

Research

# Biochemical characterization of the purple form of *Marinobacter hydrocarbonoclasticus* nitrous oxide reductase

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Nitrous oxide reductase (N<sub>2</sub>OR) catalyses the final step of the denitrification pathway—the reduction of nitrous oxide to nitrogen. The catalytic centre (CuZ) is a unique tetranuclear copper centre bridged by inorganic sulphur in a tetrahedron arrangement that can have different oxidation states. Previously, *Marinobacter hydrocarbonoclasticus* N<sub>2</sub>OR was isolated with the CuZ centre as CuZ\*, in the [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] redox state, which is redox inert and requires prolonged incubation under reductive conditions to be activated. In this work, we report, for the first time, the isolation of N<sub>2</sub>OR from *M. hydrocarbonoclasticus* in the ‘purple’ form, in which the CuZ centre is in the oxidized [2Cu<sup>2+</sup> : 2Cu<sup>+</sup>] redox state and is redox active. This form of the enzyme was isolated in the presence of oxygen from a microaerobic culture in the presence of nitrate and also from a strictly anaerobic culture. The purple form of the enzyme was biochemically characterized and was shown to be a redox active species, although it is still catalytically non-competent, as its specific activity is lower than that of the activated fully reduced enzyme and comparable with that of the enzyme with the CuZ centre in either the [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] redox state or in the redox inactive CuZ\* state.

**Keywords:** nitrous oxide reductase; *Marinobacter*; denitrification; isolation in presence of oxygen; fully oxidized CuZ centre; purple form

## 1. INTRODUCTION

Nitrous oxide (N<sub>2</sub>O) is a potent long-lived greenhouse gas, the atmospheric concentration of which has increased over past years. In addition, the potential of N<sub>2</sub>O as a global warming gas relative to that of CO<sub>2</sub> is 298 for a 100 year period, and this gas is also responsible for stratospheric ozone depletion [1].

Different sources have been responsible for this increase, such as industry and wastewater treatment, but bigger contributors are the soil and seawater because of microbial (fungal and bacterial) processes. N<sub>2</sub>O is produced by bacteria through denitrification and nitrification, which are part of the biogeochemical nitrogen cycle.

While nitrification is a biochemical pathway that takes place under aerobic conditions, denitrification occurs mainly under strictly anaerobic or microaerobic conditions. In the first case, N<sub>2</sub>O is produced as a by-product of ammonium oxidation, whereas in denitrification, bacteria reduce inorganic forms of nitrogen

and N<sub>2</sub>O is formed as an intermediate by-product, and in fact this biological process can also consume N<sub>2</sub>O. Although denitrification can decrease the level of nitrous oxide by reducing it to nitrogen gas, different environmental conditions, such as pH, oxygen and nitrogen levels, and temperature, may lead to its accumulation and release into the atmosphere [2,3]. Therefore, there is a particular interest in understanding how the reduction of nitrous oxide to inert N<sub>2</sub> is carried out in nature [4]. The enzyme involved in the denitrification pathway and responsible for catalysing this reaction is nitrous oxide reductase (N<sub>2</sub>OR), which can be isolated from  $\alpha$ -,  $\beta$ - and  $\gamma$ -proteobacteria [5].

Interestingly, another enzyme of the nitrogen biogeochemical cycle, nitrogenase, is able to carry out the *in vitro* reduction of N<sub>2</sub>O to N<sub>2</sub> [6,7] and *Escherichia coli* has also been shown to be able to reduce N<sub>2</sub>O, even if this reaction might not have any physiological implications and the enzyme responsible for the catalysis has not been identified [8]. Recently, another copper enzyme, a multi-copper oxidase, from *Pyrobaculum aerophilum* has been shown to be able to catalytically reduce N<sub>2</sub>O, although its *in vivo* function is still unclear [9].

N<sub>2</sub>OR, a periplasmic enzyme in Gram-negative bacteria, was isolated for the first time many years ago [10], but its structure has only been solved more

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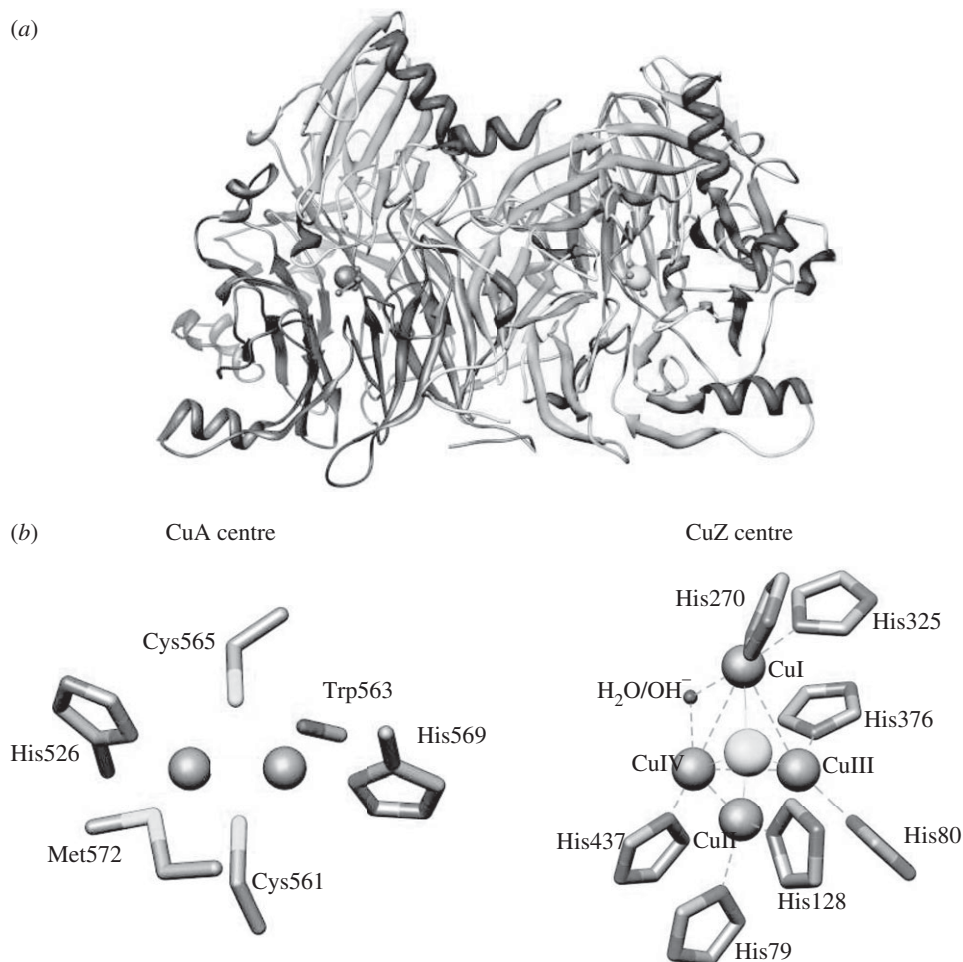


