Cu1. It shifted position towards the nearby lysine K454, forming a hydrogen bond that stabilized the [3Cu:2S] cluster (Fig. 3B). As a consequence, the S2-Cu4-S2 bond angle in the [3Cu:2S] site increased by approximately 26° with respect to the one in the native, [4Cu:2S] Cu2. In contrast, the changes to the individual bond lengths in the cluster were insignificant (Fig. 3C).

The X-band EPR spectra for both WT N2OR (Fig. 4A) and H382A variant (Fig. 4D) showed a similar 7-line hyperfine splitting pattern in the *g* region originating from the mixed-valent [Cu1.5⁺:Cu1.5⁺] state of oxidized CuA, although the
CuZ, the two oxidized coppers couple antiferromagnetically, with a total spin of $S = 0.7$. The EPR signal shown in Fig. 4B was derived from residual $[3\text{Cu}^+\text{Cu}^{2+}]$ CuZ that was described to have lost sulfide $S_{\text{Z2}}$. Interestingly, EPR spectra of both ascorbate- and dithionite-reduced H382A showed a 4-line hyperfine splitting pattern in the $g_3$ region ($g_3 = 2.18, A_3 = 66$ G) (Fig. 4E and F), indicating the presence of a Cu(II) ion ($S = 1/2$) with its ground state in a $3d_{x^2-y^2}$-derived molecular orbital, and consistent with that of typical mononuclear type 1 (T1) copper as found in plastocyanin, azurin, and cucumber basic protein, as well as copper-containing nitrite reductase (NitR), especially from fungal laccase and Fet3p, where Cu(II) is trigonal-planar coordinated by one cysteine and two histidine residues.

Reduction with dithionite (Fig. 4F) reduced the signal intensity to about 1/3 of that for the ascorbate-reduced sample (Fig. 4E), and also destabilized the cluster, as shown by the broad peak in the $g = 2.3-2.4$ region (Fig. S12†). Therefore, the $[3\text{Cu}:2\text{S}]$ cluster is very likely in a $[2\text{Cu}^+:\text{Cu}^{2+}:2\text{S}^2]$ state, with CuZ (Fig. 3B) as Cu(II), which is trigonally coordinated in the $S(\mu_3-S)$–N(His494)–S(μ3-S) plane.

Residue H326, the second ligand to Cu1 in the CuZ site (Fig. 3A), did not coordinate a metal in the $[3\text{Cu}:2\text{S}]$ cluster (Fig. 3B). Thus, we expected the $[3\text{Cu}:2\text{S}]$ cluster to be present in variant H326A as well. However, the H326A only contained a single Zn2+ (Fig. S7†), indicating the stabilizing effect of H326 and H382 to the CuZ site differs. We hypothesize that H326 is required already in the early stages of CuZ maturation, so that the site will not assemble if this histidine residue is mutated. H326, in contrast, only seems come into play once the entire cluster is assembled. Our data do not reveal whether CuZ is initially complete as a $[4\text{Cu}:2\text{S}]$ cluster that is then prone to lose Cu1 in the absence of the support by H382. Residue K454 (K397 in the case of N2OR from P. stutzeri (PDB code: 3SBQ) shows hydrogen-bonding interactions (Fig. 3B).

Beyond these questions regarding the assembly of CuZ in vivo, the $[3\text{Cu}:2\text{S}]$ cluster in PsN2OR H382A also has interesting functional implications. In the variant, the cluster retains sulfide $S_{\text{Z2}}$, but has it shifted towards residue K454, leading to UV/vis properties that fall between the forms I and II described earlier. Form II N2OR was proposed to contain a $[4\text{Cu}:2\text{S}]$ CuZ* center, requiring the loss of $S_{\text{Z2}}$ as a prerequisite for reductive activation. Nevertheless the $[4\text{Cu}:2\text{S}]$ CuZ state has been consistently isolated from cells grown under denitrifying conditions (i.e. after having turned over in vivo), and the H382A variant may now help to reconcile these seemingly incompatible results, suggesting that $S_{\text{Z2}}$ can indeed change its position from ligating Cu1 of CuZ to the nearby K454 (similar sulfur-shift mechanism was envisaged by Moura and Pauleta). This would leave both Cu1 and Cu4 with three remaining ligands, and thus with the opportunity to bind an additional

