Evidence for an iron center in the ammonia monooxygenase from *Nitrosomonas europaea*

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Received 18 July 1996; revised version received 16 September 1996

Abstract Binding of the ligand, nitric oxide, in the presence of reductant was used to identify a ferrous $S = 3/2$ signal, characteristic of a ferrous nitrosyl complex, and a $g = 2.03$ copper or iron signal in membranes of the ammonia-oxidizing bacterium, *Nitrosomonas europaea*. The same ferrous $S = 3/2$ signal is thought to be a component of the membrane-associated methane monooxygenase (pMMO) of *Methylococcus capsulatus* Bath, since it is seen in the membrane fraction of cells expressing pMMO and in the purified enzyme, but not in the membrane fraction of cells expressing the soluble MMO [Zahn, J.A. and DiSpirito, A.A. (1996) J. Bacteriol. 178, 1018–1029]. Treatment of resting membranes or cells of *N. europaea* with nitrapyrin, 2-chloro,6-trichloromethylpyridine, resulted in the increase in magnitude of a $g = 6$, high-spin ferric iron signal. In the presence of NO and reductant, nitrapyrin prevented the formation of the $S = 3/2$ nitrosyl-iron complex while increasing the intensity of the $g = 6$ signal. Nitrapyrin is a specific inhibitor of, and is reduced by, the ammonia monooxygenase (AMO) [Bédard, C. and Knowles, R. (1989) Microbiol. Rev. 53, 68–83]. Taken together the data suggest that iron capable of forming the $S = 3/2$ complex is a catalytic component of AMO of *N. europaea*, possibly a part of the oxygen-activating center. Inactivation of the membrane-associated AMO with acetylene did not diminish the $S = 3/2$ nitrosyl-iron signal, the $g = 6$ signal, or the $g = 6$ signal.

Key words: Ammonia monooxygenase; Ammonia oxidation; Nitrification; Nitrapyrin; *Nitrosomonas europaea*

1. Introduction

The ammonia monooxygenase (AMO) in nitrifying bacteria and the membrane-associated methane monooxygenase (pMMO) in methanotrophic bacteria have many enzymatic similarities and putative structural genes with strong sequence homology [1–9]. The pMMO consists of three polypeptides with molecular masses of 47,000, 27,000, and 25,000 Da, contains 2.5 non-heme iron and 14.6 copper atoms [1]. In *Paracoccus denitrificans*, the AMO consists of two subunits with molecular masses of 46,000 and 38,000 Da and is proposed to contain copper[9]. In *Nitrosomonas europaea* indirect evidence suggests that AMO is composed of at least two polypeptides with molecular masses of 27,000[9] and 43,000 Da [4,5] which contain copper [4,11–14]. Acetylene, an irreversible inhibitor of the AMO, derivatizes a 27,000 Da polypeptide [10,11]. A membrane-associated polypeptide with a molecular mass of 25,500–27,000 Da is also derivatized by treatment of whole cells, washed membranes, or isolated pMMO from several methanotrophs with [^{14}C]acetylene [1,15,16]. In both *N. europaea* and *Methylococcus capsulatus* Bath, a second membrane-associated polypeptide with a molecular mass of 43,000 and 47,000 Da, respectively, is also labeled with [^{14}C]acetylene [1,10]. As originally proposed by Prior and Dalton [14], the labeling of the 47,000 Da polypeptide probably results from an activated acetylene intermediate, such as a ketene, which diffuses from the active site and forms a covalent attachment to amino acid residues in the active site or with neighboring polypeptides.

Like acetylene, nitrapyrin (2-chloro-6-trichloromethylpyridine) specifically inhibits AMO in nitrifiers (i.e., does not inhibit hydroxylamine oxidation) and pMMO in methanotrophs (i.e., does not inhibit methanol, formaldehyde or formate oxidation) [15–19]. Under micro-aerophilic conditions, nitrapyrin can be both an inhibitor of the AMO [20] and to undergo a two-electron reductive dechloroination to 2-chloro-6-hydroxyldichloromethylpyridine [21]. Aerobically 6-chloropicolinic acid is produced, probably by the chemical hydrolysis of enzymatically produced 2-chloro-6-hydroxyldichloromethylpyridine [20].

