Interaction of the mechanism-based inactivator acetylene with ammonia monooxygenase of *Nitrosomonas europaea*

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Received 27 August 2008
Revised 15 October 2008
Accepted 15 October 2008

The ammonia monooxygenase (AMO) of *Nitrosomonas europaea* is a metalloenzyme that catalyses the oxidation of ammonia to hydroxylamine. We have identified histidine 191 of AmoA as the binding site for the oxidized mechanism-based inactivator acetylene. Binding of acetylene changed the molecular mass of His-191 from 155.15 to 197.2 Da (+42.05), providing evidence that acetylene was oxidized to ketene (CH₂CO; 42.04 Da) which binds specifically to His-191. It must be assumed that His-191 is part of the acetylene-activating site in AMO or at least directly neighbours this site.

INTRODUCTION

Under oxic growth conditions *Nitrosomonas europaea* obtains energy by oxidation of ammonia via hydroxylamine to nitrite (Rees & Nason, 1966; Hooper *et al.*, 1997). The formation of hydroxylamine is catalysed by ammonia monooxygenase (AMO), which is thought to be a heteromultimeric copper protein encoded by the *amoCAB* operon (Dua *et al.*, 1979; Anderson & Hooper, 1983; Hyman & Wood, 1985; Hyman & Arp, 1993; Hooper *et al.*, 1997; Chain *et al.*, 2003). AmoA and AmoB were shown to co-purify (McTavish *et al.*, 1993). Much of what is known about the catalytic activity of AMO originates from biochemical studies with intact bacteria or subcellular fractions, and was deduced from the very similar process of methane oxidation by (particulate) methane monooxygenase, (p)MMO. Acetylene (C₂H₂) is an irreversible inhibitor of ammonia oxidation in *Nitrosomonas* (Dua *et al.*, 1979; Anderson & Hooper, 1983; Hyman & Wood, 1985; Schmidt *et al.*, 2001), but has no effect on hydroxylamine oxidation at concentrations sufficient to completely inactivate ammonia oxidation (Hynes & Knowles, 1978). Acetylene has also been shown to be an inhibitor of other catalytically active monooxygenases (Hynes & Knowles, 1978; Prior & Dalton, 1985a; Zahn & DiSpirito, 1996; Yeager *et al.*, 1999). In AMO, acetylene binds to the 27 kDa AmoA subunit (Hyman & Arp, 1992). It was proposed that acetylene specifically inactivates catalytically active AMO as a result of an attempted oxidation of the triple bond of acetylene, forming a reactive intermediate that binds to an amino acid in or near the acetylene-activating site of AMO

(Hynes & Knowles, 1978; Hyman & Wood, 1985). Even after denaturation of AMO, acetylene (or an acetylene derivative) remains bound to the AmoA subunit. Hence, acetylene has to bind directly to the protein, and binding to a metal atom can be excluded, because metals are liberated from proteins upon denaturation. Nevertheless, the mechanism of acetylene inactivation and its binding site in AMO are unknown.

Soluble and particulate methane monooxygenases (sMMO, pMMO) catalyse the oxidation of methane to methanol. Interestingly, sMMO, pMMO and AMO are able to oxidize both ammonia and methane, although ammonia supports growth only of nitrifiers and methane only that of methanotrophs (Bedard & Knowles, 1989). As during ammonia oxidation, incorporation of one oxygen atom from O₂ into a non-activated C–H bond of methane (AMO: N–H bond of ammonia) is catalysed by sMMO and pMMO and the second oxygen atom is reduced to water (Lipscomb, 1994). It was suggested that a dinuclear ferrous cluster in sMMO (Dalton *et al.*, 1993) co-ordinates and activates oxygen. In pMMO a dinuclear or trinuclear copper centre (Lieberman & Rosenzweig, 2005a, b; Lieberman *et al.*, 2006; Chen *et al.*, 2007; Chan & Yu, 2008) or a dinuclear iron centre (Martinho *et al.*, 2007) were proposed as catalytic centres. The resulting metal oxenoid species might then abstract a hydrogen atom from the substrate, leading to a deprotonated substrate intermediate and a metal-bound hydroxyl moiety. Rebinding of the hydroxyl could then yield the product (Nesheim & Lipscomb, 1996). Because AMO and pMMO are similar on the genetic, protein and functional level such a reaction mechanism was also proposed for the copper-containing monooxygenase AMO (Hooper & Terry, 1973; Stirling &

Abbreviations: AMO, ammonia monooxygenase; pMMO and sMMO, particulate and soluble methane monooxygenase.

