Crystal structure of nitrous oxide reductase from *Paracoccus denitrificans* at 1.6 Å resolution

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N₂O is generated by denitrifying bacteria as a product of NO reduction. In denitrification, N₂O is metabolized further by the enzyme N₂O reductase (N₂OR), a multicopper protein which converts N₂O into dinitrogen and water. The structure of N₂OR remained unknown until the recent elucidation of the structure of the enzyme isolated from *Pseudomonas nautica*. In the present paper, we report the crystal structure of a blue form of the enzyme that was purified under aerobic conditions from *Paracoccus denitrificans*. N₂OR is a head-to-tail homodimer stabilized by a multitude of interactions including two calcium sites located at the intermonomeric surface. Each monomer is composed of two domains: a C-terminal cupredoxin domain that carries the dinuclear electron entry site known as Cu_A, and an N-terminal seven-bladed β-propeller domain which hosts the active-site centre Cu_Z. The electrons are transferred from Cu_A to Cu_Z across the subunit interface. Cu_Z is a tetranuclear copper cluster in which the four copper ions (Cu1 to Cu4) are ligated by seven histidine imidazoles, a hydroxyl or water oxygen and a bridging inorganic sulphide. A bound chloride ion near the Cu_Z active site

shares one of the ligand imidazoles of Cu1. This arrangement probably influences the redox potential of Cu1 so that this copper is stabilized in the cupric state. The treatment of N₂OR with H₂O₂ or cyanide causes the disappearance of the optical band at 640 nm, attributed to the Cu_Z centre. The crystal structure of the enzyme soaked with H₂O₂ or cyanide suggests that an average of one copper of the Cu_Z cluster has been lost. The lowest occupancy is observed for Cu3 and Cu4. A docking experiment suggests that N₂O binds between Cu1 and Cu4 so that the oxygen of N₂O replaces the oxygen ligand of Cu4. Certain ligand imidazoles of Cu1 and Cu2, as well as of Cu4, are located at the dimer interface. Particularly those of Cu2 and Cu4 are parts of a bonding network which couples these coppers to the Cu_A centre in the neighbouring monomer. This structure may provide an efficient electron transfer path for reduction of the bound N₂O.

Key words: bridging sulphide, chloride, copper protein, Cu_Z, Cu_A, denitrification.

INTRODUCTION

N₂O, or laughing gas, is an important intermediate of bacterial denitrification [1]. It is also a trace component of the atmosphere, where it is present at 300 parts per billion with a long lifetime of over 100 years [2,3]. Owing to a number of human activities, the atmospheric concentration of N₂O is increasing by 0.2–0.3 % per annum. A major factor behind this trend is thought to be the agricultural use of nitrogen fertilizers, which enhances microbial production of N₂O. The molecular properties of N₂O make it a potent greenhouse gas and, in spite of its low abundance in the air, there are concerns about the role of the increasing N₂O level in global warming.

During bacterial denitrification, N₂O is produced via reductive dimerization of NO in a reaction catalysed by a membrane-bound haem-containing NO reductase [1]. In the terminal step of denitrification, N₂O is reduced to N₂ in a reaction catalysed by N₂O reductase (N₂OR), a periplasmic soluble protein [1,4]. The reaction

is highly exergonic ($\Delta G^\circ = 340 \text{ kJ/mol}$). Yet N₂O is a very inert gas at room temperature, and an efficient catalyst is necessary for the reaction to occur.

Much of the current biochemical knowledge about N₂OR and its several spectroscopic forms is based on the studies of the enzyme from *Pseudomonas stutzeri* by Zumft and coworkers [1,4–7]. N₂OR is a copper-containing enzyme that has two redox-active copper centres: a binuclear electron input site called Cu_A and a catalytic site termed Cu_Z [1]. The Cu_A site is closely related to the electron entry site of cytochrome *c* oxidase [5–7]. It has been suggested that this site developed first in N₂OR and was then adopted by cytochrome *c* oxidase, the catalytic part of which evolved from an NO reductase as the atmosphere became more aerobic some two billion years ago [8]. Recently, however, a menaquinol-oxidizing NO reductase from *Bacillus azotoformans* has been shown to contain a Cu_A centre [9], raising the possibility that Cu_A also appeared first in a primordial NO reductase.

Analogously with Cu_A of cytochrome *c* oxidases, the Cu_A centre of N₂OR is thought to accept the electrons from a reducing substrate and feed them to the Cu_Z site, where N₂O is

Abbreviations used: BCA, bicinechonic acid; EPR, electron paramagnetic resonance; G, Gauss; IMAC, immobilized metal affinity chromatography; MAD, multiple wavelength anomalous dispersion; N₂OR, N₂O reductase; RMSD, root mean square deviation.

The co-ordinates of the nitrous oxide reductase structure have been deposited in the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank with the accession code 1FWX.

We dedicate this work to the memory of Matti Saraste, who died in May 2001.

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activated and reduced. Comparison of multifrequency electron paramagnetic resonance (EPR) spectra of N_2OR and cytochrome *c* oxidase led Peter Kroneck and colleagues to suggest that, in both enzymes, the EPR-detectable copper (Cu_A) forms a binuclear mixed valence [$Cu(1.5) \cdots Cu(1.5)$] centre [6,7]. Subsequently, the binuclear nature of Cu_A has been confirmed by several crystal structures [10–15]. The two copper ions are ligated by two equatorial cysteine sulphurs, two terminal histidine nitrogens and two axial ligands (a methionine sulphur and a main chain carbonyl group). The electronic properties and factors contributing to the electron transfer function of Cu_A have been studied extensively [16–18], mostly using engineered domains harbouring Cu_A as the only metal centre. These studies have led to the picture of Cu_A as a redox site with low reorganization energy, rendering it specially adapted for fast electron transfer [16].

Despite a body of spectroscopic data (reviewed in [1]), the exact nature of the Cu_z site has remained elusive. However, the recent determination of the crystal structure of N_2OR from *Pseudomonas nautica* at 2.4 Å resolution (1 Å = 0.1 nm) has clarified the molecular details of the Cu_z centre [14,19]. Cu_z is a tetranuclear copper cluster residing in the middle of a seven-bladed β -propeller domain. Earlier magnetic circular dichroism and EPR experiments on the N_2OR from *Ps. stutzeri* in its reduced form had provided evidence about a sulphur ligand of the active-site copper [20]. The presence of an inorganic sulphur bridging the four coppers of Cu_z has now been confirmed with further analytical, spectroscopic and crystallographic studies, defining Cu_z as a novel type of copper–sulphur cluster [19–23], termed μ_4 -sulphide-bridged tetranuclear Cu_z centre [24]. The mechanism by which Cu_z binds, activates and reduces N_2O remains to be understood.

We have isolated and crystallized a blue form of N_2OR from *Paracoccus denitrificans*. In the present paper, we describe the crystal structure of the enzyme at 1.6 Å resolution. In addition to the Cu_z active site, the key features of the structure include a chloride site closely associated with Cu_z and two calcium sites located near the monomer–monomer interface of the enzyme, which is a functional dimer. Numerous interactions across the dimer interface appear to stabilize the oligomeric structure and may provide the electron transfer paths from Cu_A in one monomer to the substrate bound to Cu_z in the other.

MATERIALS AND METHODS

Growth of *P. denitrificans* 1657

P. denitrificans 1657 was obtained from Henk van Verseveld, Department of Microbiology, Free University, Amsterdam, The Netherlands. It is a wild-type strain identical with the strains DSM 413 and NCIMB 8944 ([25]; R. van Spanning, Faculty of Biology, Free University, Amsterdam, The Netherlands, personal communication). The strain NCIMB 8944 is the source of the nucleotide sequence of the N_2OR gene [26].

P. denitrificans cells were grown without shaking at 30 °C for 14–16 h in 2 l Erlenmeyer flasks filled to the top with the medium of Ludwig [27], supplemented with 10 g/l KNO_3 . Each bottle was inoculated with a 150 ml culture grown aerobically at 37 °C in Luria broth for 24 h. The yield was 13–15 g of wet cells/l of culture.

