The model structure of the copper-dependent ammonia monoxygenase

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Abstract
Ammonia monoxygenase is a copper-dependent membrane-bound enzyme that catalyzes the first step of nitrification in ammonia-oxidizing bacteria to convert ammonia to hydroxylamine, through the reductive insertion of a dioxygen-derived O atom in an N–H bond. This reaction is analogous to that carried out by particulate methane monoxygenase, which catalyzes the conversion of methane to methanol. The enzymatic activity of ammonia monoxygenase must be modulated to reduce the release of nitrogen-based soil nutrients for crop production into the atmosphere or underground waters, a phenomenon known to significantly decrease the efficiency of primary production as well as increase air and water pollution. The structure of ammonia monoxygenase is not available, rendering the rational design of enzyme inhibitors impossible. This study describes a successful attempt to build a structural model of ammonia monoxygenase, and its accessory proteins AmoD and AmoE, from *Nitrosomonas europaea*, taking advantage of the high sequence similarity with particulate methane monoxygenase and the homologous PmoD protein, for which crystal structures are instead available. The results obtained not only provide the structural details of the proteins ternary and quaternary structures, but also suggest a location for the copper-containing active site for both ammonia and methane monoxygenases, as well as support a proposed structure of a CuA-analogue dinuclear copper site in AmoD and PmoD.

Graphic abstract

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Extended author information available on the last page of the article
Introduction

It has been estimated that the world population will reach 9 billion by the year 2050 [1], and that to sustain the consequential food demand, a 70–100% expansion in global agricultural production will be needed [2]. Nitrogen (N) is an essential element for life on Earth [3] as well as a critical nutrient for agriculture and food production [4]; due to its tremendous importance on agriculture, soil nitrogen fertilization must thus be carried out to increase crop yield [5]. According to the Food and Agriculture Organization of the United Nations (FAO), the world nitrogen fertilizer demand is expected to increase continuously for the period between 2017 and 2022 [6], and only in the United States of America (USA), nitrogen fertilizers use has increased more than 40 times from 1950 to 2015 [7].

However, concerns exist about human impact on the global N cycle [8, 9] and novel N management approaches are essential for sustainable soil fertilization and crop productivity [10]. In particular, unlike phosphorus, N possesses high reactivity in the environment and is prone to significant losses, being leached to underground water or released to the atmosphere as a product of nitrification, denitrification, leaching, and volatilization [3, 7, 8, 10–12]. Indeed, nowadays, almost 60% of N₂O and ca. 23% of total global NOx emissions come from agriculture, and the continuous increase of food demand, resulting in an increased use of nitrogen fertilizers, will contribute even more to the nitrogen gases emission in the coming years [10].

According to a report of the International Fertilizer Association (IFA), around 60% of all nitrogen fertilizers in use are based on urea [CO(NH₂)₂] [13], a chemical that represents 55% of the whole market [14]. Upon deposition in soil, urea is rapidly hydrolyzed to ammonium (NH₄⁺) and bicarbonate (HCO₃⁻), a process catalyzed by the nickel-dependent enzyme urease (urea aminohydrolase, EC 3.5.1.5) [15, 16] commonly found in soils used for crop production [14] both as intra- and extra-cellular enzyme [17]. This hydrolysis causes a rapid pH increase in the medium that leads to the formation of gaseous ammonia (NH₃) and consequent N loss from soil.

The NH₄⁺ ion formed upon urea hydrolysis serves as a nutrient to plants [5] as well as for aerobic respiration conducted by specific microorganisms that carry out a nitrification process that leads to the formation of nitrate (NO₃⁻) via nitrite (NO₂⁻). This is a mutualistic symbiosis involving ammonia-oxidizing bacteria (AOB) and Archaea (AOA), which convert ammonia to nitrite [5], and nitrite-oxidizing bacteria (NOB) that convert nitrite to nitrate [18, 19]; the entire process can also be carried out directly by ammonia-oxidizing (Comammox) bacteria [20, 21]. Nitrate thus formed in these processes can either be taken up by plant roots or enter an anaerobic denitrification route [22], being converted back to nitrite by the Mo-dependent nitrate reductase (NAR); nitrite is then transformed to gaseous forms of N such as nitric oxide (NO), nitrous oxide (N₂O), and eventually dinitrogen (N₂) [23], while a large portion of nitrate is also eventually leached into groundwater [5].