**Concluding remarks**

The EPR signal shown in Fig. 4B was derived from residual $[3\text{Cu}^+\text{Cu}^{2+}]$ CuZ that was described to have lost sulfide $S_{\text{Z2}}$. Interestingly, EPR spectra of both ascorbate- and dithionite-reduced H382A showed a 4-line hyperfine splitting pattern in the $g_3$ region ($g_3 = 2.18, A_3 = 66$ G) (Fig. 4E and F), indicating the presence of a Cu(II) ion ($S = 1/2$) with its ground state in a $3d_{x^2-y^2}$-derived molecular orbital, and consistent with that of typical mononuclear type 1 (T1) copper as found in plastocyanin, azurin, and cucumber basic protein. As well as copper-containing nitrite reductase (NitR), especially from fungal laccase and Fet3p, where Cu(II) is trigonally-planar coordinated by one cysteine and two histidine residues.

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Residue H326, the second ligand to Cu1 in the CuZ site (Fig. 3A), did not coordinate a metal in the $[3\text{Cu}:2\text{S}]$ cluster (Fig. 3B). Thus, we expected the $[3\text{Cu}:2\text{S}]$ cluster to be present in variant H326A as well. However, the H326A only contained a single Zn2+ (Fig. S7†), indicating the stabilizing effect of H326 and H382 to the CuZ site differs. We hypothesize that H326 is required already in the early stages of CuZ maturation, so that the site will not assemble if this histidine residue is mutated. H326, in contrast, only seems come into play once the entire cluster is assembled. Our data do not reveal whether CuZ is initially complete as a $[4\text{Cu}:2\text{S}]$ cluster that is then prone to lose Cu1 in the absence of the support by H382. Residue K454 (K397 in the case of N2OR from P. stutzeri (PDB code: 3SBQ) shows hydrogen-bonding interactions (Fig. 3B).

Beyond these questions regarding the assembly of CuZ in vivo, the $[3\text{Cu}:2\text{S}]$ cluster in PsN2OR H382A also has interesting functional implications. In the variant, the cluster retains sulfide $S_{\text{Z2}}$, but has it shifted towards residue K454, leading to UV/vis properties that fall between the forms I and II described earlier. Form II N2OR was proposed to contain a $[4\text{Cu}:2\text{S}]$ CuZ* center, requiring the loss of $S_{\text{Z2}}$ as a prerequisite for reductive activation. Nevertheless the $[4\text{Cu}:2\text{S}]$ CuZ state has been consistently isolated from cells grown under denitrifying conditions (i.e. after having turned over in vivo), and the H382A variant may now help to reconcile these seemingly incompatible results, suggesting that $S_{\text{Z2}}$ can indeed change its position from ligating Cu1 of CuZ to the nearby K454 (similar sulfur-shift mechanism was envisaged by Moura and Pauleta). This would leave both Cu1 and Cu4 with three remaining ligands, and thus with the opportunity to bind an additional
exogenous ligand, the substrate N₂O, in a 1,3-bridging fashion. This binding mode is similar to the one proposed by Moura and Solomon, but does not require dissociation of S₂₂ in accordance with our structural data. It would also imply a binding mode of N₂O that is very much compatible with the N₂O binding site we observed at Cu₂ after pressurizing crystals of the enzyme with the substrate gas.

In particular, the UV-vis properties of the H382A variant highlight the fact that preparations of the enzyme that were typically assigned to a ‘form II’, or Cu₂* species that implies a [4Cu:S] site may well be of a different nature. The loss of the charge transfer band at 550 nm that characterizes this form II may be rooted in the shift of S₂₂ towards K454, without being fully lost form the cluster. This finding is also in line with the frequent observation of a less well-defined, elongate electron density feature at the Cu¹–Cu₄ edge of the Cu₂ cluster in different published and unpublished structures. Such features were frequently interpreted as two H₂O ligands, or inspired an originally suggested binding mode for N₂O that was, however, never observed experimentally. The present data now offers an alternative rationalization for the reported spectroscopic features that may eventually lead to a unified picture of the structural and functional features of the unique and enigmatic Cu₂ site.

Conflicts of interest
There are no conflicts to declare.

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