Purification of N_2OR

Wet cells (200–300 g) were suspended in 200 ml of 100 mM Tris/HCl, pH 8.0, with 10 mM succinate and containing 0.5 mM PMSF and lysed in a pressure cell at 4 °C. The lysate was

centrifuged twice at 42000 rpm for 30 min at 4 °C (Beckman Ti45 rotor). All the chromatographic steps were performed at room temperature. The strongly coloured supernatant was applied to an XK-50/20 column containing 400 ml of DEAE-CL6B resin equilibrated in 20 mM Tris/HCl, pH 8.0. The column was washed with 1 volume of 20 mM Tris/HCl, pH 8.0, and 200 mM NaCl, at 5 ml/min and eluted at 0.9 ml/min by increasing the NaCl concentration from 200 mM to 700 mM in a volume of 918 ml. A strongly coloured blue/green fraction was eluted near the end of the gradient so that the green component becomes dominating when the salt concentration increases. The coloured fractions were analysed with SDS/PAGE (12% gels). A very well staining band slightly beneath the 66 kDa standard elutes just before a broad 66 kDa band. The smaller band correlates with a blue colour and the larger band with a dark green colour; the fractions in which the smaller band was strong were pooled and applied to a XK-26/10 QS-Sepharose High Performance column equilibrated with 20 mM Tris/HCl, pH 8.0, and 200 mM NaCl. The column was eluted at 0.3 ml/min with a 300 ml NaCl gradient (200–560 mM). The fractions were analysed with SDS/PAGE, as above. Fractions containing a 66 kDa band and blue colour were applied to a XK-16/10 column packed with 20 ml of Source-Q resin in 20 mM Tris/HCl, pH 8.0, and 200 mM NaCl. This column was eluted with a 200–360 mM NaCl gradient at 0.7 ml/min. Fractions eluting first have a blue colour, whereas those eluting later in the gradient are pinkish purple. The fractions with different colours were pooled separately and applied (at 0.5 ml/min) to a 1 ml HiTrap chelating column loaded with Cu^{2+} and equilibrated in 20 mM Na_2HPO_4 and 0.5 M NaCl, pH 8.0. Although N_2OR does not bind to the copper immobilized metal affinity chromatography (IMAC) column, most of the remaining contaminants do, resulting in purified enzyme in the flow-through fraction. Purified N_2OR was concentrated with an ultrafiltration cell to 10–15 mg of protein/ml using an Amicon XM50 membrane. The concentrated enzyme was diluted at least 10-fold with 20 mM Tris/HCl, pH 8.0, and 150 mM NaCl, and concentrated again; this procedure was repeated once. The preparation was stored in liquid nitrogen.

Spectrophotometry

UV–visible spectra were recorded with a Shimadzu UV 3000 instrument. For concentration determinations, a millimolar absorption coefficient of 97 cm^{-1} at 280 nm was used (determined as described in [28]). The millimolar absorption coefficient at 540 nm is 1.7 cm^{-1} when 850 nm is used as a reference wavelength. This value is very similar to values reported for the Cu_A centre of *P. denitrificans* cytochrome oxidase or for *Ps. stutzeri* N_2OR Cu_A [17], but it is smaller than the value reported earlier [1,4]. Protein samples were diluted in 40 mM K_2HPO_4 , pH 8.0.

EPR spectroscopy

EPR spectra were recorded with a Bruker ESP 300 spectrometer equipped with an Oxford Instruments GSF 300 liquid helium cryostat. Sample temperature was 10 K. The instrumental parameters were set as follows: modulation frequency, 100 kHz; modulation amplitude, 9.9 G (Gauss, where 10 G = 1 mT); sweep rate, 14.3 G/s; microwave frequency, 9.424 GHz; microwave power, 0.2 mW. For spin quantitations, a copper standard containing 0.5 mM $CuSO_4$, 2 M $NaClO_4$ and 10 mM HCl was used. Double integrations were performed with Bruker ESP 1600 software v.2.2. A baseline obtained with a water-filled sample tube was subtracted from all spectra prior to integration.

Table 1 Data collection statistics

PK, X-ray wavelength at the peak of the X-ray absorption spectrum (XAS) at the copper edge; PI, point of inflection of the copper-edge XAS; RE, X-ray wavelength remote from the absorption edge; $R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the mean intensity of that reflection.

Data set	PK ESRF	PI ESRF	RE ESRF	PK EMBL	PI EMBL	RE EMBL	Native EMBL	CN ⁻ Elettra	H ₂ O ₂ Elettra
Wavelength Å	1.378	1.38	1.033	1.3791	1.38080	1.000	1.000	1.000	1.000
Resolution limit Å	2.5	2.5	2.2	2.2	2.2	1.9	1.63	1.7	2.1
Total observations	292 149	295 386	451 332	498 795	367 986	548 029	552 072	722 517	704 616
Unique observations	79 867	79 683	116 891	114 208	113 933	178 946	267 664	253 256	132 948
R_{sym}	0.060	0.062	0.065	0.068	0.066	0.065	0.051	0.063	0.052
R_{sym} (last shell)	0.210	0.230	0.264	0.183	0.193	0.281	0.252	0.376	0.217
I/Σ	26.7	22.5	17.6	34.2	16.8	20.5	27.9	13.6	4.9
% Completeness (overall)	97.7	98.5	98.5	97.9	97.5	99.6	91.8	98.0	98.6
% Completeness (last shell)	96.2	95.9	95.2	59.0	50.8	99.2	84.7	89.8	99.3

Miscellaneous

Protein determinations were performed with the bicinchoninic acid (BCA) method using a kit from Pierce. Alternatively, protein concentrations of pure preparations were determined by measuring the absorbance at 280 nm using an absorption coefficient of 97 cm⁻¹·mM⁻¹. The results of the two methods agree to within 15 %. Metal determinations using the technique of total reflection X-ray fluorescence were carried out at the Institute of Inorganic Chemistry, University of Frankfurt, as described in [29]. The values were normalized to the sulphur content of N₂OR (19 methionines and 8 cysteines per monomer; the bridging sulphide was omitted) or by determining the protein concentration with the BCA method. SDS/PAGE was performed on 12 % gels using the Laemmli buffer system. N₂OR activities were measured as described [30] using proflavin in the photochemical reduction of benzyl viologen. For the cyanide treatment of N₂OR, a 500 mM KCN solution was prepared by dissolving the appropriate amount of cyanide in 1 M Tris/HCl, pH 8.0. The N-terminal sequence of the blue form of N₂OR was determined with automated Edman degradation using an Applied Biosystems 477A protein sequencer. A sample of purified enzyme preparation, applied directly to the instrument, yielded the amino acid sequence ASGDGS, showing that the 57-residue signal peptide had been cleaved off [26].

Crystallization and data collection

N₂OR crystals were grown using the vapour diffusion technique at 4 °C. The well solution (1 ml) contained 16 % (w/v) PEG-8000, 80 mM sodium cacodylate, pH 6.5, and 160 mM magnesium acetate. The drop consisted of 2 µl of the well solution and 2 µl of N₂OR stock solution (14 mg of protein/ml based on absorbance at 280 nm). Blue plate-like crystals appeared in three weeks. Typically, crystal formation was preceded by the appearance of a star-like phase transition in the drop.

For data collection, crystals were transferred to a solution containing the well solution and 20 % (v/v) glycerol as a cryo-protectant. The crystals were flash-cooled in liquid nitrogen. For the treatment with cyanide, N₂OR crystals were soaked for 4 h in 10 mM KCN solution prepared in the mother liquor, and flash frozen in the cryo-protectant solution containing 10 mM KCN. Similarly, for H₂O₂ treatment, crystals were soaked for 3 h in 1 mM H₂O₂ prepared in the well solution, and subsequently flash frozen as described for the cyanide-soaked crystals.

The CuMAD [multiple wavelength anomalous dispersion (MAD) on copper absorption edge] data were collected at the beamlines BM14 at ESRF (European Synchrotron Radiation Facility) and BW7A at the EMBL (European Molecular Biology

Laboratory)-Hamburg c/o DESY (Deutsches Elektronen-Synchrotron). Datasets from the cyanide- and H₂O₂-soaked crystals were collected at the XRD1 beamline at Elettra, Trieste, while the high-resolution native dataset was collected at the BW7A beamline at EMBL-Hamburg c/o DESY. All datasets were collected at cryogenic temperature (100 K) maintained with an Oxford cryosystem. Mar Research charge-coupled device (CCD) detectors were used at ESRF and EMBL-Hamburg beamlines, while a Mar345 imaging plate was used at Elettra for the cyanide-soaked crystals, and a MarCCD detector for the H₂O₂-soaked crystals. Data analysis was carried out using the programs Denzo and Scalepack [31] or Mosflm [32] and Scala [33]. For the CuMAD datasets, two wavelengths near the copper absorption edge, and one remote wavelength, were selected for both experiments.