As a consequence of these processes, as much as 50% of nitrogen fertilizer applied to soil is not used by crops and is lost to the environment, either as gaseous species (NH₃, NO, N₂O, N₂), some of which significantly contribute to the greenhouse effect [24] and the formation of air particulate matter [25], or as leached NO₃⁻, which is a source of eutrophication [26–28]. This loss represents a very significant economic and environmental cost to farmers specifically, and for society more generally. These considerations highlight the need for the development of efficient inhibitors of nitrification.

Currently, a handful of nitrification inhibitors are used in agricultural practice. In particular, dicyandiamide (DCD), 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin), and 3,4-dimethylpyrazole phosphate (DMPP) are most frequently used [5]. However, their mode of action is not known at the molecular level, and they are thought to act as chelators of the essential copper atom present in the active site of AMO, an unproven hypothesis, while other more potent inhibitors are known but not marketed for field applications [5]. Moreover, their efficacy to reduce nitrogen losses has been shown to be highly variable and depending on many environmental conditions [29, 30]. In any case, it is important to consider the environmental toxicity, the solubility as well as the concentrations required to modulate nitrification [31, 32]. For these reasons, the search for new inhibitors is necessary to increase the efficiency of soil nitrogen fertilization toward an environmentally sustainable agriculture.

The initial step of nitrification is the oxidation of NH₄⁺ to hydroxyl amine (NH₂OH), catalyzed by the copper-dependent ammonia monoxygenase (AMO); this step is followed by the formation of nitrite (NO₂⁻) catalyzed by the iron-dependent hydroxylamine oxidoreductase (HAO), and finally by the formation of nitrate (NO₃⁻), catalyzed by the molybdenum-dependent nitrite oxidoreductase (NIX) [5]. AMO, present both in AOA and AOB (comprising both β- and γ-proteobacteria) [33] as well as in Comammox bacteria [5, 19], is thus the key enzyme to focus on for the purpose of modulating the nitrification activity in soils. In particular, *Nitrosomonas europaea* (Ne), a β-proteobacterium, is the most...
studied example of AOB [34], and NeAMO, a heterotrimeric (αβγ)₃ transmembrane copper-dependent enzyme, will be the focus of the present study.

N. europaea presents two nearly identical functional amo operon copies composed by amoC, amoA, and amoB (amoCAB), followed downstream by two open reading frames (namely Orf4 and Orf5) [18, 35]. Differently, the functional AMO operon found in γ-AOB bacteria is present only once, and while it contains Orf5, it does not comprise Orf4 [18], inducing the designation of the highly conserved Orf5 as amoD [36]. Orf4 (also called amoE) is described as a complete gene duplication of Orf5, present in all β-AOB [18, 33]. Both genes, amoD and amoE, have a highly conserved sequence and are similarly localized in the AMO operon [36], suggesting that both genes could codify for a protein playing an important role in ammonia oxidation [18]. Genomic studies on AMO are available [37], but the problems experienced in its purification as an active enzyme has significantly hampered the expansion of the structural and mechanistic knowledge on this protein in the last 30 years [34, 35].

AMO features a high evolutionary correlation with particulate methane monoxygenase (pMMO), a heterotrimeric transmembrane copper-dependent enzyme that catalyzes the conversion of methane to methanol by insertion of an O₂-derived O atom in a C–H bond [38–40], a reaction analogous to that catalyzed by AMO, which, in turn, inserts an O atom in an N–H bond, releasing a water molecule in both cases (Scheme 1).

pMMO is composed of three subunits PmoA, PmoB, and PmoC codified by the pMMO functional operon (pmoCAB), which is found in all methanotrophs [41]. Moreover, the pMMO and the AMO operons feature exactly the same gene structure, being pmoC followed by pmoA, pmoB, and pmoD [18, 35].