The electron paramagnetic resonance spectrum of pMMO indicates the presence of a type 2 copper center ($g_\perp = 2.057$, $g_\parallel = 2.24$, and $|A_\parallel| = 172$ G), a weak high-spin iron signal ($g = 6.0$), and a broad low-field ($g = 12.5$) signal [1]. Treatment of reduced pMMO with nitric oxide produces a ferrous–nitric oxide derivative with an electron spin of $S = 3/2$ and was similar to those for other non-heme iron proteins with g values near 4 and 2 [22–24]. In the present study, EPR derivative spectra of the membrane fraction from *N. europaea* treated with nitric oxide and nitrapyrin show that iron is a component of AMO.

2. Materials and methods

2.1. Materials and general methods

Materials, culture conditions, EPR spectroscopy, labeling with

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Abbreviations: AMO, ammonia monooxygenase; DMSO, dimethyl sulfoxide; 3-[*N*-morpholino]propane-sulfonic acid; MOPS; pMMO, membrane-associated methane monooxygenase; sMMO, soluble methane monooxygenase

This paper is contribution J-17083 from the Agriculture and Home Economics Experiment Station, Ames, Iowa, USA (Project 3252).

PII S0014-5793(96)01116-7

[U^{14}-C]acetylene, propylene oxide assay, isolation of membrane fractions, and preparations of membrane suspensions were performed as previously described [1,25,26]. Stock solutions (0.4 M) of nitrapyrin (Dow Chemical) were solublized in dimethylsulfoxide (DMSO) as previously described [20,21].

2.2. Nitric oxide and nitrapyrin experiments

Reaction mixtures (2.6 ml) contained cells or membrane fractions (approx. 50–80 mg protein/ml), 0.25 mM NH_4Cl and 20 mM 3-[*N*-morpholino]propane-sulfornic acid (MOPS), pH 7.2, were incubated in 7 ml serum vials sealed with teflon/silicone crimp seals in under anaerobic conditions. The samples were incubated in the presence of 10 mM nitrapyrin/DMSO or DMSO alone for 50 min at 26°C on a rotary shaker. For acetylene-treated samples, duroquinol- or ascorbate-reduced membranes were incubated for 5 min under anaerobic conditions followed by the addition of 1 ml of unlabeled acetylene and the mixture incubated at room temperature for 50 min. Following the 50 min incubation period, the nitric oxide derivative of reduced samples was prepared using in situ reduction of the nitrite ion as the source of the nitric oxide [1] or by addition of nitric oxide to reduced membrane samples under anaerobic conditions (see figure legends for a specific description of the sample treatments). Following formation of nitrosyl derivatives, membrane samples were immediately (< 1 min) transferred to quartz EPR tubes and stored at liquid nitrogen temperature prior to spectral analysis. Experimental controls for membrane samples treated with nitrapyrin contained 25 μM DMSO per ml of sample.

3. Results

3.1. Enzyme activity of membranes

The membrane fractions used in these studies exhibited AMO (2.1 ± 0.7 nmol propylene oxidized•min^{-1}•mg protein^{-1}) activity as measured by the epoxidation of propylene to propylene oxide using duroquinol as a reductant. Propylene oxidation activity was not detected using ascorbate as a reductant. However, enzyme turnover with ascorbate as a reductant was determined by labeling with [^{14}C]acetylene. In membrane samples, the amount of label ([^{14}C]acetylene) incorporated into the 27,000 Da polypeptide using of ascorbate as a reductant was approximately 5% of the level observed using duroquinol as the reductant. Enzyme turnover with ascorbate as a reductant has also been observed with pMMO from *M. capsulatus* Bath, although the rate was less than 10% of the rate observed using duroquinol as a reductant (Zahn and DiSpirito, unpublished results).

3.2. EPR spectra of membrane fractions

The EPR spectra of the membrane fraction from *N. europaea* (Fig. 1, trace A) were quantitatively identical to the spectrum reported previously [27]. The complex spectrum near $g = 6$ was similar to putatively uncoupled heme a_3 of partially reduced cytochrome aa_3 [27–30]. Iron of high-spin membrane-associated *c*-type cytochromes may also contribute to the spectrum [31]. A weak $g = 6$ signal is also seen in purified pMMO [1]. The type II copper signal ($g_{\|} = 2.28$, $A_{\|} = 180$ G, $g_{\perp} = 2.054$) is similar to that which is observed in cytochrome aa_3 [27] as well as in pMMO [1,32]. Thus, both $g = 6$ iron signal and type II copper signal may, in theory, be associated with AMO. The addition of ascorbate and dithionite to the membrane fractions resulted in the loss of the high-spin iron and copper signals (results not shown). The addition of nitric oxide (as NO or by reduction of added nitrite) under anaerobic conditions to the ascorbate-reduced (Fig. 1, trace B) or dithionite-reduced (not shown) membrane fractions resulted in the appearance of an intermediate spin, $S = 3/2$ ($g = 3.98$), center commonly associated with reduced non-heme iron centers and a $g = 2.03$ signal. Thus, a ferrous iron center found in resting membranes becomes detectable upon reaction with NO. The source of the $g = 2.03$ signal has not been identified, but may be associated with the copper center believed to be associated with AMO [4,10–13].

