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View Journal | View Issue Conversion of nitrous oxide to nitrogen by cobalt-

Trevor D. Rapson,^{*a} Soeren Warneke,^a Mustafa M. Musameh,^b Helen Dacres,^a Ben C. T. Macdonald^a and Stephen C. Trowell^a

substituted myoglobin*

Developing technology to decrease greenhouse gas emissions is one of the greatest challenges we face in the 21st century. Nitrous oxide (N₂O) is an important greenhouse gas, which is estimated to contribute 6% of the overall global warming effect. Herein we report the use of cobalt substituted heme proteins to reduce N₂O to nitrogen (N₂). This catalysis was electrochemically driven using methyl viologen or benzyl viologen as electron transfer partners for cobalt myoglobin. Using bulk electrolysis we demonstrated the production of ¹⁵N₂ from ¹⁵N₂. This catalysis, however, was noted to be poor, most likely due to oxidative damage to the protein scaffold.

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1. Introduction

Nitrous oxide has a global warming potential approximately 300 times greater than carbon dioxide over a 100 years time horizon. In the stratosphere, N_2O is oxidized to NO and NO_2 , which catalyze the destruction of ozone.^{1,2} Unlike other greenhouse gases such as carbon dioxide, N_2O is largely produced by natural systems, predominantly as an intermediate in the denitrification pathway.³ Over the last century, there has been a 20% increase in atmospheric N_2O concentration, attributed to the use of nitrogen-based fertilizers, intensive pastoral farming and waste-water treatment. It is estimated that up to 10% of anthropogenic N_2O emissions are from the biological treatment of sewage, manure and industrial effluents.⁴ Such N_2O emissions from sewage works, are a feasible target for point source remediation.

Metalloproteins that can catalyze the reduction of N_2O to N_2 have been investigated for their potential to help reduce nitrous oxide emissions.⁵ However, nitrous oxide binds very poorly to metal ions making it difficult to activate it towards reduction. In the face of this constraint, nature has evolved a class of copper dependent N_2O reductases (N_2OR) that catalyze the final step of denitrification.⁵ N_2OR are soluble enzymes, located in the bacterial periplasm. One promising approach currently being explored is the use of N_2OR produced in genetically modified crops to 'scrub' N_2O emissions from soils.⁶ Naturally occurring N_2OR enzymes are, however, inactivated by exposure to O_2 and the activity of N_2OR is also sensitive to pH, which makes them unsuitable, without further modification, for bioremediation.⁷

In addition to copper N₂O reductases, N₂O has been reported to react with a number of other transition metal complexes,⁸ in particular the cobalt corrins found in cobalamin (Vit B12 – ESI Fig. 1†). Nitrous oxide has been shown to inhibit the activity of cobalamin dependent methionine synthase.⁹ The mechanism of inhibition has been proposed to involve a reaction between Co^{I} and N₂O. The cobalamin cofactor alone, when reduced with sodium borohydride to Co^{I} , reacts with N₂O to produce N₂ and Co^{II} .^{10,11} To date, neither methionine synthase nor cobalamin have been used in N₂O remediation, largely because of their oxygen sensitivity. In order to develop an alternative strategy for mitigating N₂O, we sought to develop a N₂OR that is not inhibited by oxygen. Other desirable characteristics are that the protein needs to be readily produced allowing large-scale production making the process commercially viable.