Structure solution and refinement

Twenty-four copper sites were located with the SHELX-97 program package [34], as well as with the program SOLVE [35]. The phase improvement was carried out using the SHARP package [36], followed by solvent-flattening procedures as implemented in SOLOMON [37]. The electron density maps showed a clear contrast between the protein and solvent regions, but were not straightforwardly interpretable. At this point, a molecular model of N₂OR from *Ps. nautica* became available [14], and we proceeded with the structure solution using molecular replacement techniques and the program AMoRe [38]. The dimer of *Ps. nautica* N₂OR was used as the template, with the metal atoms deleted from the molecular model. Data from the high-resolution native dataset were used for the cross-rotation function search in the resolution range of 10–3.8 Å. Two major peaks corresponding to two N₂ORs in the asymmetric unit were observed. The model was re-oriented according to the solutions of the cross-rotation search and used in a subsequent translational search, followed by rigid-body refinement of the two dimers. This resulted in an R_{factor} of 43.3 % and correlation coefficient of 0.409 for the diffraction data between 10 and 3.8 Å.

This model, consisting of two N₂OR dimers per asymmetric unit, was used to calculate the initial $2F_o - F_c$ and $F_o - F_c$ electron density maps (where F_o and F_c are the observed and calculated structure factors, respectively) with the diffraction data between 30 and 2.5 Å. The inspection of the difference Fourier maps clearly indicated strong positive peaks at positions where copper ions were expected. Additionally, when positions of the copper sites, as identified by the MAD experiment, were put on the same origin with respect to the molecular replacement solution, they again coincided with the locations of the highest

Table 2 Model refinement statistics

$R_{\text{factor}} = \Sigma |F_o - F_c| / \Sigma F_o$; R_{free} is the cross-validation R_{factor} computed for the test set of reflections (5% of the total) which are omitted in the refinement process; Av B, average temperature factor.

	Native
$R_{\text{factor}}/R_{\text{free}}$	0.241/0.267
Resolution range (Å)	1.63–30.0
RMSD from target values	
RMSD, bond lengths (Å)	0.007
RMSD, bond angles (°)	2.3
Av B (Å ²)	24.2
Av B (Å ²), protein	22.3
Av B (Å ²), ligands	24.2
Av B (Å ²), water	37.8

peaks of the difference map, confirming, in this way, the molecular replacement solution. The initial $2F_o - F_c$ electron density map was of good quality and allowed us to rebuild the majority of the molecular model according to the *P. denitrificans* N₂OR sequence [26] using the programs O [39] and Turbo-Frodo [40]. The model was refined with progressive increase of the outer resolution limit of data with the program suite CNS, v. 1.0 [41]. The CNS restraints used for the initial refinement steps were gradually released to allow deviations between the monomers owing to different crystal-packing environments. Final model adjustments involved correcting errors in the side-chain conformation and locating solvent molecules.

The final R_{free} and R_{factor} values are 0.241 and 0.267 respectively. These relatively high values for a structure at 1.6 Å resolution are due to the disorder of molecule D and the high B-factors of molecule C compared with those of molecules A and B. The current model consists of 24 copper, eight calcium and four chlorine atoms, 18497 non-hydrogen atoms, and 2596 solvent molecules. The final RMSD (root mean square deviation) values for the bond lengths and bond angles are 0.007 Å and 2.3° respectively.

Refinement of N₂OR soaked in cyanide and H₂O₂

For the cyanide- and H₂O₂-treated enzymes, the native model was submitted to several cycles of rigid body refinement, followed by conventional refinement with gradual increase of resolution. The inspection of $F_o - F_c$ electron density maps showed negative peaks at Cu_z sites, suggesting lower occupancy of these scatterers. The occupancies of copper atoms were therefore refined once the refinement of the atomic positions and thermal factors reached convergence. A summary of the data collection statistics is reported in Table 1, with model refinement found in Table 2.

RESULTS AND DISCUSSION

Purification and characterization of N₂OR

The enzyme was purified from the soluble fraction of *P. denitrificans* 1657 cells. Apart from including 10 mM succinate in the crude cell lysate, no attempt was made to keep the solutions anaerobic, and our preparation should be considered an aerobic one. The protocol involves three consecutive anion-exchange columns followed by an IMAC step. The IMAC column works in a negative manner, so that N₂OR does not bind to the resin, whereas many remaining contaminants do. The flow-through fraction thus contains purified N₂OR.

Figure 1 Optical and EPR spectra of N₂OR

(A) Optical spectra of N₂OR: **a**, blue form; **b**, pinkish-purple form; **c**, blue form plus 10 mM H₂O₂; **d**, blue form plus 1 mM KCN; **e**, blue form plus 10 mM ascorbate. In **b**, the preparation is only partially pure, meaning that the scale on y-axis does not apply to this spectrum. The 640 nm band is assigned to Cu₂. (B) EPR spectra: **a**, blue form; **b**, pinkish-purple form; **c**, blue form plus 10 mM H₂O₂; **d**, blue form plus 1 mM KCN; **e**, blue form plus 10 mM ascorbate. In **c**, the two bars mark the distance (19.3 mT) between the two low-field hyperfine features of type 2 copper. Instrumental parameters: modulation frequency, 100 kHz; modulation amplitude, 9.9 G; sweep rate, 14.3 G/s; microwave frequency, 9.424 GHz; microwave power, 0.2 mW; temperature 10 K. Enzyme concentration, 155 μM (based on the absorption at 280 nm). The sample of the pinkish-purple form was prepared so that it gave the same absorbance in the 480–540 nm region as the sample of the blue form.

During the Source-Q ion-exchange step, two types of enzyme fractions separate from each other. A blue fraction elutes first, followed by a less pure, pinkish-purple fraction. Both forms have a low N₂O reductase activity of 7–15 μmol of N₂O reduced/min per mg of protein (cf. [1,4,21,42,43]). The optical spectrum of the pinkish-purple fraction lacks the 640 nm band (Figure 1A, spectrum b). Moreover, the EPR spectrum of the pinkish-purple form tends to have better resolved hyperfine lines in the g_{||} region (Figure 1B, spectrum b). The blue and pinkish-purple forms appear to represent enzyme molecules whose Cu_z centre is in a different state (cf. [4,21,43] and see below). The blue fraction was used in crystallization experiments. A typical yield was 36 mg of blue N₂OR from 3600 mg of total soluble protein.

The optical spectrum of the blue N₂OR has four bands: relatively strong peaks at 480, 540 and 640 nm, and a weak one around 800 nm (Figure 1A, spectrum a). An absorption coefficient of 1700 M⁻¹·cm⁻¹ at 540 nm, using 850 nm as a reference wavelength, was obtained; this is smaller than the values

reported in [1,4], but agrees well with the values determined for Cu_A sites by Farrar et al. [17]. Addition of 100 μM ferricyanide does not change the absorption spectrum, suggesting that the enzyme cannot easily be oxidized further (cf. [21,42–44]). The absorption band at 640 nm is not present in the pinkish-purple form (Figure 1A, b). Moreover, the 640 nm band disappears upon addition of cyanide or H_2O_2 to the blue form (Figure 1A, spectra c and d), whereas the other bands disappear upon reduction with ascorbate or dithionite (Figure 1A, spectrum e). The absorption bands at 480, 540 and 800 nm behave similarly to those of the oxidized (mixed-valence) Cu_A site of cytochrome oxidase [17,45], and are therefore likely to arise from the Cu_A centre of N_2OR . In contrast, the 640 nm band can be attributed to the Cu_Z centre in its (partially) reduced state [1,4,20,21,24,43]. Loss of the band could reflect a change in the redox state of Cu_Z , but it can also result from partial depletion of copper from the tetranuclear Cu_Z centre, as evident from the X-ray structures of the active site in cyanide- or H_2O_2 -treated N_2OR (see below).

Earlier work shows that the EPR spectrum of N_2OR comprises at least two overlapping signals, one from each of the copper centres [1,20,30]. A diagnostic seven-line hyperfine pattern is often, but not always, seen near $g = 2.18$ in the signal arising from the Cu_A centre [21,30]. In contrast, the signal from Cu_Z has been reported to be broad and featureless [1,20,30], but see also [21]. The EPR spectrum of the blue form (Figure 1B, spectrum a) has traces of fine structure around $g = 2.18$; double integration of the signal yields a spin concentration of 2.3 spins per monomer. The pinkish-purple form (Figure 1B, spectrum b) has a similar intensity, but the fine structure is slightly better resolved. Yet, it is difficult to tell how many lines the hyperfine pattern contains. It has also been suggested that Cu_Z and Cu_A give rise to overlapping patterns of four and seven lines with a 70 G and 40 G spacing, respectively [21].

Adding a millimolar concentration of H_2O_2 to blue N_2OR causes the disappearance of the 640 nm band, but does not influence the optical features attributed to Cu_A (Figure 1A, spectra c and d), suggesting that H_2O_2 reacts with Cu_Z . In the EPR spectrum, a type 2 copper signal is observed (Figure 1B, spectrum c; see [4]). The spin intensity increases slightly to 2.6 spins per monomer. The crystal structure of Cu_Z in the H_2O_2 -treated N_2OR shows that the active site has lost a part of its copper (Figure 4C). H_2O_2 thus appears to cause a rearrangement within the copper cluster which may involve oxidation, loss of spin-coupling, as well as changes in co-ordination, so that a type 2 copper site with nitrogen (or oxygen) co-ordination is created.