Figure 1. (a) Representation of *Marinobacter hydrocarbonoclasticus* nitrous oxide reductase functional dimer. The dimer is coloured according to subunit, with one monomer coloured grey and the other dark grey, and CuA and CuZ centres coloured grey. (b) CuA and CuZ centres are coloured grey and in CuZ centre the copper ions are numbered I, II, III and IV. The sulphur ion in CuZ centre is shown as a light grey sphere. The figure was created with Chimera using 1QNI.

recently, revealing that it is a functional dimer, containing two copper centres per monomer (figure 1a) [11]. The CuA centre located in a cupredoxin-like domain can be assumed to be the electron-transferring centre, as it is proposed to receive electrons from small electron carrier proteins and transfer them to the catalytic centre—CuZ centre [11]. The CuA centre is a binuclear copper centre, similar to that found in cytochrome *c* oxidase [12] (figure 1b).

The CuZ centre has a unique structure as it comprises a tetranuclear copper cluster bridged by sulphur in a tetrahedral arrangement [11] (figure 1b). In the past, many studies have focused on the complex chemistry of the catalytic CuZ centre, to identify and spectroscopically characterize its different redox forms, and how these redox states are involved in the catalytic cycle.

Previously, *Marinobacter hydrocarbonoclasticus* (formerly known as *Pseudomonas nautica* [13]) N<sub>2</sub>OR was purified in the presence of oxygen from a strictly anaerobic culture and two forms of the enzyme were characterized, named purple and blue. The difference between these enzyme forms was mainly the oxidation state of the CuA centre, which was in the oxidized state ([Cu<sup>1.5+</sup>:Cu<sup>1.5+</sup>]) and

reduced state ([Cu<sup>+</sup>:Cu<sup>+</sup>]), respectively (table 1). In these isolated forms, the CuZ centre was in ‘the resting state’, i.e. the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] redox state, denoted by some authors as CuZ\* [15,16]. This form presents an absorption spectrum characterized by a band at 640 nm characteristic of the resting CuZ centre together with contributions at 480, 550 and 800 nm of the CuA centre in the [Cu<sup>1.5+</sup>:Cu<sup>1.5+</sup>] state [17]. The proportion of the contribution of CuA centre bands depended on the amount of this centre that was oxidized.

Although the spectroscopic, electronic and structural properties of the CuZ centre in the resting state have been extensively characterized [14], this state is redox inactive in the sense that it cannot be oxidized or easily reduced, and it is also catalytically inactive, as its specific activity is very low when compared with that of the enzyme with the CuZ centre in the fully reduced state [18,19].

However, this resting form of the enzyme can be activated through prolonged incubation with a strong reducing agent, such as reduced methyl viologen [18], during which the CuZ centre is completely reduced to [4Cu<sup>+</sup>]. It has been hypothesized that this long activation process corresponds to a rearrangement in the

Table 1. The different redox forms of nitrous oxide reductase (N<sub>2</sub>OR).

	CuA centre	CuZ centre	CuZ* centre
fully oxidized/as-isolated (purple form)	[Cu <sup>1.5+</sup> -Cu <sup>1.5+</sup> ] <sup>3+</sup>	[2Cu <sup>2+</sup> -2Cu <sup>+</sup> S] <sup>4+</sup>	
ascorbate-reduced	[Cu <sup>1+</sup> -Cu <sup>1+</sup> ] <sup>2+</sup>	[2Cu <sup>2+</sup> -2Cu <sup>+</sup> S] <sup>4+</sup>	
dithionite-reduced	[Cu <sup>1+</sup> -Cu <sup>1+</sup> ] <sup>2+</sup>	[1Cu <sup>2+</sup> -3Cu <sup>+</sup> S] <sup>3+</sup>	
fully oxidized/as-isolated (purple/pink form <sup>a</sup> )	[Cu <sup>1.5+</sup> -Cu <sup>1.5+</sup> ] <sup>3+</sup>		[1Cu <sup>2+</sup> -3Cu <sup>+</sup> S] <sup>3+</sup>
dithionite-reduced/ascorbate-reduced (blue form <sup>b</sup> )	[Cu <sup>1+</sup> -Cu <sup>1+</sup> ] <sup>2+</sup>		[1Cu <sup>2+</sup> -3Cu <sup>+</sup> S] <sup>3+</sup>
fully reduced/activated form <sup>c</sup>	[Cu <sup>1+</sup> -Cu <sup>1+</sup> ] <sup>2+</sup>		[4Cu <sup>+</sup> S] <sup>2+</sup>

<sup>a</sup>Also named as a purple form by some authors.