Heme proteins are attractive scaffolds for engineering, as their gas binding properties can be 'tuned' through modifying the amino acid residues surrounding the heme cofactor. In particular the binding of oxygen to the heme center is stabilized by electrostatic interactions between the polar Fe-O bond and distal pocket residues such as histidine or tyrosine.12,13 If this interaction is removed through site directed mutagenesis, the affinity of the iron heme center for oxygen dramatically decreases.^{13,14} This could be used to prevent O₂ from binding to the heme centre.¹⁵ Furthermore, the native heme group can readily be removed and the protein can be reconstituted with an artificial porphyrin or other macrocycle. For example, iron protoporphyrin IX can be substituted with copper, cobalt or manganese protoporphyrin IX16 or cobalt corrins.17 More recently it has become possible to express heme proteins with artificial metalloporphyrins.18

Given the N_2O reactivity of cobalt metal centers within corrin rings (*e.g.* cobalamin), we decided to use cobalt macrocycles to make cobalt substituted heme proteins, to determine if they

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^aCSIRO, Black Mountain, ACT, 2601, Australia. E-mail: trevor.rapson@csiro.au ^bCSIRO Clayton, VIC, 3168, Australia

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2. Experimental section

2.1 Chemicals

All chemicals were purchased from Sigma Aldrich and used as received unless stated otherwise. Cobalt protoporphryin IX was purchased from Frontier Scientific and unlabelled nitrous oxide from Coregas.¹⁵N–N₂O (100%) was purchased from Sigma Aldrich, US branch.

2.2 Preparation of cobalt myoglobin (CoMb)

The heme group was removed from horse heart myoglobin (FeMb) using the acid/butanone method described previously to generate apoMb.^{16,19} Reconstitution of apoMb with cobalt protoporphyrin following the method described by Hoffman and Petering²⁰ with a modification that sodium dithionite was not used, preparing cobalt myoglobin as Co^{III}Mb as developed by Hambright and Lemelle.²¹ Briefly apoMb was incubated with a two times excess of cobalt protoporphyrin (CoPPIX) dissolved in a minimal amount of pyridine at 4 °C overnight. Unbound CoPPIX was removed using a PD10 column from GE Healthcare eluting CoMb with of 50 mM phosphate (pH 7.4). CoMb was then concentrated to the required concentration using spin dialysis.²²

2.3 Cyclic voltammetry

Cyclic voltammetry was carried out using a BAS Epsilon potentiostat with a C3 cell stand. A three-electrode system was employed comprising a glassy carbon electrode, Pt wire counter and Ag/AgCl reference electrode. Anaerobic conditions were established through purging with argon. During experiements an argon blanket was used to maintain anaerobic conditions. Nitrous oxide was added from a saturated solution (prepared by exhaustive purging with N₂O). Buffer solutions of 50 mM phosphate (pH 7.4) and 50 mM Tris (pH 7.5) were used with either methyl viologen or benzyl viologen (at varying concentrations 5 μ M to 50 μ M) as the mediator. The use of 2-hydroxy-1,4-napthoquinone as a mediator was tested however no catalysis was observed.

For cyclic voltammetry, the enzyme electrodes were prepared firstly by polishing the glassy carbon electrode according to the manufacturers instructions (BASi). Secondly 10 μ L of a 100 μ M protein solution was dried at 4 °C and finally covered with a presoaked dialysis membrane (MW cut-off 3.5 kDa), fastened to the electrode using a rubber O-ring. All potentials are quoted *vs.* Normal Hydrogen Electrode (NHE), calculated as Ag/AgCl + 196 mV.

2.4 Bulk electrolysis

Bulk electrolysis was carried out using a homemade air-tight electrochemical cell. A carbon cloth electrode (AVCar HCB

1071 – 2 × 5 cm) was employed as the working electrode with a Ag/AgCl electrode immersed in the analyte solution while a Pt wire counter electrode was separated from the main solution by a CoralPorTM porous frit. The electrochemical solution was ~80 mL of 50 mM phosphate buffer with CoMb (15 μ M) and methyl viologen (2 mM). To remove atmospheric nitrogen the electrochemical solution was thoroughly purged with helium with stirring for 90 minutes. A mixture of 9 mL ¹⁴N–N₂O (100%) and 6 mL ¹⁵N–N₂O (100%) was added into the headspace of the electrochemical cell and the electrochemical solution was stirred for 90 minutes to ensure that equilibrium of N₂O between solution and headspace was reached.