The structures of pMMO from the methane-oxidizing bacteria *Methylococcus capsulatus* (strain ATCC 33,009 / NCIMB 11,132 / Bath) (Mc, PDB id: 1YEW, replaced by PDB id 3RGB [42]), *Methylosinus trichosporium* OB3b (Mt, PDB id: 3CHX), *Methylocystis* sp. strain M (MM, PDB id: 3RFR), *Methylocystis* sp. ATCC 49,242 (Rockwell) (MR, PDB id: 4PHZ, 4P10, and 4P12), and *Methylomonas alcaliphilum* 20Z (Ma, PDB id: 6CXH) were determined in the recent years by group of Rosenzweig [42–46] (Fig. 1). The enzyme features an homotrimer of heterotrimeric PmoABC units (PmoABC)₃ that span the bacterial membrane (Fig. 1). Three copper-binding sites have been identified, namely the so-called “monocopper” site as well as the Cu₈ and Cu₉ sites. The monomeric copper site is located in the PmoB subunit bound to the N₈ atoms of His48 and His72 as well as to the carbonyl O atom of Gln404 (Fig. 1); this site has been observed only in the pMMO structure from *M. capsulatus*, while it is not conserved in pMMO’s from other bacteria [47]. On the other hand, the Cu₈ and Cu₉ sites are conserved in all pMMO’s so far investigated. In particular, the Cu₈ center is located in the PmoB subunit and contains one Cu atom coordinated by the amino group and the imidazole N₈ atom of His33 together with the Ne atoms of His137 and His139 in a distorted tetrahedral geometry [48] (Fig. 1), while the Cu₉ site is located in the PmoC subunit and appears to feature a single Cu atom bound to Asp156 Oδ, His160 Ne, His173 Ne, and a water molecule that completes a flattened tetrahedral geometry [49] (Fig. 1). Thus far, however, the crystal structures have not fully established the location and composition of the pMMO active site [48], but all evidence points to either the Cu₈ or the Cu₉ site for this role. It is the opinion of the authors of the present study that the latter, with its labile water-bound position, should more logically constitute the enzyme active metal site.

Recently, group of Rosenzweig has also determined the structure of PmoD from *Methyllocystis* sp. ATCC 49,242 (Rockwell) (MrPmoD, PDB id: 6CPD) [35] (Fig. 2a). PmoD, a protein encoded within many pmo operons, is homologous to the AmoD proteins encoded within AOB amo operons and has been proposed to facilitate loading, assembly, and stabilization of the active sites and/or delivery of electrons and protons to pMMO [35]. The pmoD gene is adjacent or close to the genes encoding for the pMMO enzyme subunits in α-, β-, and γ-proteobacterial methane-oxidizing bacteria (α-MOB, β-MOB, and γ-MOB, respectively) [35, 50]. The same occurs for the amoD gene in AOB (including the *Nitrosomonas, Nitrosospira*, and *Nitrosovibrio* genera [18]), while in β-proteobacterial amo operons an additional gene, homologous to amoD and denoted amoEorf4, precedes amoD. The MrPmoD sequence comprises an N-terminal signal peptide followed by a periplasmic domain containing two strictly conserved cysteine residues and a C-terminal transmembrane helix. Size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) analysis suggested that the Cu-loaded periplasmic domain is present in solution both as a monomer and as a dimer [35]. The absorption spectrum of the copper-loaded MrPmoD and its dimeric form give results similar to those observed in the case of the dinuclear Cu₂ site of the cytochrome c oxidase (CcO), nitrous oxide reductase (N₂OR), and engineered Cu₂ proteins, including Cu₉ azurin [51], while the same features are not observed in the monomeric form [35]. The Cu₉ center is characterized by the presence of a mixed-valence Cu(+1.5)–Cu(+1.5) site in which two copper ions separated by ca. 2.5 Å are bound to two bridging cysteine thiolate S atoms, to yield a Cu₉S₂ core, as well as to two histidine imidazole N atoms, a methionine thioether S atom.

\[
\begin{align*}
H_3C–H + O_2 + 2H^+ + 2e^- &\rightarrow H_3C–OH + H_2O \\
H_2N–H + O_2 + 2H^+ + 2e^- &\rightarrow H_2N–OH + H_2O
\end{align*}
\]

Scheme 1 Reactions catalyzed by pMMO and by AMO.
and a backbone carbonyl O atom [52]. Unfortunately, only the monomeric form of PmoD, and not its dimeric form, could be crystallized [35], revealing the presence of a single copper atom located between the two monomers in the asymmetric unit, bound in tetrahedral geometry by two invariant Met residue S atoms from each monomer (Fig. 2a). The authors considered this site a crystallization artifact and proposed a different dinuclear CuA site for the active form of the dimeric MrPmoD based on the structure of Thermus thermophilus cytochrome c oxidase (TtCcO, PDB id: 2CUA [53]) (Fig. 2b).