Adding cyanide also abolishes the optical band at 640 nm (Figure 1A, spectrum d). However, the EPR spectrum of cyanide-treated enzyme (Figure 1B, spectrum d) is different from that of the H_2O_2 compound. The $g = 2$ signal has little fine structure, and the intensity corresponds to 2.2 spins per monomer. The crystal structure of Cu_Z in N_2OR that is treated with cyanide (see Figure 4D) suggests that the treatment has removed an average of one copper from each Cu_Z . Cu_3 and Cu_4 seem to be lost most easily.

The ascorbate-reduced form of the enzyme has a faint greenish-blue colour with the 640 nm band as the dominating optical feature (Figure 1A, spectrum e). In the EPR spectrum (Figure 1B, spectrum e), a broad signal with an intensity of 1.3 spins per monomer is seen. Clearly, even in the presence of reductant, one copper remains oxidized (or a copper pair remains in a mixed valence state) within the Cu_Z cluster. In agreement with this, Prudencio et al. [21] have found that, in the *Ps. nautica* enzyme, both the oxidized and reduced states are EPR-active with spin intensities of approx. 2 and 1 per enzyme monomer respectively

Figure 2 Overall view of the N_2OR monomer from the top side of the propeller

The β -propeller domain up to the last residue of the seventh blade of the propeller (Val⁵⁰⁹) is shown in dark grey. The following linker region up to Ser⁵¹⁹ is coloured black. The cupredoxin domain (from Val⁵²⁰ up to the C-terminus) is shown in light grey. The copper ions of Cu_A (Cu2 on the top) and Cu_Z (Cu2, Cu4 and Cu1 from left to right; Cu3 is not seen) are displayed in dark grey. The bridging inorganic sulphur and the chloride ion next to the active site are in light grey. Calcium ions 4905 (on the left above Cu_Z) and 4903 (on the right, partially masked by the protein), bound at or near the monomer–monomer interface, are coloured grey. Ca 4904, located on the surface of the propeller in molecules A, B and C only, is shown in black. The Figure was generated using the programs MolScript [55] and Raster3D [56].

(see also [43]). Moreover, a recent spectroscopic study [24] strongly suggests that Cu_Z (in the dithionite-reduced N_2OR of *Ps. nautica*) has a total spin of 0.5, with three coppers in the cuprous and one in the cupric state. Chen et al. [24] suggested that the spin is predominantly delocalized (via the sulphide bridge) over two coppers (Cu_1 and Cu_2) in a ratio of 5:2.

In conclusion, we have used an aerobic preparation of N_2OR . This blue enzyme species resembles (but is not identical with) the pink form of the *Ps. stutzeri* enzyme (N_2OR II) ([1,4]; cf. [42]) and the blue form of the *Ps. nautica* N_2OR (form B in [21]) and the aerobic (blue in colour) form of *P. pantrophus* N_2OR [43,44]. (Note that our blue N_2OR does not have the same redox state as the blue N_2OR III [1,4]; see recent discussion in [43].) Although our preparation has low activity (which, however, is in the same range as in [43]), it appears to be homogeneous also in terms of its redox state, which may be a prerequisite for crystal formation [19,44]. The blue form carries two spins per monomer: one originates from the Cu_A centre, and the other from the Cu_Z centre. In the ascorbate-reduced form, the equivalent of one copper ion within Cu_Z remains oxidized and EPR-visible. No EPR-silent state was found. A difference between the preparations of *P. denitrificans* and *Ps. nautica* N_2OR (both of which crystallize) appears to be in their redox state: the optical spectrum of the crystalline blue *Ps. nautica* enzyme is dominated by the 630 nm band, with no bands assignable to oxidized Cu_A ([19]; cf. [21,44]). In a recent report, two spectroscopic forms of the N_2OR active site, termed Cu_Z^* and Cu_Z^* , were characterized by Rasmussen et al. [43]. Based on their criteria (the optical and EPR spectra of the ascorbate-reduced enzyme), our preparation should contain mostly Cu_Z^* .

Overall structure

The N_2OR from *P. denitrificans* is a homodimer in which the monomers are held together strongly. Each subunit is composed

```

1      10      20      30      40      50      60
.MESKQEKKG.LSRRALLGATAGGAAGVAGFGRRLALGLGATGAVTAVGSG...AALAASGDGGS
.MESKELKG.LLSRALFSTATAGSAILAGTVG.....PAALSLSGAAGLATPARA...ATGADGGS
MSDKDKSKNTPOVPEKLGLSRRRGFLGASAVTGAAVA.ATALGGAVMTRESWAQAVK.....BSKQKIH

MSDSNNIKG.VSRRTLGGTTAAAGVGLAGGAVVTKDGAQFVSTADAQTKSAAPKAPPAR.PAVQKTE
MSNEETKMR.LNRRQMLGGTATFMAAAGV.AVGAGG.ALTLGGGTATPARAQETSGS.....SYE
MSDKQTDKDK...EXTGLSRRRGFLGASALTRSAYA.ASLGVGGVMTDRSWAAAAAK...EAQQKIH
MSDKKQDQVPGAVEAPRGVSRRSFELGTGAVTGAVLAGATALGAGTFTRESWAAAAAK...BAKSKIH
MSDKKQDQVPGAVEAPRGVSRRSFELGTGAVTGAVLAGATALGAGTFTRESWAAAAAK...BAKSKIH
                                     1      10

```

Paracoccus denitrificans
Achromobacter cycloclastes
Pseudomonas stutzeri
Rhodobacter sphaeroides
Bradyrhizobium japonicum
Rhizobium meliloti
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas nautica

70 80 90 100 110 120 130
 V A P G L D D Y Y G F W S G G Q S G E M R I L G I P S M R E L M R V P V F N R C S A T G W G T N E S V R I H E R T M S E R T K K F L A A
 V A P G L D D Y Y G F W S G G Q T G E M R I L G I P S M R E L M R V P V F N R C S A T G W G T N E S I R I H Q R T M T E K T K K Q L A A
 V G P G L D D Y Y G F W S G G H Q G E V R V L G V P S M R E L M R I P V F N V D S A T G W L T N E S R H I M G D . . . S A K P L .
 . . P G L D D Y Y G F W S S Q T G E L R I L G I P S M R E L M R V P V F N R C S A T G W G T N E S L R I M T E N M S D K T R A F L Q A
 V A P G L D D Y Y G F W S G G Q S G E M R I L G I P S M R E L M R V P V F N R C S A T G W G T N E S L K V L T E G M Q P A T R E F L K N
 V K P G L D D Y Y G F W S G G Q S G E I R I V G A P S M R E M M R I P V F N R C S A T G W G T N E S R K V M T E G M L P S T V E F L K N
 V A P G L D D Y Y G F W S G G H Q G E V R V L G V P S M R E L M R I P V F N V D S A T G W G T N E S R H I L G D . . . T A K P L .
 V G P G L D D Y Y G F W S G G H Q G E V R V L G V P S M R E L M R I P V F N V D S A T G W L T N E S K R I L G D . . . S A K Y Q .
 V A P G L D D Y Y G F W S G G H Q G E V R V L G V P S M R E L M R I P V F N V D S A T G W G T N E S K E I L G G . . . D Q Q Y L .
 20 30 40 50 60 70

Paracoccus denitrificans
Achromobacter cycloclastes
Pseudomonas stutzeri
Rhodobacter sphaeroides
Bradyrhizobium japonicum
Rhizobium meliloti
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas nautica

140 150 160 170 180 190 200
NGKRIHD NGDLHHVHMSFTDG KYDGRYLFPMNDKANTRVARVRCDVMKKDAILEITPNAKGIHGLERPKQKWER
NGKKIHD NGDLHHVHMSFTDG KYDGRYLFPMNDKANTRVGARVRCDVMKKDAILEITPNAKGIHGLERPKQKWER
..... NGDCCHHPHISMTDG KYDGRYLFPMNDKANSRVARIRLDIMKCDKMITVFNVAIHGSLRLQKVPFH
NGKKVHD NGDLHHVHMSFTDG KYDGRYLFPMNDKANTRVARVRCDVMKKDAILEITPNAKGIHGLERPKQKWER
RG.GTFM NGDLHHHPHVSFTDG TYDGRYAFPMNDKANSRVARVRDVMKCDKILBLPNQHTVHGLRLQKVPFR
QG.GLYL NGDLHHHPHVSFTDG TYDGRYLFYANDKNSRVCRIRLDVMKCDKIIQLPNQHTVHGLERQKVPFR
..... NGDCCHHPHISMTDG KYDGRYLFPMNDKANSRVARIRLDIMKCDKMITVFNVAIHGSLRLQKVPFH
..... NGDCCHHPHISMTDG KYDGRYLFPMNDKANSRVARIRLDIMKCDKMITVFNVAIHGSLRLQKVPY
..... NGDCCHHPHISMTDG KYDGRYLFPMNDKANTRVARIRLDIMKCDKITHITVFNVAIHGSLRLQKVPK
----- 80 90 100 110 120 130
▲ ▼