Controlled potential was applied to the carbon cloth electrode ($-600 \text{ mV} \nu s$. NHE) using a BAS Epsilon potentiostat. 1 mL samples of the headspace were removed using an airtight syringe and immediately placed into a sample tube filled with helium. To avoid a negative pressure within the bulk electrochemical cell, 1 mL of helium gas was immediately injected following sampling. The concentration of $^{14}N-N_2$, $^{15}N-N_2$, $^{14}N-N_2O$ and $^{15}N-N_2O$ was determined at the UC Davis Isotope Analysis facility (full details in ESI†). Due to the expense of $^{15}N-N_2O$ this experiment could only be carried out once.

3. Results and discussion

Co^{III}Mb prepared was characterized using UV-Visible spectroscopy and was similar to that reported by Hambright and Lemelle (Fig. 1, λ_{max} 425 nm).^{21,23} The Co^{II}Mb form could be readily obtained with the addition of sodium dithionite (Fig. 1, λ_{max} 406 nm).

The nitrous oxide reductase activity of CoMb was subsequently tested using mediated catalytic voltammetry. Methyl viologen (-456 mV vs. normal hydrogen electrode – NHE) was chosen as the mediator, as it is typically used in N₂OR activity assays,²⁴ and to generate Co^I in cobalamin dependent methionine synthase.⁹ The enzyme electrodes were prepared by immobilizing CoMb under a dialysis membrane covering a glassy carbon electrode as described by Bernhardt and coworkers.²⁵ Experiments were initially conducted under anaerobic conditions using an argon blanket. Nitrous oxide was



Fig. 1 The UV-Vis spectrum of $Co^{III}Mb$ (solid line) as prepared by reconstitution and $Co^{III}Mb$ (dotted line) obtained by the reduction of $Co^{III}Mb$ with sodium dithionite.



Fig. 2 Catalytic conversion of N₂O to N₂. The voltammetric response before (dashed line) and after the addition of N₂O is shown (solid line) for: (A) cobalt myoglobin (CoMb) mediated by methyl viologen (MV – 40 μ M). (B) CoMb mediated by benzyl viologen (BV – 40 μ M) and (C) Iron myoglobin (FeMb) with benzyl viologen (BV – 40 μ M, scan rate = 5 mV s⁻¹). The arrow indicates the increase in current with the addition of N₂O.

added to the electrochemical cell by purging the solution with anaerobic N_2O gas to yield a saturated N_2O solution (~25 mM).

Addition of N₂O to the electrochemical cell resulted in significant amplification of the reductive current and the waveform changed from a peak-shaped transient voltammogram to a sigmoidal steady-state voltammogram (Fig. 2A). A catalytic response from CoMb was also observed when an alternative mediator, benzyl viologen ($-370 \text{ mV} \nu s$. NHE) was used. In this case the catalytic current was only 60% of that obtained using methyl viologen (Fig. 2B). A higher potential mediator 2-hydroxy-1,4-napthoquionone ($-137 \text{ mV} \nu s$. NHE) was tested, however no catalytic currents were observed (data not shown).

The pronounced catalytic current observed for CoMb was in contrast to the absence of catalysis with native FeMb (Fig. 2C). Furthermore, no changes were noted when N_2O was added to an electrochemical cell with only methyl viologen or benzyl viologen and no CoMb or with the protein alone without a mediator (ESI Fig. 2†).

The results observed for CoMb can be interpreted as shown in Scheme 1. Methyl viologen is reduced at the electrode through heterogeneous electron transfer (Step 1). CoMb in turn is reduced by methyl viologen to Co^{I} (Step 2) which is able to reduce N₂O to N₂ (Step 3). The amplified catalytic current noted with the addition of N₂O, is due to the rapid reduction of methyl viologen driving the enzymatic conversion of N₂O to N₂.