Figure 1: Ribbon scheme and molecular surface of Mc-pMMO subunits (PmoA, Pmo, and PmoC), trimer (PmoABC), and trimer of trimers ([PmoABC]3) (PDB id 3RGB [42]). The ribbons are colored from white in correspondence of the N-terminals to dark green, dark blue, and orange in correspondence of the C-terminals for PmoA, PmoB, and PmoC, respectively. The positions (blue dots) and the schemes of the copper sites (“monocopper”, CuB, and CuC) are also reported.

Fig. 1 Ribbon scheme and molecular surface of Mc-pMMO subunits (PmoA, Pmo, and PmoC), trimer (PmoABC), and trimer of trimers ([PmoABC]3) (PDB id 3RGB [42]). The ribbons are colored from white in correspondence of the N-terminals to dark green, dark blue, and orange in correspondence of the C-terminals for PmoA, PmoB, and PmoC, respectively. The positions (blue dots) and the schemes of the copper sites (“monocopper”, CuB, and CuC) are also reported. The CuB and the CuC sites have been reported accordingly to the recent literature (see Ref [48] and [49], respectively). The orientation of the (PmoABC)3 in the bottom-right panel has been rotated by 90° around the horizontal axis with respect to the orientation in the upper right panel. The membrane position is indicated in the upper right panel by a gray band.

To obtain structural information on AMO and its accessory protein, and taking advantage of the high sequence identity between pMMO and AMO [54], as well as between PmoD and AmoD/AmoE [35], the present study was undertaken, using homology modelling to predict the model structure of AMO, AmoD, and AmoE from Nitrosomonas europaea based on the structures of pMMO and PmoD available in the Protein Data Bank. The obtained structural models will be critical for the rationalization of the modulation of AMO activity by the currently known enzyme inhibitors as well as for the design of new strategies for the development of new and more efficient nitrification inhibitors.

Materials and methods

Homology modelling of AMO from Nitrosomonas europaea

Template searches for each of the three subunits of AMO from Nitrosomonas europaea (NeAmoA, NeAmoB, and NeAmoC, UniProtKB id: Q04507, Q04508, and H2VFU7, respectively) were performed using the HHsearch method implemented in the HHpred server [55]. HHsearch accomplishes up to eight iterative PSI-BLAST [56] searches...
through filtered versions of the non-redundant (nr) database from NCBI. Using the final target alignment, a hidden Markov model (HMM) [57] profile is calculated. Homologous templates are identified by searching through a database containing HMMs for a representative subset of PDB sequences. HHsearch ranks the database matches based on the probability of the match to be homologous to the target sequence to distinguish homologous from non-homologous matches.

The most reliable templates were aligned with the target sequences of NeAmoA, NeAmoB, and NeAmoC using the Promals3D server [58]. The obtained alignment was then used to calculate 100 structures using as templates the available crystal structures of McPmoA and MaPmoA for NeAmoA (PDB id 3RGB and 6CXH, respectively), McPmoB (PDB id 3RGB) for NeAmoB, while McPmoC and MRpMoC (PDB id 3RGB and 4P12, respectively) were used to model NeAmoC. The Modeller 9.18 software [59] was used for all the computations. Symmetry restraints were included to grant the C₃ᵥ symmetry of the quaternary structure of the AMO trimer of trimers, while secondary structure restraints were used when needed accordingly to the prediction done with the PSIPRED 4.0 webserver [60, 61]. The best model was selected using the DOPE potential function built into Modeller [62]. A loop optimization routine was used to refine the regions that showed higher than average energy as calculated using the DOPE potential function. The Cu₈ and Cu₉ copper centers were included in the modelling following an established procedure that takes the advantage of the loop optimization routines implemented in Modeller [63–65]. The copper ions were considered always in the oxidized Cu(II) form. In particular, the van der Waals parameters for the Cu(II) ions were derived from the Zn(II) parameters included in the CHARMM22 force field [66] implemented in the Modeller v9.18 package by applying a scale factor of 1.01 calculated on the basis of the Cu(II) ionic radius. In all modelling calculations that included Cu(II) ions, constraints were imposed using a Gaussian-shaped energy potential for distances, angles, and dihedrals to correctly position the Cu(II) ions with respect to the experimentally identified ligated residues.