<sup>b</sup>In the 'blue form' of N<sub>2</sub>OR, the CuZ centre is found mainly in the CuZ\* state, which has also been named 'resting CuZ' [14]. The two dithionite-reduced forms of N<sub>2</sub>OR are different, although both copper centres have the same redox state.

<sup>c</sup>This form has only been shown to be formed from CuZ\*.

Table 2. Properties of the purple form of nitrous oxide reductase (N<sub>2</sub>OR) isolated from different organisms.

	<i>Paracoccus denitrificans</i>	<i>Paracoccus pantotrophus</i>	<i>Pseudomonas stutzeri</i>	<i>Achromobacter xylosoxidans</i>
anaerobic purification	yes	yes	yes	yes
visible spectrum				
ε <sub>550nm</sub> <sup>a</sup> (M <sup>-1</sup> cm <sup>-1</sup> ) (dimer)	10 600	8000	14 000	10 500
A <sub>550nm</sub> /A <sub>640nm</sub>	1.7	1.9	2.1	1.8
EPR				
g <sub>  </sub>	2.224	2.181	2.180	2.180
g <sub>⊥</sub>	2.042	2.038	2.030	2.019
A (gauss)	35	38	38	40
specific activity (U mg <sup>-1</sup> ) <sup>b</sup>	122	3	4	6
reference	[22]	[15]	[21]	[23]

<sup>a</sup>Value determined based on the total protein content for the fully oxidized purple N<sub>2</sub>OR.

<sup>b</sup>The specific activity was determined using benzyl viologen (*Pseudomonas stutzeri* and *Paracoccus denitrificans*) or methyl viologen (*Paracoccus pantotrophus* and *Achromobacter xylosoxidans*) as electron donor.

coordination sphere of the CuZ centre, which is required for full activity [14].

Recently, an intermediate in the catalytic cycle of the enzyme has been identified, CuZ<sup>o</sup>, which is proposed to have the CuZ centre in the same redox state as the resting state [1Cu<sup>2+</sup>:3Cu<sup>+</sup>], but differs by exhibiting full activity. This species has a short half-life, with a rate of decay to the resting form of 0.3 min<sup>-1</sup> [20].

However, from the first purifications of *Pseudomonas stutzeri* N<sub>2</sub>OR, it became clear that this enzyme can be isolated in different forms with the CuZ centre in different redox states depending on the purification conditions [21]. N<sub>2</sub>OR purified in the absence of oxygen from *Pseudomonas stutzeri*, *Achromobacter xylosoxidans*, *Paracoccus denitrificans* and *Paracoccus pantotrophus* [15,21–23] exhibits an intense absorption at 550 nm. This form, named 'purple' (because of its colour), presents an oxidized CuA centre because the electron paramagnetic resonance (EPR) spectra exhibit the typical seven-line hyperfine pattern in the g<sub>||</sub> region, and the CuZ centre in the oxidized [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state (table 2). In contrast, N<sub>2</sub>OR purified in the presence of oxygen from these bacterial sources is in a different form, named 'pink' [24], which is similar to *M. hydrocarbonoclasticus* N<sub>2</sub>OR also isolated in the presence of oxygen [17].

The reduction of N<sub>2</sub>OR in the purple form by sodium dithionite proceeds in two kinetic steps: a fast

phase in which the absorbance at 540 nm disappears almost within seconds, owing to the reduction of the CuA centre (this form can also be obtained by reduction of the purple form with sodium ascorbate), and a slower phase that takes place over the course of minutes or hours, in which the CuZ centre, as [2Cu<sup>2+</sup>-2Cu<sup>+</sup>], is reduced to [1Cu<sup>2+</sup>-3Cu<sup>+</sup>] [5,25].

The EPR spectrum of the purple form (CuA centre oxidized and CuZ centre as [2Cu<sup>2+</sup>-2Cu<sup>+</sup>]) exhibits a well-defined seven-line hyperfine split axial signal, whereas that of the enzyme with CuA reduced and the CuZ centre as CuZ\* (redox inactive [1Cu<sup>2+</sup>-3Cu<sup>+</sup>] state) has a broad and poorly resolved four-line hyperfine split axial signal [26]. The purple form of N<sub>2</sub>OR after reduction of the CuA centre (with the CuZ centre in the [2Cu<sup>2+</sup>-2Cu<sup>+</sup>] state) shows an extremely weak EPR signal and must be considered EPR-silent, although it still exhibits an absorption band at 540 nm and a small contribution at 640 nm. In fact, magnetic circular dichroism (MCD) data confirmed that the CuZ centre in that form is not a ferromagnetically coupled (S = 1) centre but must be anti-ferromagnetically coupled (S = 0) [16].

It is also important to mention that *Paracoccus pantotrophus* N<sub>2</sub>OR purified in the presence of oxygen exhibits a CuZ\*/CuZ<sub>total</sub> ratio of 0.66, whereas this ratio is 0.29 when the enzyme is isolated in the absence of oxygen [15]. Therefore, it seems that

CuZ centre has always been present as CuZ\* in all the reported preparations of N<sub>2</sub>OR, regardless of the presence or absence of oxygen during purification.

It has been argued that the oxidized form of the CuZ centre (in the [2Cu<sup>2+</sup>–2Cu<sup>+</sup>] state) is catalytically important, and its redox potential was estimated to be  $E'^{\circ} = +60$  mV, for the reduction of [2Cu<sup>2+</sup> : 2Cu<sup>+</sup>] to [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>]. However, this is still a subject of discussion and contradictions, as its catalytic activity seems to be very low, and similar to that of N<sub>2</sub>OR with the CuZ centre either in the dithionite-reduced state (redox active [1Cu<sup>2+</sup>–3Cu<sup>+</sup>]) or as CuZ\* (redox inactive [1Cu<sup>2+</sup>–3Cu<sup>+</sup>]) [15].