Dalton, 1977; Prior & Dalton, 1985b; Shears & Wood, 1985; Ensign *et al.*, 1993; Zahn *et al.*, 1996; Lieberman & Rosenzweig, 2005a).

The present study started from the assumption that acetylene, or rather an activated acetylene derivative, binds to a specific amino acid of AmoA. We have identified ketene as the product of acetylene activation at AMO and His-191 in AmoA as the binding site for ketene.

METHODS

Organism and growth conditions. Stock cultures of *N. europaea* (ATCC 19718) were grown aerobically in 1 l Erlenmeyer flasks containing 400 ml mineral medium (Schmidt & Bock, 1997). Cultures were grown in the dark at 28 °C with shaking at 50 r.p.m. Cultures in the late exponential growth phase were used to inoculate a 30 l fermenter (Braun). Biomass was produced under discontinuous conditions with 20 l mineral medium supplemented with 50 mM NH₄Cl. The medium was stirred at 50–200 r.p.m. and aerated with 0.2–8 l air min⁻¹ to maintain a dissolved oxygen concentration of 5 ± 0.2 mg l⁻¹. The temperature was 28 °C and the pH value was kept at 7.3 [20% (w/v) Na₂CO₃]. Bacteria were harvested in the late exponential growth phase at a culture density of $2 \times 10^8 \pm 3 \times 10^7$ cells ml⁻¹, concentrated by cross-flow filtration (Haemoflow HF80S, Fresenius Medical Care), and sedimented by low-speed centrifugation. The sediment was washed twice by centrifugation in mineral medium without ammonium.

Production of [¹⁴C]acetylene and ¹⁴C-labelled AMO. Acetylene was produced from barium carbide (BaC₂) according to Hyman & Arp (1990) in a closed glass bottle upon addition of water, dissolved in DMSO, which was placed in a second compartment of the bottle, and stored at -80 °C. Ba¹⁴C₂ (108 mCi mmol⁻¹, 3996 MBq mmol⁻¹; Biotrend) was composed of Ba^{14,14}C₂, Ba^{14,12}C₂ and Ba^{12,12}C₂ such that on average one carbon per BaC₂ was a ¹⁴C isotope. To produce [¹⁴C]acetylene, 90% unlabelled BaC₂ was mixed with 10% ¹⁴C-labelled BaC₂. Hence, the [¹⁴C]acetylene produced was composed of about 92.5% [^{12,12}C]acetylene (26.04 Da), 5.0% [^{14,12}C]acetylene (28.04 Da) and 2.5% [^{14,14}C]acetylene (30.04 Da). Throughout this paper the term [¹⁴C]acetylene is used for this mixture.

To label AMO, fresh *N. europaea* cells (1 g) suspended in mineral medium without ammonium (4 ml) were transferred into a 25 ml serum flask sealed with a butyl rubber stopper. NH₄Cl was added from a 1 M stock solution to a final concentration of 10 mM and acetylene from 2.3 µM [¹⁴C]acetylene solution in DMSO to a final concentration of 5.8 nM (63 µCi, 2.3 MBq). Bacteria were incubated for 1 h at 28 °C on a reciprocal shaker at 40 r.p.m. Labelling (inhibition) of AMO was considered complete when the nitrite concentration remained constant for 10 min. To remove unbound acetylene, bacteria were washed five times by centrifugation in 50 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.3).

Electrophoresis. Protein concentration was estimated by the biuret assay (Bonner & Laskey, 1974) after solubilizing protein in 3 M NaOH for 30 min at 60 °C and removal of insoluble material by centrifugation (10 000 g for 5 min). BSA was employed for standardization. Denaturing PAGE (SDS-PAGE) was performed in a Mini-Protean II cell vertical gel electrophoresis chamber (Bio-Rad) on 12% gels according to Laemmli (1970) at 10–30 mA for about 14 h. Prior to loading, protein samples (100 µg protein) were supplemented with an equal volume of buffer containing 125 mM Tris/HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol and 0.008%

(w/v) bromophenol blue. To prevent temperature-dependent aggregation of AmoA (Hyman & Arp, 1993), samples were incubated for 10 min at room temperature, centrifuged (10 min at 8000 g) and loaded onto the gels. Molecular mass standards (PepLab) were included on each gel to determine the apparent molecular masses of the polypeptides. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in aqueous solution with 5% (v/v) acetic acid and 25% (v/v) methanol. To analyse peptides smaller than 30 kDa, Tricine-SDS-PAGE was applied according to Schagger & von Jagow (1987).