**Homology modelling of NeAmoE and NeAmoD**

The same template search procedure followed by a multiple sequence alignment step used in the case of AMO was repeated for the modelling of NeAmoE and NeAmoD. The modelling procedure was identical, except for the fact that the template used here was the MrPmoD dimeric structure (PDB id: 6CPD). The Cu₈ site proposed for MrPmoD was modelled in the NeAmoE model structure using the same procedure used above for the AMO copper sites.

**Fig. 2** a Ribbon scheme and molecular surface of MrPmoD (PDB id: 6CPD) [35]. The ribbons are colored from white in correspondence of the N-terminal to brown in correspondence of the C-terminal. The copper ion is represented with a cyan sphere, while the copper-binding residues are reported as sticks colored accordingly to the atom type. b Scheme of the proposed PmoD Cu₈ copper site [35]
Model analysis

The stereo-chemical quality of the final model structures was established using ProCheck [67] and the Prosa-web server [68, 69] to confirm the reliability of the model structures. The obtained molecular models and their molecular surfaces were displayed using UCSF Chimera [70] and UCSF ChimeraX [71].

Results and discussion

Homology modelling of AMO from *Nitrosomonas europaea*

The search for possible templates useful for the modelling of AMO from *Nitrosomonas europaea* yielded the pMMO structures listed in Table 1. In particular, the pMMO structure from *Methylococcus capsulatus* (strain ATCC 33,009 / NCIMB 11,132/Bath) (PDB id 3RGB) resulted as the best template for all the AMO subunits. On the other hand, the *Mc-pMMO* structure shows large disordered regions that have not been solved in the crystal structure (see Figs. S1–S3 in the Supplementary Information): residues 1–6, 192–222, and 246–247 in *McPmoA*, and residues 1–44, 225–253, and 287–298 in *McAmoC* are indeed absent. In the case of *McPmoB*, the first 32 residues at the N-terminal are missing, but this is due to a 5′ untranslated region [72] required for the correct localization of the protein, thus the functional form of the subunit has been fully solved in the crystal structure. To gain structural information on the missing regions in the *Mc-pMMO* structure, and considering the multiple sequence alignment carried out using the Promals3D server [58] (see Fig. S1–S3), the crystal structures of *MaPmoA* (PDB id 6CXH) and *MRPmoC* (PDB id 4PI2) were included in the modelling procedure. Indeed, the *MaPmoA* structure has only three unresolved residues at the N-terminal and at the C-terminal, while, in the case of *MrPmoC*, the presence of one Zn(II) ion enabled the resolution of at least one part of the central region of the protein aligning with the *McAmoC* 205–233 portion, thus leaving only 13 residues without a template structure. To obviate this setback and calculate a reasonable model structure for this region, secondary structure restraints based on the prediction performed using the PSIPRED 4.0 websolver [60, 61] were included in the computation (see Fig. S1–S3).

