Mutagenesis of the hydrocarbon monooxygenase indicates a metal centre in subunit-C, and not subunit-B, is essential for copper-containing membrane monooxygenase activity

Elissa F. Liew, Daochen Tong, Nicholas V. Coleman and Andrew J. Holmes

The hydrocarbon monooxygenase (HMO) of Mycobacterium NBB4 is a member of the copper-containing membrane monooxygenase (CuMMO) superfamily, which also contains particulate methane monooxygenases (pMMOs) and ammonia monooxygenases (AMOs). CuMMOs have broad applications due to their ability to catalyse the oxidation of difficult substrates of environmental and industrial relevance. Most of our understanding of CuMMO biochemistry is based on pMMOs and AMOs as models. All three available structures are from pMMOs. These share two metal sites: a dicopper centre coordinated by histidine residues in subunit-B and a ‘variable-metal’ site coordinated by carboxylate and histidine residues from subunit-C. The exact nature and role of these sites is strongly debated. Significant barriers to progress have been the physiologically specialized nature of methanotrophs and autotrophic ammonia-oxidizers, lack of a recombinant expression system for either enzyme and difficulty in purification of active protein. In this study we use the newly developed HMO model system to perform site-directed mutagenesis on the predicted metal-binding residues in the HmoB and HmoC of NBB4 HMO. All mutations of predicted HmoC metal centre ligands abolished enzyme activity. Mutation of a predicted copper-binding residue of HmoB (B-H155V) reduced activity by 81%. Mutation of a site that shows conservation within physiologically defined subgroups of CuMMOs was shown to reduce relative HMO activity towards larger alkanes. The study demonstrates that the modelled dicopper site of subunit-B is not sufficient for HMO activity and that a metal centre predicted to be coordinated by residues in subunit-C is essential for activity.

INTRODUCTION

The copper membrane monooxygenases (CuMMOs) are an enzyme superfamily of broad significance (Culpepper & Rosenzweig, 2012; Dalton, 2005; Semrau et al., 2010). CuMMOs have a central role in the microbial oxidation of a wide variety of difficult substrates including ammonia, methane, other small alkanes and chlorinated hydrocarbons (Bédard & Knowles, 1989; Conrad, 1996; Jiang et al., 2010). CuMMO-containing microbes dominate key steps in the biogeochemical cycles of methane and nitrogen, making the genes useful targets as environmental indicators (Holmes et al., 1995, 1999; Menyailo et al., 2008; Nazaries et al., 2013; Op den Camp et al., 2009; Singh et al., 2010). Their activities give rise to a broad range of biotechnology applications, such as in the removal of excess nitrogen from wastewater (Abell et al., 2011; Hatzenpichler, 2012; Mußmann et al., 2011), sustainably exploiting methane for alternative fuel production (Dalton, 2005; Lieberman & Rosenzweig, 2005; Singh et al., 2010), and remediating chlorinated pollutants that persist in groundwater and soil (Le & Coleman, 2011; Semrau et al., 2010).

CuMMOs are present in diverse biological contexts. To date, they have been found in Archaea (Thaumarchaeota) and in members of four bacterial phyla (Proteobacteria, Verrucomicrobia, Actinobacteria, candidate division NC10). There is considerable divergence between the archaeal and bacterial forms (Hatzenpichler, 2012; Stahl & de la Torre, 2012). The bacterial CuMMOs encompass three known physiological roles: methane hydroxylation via particulate methane monooxygenases (pMMOs), ammonia hydroxylation via ammonia monooxygenases (AMOs) and C₂–C₄ hydrocarbon hydroxylation via hydrocarbon monooxygenases (HMOs) (Coleman et al., 2012). The AMOs and pMMOs are restricted to highly physiologically specialized...
host organisms (autotrophic ammonia oxidizers and methanotrophs), whereas the recently described HMOs occur in more physiologically versatile organisms including various Actinobacteria and Proteobacteria. These organisms are also able to grow on a wide range of other carbon sources that do not involve CuMMO activity.