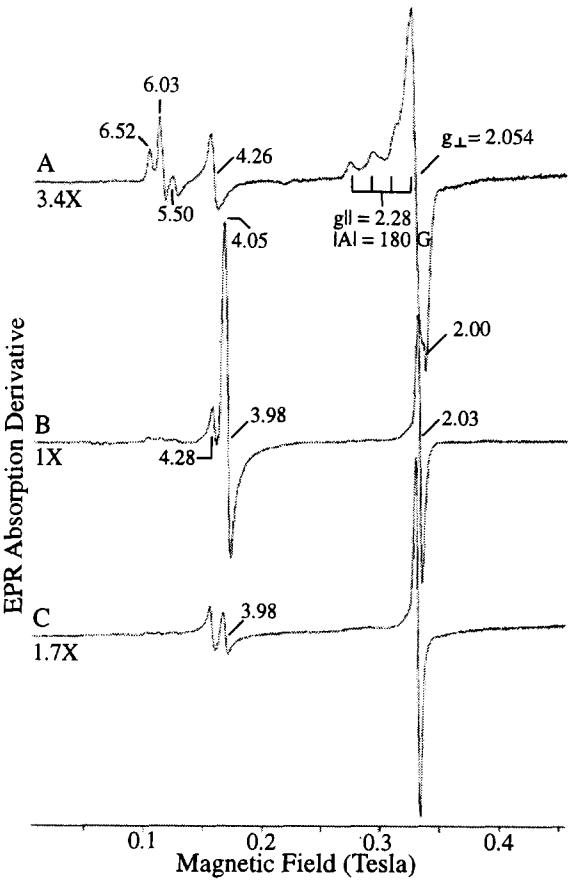

Fig. 1. EPR spectra of the membrane fraction of *N. europaea*. A: Resting membrane fraction. B: In situ reduction of nitrite to nitric oxide: membrane fraction plus ascorbate and $NaNO_2$ under anaerobic conditions. C: Sample B following aeration. Protein concentration was 51 mg/ml in 20 mM MOPS, pH 7.2, at 7.7°K. Operating parameters were: modulation frequency, 100 kHz; modulation amplitude, 0.4 mT; time constant, 100 ms. The microwave frequency was 9.421 GHz and the microwave power was 0.64 mW.

Long incubation periods or the addition of oxygen to the ascorbate- or dithionite-reduced and nitric oxide-derivatized membrane fraction resulted in the loss of the $S = 3/2$ nitrosyl–iron complex but not the $g = 2.03$ signal (Fig. 1, trace C). The reason for the loss of the nitrosyl–ferrous iron complex is presently unknown. It might have resulted from reduction of the NO by AMO or a nitric oxide reductase. The presence of the latter is suggested by the ability of cells to produce N_2O from nitrite [33].

3.3. Effect of nitrapyrin on EPR spectra of cells or membranes

Cells (results not shown) and cell membranes (Fig. 2) of *N. europaea* under anaerobic conditions in the presence of ammonia exhibited high-spin iron signals: $g = 6.52$ and $g = 6.0$. The addition of nitrapyrin resulted in the selective increase in the $g = 6.01$ signal. EPR signals associated with the small fraction of HAO found in the membrane [34] also increased in the presence of nitrapyrin. Thus, the presence of nitrapyrin results

in increased oxidation of a iron center seen at $g = 6.01$ and a small amount of HAO.