| AMO | Structure | Biological source | Sequence identity | Resolved regions/total length |
|-----|-----------|-------------------|------------------|----------------------------|
| NeAmoA | 3RGB,B/F/G | *Mc* (2.8) | 50% 1–6, 192–222, 246–247 | 247 |
| 6CXH,B/F/G | *Ma* (2.7) | 48% 1–3, 245–247 | 247 |
| 4PHZ,B/F/G | *MR* (2.6) | 46% 1–8 | 252 |
| 4PI0,B/F/G | *MR* (3.2) | 46% 1–8 | 252 |
| 4PI2,B/F/G | *MR* (3.3) | 46% 1–8 | 252 |
| 3RFR,B/F/G | *MM* (2.68) | 50% 1–10 | 252 |
| 3CHX,B/F/G | *Mr* (3.9) | 47% 1–11, 250–252 | 252 |
| NeAmoB | 3RGB,A/E/I | *Mc* | 43% 1–32a | 414 |
| 6CXH,A/E/I | *Ma* | 43% 1–32 | 414 |
| 4PHZ,A/E/I | *MR* | 40% 1–28a, 417–420 | 420 |
| 4PI0,A/E/I | *MR* | 40% 1–28a, 419–420 | 420 |
| 4PI2,A/E/I | *MR* | 40% 1–28a, 419–420 | 420 |
| 3RFR,A/E/I | *MM* | 40% 1–28a, 415–419 | 419 |
| 3CHX,A/E/I | *Mt* | 39% 1–40a, 284–294, 318–327, 347–350, 427–431 | 431 |
| NeAmoC | 3RGB,C/G/K | *Mc* | 46% 1–44, 225–253, 287–289 | 289 |
| 6CXH,C/G/K | *Ma* | 50% 1–89, 123–156, 193–218 | 250 |
| 4PHZ,C/G/K | *MR* | 46% 1–15, 138–165, 198–225 | 256 |
| 4PI0,C/G/K | *MR* | 46% 1–18, 200–223 | 256 |
| 4PI2,C/G/K | *MR* | 46% 1–15, 211–223 | 256 |
| 3RFR,C/G/K | *MM* | 48% 1–15, 198–225 | 256 |
| 3CHX,C/G/K | *Mt* | 46% 1–17, 177–256 | 256 |

The biological sources have been shortened as follows: *Methylococcus capsulatus* (strain ATCC 33,009 / NCIMB 11,132 / Bath) (*Mc*); *Methylosinus trichosporium* OB3b (*Mt*); *Methylocystis* sp. strain *M* (*MM*); *Methylocystis* sp. ATCC 49,242 (Rockwell) (*MR*); and *Methylomicrobium alcaliphilum* 20Z (*Ma*)

*5′* untranslated region [72]
The AMO metal-binding sites were modelled accordingly to the most recent findings on pMMO and on the conservation of pMMO metal-binding residues in the AMO sequence. In particular, the “monocopper” site is not conserved in pMMO nor in NeAMO (Fig. S2). Moreover, of the three copper-binding residues observed for this site in the case of pMMO, only McPmoB His72 is fully conserved, while His48 is substituted with a glutamine or an asparagine and Glu404 is replaced with a serine in the NeAMO sequence. Thus, the “monocopper” site was not included in the modelling procedure. The CuB copper site is instead fully conserved both in pMMO and in AMO (Fig. S2) and was modelled considering one copper atom bound to the N-terminal amino group and to His38 Nδ, His143 Ne, and His142 Ne. The Cu–N distances (Table 2) for the AMO CuB site model were taken from the recent crystallographic refinement of the electron density enhanced with quantum–mechanical calculations carried out on the Mc-pMMO crystal structure [48]. The latter study suggested the presence of a mononuclear copper site in a flattened tetrahedral geometry, as confirmed by electron paramagnetic resonance (EPR) spectroscopic studies [49]. Finally, the CuC site in the AmoC subunit was modelled accordingly to the coordination geometry proposed for McPmoC [49], namely Asp156, His160, and His173 (corresponding to NeAmoC Asp136, His140, and His153) in a distorted tetrahedral geometry comprising a water molecule as a fourth Cu(II) ligand. All the residues in the CuC copper site are fully conserved. The bond distances for the CuC site have been taken from model compounds [73]. The Cu(II) ion in the crystal structures remains unclear. On the basis of the quantum–mechanical structural refinements [48] and the EPR spectra [49], we opted for oxidized Cu(II) ions in all cases. As for the Zn-binding sites found in the pMMO structures, these are not conserved in the AMO sequence and thus were not included in the present modelling procedure.

The resulting Ne AMO model structure was analyzed using ProCheck [67] and Prosa [68, 69], and the results are reported in Table S1 and Fig. S4, together with a comparison with the structural parameters of the main template (Mc-pMMO, PDB id 3RGB, resolution 2.8 Å). The quality of the model is comparable to that of the template crystal structure. As expected by a homology model, the structural parameters for the backbone are better than the template structure (in particular for the Ramachandran plot analysis), while the overall structural parameters are slightly poorer [74, 75]. The structural analysis is overall satisfactory for a relatively low-resolution model as the one presented here for AMO.