The vast majority of biophysical and biochemical work has been on AMO or pMMO. Both systems present challenges due to the physiological specialization of ammonia oxidizers and methanotrophs and the difficulty of purifying these membrane-associated proteins. There are currently three crystallographic pMMO structures available, from Methylococcus capsulatus Bath, Methylosinus trichosporium OB3b and Methylocystis strain M (Hakemian et al., 2008; Lieberman & Rosenzweig, 2005; Smith et al., 2011). All three comprise a three-polypeptide protomer (subunits-A, -B and -C) arranged in a trimer of $\alpha_3\beta_3\gamma_3$ configuration. There are two metal centres shared across all three published structures (Fig. 1), but details of the metals occupying these sites vary between protomers, or between structures. A metal centre in PmoB (henceforth referred to as the subunit-B site) is coordinated by three histidine residues (His33, His137 and His139 in Methylococcus capsulatus Bath) and was modelled as a dinuclear or mononuclear copper site in different protomers of the three structures. A second metal centre located within the membrane-spanning regions (henceforth referred to as the subunit-C site) was originally modelled as coordinated by ligands from PmoA (Glu195) and PmoC (Asp153, His160 and His173) and contained zinc or copper (Lieberman & Rosenzweig, 2005). Based on the higher resolution Methylocystis strain M structure, an alternate model of this site was proposed involving only PmoC residues (Culpepper & Rosenzweig, 2012; Smith et al., 2011). Alternative models for tricopper and di-iron metal centres have also been proposed by Chan et al. (2007) and Martinho et al. (2007), respectively. Given the variation between models and the low activity of pMMO protein preparations, the biological relevance of the metal centre(s) in the active pMMO is still contested (reviewed by Culpepper & Rosenzweig, 2012; Semrau et al., 2010).

Convincing evidence for the nature and importance of the subunit-B site derives from the recombinant expression of soluble portions of PmoB (spmoB) in Escherichia coli and the observation that this protein was sufficient for activity (Balasubramanian et al., 2010). spmoB showed that methane oxidation activity was lost after mutagenesis of the histidine residues observed to coordinate the dicopper centre. This activity in the absence of the subunit-C site, together with reports that structurally similar synthetic copper-containing molecules also catalyse methane oxidation (Himes & Karlin, 2009; Woertink et al., 2009), led Balasubramanian and co-workers to propose that only the subunit-B site is essential for activity. This model raises some anomalies, however, as the subunit-B site is absent from the verrucomicrobial methanotroph pMMOs (Dunfield et al., 2007; Pol et al., 2007; Semrau et al., 2008). Despite the subunit-B site being strongly conserved across most CuMMOs, including AMOs and HMOs, it is difficult to reconcile this with it being essential for activity. In contrast, direct evidence for the catalytic importance of the subunit-C site has proven harder to obtain, and there is even more uncertainty regarding the nature of the metal centre at this site. Alternative models for metal occupancy at this site have been proposed that include ligands from the A-subunit (Martinho et al., 2007; Semrau et al., 2010). It is noteworthy that $^{14}$C-labelled acetylene, a known suicide inhibitor, irreversibly binds to the subunit-A of all tested CuMMO physiological subtypes (Gilch et al., 2009; Hamamura et al., 1999; Hyman & Wood, 1985; Prior & Dalton, 1985). The proposed ligands of this subunit-C site and other residues in the structural vicinity are also strongly conserved across all known CuMMOs (Fig. 1). Collectively, these observations argue that this site has a fundamental catalytic role (Semrau et al., 2010).

One of the major constraints to clarifying the role of these two metal sites and understanding AMO or pMMO biochemistry has been the absence of a flexible recombinant expression system. The strongly conserved protein sequences across the bacterial CuMMOs, as well as their overlapping substrate ranges and shared inhibitor profiles (Bédard & Knowles, 1989; Culpepper & Rosenzweig, 2012; Holmes et al., 1995; Op den Camp et al., 2009; Semrau et al., 2010), validate the use of the more genetically amenable HMOs as a useful alternative model to investigate CuMMO biochemistry (Coleman et al., 2012). Here we use the HMO expression system as a model to test the biological importance of the subunit-B and -C sites. Site-directed mutagenesis (SDM) was used to systematically replace homologous residues in HmoB and C (Fig. 1). We demonstrate that in HMO the subunit-B site strongly influences activity and kinetic parameters, although only the subunit-C site is essential for CuMMO functionality, and that a pMMO-specific residue in the neighbourhood of the subunit-C site influences substrate preference of the HMO.

**METHODS**

**Bacterial strains, culture conditions and plasmids.** E. coli EPI300 [F' mcrA Δ(mrr-hsdRMS-mcrBC) q80lacZAM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK ΔrpsL (Sta8) nupG trpA dhfr (Epicentre Biotechnologies) and Mycobacterium smegmatis mc²-155 (ATCC 700084) (Snapper et al., 1990)] were used as cloning and expression hosts, respectively. E. coli was grown aerobically at 37 °C in LB (Sambrook & Russell, 2001). Mycobacterium smegmatis was grown aerobically at 30 °C in TSB [3 g trypticase soy broth (Oxoid) l⁻¹, 1% (w/v) glucose] or minimal MSM medium (Coleman et al., 2002) supplemented with 20 mM glucose. Kanamycin (Km) was added at 50 µg ml⁻¹ for E. coli and 20 µg ml⁻¹ for mc²-155 cultures for vector maintenance. All broths where shaken at 9 g and Tween 80 (0.05%, w/v) was added to mc²-155 cultures to reduce cell clumping. Mycobacterium–E. coli shuttle vector pMycoFos (12.5 kb, Km²) (Ly et al., 2011) was used to clone and express mutant hmoCAB gene clusters. All mutations were made upon a base
construct termed pHMOWT that contains a single copy of the hmoCAB operon of Mycobacterium strain NBB4 cloned into pMycoFos.