3.4. *Effect of nitrapyrin or acetylene on the S = 3/2 nitrosyl-iron complex*

The addition of nitrapyrin and ammonia under anaerobic conditions to cell membranes prior to the addition of nitric oxide inhibited the formation of both the nitrosyl iron complex and the $g = 2.03$ signal (Fig. 3, trace A) and resulted in the increase of the high-spin iron ($g = 6.01$) signal (Fig. 3, traces A and C). The addition of acetylene and ammonia under anaerobic conditions to cell membranes of *N. europaea* prior to the addition of nitric oxide did not change the extent of the formation of the nitrosyl iron complex (data not shown).

4. Discussion

Nitric oxide has been used to form EPR-active nitrosyl iron

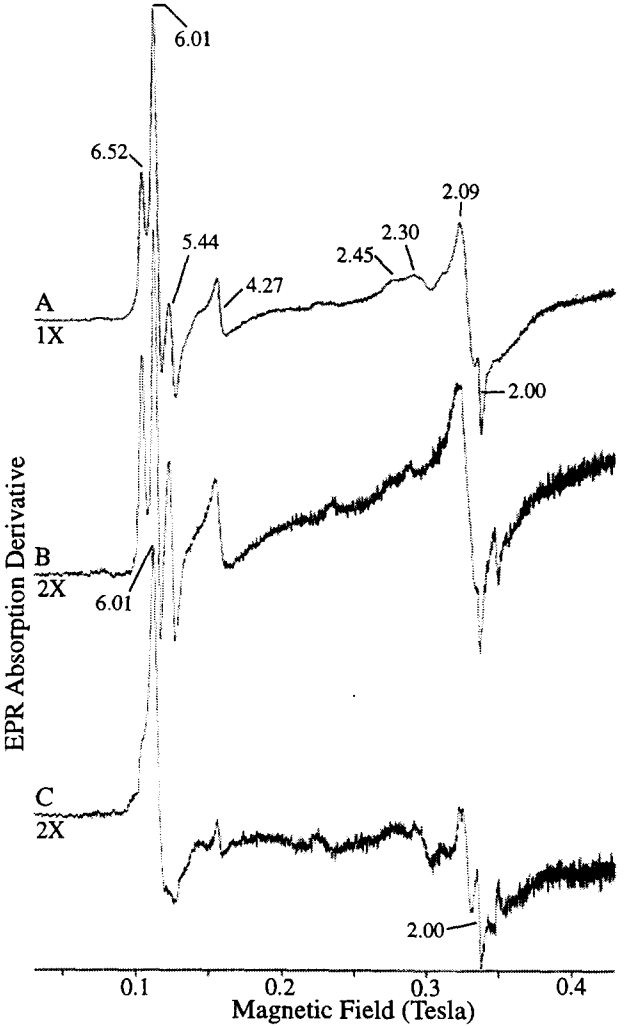

Fig. 2. EPR spectra of *N. europaea* cells plus ammonia under anaerobic conditions. A: Nitrapyrin-treated whole-cell fraction: whole-cell fraction plus 0.25 mM NH_4Cl and 10 mM nitrapyrin. B: DMSO control: whole-cell fraction plus 0.25 mM NH_4Cl and 25 μM DMSO. C: Spectrum A minus spectrum B. Protein concentration was 40 mg/ml in 20 mM MOPS, pH 7.2, at 7.7°K. Operating parameters were: modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; time constant, 100 ms. The microwave frequency was 9.421 GHz and the microwave power was 2.2 mW.