The redox active purple form, in which the CuZ can be oxidized and reduced between the [2Cu<sup>2+</sup> : 2Cu<sup>+</sup>] and [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] states, had not been observed in *M. hydrocarbonoclasticus*. Here, we report, for the first time, the isolation of the purple form of N<sub>2</sub>OR from this organism. Moreover, N<sub>2</sub>OR was isolated not only from a microaerobic culture in the presence of nitrate but also from a strictly anaerobic culture purified in the presence of oxygen. This is the first report of a purple N<sub>2</sub>OR isolated in the presence of oxygen. The purple form of *M. hydrocarbonoclasticus* N<sub>2</sub>OR was characterized using visible and EPR spectroscopies and the catalytic activity of the enzyme with the CuZ centre in different redox states was determined.

## 2. MATERIAL AND METHODS

### (a) Preparation of the different nitrous oxide reductase forms

*Marinobacter hydrocarbonoclasticus* 617 (previously known as *Pseudomonas nautica* 617 [13]) was grown in a 10 l fermenter at 30°C in artificial seawater, with yeast extract (0.1%) and lactate (6%) as carbon and energy sources [27]. The medium was supplemented with a Starkey oligoelement solution [28] (0.2 ml l<sup>-1</sup> of culture) and 0.2 mM sodium nitrate (I. Fernandes, I. Moura, P. Paes de Sousa & S. R. Pauleta 2011, unpublished data). The bacterium was grown for 48 h with pH being continuously monitored and maintained at 7.5. A low aeration rate (0.2 v/v min) and an agitation speed of 150 rpm (central axis with flat-blade turbines) were used.

The periplasmic extract was obtained by diluting five times the resuspended cells into Tris–HCl (10 mM; pH 7.6), EDTA (0.5 mM) for 30 min. The soluble extract was obtained by centrifugation for 45 min at 125 000g (Beckman L-70 ultracentrifuge), and loaded onto a DE52 Fast Flow chromatographic column (GE Healthcare), equilibrated with Tris–HCl (10 mM; pH 7.6). The proteins were eluted with a gradient between 0 and 500 mM NaCl in Tris–HCl (10 mM; pH 7.6). The fractions containing N<sub>2</sub>OR were combined and concentrated in a diaflo apparatus (Millipore) over a 30 kDa cut-off membrane. This fraction was then loaded onto a gel filtration column (Superdex 200, GE Healthcare) equilibrated with Tris–HCl (300 mM; pH 7.6). The fractions containing the pure N<sub>2</sub>OR were combined, concentrated and stored at –80°C until further use.

The purity of the fractions was checked throughout the purification by SDS–PAGE, and N<sub>2</sub>OR concentration was determined using an extinction coefficient

at 640 nm, for the dithionite-reduced form, of 7.1 mM<sup>-1</sup> cm<sup>-1</sup>, for the sample named 'anaerobic2' [17]. Total protein was quantified using the bicinchoninic acid (BCA) assay (Sigma) and bovine serum albumin as protein standard [29]; the total copper content was determined using the 2,2'-biquinoline assay [30].

N<sub>2</sub>OR from different anaerobic cultures was prepared and purified in the presence of oxygen as previously reported [17].

### (b) Spectroscopic methods

N<sub>2</sub>OR in different redox states was prepared by reduction with sodium ascorbate or sodium dithionite or by oxidation with potassium ferricyanide in Tris–HCl (100 mM; pH 7.6).

UV–visible absorption spectra were recorded in a Shimadzu UV-1800 spectrophotometer using 1 cm quartz cells. X-band EPR spectra were recorded on a Bruker EMX spectrometer equipped with a rectangular cavity (model ER 4102ST) and an Oxford Instruments continuous-flow cryostat at 30 K temperature. EPR spectra were simulated using the program WIN-EPR SIMFONIA (v. 1.2) from Bruker Instruments.

Samples for EPR spectroscopy were prepared with 70 μM enzyme in Tris–HCl (100 mM; pH 7.6). Spin quantification was performed at the same temperature under non-saturating conditions, using a sample of dithionite-reduced N<sub>2</sub>OR (1 spin). Experimental conditions are described in the figure legends.

### (c) Activity assay

The activity assay was performed using the procedure previously described [31], by adding N<sub>2</sub>OR (100 nM) in different redox forms as the last reactant into a cuvette already containing reduced methyl viologen and N<sub>2</sub>O in Tris–HCl (100 mM, pH 7.6). The specific activity of the enzyme is expressed in units per milligram, which corresponds to micromoles of N<sub>2</sub>O reduced per minute per milligram of N<sub>2</sub>OR dimer.

## 3. RESULTS AND DISCUSSION

### (a) Microaerobic culture of *Marinobacter hydrocarbonoclasticus*

The periplasmic fraction of *M. hydrocarbonoclasticus* grown under microaerobic conditions in the presence of nitrate, contained a large amount of nitrite reductase cytochrome *cd*<sub>1</sub> and N<sub>2</sub>OR, thus indicating that this bacterium can be classified as an aerobic denitrifier, similar to *Paracoccus denitrificans* and *Pseudomonas stutzeri* [32,33].

### (b) Characterization of the purple form of nitrous oxide reductase from *Marinobacter hydrocarbonoclasticus*

#### (i) Spectroscopic characterization

The 'purple' form of *M. hydrocarbonoclasticus* N<sub>2</sub>OR was purified in the presence of oxygen from two different cultures. In one case, *M. hydrocarbonoclasticus* was grown under microaerobic conditions in the presence of nitrate ('aerobic sample'), and in the other this bacterium was grown under strict anaerobic conditions ('anaerobic1 sample'). The purified enzyme from these two cultures has similar features (see below).