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Rhodobacter sphaeroides
Bradyrhizobium japonicum
Rhizobium meliloti
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas nautica

210 220 230 240 250 260 270
 SNVYFCNGREDETPLVNDGTNMDVA.NYVNVFETAVDADKWEVAQVYKLVGGLNDNCDAVYEGKWAFFSTYN
 SNVYFCNGREDDEAPLVNDGSTMEDVA.TYVNIFETAVDADKWEVAQVYKLVGGLNDNCDAVYEGKWAFFSSSYN
 TKYVFCNAREPIIPHPNDGKGVFDLDGNSYTMFYAVDAETMEMAQCVCYKLVGGLNDNTDAVYEGKGFPAATSYN
 TNYVFCNAREGDEAPLVNDGKGVLDLDFS.QYVNIFETAVDADRMKVAQCVCYKLVGGLNDNTDAVYEGKYFAFSTSYN
 TGYVFCNGREDGVPPLVNDGKGVLDLDPK.EYHSIFETALDGDGMTKVAQCVCYKLVGGLNDNVDSYQYKGYCFSTSYN
 TGYVFCNGREDDAVFPVNDGKGTMGDKN.SYQAIPTAVDGTETMEVAQCVCYKLVGGLNDNVDSYQYKGYCFATSYN
 TKYVFCNAREPIIPHPNDGHTDLDBDENSPTMYFAVDAETMEVAQCVCYKLVGGLNDNTDAVYEGRFTAATSYN
 TKYVFCNAREPVPVNDGHTGFDLDKNSPTMFYAVDAEKMMEVAQCVCYKLVGGLNDNSDAVYEGKYAATSYN
 TNYVFCNAREPVPVNDGTDPSLD.NSYTMTFAVDAETMDVAQCVCYKLVGGLNDNTDAVYEGKYATSTSYN
 140 150 160 170 180 190 200

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Rhizobium meliloti
Pseudomonas aeruginosa
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Pseudomonas nautica

280 290 300 310 320 330
 SEKGMTLPENTAAEMDHVVFENIARIEKIAAAGDYQELNG..VKVVDGRKEA..SSLPTRYETIANNPHG
 SEMGMTLEMTKSMDDHVVFENIARIEKPLRRGQYQELNG..VKVVDGRKEA..KSLPTRYETIANNPHG
 SEKAPDLGGMNRNERDDHVVFEDIHAAEAAVKAQDFITILDGSKTPVLDGRKKDKDGKSKFTRYETIANNPHG
 SEMGTLGEMMAARDDHVVFENIPRIEKGIAAGDYQELNG..VKVLDGRKGR..NKNTTRYETIANNPHG
 AEEGFTLAEMTANEDDHWVFENLKRIEEAVKKGDQFEMNG..VPVLDGRKG...SPYTRYETIANNPHG
 SEEGFTLADMMAERDDHVVFENLKRIEEAVKGDQYKEGNG..VPVLDGRKG...SPYTRYETIANNPHG
 SEKAPDLGGMNRNERDDHVVFEDIHAAEAAVKAQDFITILDGSKTPVLDGRKKDKGDSVTRYETIANNPHG
 SEKAYDLGGMNRNERDDHVVFENILIRIAAKAKSFITILDGSKTPVLDGRKTDGKDSFTRYETIANNPHG
 SERAYDLAGTMNRNDDHVVFENVRIAAAVKAGNFKITIGDSKTPVLDGRG...ESETRYETIANNPHG
 210 220 230 240 250 260 270

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Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas nautica

340 350 360 370 380 390 400

CNMAPDKKHLCAVAGKLSPTATVLDVTRFDVAFYENADPEPSAVVAEPELGLGLPLHTAFDGRGNAYTSLFL
CNMAPDKRHLCAVAGKLSPTVTVLVDVTRFDVAFYDNADPEPSAVVAEPELGLGLPLHTAFDGRGNAYTSLFL
CNTSSDGKYFIAAGKLSPTCSMIAIDPLPDLFAGKLADPRDVVVAEPELGLGLPLHTTFDGRGNAYTSLFL
CNMAPDKLHLCINAGKLSPTVTVIDVRKLDLAFYEGAEPKSTVVAEPELGLGLPLHTAFDGCAGNAYTSLFL
MNTAPDGIHIVAAAGKLSPTVTVMYDVRLEFNQLEFDDTKPKPRDVVVAEPELGLGLPLHTAFDGCAGNAYTSLFL
INTAPDGIHVVAAAGKLSPTVTVFVDKRFDDLEFDDTKPKQARDTVVAEPELGLGLPLHTAFDGCAGNAYTSLFL
LNTSGDKGYFIANGKLSPTCSMIAIDPLPDLFAGKLADPRDVVVAEPELGLGLPLHTTFDGRGNAYTSLFL
LNTSSDGKYFIANGKLSPTVSMIAIDRLDDLEFNDRYKDPREVIVAAEPELGLGLPLHTTFDGRGNAYTSLFL
LNTSPDGIHIVAAAGKLSPTVSVIAIDKLDDLEFEDTKPKEDTIVAAEPELGLGLPLHTTFDGRGNAYTSLFL

280 290 300 310 320 330 340

▼▼ ★

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Pseudomonas aeruginosa
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Pseudomonas nautica

410 420 430 440 450 460 470

DSQVWKKNIEDAIRAYAGEKVDPIKDKLDVHYQPGHLKTVMGTELTADNDWLVCLSKPFSKDRFLNVGPLK
DSQVWKKNIEDAIRAYAGEKINDPKDKLDVHYQPGHLTKTVMGTELTADNDWLVCLSKPFSKDRFLNVGPLK
DSQVWKKNMEEAIRAYKGEKVNYKQKLDVHYQPGHLHASLCTNEADGKWLVALSKPFSKDRFLPVGPLH
DSQVWKKNIEDAIRAYNGEKVDPIKDKLDVHYQPGHLNSTMGTELTADGKWLVALSKPFSKDRFLNVGPLK
DSQVWKKNIIDLAKRAFKGEKVDPIKQKLDVHYQPGHNHSSMGQTEADGKWLVALSKPFSKDRFLNVGPLK
DSQVWKKNIEDAKRAYAGEKVDPIRKLDVHYQPGHNHSTMGQTEADGKWLVALSKPFSKDRFLNVGPLK
DSQVWKKNMEEAIRAYKGEKVNYKQKLDVHYQPGHLHASLCTNEADGKWLVALSKPFSKDRFLPTGPLH
DSQVWKKNMDEAIRAYKGEKVNYKQKLDVHYQPGHNHASLSTNEADGKWLVLVALSKPFSKDRFLPTGPLH
DSQVWKKNIADAIKHYNDRVNYIRQKLDVQYQPGHNHASLSTESRDADGKWLVLVALSKPFSKDRFLPTVGPLH

350 360 370 380 390 400 410

▲

Figure 3 For legend, see facing page

of two contiguous segments in the amino acid sequence: the N-terminal catalytic domain with the Cu_Z centre and the chloride site associated with it (amino acid residues 58–509, coloured dark grey in Figure 2), and the C-terminal Cu_A domain that is responsible for the uptake of the electrons needed for reduction of N_2O (residues 520–652 shown in light grey in Figure 2). Two tightly bound calcium ions (shown in grey) are located in each monomer at or near the surface facing the other monomer.

The N-terminal domain is a seven-bladed β -propeller; the active site resides on the propeller axis. Using the program Dali [46], a similar fold can be found in several proteins. In spite of this similarity, the amino acid sequence identity between N_2OR and the highest-scoring fold analogue (transcriptional repressor *tup1* yielding a Dali Z score of 15.0) is only 11%. In line with this, the superposition of C^α atoms gives an RMSD of 2.2 Å. With the lower scoring hits, such as prolyl oligopeptidase, methylamine dehydrogenase and galactose oxidase, the RMSDs range from 3.5 to 4.0 Å. In general, β -propellers are involved in diverse functions such as cell-cycle control, signalling and catalysis [47]. In those β -propellers which are enzymes, the catalytic site resides in the central channel of the propeller, while the activities involving protein–protein interactions map to the top or side surface of the propeller molecule. In N_2OR , the propeller domain seems to be engaged both in the formation of the active site and in protein–protein interactions that are essential for dimerization.