Gas chromatography. Methane (>99.0% purity), ethane (>99% purity), propane (99% purity) and butane (99% purity) were obtained from Sigma-Aldrich. Alkanes were quantitatively analysed by gas chromatography.
by GC of 100 μl headspace samples using an HP 5890 series II Plus chromatograph (Hewlett Packard), PLOT Q column and flame-ionization detector with helium as carrier gas. Gas concentrations were calculated from external standard curves of known alkane amounts using a 10 min isocratic run (injector and oven, 200 °C; detector, 250 °C) and expressed as total micromoles per vial.

**PCR.** Routine PCRs used Taq polymerase [New England Biolabs (NEB)] according to the manufacturer instructions. PCRs for vector construction were conducted with high-fidelity polymerases: either high-fidelity Phusion (NEB) according to the manufacturer’s instructions or Pfu polymerase (0.2 mM each) and primers (0.5 μM each). All primers and thermocycler conditions are described in Tables S1 and S2 (available in the online Supplementary Material).

**Construction of hmoCAB mutants.** Six HMO mutants (B-H155V, C-D139A, C-H143V, C-D149A, C-A151D and C-H156A) were made by overlap extension (Ho et al., 1989) using pHMOvec as template. Mutants were named using the following convention: subunit letter; dash; wild-type residue letter; residue position in HMO subunit; mutated residue letter. Right-hand side (RHS) amplicons were produced using reverse primer NV308 (5′-TTTATTAACT-TATTGGGTTCGATCGAGG-3′) containing an SwaI site (underlined) with one of six forward primers incorporating a single codon change (shown in bold; nucleotides altered from the wild-type are italicized): 5′-GGTCAGGTGCAGTGCGCGCCATGGGGAGATG-3′ for mutation B-H155V, 5′-GGGCGCCTTGTGCGACCATCGCTCAGC-3′ for C-D139A, 5′-GAGCCAGCCCTTCGACCGTC-3′ for C-H143V, 5′-CTCAAGCCCGGGCCGCCGT-3′ for C-D149A, 5′-GAGCTCACCCCAAGCGCCAGGCGGTAGTCCGACTTGTC-3′ for C-A151D and 5′-CAGCCACCGGACGACTTGTTCGAC-3′ for C-H156A. Left-hand side (LHS) amplicons were generated using forward primer NV307 (5′-TTTTATTAATCTGGCAACCTACTGTCG-3′) with 5′-ATTTAGTGCGCAGAATGGCGATG-3′, 5′-GAGCAGCGGTCCTGCTGAGG-3′, 5′-GCCGTTGCGGAGA-3′, 5′-GAGGCGATTGTGACCCCAACTCACCCAC-3′ for C-D139A, 5′-GAGCCAGGCTTGTGCGACCGTC-3′ for C-H143V, 5′-GAGCTCACCCCAAGCGCCAGGAGTCCGTCC-3′ for C-A151D and 5′-CAGCCACCGGACGACTTGTTCGAC-3′ for C-H156A. Plasmids were confirmed by restriction digest (Australian Research Genome Facility).

**Expression of hmoCAB mutants in Mycobacterium smegmatis mc²-155.** Constructs were transformed into electroporation competent mc²-155 (Seidman, 1994) by electroporation (Bio-Rad Gene Pulser; 800 Ω, 2.5 kV, 25 μF), recovered in 1 ml TSG for 4 h and plated onto TSG-Km. Transformants were plated onto LB-Km agar and positive clones were selected. DNA was purified using forward primer NV308 (5′-TTTATTAACT-TATTGGGTTCGATCGAGG-3′) with 5′-ATTTAGTGCGCAGAATGGCGATG-3′, 5′-GAGCAGCGGTCCTGCTGAGG-3′, 5′-GCCGTTGCGGAGA-3′, 5′-GAGGCGATTGTGACCCCAACTCACCCAC-3′ for C-D139A, 5′-GAGCCAGGCTTGTGCGACCGTC-3′ for C-H143V, 5′-GAGCTCACCCCAAGCGCCAGGAGTCCGTCC-3′ for C-A151D and 5′-CAGCCACCGGACGACTTGTTCGAC-3′ for C-H156A. SDS-PAGE bands were processed for DNA sequencing (Australian Research Genome Facility).