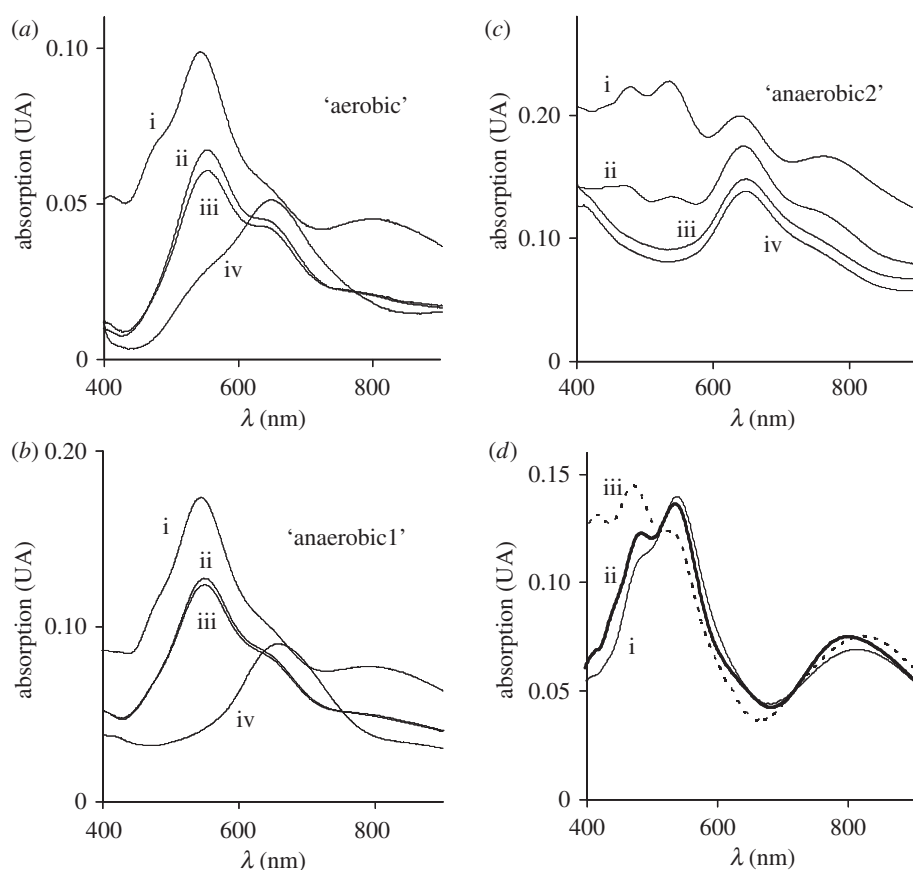


Figure 2. UV-visible spectra of nitrous oxide reductase purified in the presence of oxygen from (a) an aerobic culture; (b) an 'anaerobic1' culture and (c) an 'anaerobic2' culture. In each panel the spectra of the (i) fully oxidized, (ii) as-isolated, (iii) ascorbate-reduced and (iv) dithionite-reduced forms of the enzyme are presented. (d) Difference spectra between fully oxidized and the ascorbate-reduced forms for the (i) 'aerobic' (solid line) (ii) 'anaerobic1' (thick line) and (iii) 'anaerobic2' (dotted line) N<sub>2</sub>OR samples.

These N<sub>2</sub>OR preparations present a UV-visible spectrum characterized by an absorption maximum at 550 nm with a shoulder at 635 nm (figure 2*a(ii)*, *b(ii)*) and a very small absorption band around 800 nm. After oxidation with potassium ferricyanide, the absorption maximum at 550 nm shifts to 540 nm and the absorption band at 800 nm becomes more intense (figure 2*a(i)*, *b(i)*). This is evidence for the CuA centre being almost completely reduced in as-isolated N<sub>2</sub>OR (approx. 10% and 3% of CuA centre is oxidized in as-isolated 'aerobic' and 'anaerobic1' N<sub>2</sub>OR, respectively), and becoming oxidized upon addition of potassium ferricyanide.

The absorption spectrum of the CuA centre of N<sub>2</sub>OR can be obtained by subtracting the ascorbate-reduced N<sub>2</sub>OR spectrum from the ferricyanide-oxidized N<sub>2</sub>OR spectrum (figure 2*d*). Analysis of these spectra shows that the CuA centre has three main absorption bands at 480, 540 and 800 nm, and that the absorption bands observed at 550 and 635 nm in the as-isolated spectrum of N<sub>2</sub>OR correspond to the CuZ centre in the [2Cu<sup>2+</sup> : 2Cu<sup>+</sup>] oxidation state (see below). Hence, this is the first time that this redox form of N<sub>2</sub>OR (the purple form) has been isolated in the presence of oxygen and also from a microaerobic culture in the presence of nitrate. In fact, even though *Alcaligenes xylosoxidans* and *Paracoccus denitrificans* N<sub>2</sub>OR have also been isolated with the CuA centre reduced and the CuZ centre in the [2Cu<sup>2+</sup> : 2Cu<sup>+</sup>] redox state, in these cases the enzymes

were isolated under exclusion of oxygen from bacteria grown under anaerobic denitrifying conditions and purified under exclusion of oxygen [22,23].

The spectrum of the as-isolated purple N<sub>2</sub>OR after 1 min reduction with sodium ascorbate exhibits absorption bands at 550 nm with a shoulder at 635 nm (figure 2*a(iii)*, *b(iii)*), which is similar to that described for the equivalent N<sub>2</sub>OR forms from *Pseudomonas stutzeri* or *Paracoccus pantotrophus* N<sub>2</sub>OR [15,21].

The reduction of the purple *M. hydrocarbonoclasticus* N<sub>2</sub>OR with sodium dithionite reduces the CuA centre to [Cu<sup>+</sup> : Cu<sup>+</sup>] and the CuZ centre to [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] (figure 2*a(iv)*, *b(iv)*). The visible spectrum of this form, with an absorption band with maximum at 655 nm ('aerobic' sample) or at 660 nm ('anaerobic1' sample), is different from that of the enzyme with CuZ\* in the [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] redox state (figure 2*c(iii)*; maximum at 640 nm). Moreover, the absorption band is also broader in the spectrum of the enzyme with a [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] CuZ centre than that of [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] CuZ\*, with an increased contribution at higher wavelengths.