The voltammetric sweep rate is a powerful variable to probe the mechanisms of electrochemical processes. For both methyl viologen and benzyl viologen mediated experiments, the scan rate was varied between 5 mV s⁻¹ and 500 mV s⁻¹. It was clearly observed, for both methyl viologen and benzyl viologen that, as the scan rate increased, the heterogeneous reduction and oxidation of the mediator became too fast for homogenous electron transfer between the mediator and CoMb. At low scan rates (5 mV s⁻¹) sigmoidal catalytic responses were noted, as the scan rate was increased the voltammograms became more peak shaped (Fig. 3). This result provided strong evidence that an enzymatic response due to the reduction of N₂O by CoMb was being observed at slow scan rates (<20 mV s⁻¹).

The N₂O concentration dependence of the catalytic reaction was investigated by incrementally adding aliquots from a mediator solution saturated with N₂O (25 mM) to the electrochemical cell. There was an increase in the catalytic current with increasing N_2O concentrations (Fig. 4), however the catalytic current did not appear to follow standard Michaelis-Menten kinetics. The plot of catalytic current vs. N₂O concentration could be fitted to a sigmoidal curve (ESI Fig. 3[†]). The origin of this unusual concentration dependence is unclear. There are a number of reports which indicate that N₂O binds to several non-iron sites within heme proteins such as myoglobin and hemoglobin.^{26,27} It is possible that binding of N₂O to such non-heme sites gives rise to an allosteric effect on the N2O reductase activity of CoMb. Alternatively, the enzyme electrode was prepared by immobilizing CoMb under a dialysis membrane. This immobilization was required to obtain catalytic currents with N₂O, but the use of a membrane could



Scheme 1 The proposed pathway of electrochemical enzymatic catalysis whereby the mediator (either methyl viologen or benzyl viologen) supplies electrons to cobalt myoglobin (CoMb).



Fig. 3 The effect of scan rate on the catalytic voltammogram obtained for CoMb in the presence of N₂O (~25 mM) with benzyl viologen as a mediator (40 μ M).

interfere with the diffusion of N_2O from the solution to the enzyme electrode.

In order to confirm that CoMb reduces N₂O to N₂ we characterized the reaction products from a bulk electrolysis of isotopically labelled N2O (approximately 34% 15N-N2O) carried out in a helium atmosphere. A potential of -600 mV vs. NHE was applied to the carbon-cloth working electrode. Samples from the headspace were removed at different times and were analyzed for concentrations of ¹⁵N-N₂ and N₂. Over the course of the experiment, there was an increase in the levels of total N₂ (11.3 to 19.6 μ mol – Fig. 5) and ¹⁵N–N₂ (41 to 72 nmol – ESI Table 1^{\dagger}). There was also a doubling in the ratio of ¹⁵N–N₂ (a product of the enzymatic reaction) to ¹⁴N-N₂ (ESI Table 1[†]) from 1.57×10^{-5} to 3.42×10^{-5} . The atom percentage of 15 N–N $_2$ however was low (\sim 0.36%) indicating that there was a high level of contamination with atmospheric nitrogen. This contamination unfortunately was unavoidable due to the experimental constraints in using syringes to sample the headspace of the



Fig. 4 The effect of N₂O concentration on the voltammetric response from CoMb. Methyl viologen (40 μ M) was used as the mediator with a scan rate of 5 mV s⁻¹. N₂O concentration was varied by adding aliquots of a mediator solution saturated with N₂O.

bulk electrolysis cell rather than using a sampling loop attached directly to a mass spectrometer.

The pronounced catalytic currents observed upon the addition of N_2O when CoMb is immobilized on a glassy carbon electrode indicate that through a simple iron to cobalt substitution, myoglobin can be turned into a N_2O -reductase. No catalytic currents were noted when FeMb was used rather than CoMb or in the presence of the mediators alone, confirming that CoMb is the active center for the reduction of N_2O and the mediator.