The C-terminal domain of the enzyme has a cupredoxin fold observed previously, in addition to N_2OR of *Ps. nautica* [14], in type 1 copper proteins and in subunit II of cytochrome oxidases [10–15,48]. A structural comparison with the cupredoxin domains from *P. denitrificans* and *T. thermophilus* gives RMSDs of 1.4 Å for 78 and 93 superimposable C^α atoms respectively. We conclude that the canonical cupredoxin fold is well conserved in N_2OR .

Comparison with N_2OR from *Ps. nautica*

Amino-acid-sequence comparison of known N_2OR s shows that the enzyme is fairly conserved among bacterial species (Figure 3). The N_2OR from *P. denitrificans* has a 61% amino acid sequence identity with that of *Ps. nautica*. This is clearly reflected in a high structural similarity between the two enzymes: superposition of 561 C^α atoms of the monomers produces an RMSD of 0.71 Å. The major difference between the two bacterial N_2OR s resides in the 17-amino-acid-residue insertion located in the loop which connects the first and the second blade of the propeller (inter-strand insertion I; Figure 3). Part of the insert folds into an α -helix, which is involved in the stabilization of the dimer by making intimate contacts with residues Phe⁶¹⁸–Asn⁶²² of the other subunit. Structural comparison of the *Ps. nautica* and *P. denitrificans* N_2OR dimers gives an RMSD of 0.75 Å upon superposition of 1121 equivalent C^α atoms, indicating that the quaternary structures of the enzymes are also highly similar.

Figure 3 Amino acid sequence alignment of N_2OR s of various bacteria

Sequences are given for *P. denitrificans*, *Achromobacter cycloclastes*, *Ps. stutzeri*, *Rhodobacter sphaeroides*, *Bradyrhizobium japonicum*, *Rhizobium meliloti*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Ps. nautica*. The numbering of the amino acid residues above the sequences is that of the *P. denitrificans* N_2OR . *Ps. nautica* numbering is shown beneath the sequences. Strictly conserved amino acid residues are shown with a black background. Cu_Z ligands are indicated with triangles, Cu_A ligands near the C-terminus are marked with ovals. Ligands of the two tightly bound calcium ions are marked with grey and black rectangles respectively. Chloride ligands are indicated with inverted triangles. His³⁹² (both Cu_Z and Cl^- ligand) is indicated with an asterisk. Note that the main chain amide of Cys³³⁹ is a chloride ion ligand. The position of the interstrand insertion I, which connects the first and the second blade of the propeller, is underlined with a broken line, and the region of the insertion which adopts helical structure (facing the Cu_A domain in Figure 2) is indicated with a helix symbol. The Figure was created with ESPript (<http://www.ipbs.fr/ESPript>).

Table 3 Residues at the monomer–monomer interface

Listed are the amino acid residues in monomer A which have an atom nearer than 3.5 Å to an atom in monomer B and vice versa. Residues which are interfacial only in molecule A are in parentheses; residues which are interfacial only in B are in italics. Metal ligands are in bold; copper ligands are also underlined. Invariant residues are marked with an asterisk.

Interface residue in A and B	Notes
(Asp ⁶¹ *, Pro ⁶⁶ *, Gly ⁶⁷ *, Leu ⁶⁹ *, Asp ⁷⁰ *, Tyr ⁷² *, Tyr ⁷³ *, Gln ⁸⁰ , Ser ⁸¹ , <i>Gly</i> ⁸² *, Glu ⁸³ *, Arg ⁸⁵ *, Ile ⁸⁶ , Gly ⁸⁸ *, Arg ⁹³ *, Glu ⁹⁴ *, Leu ⁹⁵ , Met ⁹⁶ *, Arg ⁹⁷ *, Asn ¹⁰² *, Arg ¹⁰³ , Cys ¹⁰³ , Ser ¹⁰⁴ *, Ala ¹⁰⁵ *, Thr ¹⁰⁶ *, Gln ¹¹¹ , Glu ¹²⁵ , (Lys ¹²⁹),	Helical insertion between blades 1 and 2. Interact with residues 618–622 of the other monomer Helical insertion between blades 1 and 2. Interact with residues 618–622 of the other monomer
Phe ¹³⁰ , Ala ¹³³ ,	
Asn ¹³⁴ , Gly ¹³⁵ , Lys ¹³⁶ , His ¹³⁹ , Asp ¹⁴⁰ , Asn ¹⁴¹ *, Gly ¹⁴² *, Asp ¹⁴³ *, Lys ¹⁶⁶ *, Ala ¹⁶⁷ , Asn ¹⁶⁸ *, Thr ¹⁶⁹ , (Asp ¹⁷⁷)*, Val ¹⁷⁸ , Met ¹⁷⁹ *, Lys ¹⁸⁰ *, Lys ¹⁹¹ , His¹⁹⁴* , Glu ²¹² *, Asp ²¹³ , Glu ²¹⁴ , Thr ²¹⁵ , Pro ²¹⁵ , Asn ²¹⁹ *, Asp ²²⁰ *, Gly ²²¹ *, Thr ²²² , Met ²²⁴ , <i>Glu</i> ²²⁵ , Val ²²⁷ , Tyr ²³⁰ , Asn ²⁵³ *, Asp ²⁵⁵ *, Tyr²⁷¹* , Asn ²⁷² *, Met ²⁷⁷ , Thr ²⁷⁸ , Leu ²⁷⁹ *, Met²⁸² , Thr ²⁸³ , Asn³³⁵ , His³³⁷* , Lys ³⁵³ *, Leu ³⁵⁴ *, Leu ³⁹¹ *, Phe ⁴⁰⁶ *, Leu ⁴⁰⁷ , Lys⁴⁶⁴* , Phe ⁴⁶⁵ *, Ser ⁴⁶⁶ *, Lys ⁴⁶⁷ *, Asp ⁴⁶⁸ *, Arg ⁴⁶⁹ *, Phe ⁴⁷⁰ , Leu ⁴⁷¹ , Asn ⁴⁷² , Val ⁴⁷³ , Gly ⁴⁷⁴ *, Pro ⁴⁷⁵ *, Leu ⁴⁷⁶ *, Lys ⁴⁷⁷ , Pro ⁴⁷⁸ *, Glu⁴⁷⁹* , Leu ⁴⁹³ *, Val ⁴⁹⁴ *, His ⁴⁹⁵ *, Asp ⁴⁹⁶ *, Gly ⁴⁹⁷ , Pro ⁴⁹⁸ *, Phe ⁵⁰⁰ , Ala ⁵⁰¹ *, Glu ⁵⁰² *, His⁵⁰⁴* , Pro ⁵¹¹ , Lys ⁵¹⁸ , Ser ⁵¹⁹ , Val ⁵²⁰ , Trp ⁵²¹ , Asp ⁵²² , Ala ⁵⁶⁴ , Pro ⁵⁶⁵ *, Glu ⁵⁷⁰ , Asp ⁵⁹¹ , Asp ⁵⁹² *, Leu ⁵⁹³ ,	For Asp ¹⁴³ , see Figure 4B Cu ₂ /Cu _z ligand 4903 Ca ²⁺ ligand 4903 Ca ²⁺ ligand 4903 Ca ²⁺ ligand Cu ₁ /Cu _z ligand 4905 Ca ²⁺ ligand; an active site residue in Figure 4A 4905 Ca ²⁺ ligand Cu ₄ /Cu _z ligand

Table 3 (contd.)