These spectral characteristics are different from those presented by dithionite-reduced N<sub>2</sub>OR isolated in the presence of oxygen from different bacterial sources [15,21] and also different from those previously reported for *M. hydrocarbonoclasticus* N<sub>2</sub>OR (which had the CuZ centre in the [1Cu<sup>2+</sup> : 3Cu<sup>+</sup>] CuZ\* state and differed in the amount of oxidized CuA centre) [17]. In fact, as previously shown by

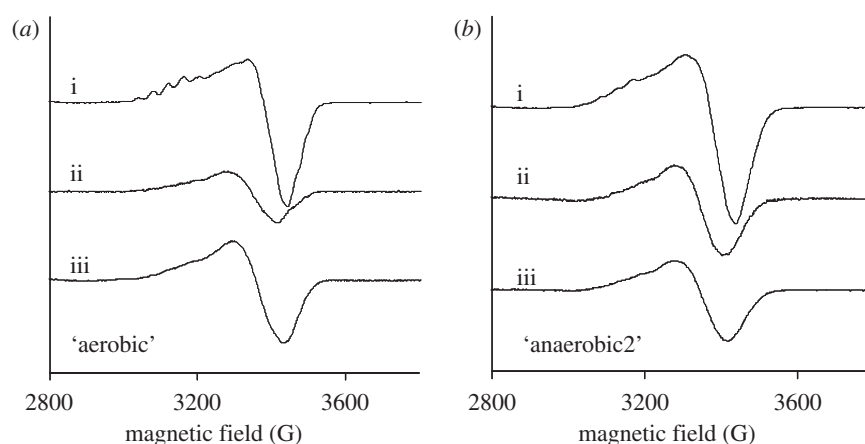


Figure 3. EPR spectra of N<sub>2</sub>OR purified in the presence of oxygen from (a) an 'aerobic' and (b) an 'anaerobic2' culture. In each panel the spectra of the (i) fully oxidized, (ii) as-isolated minus the CuA centre contribution and (iii) dithionite-reduced forms of the enzyme are presented. Instrumental parameters: modulation amplitude (5 G); microwave frequency (9.66 GHz); temperature (30 K).

Prudencio *et al.* [17], N<sub>2</sub>OR isolated in the presence of oxygen and obtained from an anaerobic culture of *M. hydrocarbonoclasticus* shows absorption bands at 480, 540, 640 and 800 nm when oxidized with ferricyanide. Thus, the CuA centre is partially oxidized (480, 540 and 800 nm absorption bands, see above) and the CuZ centre is in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] CuZ\* state (640 nm) (figure 2c(ii)). This form of N<sub>2</sub>OR has been named 'pink' owing to its colour, and in the present work corresponds to the 'anaerobic2' N<sub>2</sub>OR sample.

Regarding the redox behaviour of the 'anaerobic2' N<sub>2</sub>OR, sodium ferricyanide is able to fully oxidize the CuA centre (figure 2c(i)) which is 40 per cent oxidized in the as-isolated form, whereas both sodium ascorbate and sodium dithionite fully reduce the CuA centre, with the CuZ centre remaining unchanged and in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state (figure 2c(iii)(iv)). It is also important to mention that the degree of oxidation of the CuA centre varies among different preparations, whereas the CuZ centre has always been found mainly in the resting state.

Nevertheless, a N<sub>2</sub>OR preparation from another anaerobic culture/purification in the presence of oxygen ('anaerobic1' N<sub>2</sub>OR sample) shows the typical spectroscopic features of the 'purple' form, as described above (figure 2b). In this preparation, the fully oxidized form (figure 2b(i)) has a 550/640 nm absorbance ratio of 1.7, a value identical to that obtained for the 'aerobic' preparation and higher than the absorbance ratio of the 'anaerobic2' sample of 1.1 (figure 2c). A similar absorbance ratio was reported for *Achromobacter xylosoxidans* purple N<sub>2</sub>OR (1.8) [23] and for *Paracoccus pantotrophus* purple N<sub>2</sub>OR (1.9) [15] (table 2).

Moreover, the dithionite-reduced purple forms of *M. hydrocarbonoclasticus* N<sub>2</sub>OR not only present a distinct visible spectrum (as mentioned) but also have different redox behaviour. Although complete reduction with sodium dithionite is very slow, as observed for purple N<sub>2</sub>ORs isolated from other organisms [5,15], this reduction is reversible (data not shown), as addition of potassium ferricyanide allows fast regeneration of the fully oxidized purple form characterized by the spectrum in figure 2a(i). This

Table 3. Spin quantification of the *Marinobacter* nitrous oxide reductase (N<sub>2</sub>OR) EPR spectra.

	'aerobic' N <sub>2</sub> OR	'anaerobic2' N <sub>2</sub> OR
fully oxidized	1.4	2.2
as-isolated CuA contribution	0.4	1.0
dithionite reduced	1.0	1.0

indicates that the CuZ centre of purple N<sub>2</sub>OR can be reduced and oxidized between [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] and [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] redox states, in contrast to the CuZ centre in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state, which cannot be oxidized.

The EPR spectra of the different redox states of the two distinct N<sub>2</sub>OR preparations ('aerobic' and 'anaerobic2' N<sub>2</sub>ORs) were analysed (figure 3). The total spin was quantified for each redox state (table 3), assuming that dithionite-reduced N<sub>2</sub>OR presents a total spin of 1. This assumption is based on the fact that in this state the CuZ centre contains one unpaired electron and is in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state, whereas the CuA centre is reduced [Cu<sup>1+</sup>:Cu<sup>1+</sup>] and does not contribute to the EPR spectrum [34].