Figure 3a, b shows the obtained model structure of NeAMO. As expected, the structure is similar to those of the template pMMO structures, with some remarkable differences. In particular, the α-helix formed by NeAmoC residues 211–251 and not present in the template pMMO structure has been fully included in the NeAMO model. NeAmoC residues 186–200, 205–221, 231–252 were restrained to form α-helices, accordingly to the secondary structure prediction provided by the SPIPRED server [60, 61]. Interestingly, the α-helix formed by residues 205–222 is found in the center of the AMO homotrimer of heterotrimers and, together with the subsequent loop (residues 223–231), interacts with the analogous α-helix in the other NeAmoC monomers (Fig. 3C). In particular, Ser221 forms an H-bond with Glu217, and van der Waals interactions are formed between residues Leu206, Trp209, Gly210, His211, Phe213, Trp214, and Glu217 from one chain and Trp214, Phe215, Glu218, Ser221, Ala222, Leu224, and Trp226 from an adjacent chain. The CuB and CuC metal-binding sites were modelled as described above and the results are reported in Fig. 3. The CuB site was found at the bottom of a narrow cleft formed by the interfaces of NeAmoB and NeAmoC (Fig. 3d). The Cu(II) ion was found in a distorted square planar geometry with a root-mean-square deviation (rmsd) from the ideal coordination geometry of 0.45 Å (Fig. 3e). The CuC site is instead located in a solvent-accessible cave formed by the interaction between the NeAmoC α-helices at the trimer of trimers interface at about one half-height of the complex. This cave is lined on the cytoplasmic side by a surface made of the interaction of the three NeAmoB subunits and is closed on the

| Constrained atoms | Distance |
|-------------------|----------|
| CuB(II)-His38(N)  | 2.2 ± 0.1 |
| CuB(II)-His38(Nδ) | 1.8 ± 0.1 |
| CuB(II)-His142(Ne) | 2.1 ± 0.1 |
| CuC(II)-His144(Ne) | 1.9 ± 0.1 |
| CuC(II)-Asp136(O61) | 2.0 ± 0.1 |
| CuC(II)-Hid140/153(Ne) | 1.9 ± 0.1 |
| CuC(II)-Water(O)  | 1.9 ± 0.1 |

All constraints in the form “average distance ± 1 standard deviation.” Distances are in Angstroms while angles and dihedrals are in degrees.
intracellular side by the NeAmoC α-helices (Fig. 3f). The Cu(II) ion in the CuC site is in a slightly distorted tetra-
hedr al geometry (rmsd from the ideal geometry = 0.14 Å, Fig. 6g).

**Homology modelling of NeAmoD and NeAmoE**

A sequence database search for putative templates usable for the modelling of both AmoD and AmoE from *Nitroso-
monas europaea* resulted in the available *Methylcystis* sp. ATCC 49,242 (Rockwell) (MrPmoD, PDB id: 6CPD) [35] (Figure S5). In particular, NeAmoD and NeAmoE resulted
Fig. 4 Ribbon scheme and molecular surface of NeAmoD (a) and NeAmoE (b) model structures. For each monomer, the ribbons are colored from white in correspondence of the N-terminal to brown in correspondence of the C-terminal. The structures in the right panels are rotated by 90° around the horizontal axis with respect to the orientation in the left panels. c Detail of the copper-binding site in the NeAmoE model. The copper ions are represented with cyan spheres, while the copper-binding residues are reported as sticks colored accordingly to the atom type.
Table 3 Distances, angles and dihedral constraints used in the modelling of copper-binding site in the NeAmoE model structure

| Constrained atoms | Distance         |
|-------------------|------------------|
| Cu1-Cys58A(Sγ)    | 2.30 ± 0.10      |
| Cu1-Cys58B(Sγ)    | 2.30 ± 0.10      |
| Cu2-Cys58A(Sγ)    | 2.30 ± 0.10      |
| Cu2-Cys58B(Sγ)    | 2.30 ± 0.10      |
| Cu1-Met66A(Sδ)    | 2.50 ± 0.10      |
| Cu2-Met66B(Sδ)    | 2.50 ± 0.10      |
| Cu1-His68A(Nδ)    | 2.10 ± 0.10      |
| Cu2-His68B(Nδ)    | 2.10 ± 0.10      |
| Cu1-Cu2           | 2.50 ± 0.10      |