Interface residue in A and B	Notes
Thr ⁵⁹⁴ , Thr ⁵⁹⁸ , Gly ⁶⁰⁰ , Asn ⁶⁰¹ *, Tyr ⁶⁰² , Gly ⁶⁰³ *, Val ⁶⁰⁴ , Ala ⁶⁰⁵ , Glu ⁶⁰⁷ *, Pro ⁶¹⁰ *, Phe ⁶¹⁸ *, Val ⁶¹⁹ ,	Interact with the helical insertion in the other monomer (residues 125–133) Interact with the helical insertion in the other monomer (residues 125–133) Interact with the helical insertion in the other monomer (residues 125–133)
Ala ⁶²⁰ *, Ala ⁶²¹ ,	
Asn ⁶²² ,	
Pro ⁶²³ , Gly ⁶²⁴ , Val ⁶²⁵ , Tyr ⁶²⁶ , Trp ⁶²⁷ *, Tyr ⁶²⁹ *, Gln ⁶³¹ , Trp⁶³²* , Phe ⁶³³ *, Cys⁶³⁴* , His ⁶³⁵ *, Ala ⁶³⁶ *, Leu ⁶³⁷ , His⁶³⁸* , Met ⁶³⁹ *, Glu ⁶⁴⁰ *, Arg ⁶⁴² , Arg ⁶⁴⁴ *, Leu ⁶⁴⁶ , Val ⁶⁴⁷ *, Glu ⁶⁴⁸ *, 4905Ca ²⁺	Cu ₂ /Cu _A ligand Cu _A bridge ligand Cu ₂ /Cu _A ligand

N₂OR dimer

The Cu_A–Cu_z distance within a monomer is 40 Å, which appears too long for efficient electron transfer. In contrast, the corresponding intermonomeric distance in the dimer is 10 Å, well within the range for productive electron transfer in a protein medium [49]. Consequently, it appears that the dimeric structure is essential for catalytic function. The dimer interface extends across 26.4 % of the solvent-accessible area of a subunit, corresponding to 6250 Å². This value is well above the mean of 1685 Å² of solvent-accessible surface area buried upon formation of several other homodimers [50], although one should keep in mind that all the proteins analysed in [50] are smaller than N₂OR. In accordance with the functional importance of dimerization (which may be a common feature of all N₂ORs), the residues at the dimer interface tend to be highly conserved (see Table 3). To characterize further the intermonomeric surface of the dimer, we have measured the complementarity between the interacting surfaces using a gap index, defined as the volume enclosed between any two molecules divided by the surface area involved in the interaction [50]. For the N₂OR dimer, the gap index value is 0.92 Å (compared with the average value of 2.20 Å for homodimers), indicative of a highly complementary intermonomeric surface. Several unusual side chain rotamers can be found on the interface. Both side chain and backbone atoms participate in over 40 polar contacts shorter than 3.0 Å at the dimer interface. In addition, a number of solvent molecules participate in extensive hydrogen bonding networks across the dimer interface. Van der Waals interactions between hydrophobic residues also contribute to the stability of the N₂OR dimer; 64 % of the atoms involved in the dimer interface are non-polar. The sequence-conserved amino acid residues Phe⁶³³ and Met⁶³⁹ (Figure 3) are positioned in the dimer interface between Cu_A and Cu_z and may be involved in the electron transfer between the two monomers [14]. The residues involved in intermonomeric contacts are listed in Table 3.

Figure 4 Structure of the active site of N_2OR

(A) The active site of N_2OR with a substrate molecule docked between Cu1 and Cu4. Depicted are the four copper ions (orange spheres) and their ligand imidazoles labelled with their residue numbers. The bridging sulphur is in green. An N_2O molecule has been docked next to Cu1 and Cu4 with the O end (coloured red) towards the latter. The bound N_2O replaces the hydroxide or water ligand found between Cu1 and Cu4 [see (B)]. The O end of the substrate is at 2.9 Å from the amine nitrogen of Lys⁴⁶⁴, which might provide the water protons during catalysis. The N end (light blue) of the substrate may be hydrogen-bonded (at 3.3 Å) to the main chain amide of His⁴⁴³, a ligand of Cu1. (B) Structure of the active site. Shown are the Cu_2 cluster and the

The two tightly bound calcium ions in each monomer appear to have a role in dimer formation (Figures 2 and 3; Table 3). Recent work has shown that the *Ps. nautica* enzyme binds six coppers, two calciums and one chloride [14,19]. A metal analysis of our enzyme preparation using total reflection X-ray fluorescence (TXRF; [29]) suggests that there are indeed six (5.25–6.31) coppers, two (1.98–2.38) calciums and one (1.22–1.47) chloride per N_2OR monomer. It should be noted that the enzyme used here was prepared without EDTA. In molecules A, B and C of the asymmetric unit, a third bound Ca^{2+} (Ca 4904 in molecule A; coloured black in Figure 2) seems to reside on the surface of the protein, located at about 4 Å from Asp⁴³³ and Lys⁴⁹⁰. The nearest neighbours of this Ca^{2+} are solvent molecules. Finally, it is interesting that a recent structure of a trinuclear copper site [51] suggests the presence of a chloride ion near one of the coppers. The 4905 Ca^{2+} is co-ordinated by the carboxylate of Glu⁴⁷⁹, by the main-chain carbonyl oxygen of Lys⁴⁶⁴, and by four water molecules. The ligand residues of this Ca^{2+} as well as all except one of those of the other calcium (4903 Ca^{2+}) are invariant (Figure 3). One of the co-ordinating waters (W3000) of 4905 Ca^{2+} makes a short hydrogen bond to Trp⁶³² NE2 atom of the neighbouring subunit. In Cu_A domains of cytochrome *c* oxidases, the position of this tryptophan residue is occupied by a glutamic residue (a glutamine residue in [15,48]), which is co-ordinated to Cu2 of Cu_A via its main-chain carbonyl group [10,11]. The side chain of this glutamic residue is a ligand of Mg^{2+} , which is bound to the interface between subunits I and II of cytochrome *c* oxidase [11]. In the Cu_A domain of N_2OR , Trp⁶³² performs a similar dual role: its main-chain carbonyl group is a ligand for the Cu2 of Cu_A , whereas its side chain is part of the hydrogen-bonding network that connects the two subunits.

The second tightly bound calcium ion, 4903 Ca^{2+} , is co-ordinated by the side-chain carboxylates of residues Glu²⁷⁴ and Asp²⁸⁸, two main chain carbonyl groups from residues Met²⁸² and Tyr²⁷¹ respectively, and by OD1 atom of residue Asn³³⁵. The latter interaction stabilizes the Asn³³⁵ side chain in the appropriate position to make a productive hydrogen bond with the Glu¹²⁰ side chain of the opposing subunit.

Taken together, these results indicate that both highly specific polar and non-polar, as well as chelating interactions, contribute to the stability of the functionally essential N_2OR dimer. This multitude of interactions resembles the situation of the *Rhodobacter capsulatus* porin trimer that is stabilized, in addition to other forces, by tightly bound calcium ions residing at each of the monomer–monomer interfaces [52]. From the functional point of

associated chloride ion site and their ligands, as well as Asp¹⁴³ and Asp²⁵⁵ and a few important water molecules (red spheres). The sulphide bridges the four copper ions with bonding distances ranging from 2.22 to 2.36 Å. A complex network of hydrogen bonds appears to determine the orientation of the imidazole side chains. The carboxylate of Asp²⁵⁵ fixes in place His¹⁹⁴ and His³³⁷, whereas Asp¹⁴³ stabilizes the position of the ring of His¹⁴⁵. His¹⁴⁶ ND1 makes a hydrogen bond with the carbonyl group of His¹⁹⁴, while the orientations of His⁴⁴³ and His⁵⁰⁴ are stabilized via interactions with solvent molecules further hydrogen-bonded with other atoms in the structure. The electrons from Cu_A may enter Cu_2 from the direction of the two water molecules near Asp¹⁴³. These and a few other waters are connected to the ligands His⁵⁰⁴ (Cu4) and His¹⁴⁵ (Cu2) and also hydrogen-bonded with the Cu_A ligand His⁶³⁸ in the neighbouring monomer. Note that His³⁹² is a ligand of both Cu1 and chloride ions, a probable mechanism for lowering the redox potential of Cu1 so that it remains cupric when the other coppers are cuprous [24]. The other chloride ion ligands include the side chain nitrogens of Arg¹⁹⁷ and Asn³⁴⁰ and the main-chain amide of Cys³³⁹. (C) Structure of Cu_2 in crystals soaked with H_2O_2 along with the F_0-F_c electron density map calculated with the contribution of the fully occupied copper atoms as in the native model. The contouring levels are: -6.0σ (red), -8.0σ (green) and -10.0σ (blue). (D) Structure of Cu_2 in crystals soaked with cyanide along with the F_0-F_c electron density map calculated with the contribution of fully occupied copper atoms, as in the native model. The contouring levels are: -6.0σ (red), -12.0σ (green) and -15.0σ (blue). Atoms are coloured according to the atom type (C = yellow; N = cyan; O = red; S = green; Cu = blue). The Figure was prepared with Turbo-Frodo [40].

Figure 5 Structure of the Cu_A site

The Cu_2S_2 (cysteine) core shows centre of inversion symmetry so that the bonds Cu1-Cys^{630} and Cu2-Cys^{634} are both 2.26 Å long, whereas those of Cu1-Cys^{634} and Cu2-Cys^{630} are both 2.38 Å long. The terminal histidine-copper bond lengths are 2.03 Å (Cu1-His^{595}) and 1.98 Å (Cu2-His^{638}). The angle between the histidine ring planes is about 27°. The axial thioether sulphur of Met^{641} is at 2.45 Å from Cu1 , whereas the other axial ligand, the backbone carbonyl oxygen of residue Trp^{632} , is at 2.57 Å from Cu2 . The Cu-Cu distance is 2.51 Å. Hydrogen bonds with water molecules couple His^{638} to certain Cu_2 ligands across the monomer-monomer interface. Trp^{632} is also hydrogen-bonded across the interface. The Figure was prepared with Turbo-Frodo [40].

view, it is of interest that several Cu_A and Cu_Z ligands participate in intermonomeric contacts (see Table 3). The interfacially located ligands may provide an efficient electron transfer path from Cu_A to the substrate bound to the active site.