In all preparations, the fully oxidized form of N<sub>2</sub>OR has an EPR spectrum dominated by the features of the CuA centre (figure 3a,b(i)), as observed in the visible spectrum of these samples. This EPR spectrum is characterized by the typical seven-line hyperfine splitting pattern, with  $g_{\parallel} = 2.18$ ,  $g_{\perp} = 2.04$  and  $A_{\parallel} \approx 40$  G, regardless of the preparation. In the case of the 'anaerobic2' N<sub>2</sub>OR sample, the seven-line hyperfine splitting is less resolved as a result of the larger contribution of the resting CuZ centre characterized by a four-line hyperfine split axial EPR signal [5] (see below).

The dithionite-reduced N<sub>2</sub>OR has a broad axial EPR signal (figure 3a,b(iii)). The EPR spectrum of the N<sub>2</sub>OR with mainly CuZ\* ('anaerobic2') is characterized by  $g_{\parallel} = 2.16$ ,  $g_{\perp} = 2.04$  and  $A_{\parallel} \approx 60$  G [34]. At this stage, without further deconvolution of the

Table 4. Specific activity of *Marinobacter* nitrous oxide reductase (N<sub>2</sub>OR) isolated from the different growth conditions. The table also shows the main redox state of the CuZ centre in each redox state. asc, ascorbate; dth, dithionite.

redox state	'aerobic'		'anaerobic1'		'anaerobic2'	
	activity (U mg <sup>-1</sup> )	redox state CuZ centre <sup>a</sup>	activity (U mg <sup>-1</sup> )	redox state CuZ centre <sup>a</sup>	activity (U mg <sup>-1</sup> )	redox state CuZ centre <sup>a</sup>
fully oxidized	0.4	60% [2Cu <sup>2+</sup> :2Cu <sup>+</sup> ]	0.4	60% [2Cu <sup>2+</sup> :2Cu <sup>+</sup> ]	1.1	[1Cu <sup>2+</sup> :3Cu <sup>+</sup> ]
asc-reduced	0.3	60% [2Cu <sup>2+</sup> :2Cu <sup>+</sup> ]	0.3	60% [2Cu <sup>2+</sup> :2Cu <sup>+</sup> ]	0.9	[1Cu <sup>2+</sup> :3Cu <sup>+</sup> ]
dth-reduced	0.2	[1Cu <sup>2+</sup> :3Cu <sup>+</sup> ]	0.3	[1Cu <sup>2+</sup> :3Cu <sup>+</sup> ]	0.2	[1Cu <sup>2+</sup> :3Cu <sup>+</sup> ]
fully reduced	88.0	[4Cu <sup>+</sup> ]	96.1	[4Cu <sup>+</sup> ]	92.4	[4Cu <sup>+</sup> ]

<sup>a</sup>The CuZ centre content for 'aerobic' and 'anaerobic2' N<sub>2</sub>OR samples was determined based on the EPR spectra, whereas the one indicated for 'anaerobic1' was estimated by comparing its visible spectral features with those of the 'aerobic' sample.

spectrum of the dithionite-reduced N<sub>2</sub>OR sample with 60 per cent CuZ and 40 per cent CuZ\*, it is difficult to determine the characteristic spectrum of CuZ in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state, although it should be similar to that reported by Oganessian *et al.* [35].

The EPR spectrum of the CuA centre can be obtained by subtracting the EPR spectrum of the fully oxidized N<sub>2</sub>OR from that of the as-isolated enzyme. The contribution of the CuA centre was then subtracted from the EPR spectrum of the as-isolated purple N<sub>2</sub>OR. For the 'aerobic' N<sub>2</sub>OR sample, this difference spectrum is shown in figure 3a(ii), and has a spin quantification of 0.4 (table 3). Taking into account that this N<sub>2</sub>OR sample is proposed to have the CuZ centre mainly in the [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state, and that this state is diamagnetic, the amount of spin that is quantified must correspond to the amount of CuZ centre in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state. Therefore, the purple form of N<sub>2</sub>OR isolated in the presence of oxygen ('aerobic' N<sub>2</sub>OR) has 40 per cent of the CuZ centre as the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state, and 60 per cent in the CuZ [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state. As observed in other anaerobic preparations of purple N<sub>2</sub>OR from other organisms, the CuZ centre is not in a single redox state and the amount of CuZ centre in the paramagnetic [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state is comparable with that reported in those studies [15].

In contrast, the spin contribution of the CuA centre to the fully oxidized EPR spectrum of 'anaerobic2' N<sub>2</sub>OR is 1, and thus in this sample the CuZ centre is present almost completely in the paramagnetic CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state, as previously observed [17].

#### (ii) Specific activity of the different redox forms of N<sub>2</sub>OR

In addition to the spectroscopic characterization, it is also important to evaluate the specific activity that is associated with the CuZ centre in the [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] redox state. This state is redox active, in contrast to the redox inactive CuZ centre in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state.

The modified activity assay described by Dell'Acqua *et al.* [31] enables the separation of the slow activation (complete reduction of CuZ centre to [4Cu<sup>+</sup>]) accomplished with reduced methyl viologen from the catalytic activity. Therefore, with this assay, it will be possible to determine the specific activity of N<sub>2</sub>OR in the different redox states: fully oxidized

(CuA as [Cu<sup>1.5+</sup>:Cu<sup>1.5+</sup>] and CuZ as [2Cu<sup>2+</sup>:2Cu<sup>+</sup>]); ascorbate-reduced (CuA as [Cu<sup>+</sup>:Cu<sup>+</sup>] and CuZ as [2Cu<sup>2+</sup>:2Cu<sup>+</sup>]); dithionite-reduced (CuA as [Cu<sup>+</sup>:Cu<sup>+</sup>] and CuZ as [1Cu<sup>2+</sup>:3Cu<sup>+</sup>]) and fully reduced (CuA as [Cu<sup>+</sup>:Cu<sup>+</sup>] and CuZ as [4Cu<sup>+</sup>]). The specific activity of N<sub>2</sub>OR in these different redox forms is shown in table 4.