The current electrochemical setup does not allow the oxygen sensitivity of CoMb to be fully examined, as the redox properties of the low potential mediators used are adversely affected by the presence of oxygen. In order to investigate this further, we are exploring options to achieve direct electron transfer between the cobalt metal center and an electrode, using options such as protein film voltammetry^{28,29} or site-specific covalent attachment to gold electrodes.³⁰ There have been some reports of direct electron transfer to CoMb on pyrolytic graphite electrodes^{31,32} however these experiments employed surfactant films, which have been shown to cause the release of heme from myoglobin.³³

It is important to note that, unlike naturally occurring N_2OR , CoMb is not inactivated by oxygen. Before enzyme assays can be performed with naturally occurring N_2OR , an activation step



Fig. 5 Reaction product analysis from the headspace of the bulk electrolysis of CoMb in the presence of ¹⁵N-labelled N₂O. A constant potential of -600 mV vs. NHE was applied to a carbon cloth electrode with methyl viologen (2 mM) as mediator.

needs to be carried out whereby the enzyme is reduced using high concentrations of methyl viologen (3 mM) and dithionite (1.5 mM) for 3 h.²⁴ Here, no pre-treatment was required for CoMb; the protein was prepared and used as $Co^{III}Mb$. The reactive Co^{I} state required to reduce N₂O was generated electrochemically during cyclic voltammetry.

There have been reports previously of heme proteins, such as cytochrome P450 and myoglobin, reducing N₂O.^{34,35} These examples required the production of Fe^I to reduce N₂O and therefore catalysis was only noted at large over-potentials \sim -850 mV *vs.* NHE. Using CoMb, the potential required for catalysis is -450 mV, 400 mV higher, which avoids non-specific reduction processes.

While cyclic voltammetry suggested that CoMb is able to reduce N_2O , bulk electrolysis was carried out to confirm the production of N_2 from the enzymatic reaction. To overcome contamination with atmospheric N_2 , ¹⁵N labelled nitrous oxide was used with a 40% ¹⁵N enrichment. Through the course of bulk electrolysis both an increase in ¹⁵N–N₂ (Fig. 5) and a doubling in the ratio of ¹⁵N–N₂ to ¹⁴N–N₂ (Table 1†). This result shows that CoMb is able to convert N_2O to N_2 .

There are two possible explanations for the relatively low amount of ¹⁵N-N₂ measured in the headspace in relation to ¹⁴N-N₂. The first is that CoMb selectively reduces ¹⁴N₂O over $^{15}N_2O$. Given that in our experiment a mix of $^{14}N_2O$ (60%) and $^{15}N_2O$ (40%), the larger increase in $^{14}N_2$ (~8 µmol, Table 1†) noted could be due to the enzymatic conversion of ¹⁴N₂O to ¹⁴N₂. The second and more likely explanation is that while CoMb is able to reduce N₂O to N₂, the enzyme is not very efficient. In the case of cobalamin dependent methionine synthase, Drummond and Matthews observed that the reduction of N₂O by cobalamin produces a potent oxidant, which damages the protein structure proximal to the cobalamin cofactor.^{9,36} They propose that N₂O undergoes a single electron transfer with cobalamin resulting in a hydroxyl radical. It is likely that in the case of CoMb a similar one-electron reduction of N2O takes places which damages CoMb preventing further catalysis. We plan to continue to investigate if indeed oxidative damage is occurring to CoMb during the conversion of N₂O to N₂ and will investigate using alternative heme protein scaffolds which might be more resistant to oxidative damage.37,38

4. Conclusion

Here we demonstrate that the use of cobalt-substituted myoglobin represents a new option for the development of N_2O mitigation strategies, based on reducing N_2O to N_2 . To continue this research both the effect of oxygen on the N_2O activity of CoMb and whether CoMb is indeed oxidatively damaged during catalysis needs to be explored further.

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