Cu_Z – the active site

The Cu_Z centre of N_2OR comprises four copper ions in a distorted tetrahedral arrangement, together with seven histidine residues, an oxygen and a bridging sulphide as the ligands to the coppers (Figure 4A). Cu1 , 2 and 3 are co-ordinated by histidines 337 and 392, 145 and 194, and 146 and 443 respectively, whereas Cu4 is ligated by His^{504} and a water or hydroxyl oxygen. His^{504} is within van der Waals distance (3 Å) from a solvent molecule that makes a short hydrogen bond to His^{638} , which is a ligand of Cu2 of Cu_A in the neighbouring monomer. In this way, a water molecule connects the co-ordination spheres of Cu_Z and Cu_A . A structural interaction between these metal sites is also supported by a Cu_A ligand mutagenesis study [45], showing that mutations of the equivalents of His^{638} and Cys^{630} (see Figure 5) lead to the loss of both Cu_A and Cu_Z , whereas mutating the counterparts of His^{595} and Cys^{634} lead to spectroscopic changes of Cu_Z . An interesting feature of the structure is a chloride ion found in the proximity of the Cu_Z site (Figure 4B). Intriguingly, one of the ligands to the chloride ion is His^{392} , which is also a ligand of Cu1 in Cu_Z . The other chloride ion ligands are the guanidinium group of Arg^{197} , as well as the side chain of Asn^{340} , both of which seem to be invariant (Figure 3). In addition, the main-chain amide of the non-conserved residue Cys^{339} interacts with the chloride ion. (It should be noted that in the report describing the structure of the *Ps. nautica* N_2OR [14], the identity of the equivalent residue

is ambiguous: it is said to be Asn^{272} in the text, whereas in the sequence shown it is a leucine residue. In the PDB file 1QNI, it is a leucine residue, suggesting that there is a typographical error in the text in [14].) Apart from a structural role, the bound chloride may function in tuning the redox potential of CuI so that this copper prefers to remain cupric (and therefore capable of binding of N_2O , as shown in Figure 4A), while the other coppers are cuprous [24], a configuration likely to be essential for catalysis.

The active site is located on the verge of the central channel of the β -propeller, but it is not solvent-exposed from either of the flat surfaces of the propeller. The distance from the bottom surface is 26 Å along the channel axis, whereas, on the top side of the propeller, it is protected from the solvent by the cupredoxin domain of the other subunit of the dimer. The minimum distance from the Cu_Z centre to the top surface is around 12 Å, suggesting that the substrate approaches the active site from the top side.

A number of buried water molecules can be located both in the channel leading from the bottom side to the Cu_Z centre, as well as in the subunit interface close to the active site. The solubility of N_2O in water is 24 mM at 101.325 kPa and 25 °C (in comparison, O_2 solubility is about 1.3 mM) [53], suggesting that the access of the substrate to the active site may not require an extremely hydrophobic environment. Together with the fact that Cu_Z is located closer to the top surface of the propeller, the positions of the buried solvent molecules around the central channel might indicate that N_2O reaches the Cu_Z centre from the top side of the molecule, i.e. from the direction of the Cu_A site of the neighbouring monomer.

Cyanide is among the known inhibitors of N_2OR [1]. The structure of N_2OR determined using crystals soaked with cyanide revealed that the cyanide ion partially depletes the active site of copper ions. The positions most affected are those of S^{2-} , Cu4 and Cu3 , where the occupancies drop to 0.25, 0.25 and 0.35, respectively (Figure 4D). We do not observe any clear electron density for the bound cyanide ion. Similar behaviour is observed for crystals soaked in H_2O_2 , where the occupancies of the three atoms fall to similar values (Figure 4C). There is no evidence of peroxide bound to Cu_Z .

Cu_A – the electron entry site

In the N_2O dimer, the Cu_A centre is located close to the intermonomeric surface so that several copper ligands (e.g., His^{638}) are classified as being interfacial in Table 3. The residues that bind the copper ions (Figure 5) originate from two loops of the β -sandwich of the cupredoxin domain located near the C-terminus of the primary structure (Figure 3). The structure is very closely related to the Cu_A site in cytochrome oxidases. As mentioned above, a minor difference is found in one of axial ligands: the main-chain carbonyl group ligand is contributed by Trp^{632} , not by a glutamic or glutamine residue as in the oxidases.

As discussed in [12,16], there appears to be a subtle interplay between the positions and bond lengths of the equatorial, terminal and axial ligands of Cu_A . It is important to note that there are slight structural differences among the structurally characterized Cu_A centres [10–15,48] and that these differences contribute to the fine-tuning of the electronic symmetry, spin delocalization, reorganization energy and metal-metal distance of Cu_A [16–18]. In N_2OR , it appears that the overall structure of the Cu_A site is very closely related to Cu_A of native cytochrome *c* oxidases. In contrast, the structures of Cu_A in the engineered cupredoxin domains [12,13,15] are slightly, but significantly, different from Cu_A in N_2OR . The most notable differences involve the distances and positions of the terminal imidazoles as

well as the axial ligand bond lengths [12]. It is conceivable that the structural details of Cu_A sites in N₂OR and in the native oxidases (as opposed to those in the engineered domains) reflect the common requirement of electron transfer, i.e. that of first being able to accept the reducing equivalents and then being able to feed them rapidly further into the active site.

Why does N₂OR need a Cu_A site? In line with the analyses of the electronic structure of Cu_A [16], a kinetic study shows that an engineered Cu_A site is a more efficient electron transfer centre than is a mononuclear type 1 copper site [54]. In cytochrome *c* oxidase, Cu_A transmits electrons one by one from cytochrome *c* to the active site of the oxidase for dioxygen reduction that requires four electrons. It appears that also in N₂OR, catalysis requires fast electron transfer to the Cu_Z active site, even though one turnover of the enzyme consumes only two electrons.

Conclusion

Although the conversion of N₂O into N₂ and H₂O is a highly exergonic reaction, the uncatalysed reaction does not take place at room temperature. How does N₂OR activate the otherwise inert N₂O molecule? The structure suggests that the substrate may bind between Cu1 and Cu4 perhaps replacing the oxygen ligand of Cu4 (cf. Figures 4A and B). Whether N₂O binds to copper from its N or O end (or from both in a bridging position, as in Figure 4A) is not known, but binding from the O end has been proposed [14]. Analysis in [24] suggests that Cu1 is in the cupric state, whereas Cu4 is cuprous and could provide one of the two electrons required by the reaction. The second electron could come from the cuprous Cu2, which is strongly coupled to Cu1 via the bridging sulphide. Furthermore, the ligands His¹⁴⁵ (Cu2) and His⁵⁰⁴ (Cu4) are members of a bonding network which couples these coppers structurally across the monomer–monomer interface to the Cu_A site in the adjacent monomer. This coupling may provide an efficient pathway for re-reduction of Cu2 and Cu4. It is also noteworthy that the planes of the imidazole rings of His¹⁴⁵ and His⁵⁰⁴ are almost parallel at about 3.3 Å from each other (Figure 4B).

Regarding the role of the bridging sulphur, Rasmussen et al. [22] have speculated that, during catalysis, it could accept the oxo group from the substrate to form a sulphoxide intermediate. An alternative idea is that the bridging sulphur renders the Cu_Z site highly covalent, a property which could be advantageous for rapid consecutive electron transfers to the bound N₂O [24]. Another interesting matter deals with the number of reducing equivalents in the enzyme: it appears that Cu_Z contains three electrons [24]. Since Cu_A is a one-electron carrier, N₂OR can store a total of four electrons, twice the amount needed for one turnover. Whether or not this has any functional significance, remains to be elucidated; it is also possible that one copper in Cu_Z needs to be in a stable reduced state because it is important for the active site geometry. It is clear that, in order to determine the chemical mechanism of N₂OR, further structural and spectroscopic studies of N₂OR with substrate or inhibitor bound to the active site are necessary.

Note added in proof (received 15 November 2002)

Chen et al. [57] discuss the binding of N₂O to Cu_Z. They seem to favour a bridging binding mode in which the oxygen atom of N₂O interacts with Cu1 of Cu_Z (cf. Figure 4A).

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