The results obtained clearly show that none of these redox forms has an activity comparable with the specific activity that is reached with the fully reduced form of the enzyme, and that there are no significant differences between the redox forms of the different preparations of N<sub>2</sub>OR.

A similar result was obtained for N<sub>2</sub>OR from the organisms listed in table 2—as the specific activity of these enzymes is very low compared with the high specific activity of the fully reduced enzyme—with the exception of *Paracoccus denitrificans* purple N<sub>2</sub>OR in which the as-isolated enzyme has a high specific activity [22].

These results show that the [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state of the CuZ centre, that is the main state of the CuZ centre in the two preparations of *M. hydrocarbonoclasticus* purple N<sub>2</sub>OR, is not catalytically competent, even though the CuZ centre is in state that is redox active and thus can be reversibly reduced to [1Cu<sup>2+</sup>:3Cu<sup>+</sup>]. A similar conclusion has also been reached by Thomson and co-workers [15], although in that case the purple N<sub>2</sub>OR form was isolated in the absence of oxygen.

It is biologically relevant to correlate these different redox forms with the *in vivo* cellular capacity of N<sub>2</sub>OR to reduce N<sub>2</sub>O. In the recent work of Zumft *et al.* [36], it is reported that in *Paracoccus denitrificans*, the *in vitro* N<sub>2</sub>O reducing capacity is higher in cells from which N<sub>2</sub>OR is isolated with the CuZ centre mainly as CuZ [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] than when it is mainly in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state. However, after isolation, both enzyme forms have similarly low specific activities (3.6 U mg<sup>-1</sup>). Therefore, at this moment, it is still not possible to conclusively say which of the enzyme forms is the active one *in vivo*, because the fully reduced form still has higher specific activity than any of the as-isolated forms (when the activity of the purified enzyme is compared using the same assay).

In conclusion, N<sub>2</sub>OR purified either in the absence or in the presence of oxygen and with CuZ centre in either the [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] or [1Cu<sup>2+</sup>:3Cu<sup>+</sup>]

redox states, is not fully active. An activation process involving the complete reduction of the CuZ centre is required to turn the enzyme into an active state. It is possible to hypothesize that N<sub>2</sub>OR might be inactivated by a conformational rearrangement during the purification process, which makes it imperative to obtain structural information on the active fully reduced form of N<sub>2</sub>OR to be able to identify these conformational changes, different coordination spheres or rearrangements.

#### 4. CONCLUSION

Here, we present the first characterization and purification of a purple form of N<sub>2</sub>OR isolated from *M. hydrocarbonoclasticus*. For the first time, N<sub>2</sub>OR from this bacterium was isolated from a culture grown under non-strictly anaerobic conditions, which indicates that the denitrification pathway can occur in the presence of oxygen and thus that this bacterium can be classified as an aerobic denitrifier.

Moreover, the spectroscopic properties of the purple form of *M. hydrocarbonoclasticus* N<sub>2</sub>OR were compared with those from other bacterial sources, showing that the catalytic centre, CuZ, is present as a mixture of two species: 60 per cent as CuZ in the [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state and 40 per cent as CuZ\* in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state.

The activity assay allowed us to conclude that also the oxidized [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state of the CuZ centre is not catalytically competent, because the activity of the enzyme with CuZ centre mainly in that state (either fully oxidized, as-isolated or ascorbate-reduced) is much smaller than that of the enzyme with the CuZ centre in the fully reduced state and of the same order of magnitude of specific activity as that of the enzyme with the CuZ centre in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state.

Up to now, only the fully reduced state [4Cu<sup>+</sup>] and the CuZ<sup>0</sup> states have been identified as being catalytically competent and not requiring activation, CuZ<sup>0</sup> being an intermediate in the catalytic cycle of N<sub>2</sub>OR [20]. However, it is important to point out that in the proposed catalytic cycle of N<sub>2</sub>OR, there is indeed a [2Cu<sup>2+</sup>:2Cu<sup>+</sup>] state of the CuZ centre, as the first intermediate after N<sub>2</sub>O release [37], which has neither yet been observed nor characterized.

This study also shows that in order to obtain N<sub>2</sub>OR in the purple form, it does not have to be purified in the absence of oxygen and that the enzyme is not damaged by its presence, as upon activation the specific activities of the enzyme which was isolated with the CuZ centre mainly as CuZ ('aerobic') or as CuZ\* ('anaerobic2') are similar.

However, we believe that the storage conditions of the cellular extract have an influence on the enzyme redox state (even they are if stored at -80 °C), as N<sub>2</sub>OR purified from cellular extracts stored for long periods at -80 °C have the CuZ centre mainly in the [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state (compare 'anaerobic1' and 'anaerobic2'). In addition, the number of purification steps and duration of these steps also influence the redox state of the enzyme, in particular the CuZ centre.

These differences seem to indicate a conformational change of the enzyme, especially around the CuZ centre, to a resting non-active state upon storage. This conformational change could be the result of a rearrangement in the coordination sphere of CuA or CuZ centres or in the pathway between these two, which might be identified by comparing the structures of N<sub>2</sub>OR in the different redox states, because the presently available three-dimensional structures have the CuZ centre in the CuZ\* [1Cu<sup>2+</sup>:3Cu<sup>+</sup>] state.

During revision of this manuscript, the structure of a purple form of N<sub>2</sub>OR from *Pseudomonas stutzeri* was published [38]. This structure shows an additional sulphur bridging CuI and CuIV atoms, forming a [4Cu:2S] cluster. According to these new data, we can now assume that the differences of the properties between CuZ and CuZ\* might be partly due to a different cluster composition.

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