Scintillation autoradiography (fluorography) of ¹⁴C-labelled polypeptides. Gels stained for protein were destained, water was replaced by DMSO, and gels were soaked in 4 vols 20% (w/w) 2,5-diphenyloxazol (PPO) in DMSO for 3 h. PPO in the gels was crystallized by immersion in 20 vols cold water for 1 h. Gels were then dried on Whatman filter paper (vacuum, 1 h at 80 °C) and placed in an X-ray cassette with a high-performance chemoluminescence film (Hyperfilm ECL, Amersham Biosciences). The cassette was stored at -80 °C for 10–14 days before developing the autoradiograph.

Identification of AmoA. Polypeptides after SDS-PAGE were prepared and then analysed by MALDI-TOF mass spectrometry as described before (Schmidt *et al.*, 2004). Polypeptides were identified via peptide mass fingerprinting (Mascot 1.7.1) with calculated *m/z* values from the *N. europaea* genome database. The significance of identification was evaluated by calculating the probability-based Mowse scores (Schmidt *et al.*, 2004).

Enzymic and chemical protein cleavage. ¹⁴C-labelled AmoA after Tricine-SDS-PAGE was cut from the gel and eluted in an electro-eluter equipped with a dialysis membrane (cut-off 10 kDa) applying a buffer containing 14% (w/v) glycine, 1% (w/v) SDS, 0.3% (w/v) Tris/HCl. The eluter was operated at 50 mA for 3.5 h at 4 °C. The buffer was then replaced by the same buffer but without SDS and elution was continued for 2 h. Eluted AmoA was concentrated to 1 mg ml⁻¹ by freeze-drying and the solution was immediately digested or stored at -80 °C.

AmoA was digested with 20 µg ml⁻¹ sequence-grade trypsin (Promega) or 30 µg ml⁻¹ endoproteinase Pro-C (Sigma-Aldrich) for 10 h at room temperature. The digest was stopped by addition of 1 µl 0.1 M PMSF. Cyanogen bromide (Sigma-Aldrich) was used for AmoA cleavage according to Smith (1994). Cyanogen bromide was added in about 50-fold molar excess over the expected methionyl residues and samples were incubated for 24 h at room temperature. The digest was terminated by drying down under vacuum. The dried samples were resolved in Tris/HCl buffer (pH 7.4). Complete hydrolysis of peptides into amino acids was performed with hydrochloric acid. Peptide samples were transferred in tubes containing hydrochloric acid (6 M) in a nitrogen atmosphere and heated to 110 °C for 24 h. After cleavage, samples were stored at -80 °C.

Peptide and amino acid separation and identification. For N-terminal sequencing, peptides were separated by Tricine-SDS-PAGE, blotted on a PVDF membrane and the amino acid sequence was analysed by Edman degradation (analysis done by SeqLab).

To identify the amino acid composition of the trypsin-cleaved ¹⁴C-labelled peptide, it was further digested with cyanogen bromide, and resulting peptides were separated on a Series 900 HPLC system (Jasco) using a 250 × 3 mm ReproSil-Pur ODS-3 column (Dr Maisch, Ammerbuch, Germany) and acetonitrile (ACN) in water containing 0.1% (v/v) trifluoroacetic acid (TFA) [10% (v/v) ACN for 2 min followed by a linear gradient of 10–80% ACN within 35 min at a flow rate of 0.4 ml min⁻¹]. Peptides were monitored at 214 nm and were collected separately. The fraction containing the ¹⁴C-labelled peptide