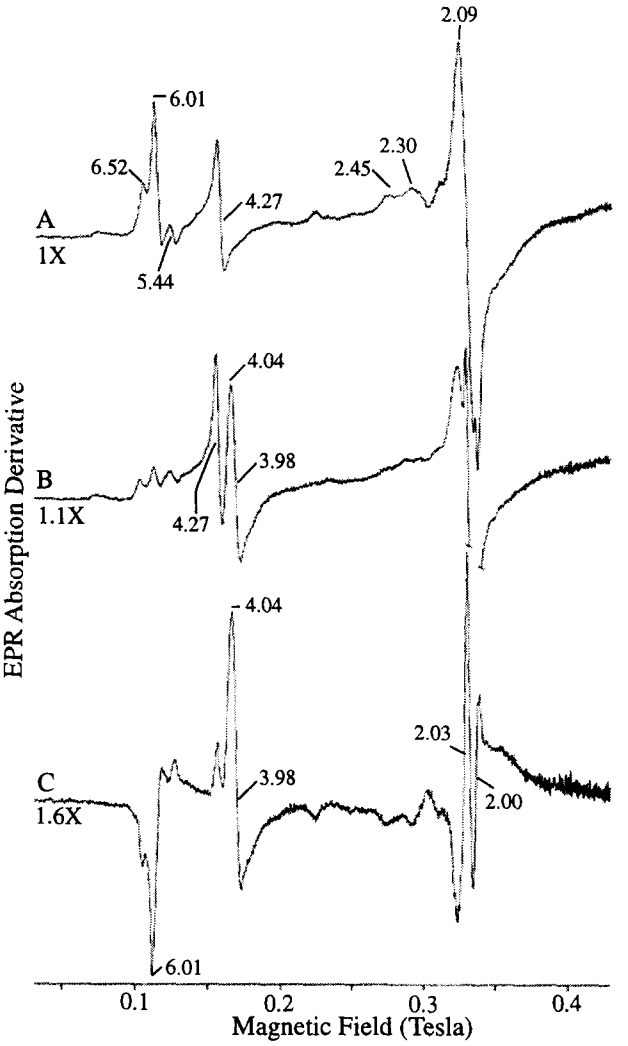

Fig. 3. EPR spectra of the NO and ammonia-treated membrane fraction from *N. europaea*. A: Nitrapyrin-treated membrane fraction: membrane fraction plus NO and 0.25 mM NH_4Cl. B: DMSO control: membrane fraction plus NO, 0.25 mM NH_4Cl and 25 μM DMSO. C: Spectrum A minus spectrum B. Protein concentration was 40 mg/ml in 20 mM MOPS, pH 7.2 at 7.7°K. Operating parameters were: modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; time constant 100 ms. The microwave frequency was 9.421 GHz and the microwave power was 2.02 mW.

complexes of otherwise spectroscopically transparent non-heme iron centers [1,11,12,23,24]. The nitric oxide derivative EPR spectrum of the membrane fraction from cells of *M. capsulatus* Bath was previously used to identify a species of iron which increased in the membrane fraction upon induction of pMMO and was associated with purified pMMO [1]. The present work shows exposure to nitric oxide of reduced membrane fractions from *N. europaea* also resulted in a EPR signal of a non-heme iron species with a spin state of $S = 3/2$. The nitrosyl iron complex was also observed when the ammonia-derived electrons (presumably originating from the successive oxidation of ammonia and hydroxylamine) were substituted for ascorbate or dithionite although the intensity of the signal was lower. The presence of an $S = 3/2$ iron nitrosyl signal in purified pMMO and in membranes of *N. europaea*, taken together with the biochemical and genetic similarities

between AMO and pMMO [2,3], suggest that the NO-reactive iron is found in AMO.

Resting membranes of *N. europaea* exhibited high-spin iron EPR signals at $g = 6.01$ and 6.52. Nitrapyrin is thought to bind specifically in the active site of AMO and is capable of reoxidating the metal center which is responsible for activation of oxygen during aerobic turnover of AMO [25]. As shown here the presence of nitrapyrin results in the apparent oxidation of an iron which then presents a $g = 6.01$ signal. Other interpretations for the increased intensity of the $g = 6.01$ high-spin iron signal include the disruption of a spin-coupled center. Although the present evidence does not establish that nitrapyrin reacts directly with iron, the result is what would be expected if the center were the oxygen- and nitrapyrin-reactive moiety. The fact that nitrapyrin prevents the formation of the $S = 3/2$ ferrous–NO complex further supports the relationship of the NO-reactive iron to AMO. Additionally, the concomitant increase in the $g = 6.01$ signal suggests that the iron(s) responsible for the $g = 6.01$ and the $S = 3/2$ nitrosyl signal are closely related or have long-range interactions.

Acetylene specifically inactivates the enzyme and derivatizes the subunit AMO-A. The lack of an effect of derivitization by acetylene on the $g = 6.01$ and $S = 3/2$ iron nitrosyl EPR signals suggests that the oxygen-activating and substrate-binding sites are significantly separated or that acetylene does not bind to the active site.

Acknowledgements: We thank C.L. Rohwer (ISU) and J. Nott (ISU Protein Facility) for technical assistance. This work was supported by grants from Iowa State University Office of Biotechnology (A.D.S.), Department of Energy DOE-FG02-96ER20237 (A.D.S.), Department of Energy DOE/DE-FGO2-95ER20191 (A.B.H.) and Iowa State University Professional Advancement Grant (J.A.Z.).

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