Bonded atoms Constrained atoms Angle
Cu(II)-Cys(Sγ) Cu(II)-Cys(Sγ)-Cys(威尼斯| 109 ± 5
Cu(II)-Met(Sδ) Cu(II)-Met(Sδ) Met(Cβ) 109 ± 5
Cu(II)-His(Nδ) Cu(II)-His(Nδ) His(Cγ) 120 ± 10
Cu(II)-His(Nδ) Cu(II)-His(Nδ) His(Chinese) 120 ± 10

Bonded atoms Constrained atoms Dihedral
Cu(II)-His(Nδ) Cu(II)-His(Nδ) His(Cε) - His(Nε) 180 ± 10
Cu(II)-His(Nδ) Cu(II)-His(Nδ) His(Cγ) - His(Cδ) 180 ± 10

Cu₂S₂ Cu1- Cys58A(Sγ)-Cu2- Cys58B(Sγ) 0 ± 10
Cu2- Cys58B(Sγ)-Cu1- Cys58A(Sγ) 0 ± 10

All constraints in the form “average distance ± 1 standard deviation”. Distances are in Angstroms, while angles and dihedrals are in degrees.

to have a sequence identity of 38% and 28% with respect to MrPmoD, respectively. The Cu-binding methionine residues found in the MrPmoD crystal structure are not conserved in NeAmoD nor in NeAmoE (Fig. S5). Instead, the proposed copper-binding residues in the dimeric form of MrPmoD (Fig. 2b) are fully conserved in NeAmoE and only partially conserved (two residues out of three) in NeAmoD (Fig. S5). The sequence alignments with MrPmoD were used to generate models of dimeric NeAmoD and NeAmoE in the metal free form (Fig. 4a). The copper-binding site was modelled only in the case of NeAmoE, due to the complete residue conservation. The modelling was performed on the apo-NeAmoE model structure using the same procedure employed for the AMO copper-binding sites and the structure of Thermus thermophilus CcO (TrCcO, PDB id: 2CUA [53]) for the Cu-ligand distances (Table 3). The resulting NeAmoD and NeAmoE model structures were analyzed using ProCheck [67] and Prosa [68, 69], and the results are reported in Table S2 (and Fig. S6) and were considered fully satisfactory. As in the template structures, each monomer is composed of two antiparallel β-sheet, each composed by four β-strands, and by two short α-helices. The results of the Cu site modelling are reported in Fig. 4b, and show two Cu(II) ions (Cu1 and Cu2, hereafter) separated by 2.55 Å. The atoms of the Cu₂S₂ rhombus deviate from the plane by 0.2 Å, and the angle between the two Cu₃S₂ planes is 170.1°. Cu1 is bound to Met66A, His68A, and Cys58 from both chains in a distorted tetrahedral geometry (rmsd from ideal geometry = 0.22 Å), while Cu2 is bound to Met66B, His68B, and Cys58 from both chains, again in a distorted tetrahedral geometry (rmsd from ideal geometry = 0.30 Å). The formation of the copper complex at the NeAmoE dimer interface appears to induce a conformational change of the N-terminal regions. This change appears to close the cleft formed by the loop between the first two β-strands of each monomer (see the NeAmoD model structure in Fig. 4) with a consequent reduction of the protein–protein interaction surface (from 875 to 540 Å² going from the apo-NeAmoE to the holo-NeAmoE).

Conclusions

The challenge to obtain the structure of the active AMO enzyme using homology modelling of the heterotrimetric enzyme and its accessory proteins AmoD and AmoE was performed based on its high sequence identity with pMMO and PmoD, respectively. The final model must, of course, be validated using experimental data possibly obtained using X-ray crystallography and/or cryo-electron microscopy. The results thus obtained provide crucial hints onto the structural framework of AMO, its quaternary, ternary, and secondary structure, as well as on the coordination environment of its metal centers. All structural findings present strong implications for its possible reaction mechanisms. Developments in this field will allow us and others to carry out the different stages of drug design and discovery that could lead to the attainment and development of new and efficient nitrification inhibitors, decreasing nitrogen losses from soil using two different and complementary fronts, namely the main active enzyme or the accessory AmoD and AmoE proteins as a target for a virtual screening.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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