was identified by liquid scintillation counting (BETAmatic BASIC, Kontron Analytical) and then subjected to complete hydrochloric acid hydrolysis. The masses of the amino acids were analysed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) according to Eckerskorn *et al.* (1997). Amino acids were separated by reverse-phase HPLC using as mobile phase 5% (v/v) ACN and 0.1% (v/v) TFA in water followed by a linear gradient to 75% (v/v) ACN and 0.1% (v/v) TFA in water within 40 min (flow rate 0.5 ml min⁻¹). Fractions corresponding to each amino acid were collected, concentrated under vacuum to dryness and resolved in methanol/water (50:50, v/v) for ESI-MS analysis. Molecular masses were determined using a Quattro-BQ triple quadrupole mass spectrometer (VG Biotech) equipped with a standard electrospray ionization source. Spectra were obtained with a capillary voltage of 3.5 kV (source temperature 80 °C). The average molecular mass values were calculated from *m/z* values in the charged ion distribution profiles of the multiply charged ions (Covey *et al.*, 1988).

RESULTS AND DISCUSSION

¹⁴C-labelling and identification of AmoA

[¹⁴C]Acetylene was added to ammonia-oxidizing *N. europaea* cells, where it selectively bound to the AmoA subunit of biologically functional AMO. ¹⁴C-labelled AmoA was isolated from cell protein by SDS-PAGE and analysed by fluorography (Fig. 1). As expected, one ¹⁴C-labelled polypeptide band was detectable with an apparent molecular mass of 27 kDa (predicted 32 kDa, Chain *et al.*, 2003) and the identity of the labelled polypeptide as AmoA was ascertained by MALDI-TOF analysis. The identification of AmoA (gi_30248947, gi_30250003) was highly significant, with a sequence coverage of 52% and an *E*-value of 6×10^{-98} .

Fig. 1. SDS-PAGE stained for protein and fluorogram of [¹⁴C]acetylene-treated *N. europaea* protein. Lane 1, protein molecular mass standards. Lane 2, Coomassie-stained protein. Lane 3, fluorogram of lane 2. The ¹⁴C-labelled polypeptide at 27 kDa was identified as AmoA by MALDI-TOF.

Identification of the [¹⁴C]acetylene-binding site in AmoA

¹⁴C-labelled AmoA was eluted from SDS-PAGE gels and aliquots of the solution were supplemented with trypsin, Pro-C or cyanogen bromide to digest the polypeptide. The resulting peptides were separated by Tricine-SDS-PAGE (Fig. 2).

Cleavage of AmoA with trypsin or Pro-C resulted in ¹⁴C-labelled peptides with molecular masses of 5.6 ± 1.1 kDa or 2.6 ± 0.9 kDa, respectively (Fig. 2, peptides A and B). Application of cyanogen bromide produced three labelled peptides; the strongest signal in the fluorogram originated from the smallest 4.5 ± 1.0 kDa ¹⁴C-labelled peptide (Fig. 2, peptide C). The amount of larger ¹⁴C-labelled peptides increased when digestion with cyanogen bromide was performed for a shorter period of time (12 or 18 h). Therefore, peptide C represents the ¹⁴C-labelled AmoA fragment after complete cleavage and signals at higher molecular masses originate from incompletely cleaved peptides. The observation that complete cleavage of AmoA with trypsin, Pro-C or cyanogen bromide always resulted in one ¹⁴C-labelled peptide provided evidence that binding of [¹⁴C]acetylene occurs at a certain part of the AmoA subunit.

Other proteases were tested for digestion of AmoA as well, but cleavage did not result in protein fragments appropriate for identification of the acetylene-binding site. Application of Asp-N, Glu-C, and Lys-C produced ¹⁴C-labelled peptides of more than 10 kDa and after cleavage with chymotrypsin the ¹⁴C-labelled peptide was too small to be separated by Tricine-SDS-PAGE.

The [¹⁴C]acetylene-binding peptides A to C (Fig. 2) were sequenced (Table 1, sequences A to C). As controls the same peptides, but without bound acetylene, were blotted from Tricine-SDS-PAGE and also subjected to N-terminal amino acid sequencing.

The sequences of labelled and unlabelled peptides A and B were identical, but in the ¹⁴C-labelled peptide C, His-191 could not be identified. The amino acid at this position had a significantly different retention time compared to histidine when analysed by HPLC during Edman degradation. In Fig. 3 the sequences of the three identified peptides are marked in the AmoA amino acid sequence. Because all three peptides were ¹⁴C-labelled (Fig. 2), acetylene has to be bound to a part of AmoA where the sequences of peptides A, B and C overlap. The common amino acid sequence of these peptides is G₁₉₀HLYVR. The modified mass of His-191 and the overlapping sequences of the three peptides provided the first evidence for His-191 as the acetylene-binding site in AmoA.

Identification and mass analysis of the ¹⁴C-labelled amino acid in AmoA

To prepare the ¹⁴C-labelled peptide with the amino acid sequence G₁₉₀HLYVR, AmoA was digested with trypsin

Fig. 2. Tricine-SDS-PAGE stained for protein and fluorogram of AmoA (lanes 2, 3), AmoA digested with trypsin (lanes 4, 5), Pro-C (lanes 6, 7) or cyanogen bromide (lanes 8, 9), and protein molecular mass standards (lane 1). Coomassie-stained protein is shown in lanes 2, 4, 6 and 8, and corresponding fluorograms in lanes 3, 5, 7 and 9. The fluorograms after trypsin or Pro-C cleavage (lanes 5, 7) show one ^{14}C -labelled peptide (A, B) and after cleavage with cyanogen bromide (lane 9) the smallest ^{14}C -labelled peptide gave the strongest signal (C). Weaker signals at higher molecular masses (lane 9) represent ^{14}C -labelled peptides after incomplete cleavage.

and cyanogen bromide. The ^{14}C -labelled peptide was then purified by HPLC and subjected to complete hydrolysis with hydrochloric acid. The molecular masses of the resulting amino acids were analysed by mass spectrometry. The results are given in Table 2.

The expected molecular masses of five amino acids (Gly, Leu, Tyr, Val, Arg) of the hexapeptide were identifiable after hydrolysis, but the molecular mass of histidine (155.15 Da) was not detectable. Instead a molecular mass of 197.2 Da (molecular mass of histidine plus 42.05) was measured. The six amino acid samples were subjected to liquid scintillation counting. Only the sample containing the modified histidine was radioactive, confirming His-191 as binding site for a ^{14}C -labelled acetylene derivative with a molecular mass of 42.05 Da.

The sum of the molecular masses of the six amino acids (Table 2) is 876.1 Da. Subtracting a molecular mass of 90 Da (5 H_2O ; five peptide bonds), a molecular mass of

786.1 Da has to be expected for the ^{14}C -labelled hexapeptide. In fact, for the purified hexapeptide a molecular mass of 786.1 Da was measured (as well as masses of 788.1 and 790.1 Da according to the isotope balance of the acetylene used). This result provides evidence that the treatment with hydrochloric acid hydrolysed the hexapeptide, but a further chemical modification of the amino acids including the histidine-acetylene derivative did not occur.

The histidine-acetylene derivative formed had molecular masses of 197.2, 199.2 and 201.2 Da. The sum of the molecular masses of histidine (155.15 Da) plus [^{14}C]acetylene ([$^{12,12}\text{C}$], 26.04; [$^{14,12}\text{C}$], 28.04; and [$^{14,14}\text{C}$], 30.04 Da) would be too small to explain the observed molecular mass of the histidine-acetylene derivative (Table 2). In general, monooxygenases introduce an oxygen atom into their substrate and the oxidation of acetylene by AMO obviously results in an activated acetylene derivative (intermediate) which then binds

Table 1. N-terminal sequences of ^{14}C -labelled peptides after cleavage with trypsin (peptide A), Pro-C (peptide B) and cyanogen bromide (peptide C)

As control, peptides without bound acetylene were sequenced. The amino acids after Edman degradation were identified by their retention time during HPLC. Lysine and leucine as well as glutamine and threonine had the same retention time and discrimination between these amino acids was not possible. The non-expected amino acids according to the AMO sequence are indicated by small letters. The subscript after the first amino acid indicates the position of the amino acid in the AmoA sequence. X, amino acid could not be identified.

Peptide	Labelled with acetylene	N-terminal sequence
A	+	N ₁₄₅ -W-L/k-V-T/q-A-L/k-V-G-G-G-F-F-G
A	-	N ₁₄₅ -W-L/k-V-T/q-A-L/k-V-G-G-G-F-F-G
B	+	I ₁₇₆ -V-V-E-G-T/q-L/k-L/k-S-M-A-D
B	-	I ₁₇₆ -V-V-E-G-T/q-L/k-L/k-S-M-A-D
C	+	G ₁₉₀ -X-L/k-Y-V-R-T/q-G-T/q-P-E
C	-	G ₁₉₀ -H-L/k-Y-V-R-T/q-G-T/q-P-E

121 INFVTPGIML PGALMLDFTL YLTRNWLVT**A** LVGGGFFGLL
 161 FYPGNWPIFG PTHLP**B**IVVEG TLLSMADYMG HLYVRTGTPE
 201 YVRHIEQGSL RTFGGHTTVI AAFSAFVSM LMFTVWWYLG

Fig. 3. Amino acid sequences of peptides after cleavage of AmoA with trypsin (A; peptide underlined with a black bar), Pro-C (B; peptide underlined with a white bar), or cyanogen bromide (C; peptide underlined with a grey bar). The amino acid sequence of AmoA from amino acids 121 to 240 is diagrammed. Sequenced N-termini are indicated with bold letters. C-termini were determined according to the cleavage characteristics of trypsin, Pro-C and cyanogen bromide. All three ^{14}C -labelled peptide sequences overlap in the sequence G₁₉₀HLYVR, providing evidence that the [^{14}C]acetylene-binding site is located in this hexapeptide.

covalently to His-191 of AmoA (Table 2, Fig. 3). Fomina *et al.* (2002) and Chan *et al.* (2004) suggested a mechanism for suicide inactivation of pMMO by acetylene, where acetylene reacts with an activated (reduced) copper centre initially forming a ketene, which then further reacts with a nucleophile at the active site. Histidine was proposed as suitable residue for binding ketene (Chen & Chan, 2006; Chen *et al.*, 2007). Due to the isotope composition of the [^{14}C]acetylene used in this study, its oxidation to a ketene results in molecular masses of 42.04 ([$^{12,12}\text{C}$]ketene), 44.04 ([$^{14,12}\text{C}$]ketene) and 46.04 ([$^{14,14}\text{C}$]ketene) Da. Consequently, histidine-ketene derivatives with molecular masses of 197.2, 199.2 and 201.2 Da [mass of histidine (155.15) plus 42.05, 44.05 or 46.05 Da] have to be expected and were in fact measured when analysing the His-191 derivative (Table 2).

Table 2. Molecular masses of amino acids in the acetylene-binding peptide G₁₉₀HLYVR

The hexapeptide was prepared via combined cleavage of AmoA with trypsin and cyanogen bromide and was then completely hydrolysed with hydrochloric acid. The molecular masses are listed in ascending order; the amino acids were assigned according to their molecular masses.

No.	Molecular mass (Da)	Amino acid
1	75.1 ± 0.5	G
2	117.2 ± 0.4	V
3	131.2 ± 0.3	L
4	174.2 ± 0.4	R
5	181.2 ± 0.4	Y
6	197.2* ± 0.2	?

*In addition small amounts of molecular masses 199.2 and 201.2 Da were detectable. According to the isotope balance of the acetylene used (92.5 % [$^{12,12}\text{C}$]acetylene, 5.0 % [$^{14,12}\text{C}$]acetylene and 2.5 % [$^{14,14}\text{C}$]acetylene) the proportion of the masses 197.2, 199.2 and 201.2 Da was approximately 90:7:3.

The molar ratio between bound ^{14}C -label and AmoA or hexapeptide, respectively, was approximately 1:1 (Figs 2 and 3, Table 2). [^{14}C]Acetylene binds exclusively to AmoA, and within AmoA only the hexapeptide G₁₉₀HLYVR, and more specifically His-191, was found to be ^{14}C -labelled (Table 2). After activation [^{14}C]acetylene apparently binds specifically to His-191 and does not diffuse away from the activation site, which would result in binding at random positions and in substoichiometric amounts of ^{14}C -label at the hexapeptide and at His-191. It must be assumed that His-191 is part of the acetylene-activating site in AMO or at least directly neighbours this site. It can be expected that the activation of the substrate ammonia occurs at the same active (metal) site.

ACKNOWLEDGEMENTS

This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG). We thank Peter Steenbakkers, Huub op den Camp and Mike Jetten, Radboud University Nijmegen for protein identification via MALDI-TOF and Matthias Schlotter for technical assistance.

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Edited by: D. J. Arp