**Preparation of membrane fractions and SDS-PAGE.** Starter cultures were used to inoculate MSM-Km acetamide broth and grown to an OD₆₀₀ of 2.0. Cells were washed with KP and lysed at 4 °C in KP (without Tween 80) by sonication (Branson Sonifier 250 and micro-tip; four 30 s pulses, 90 % output). Large particles of cell debris were removed by centrifugation (2600 g, 5 min, 4 °C) and the supernatant was incubated with 86 mM Na₂CO₃ (1 h, 4 °C, 2.2 g) to solubilize loosely membrane-bound proteins (Molloy, 2008). Supernatants were ultracentrifuged at 45'000 g for 1 h at 4 °C (Optima L-100 XP; Beckman Coulter), and the membrane pellet was washed by sonication (two 30 s pulses, 4 °C in KP) and ultracentrifuged once more. Membrane fractions were resuspended in loading buffer [0.125 M Tris/HCl, 4 % (w/v) SDS, 20 % (w/v) glycerol, 0.2 M DTT, 0.02 % (w/v) bromophenol blue, pH 8.3], denatured (95 °C, 5 min) and loaded onto 15 % (w/v) polyacrylamide gels at equal protein amounts. Protein concentration was quantified by spectrophotometry (Nanodrop 1000; ThermoScientific). Gels were stained using Coomassie blue (0.025 %, w/v) and destained with methanol (40–10 %, v/v) and acetic acid (10 %, v/v).

**Peptide identification by MS.** SDS-PAGE bands were processed for peptide mass fingerprinting as described by Scott et al. (2010). Briefly, gel bands were excised using sterile blades, washed in destain solution [40 % (v/v) acetonitrile, 24 mM ammonium bicarbonate, pH 7.8, 1 h, 25 °C], drained, vacuum-dried (1 h) and rehydrated in trypsin solution [12 ng sequencing-grade modified trypsin (Promega) μl⁻¹ in 40 mM ammonium bicarbonate, 1 h, 4 °C]. Excess trypsin was removed and the gel pieces were incubated overnight at 37 °C. Peptides were concentrated and desalted with C₁₈ Perfect Pure Tips (Eppendorf) and eluted in matrix [8 mg z-cyano-4-hydroxycinnamic acid μl⁻¹ (Sigma-Aldrich), 70 % (v/v) acetonitrile, 1 % (v/v) formic acid] directly onto target plates. Peptide mass maps were generated by MALDI-TOF MS using a Voyager DE-STR spectrometer (Applied Biosystems). Mass calibration used trypsin autolysis peaks m/z 2211.11 and m/z 842.51 as internal standards. Fingerprint data were used to search NCBI via Mascot (www.matrixscience.com) (MatrixScience, 2013). Search parameters included ±0.2 Da peptide mass tolerance and one missed cleavage per peptide and identifications based on Mascot score, E-value, number of peptide mass matches and total percentage sequence coverage of peptides.

**Resting cell suspension assays.** HMO activity was assayed in 16 ml crimp-sealed serum vials with butyl rubber septa. Alkanes were injected into vials containing 3 ml KP with 20 mM glucose and equilibrated for 30 min (30 °C, with shaking) prior to addition of cells. One millilitre induced and concentrated mc²-155 suspensions (OD₆₀₀ ~40) were injected into vials and shaken vigorously for 30 s before sampling headspace for r=0 GC analysis. Substrate depletion was monitored by further headspace sampling over 6 h and corrected for substrate loss due to headspace removal. Degradation rates were calculated using the linear section of depletion curves and converted to apparent specific activity (nmol substrate min⁻¹ mg⁻¹) by dividing at −80 °C for single use. To prepare cells for activity assays, 300 μl starter cultures were used to inoculate 300 ml MSM-Km broths with 0.2 % (w/v) acetamide (to induce HMO expression; Ly et al., 2011) and grown to an OD₆₀₀ ~1.0 (2–4 days). Cells were then harvested by centrifugation, washed twice in KP and resuspended in KP buffer to an OD₆₀₀ of approximately 40 (estimated from measuring the OD₆₀₀ of 10⁻² dilutions in KP). These concentrated cell suspensions were used to set up resting cell suspension (RCS) assays (see below). Experimental replicates originated from independently grown induction cultures inoculated from the same batch of starter cultures (1 litre). Optical densities were measured using an Eppendorf BioPhotometer at 600 nm, 8.5 mM light path and polystyrene cuvettes (Saransted).
by the total cell protein amount per assay vial. Total protein for mc2-155 was calculated using a standard curve relating whole-cell protein concentration to cell density (µg protein ml⁻¹ = 99.37 × OD600 - 32.42) (Coleman et al., 2002; Le & Coleman, 2011). The cell density in each vial was measured after assay completion by sampling 100 µl of the cell suspension, diluting to 10⁻¹ in KP and measuring OD600. Assay vials with a final OD600 > 13.5 were excluded from further analysis due to the reduction of rates caused by gas transfer limitations into high cell biomass solution (Fig. S1). For testing the inhibitory effects of N-allylthiourea (ATU), the 1 ml concentrated mc²-155 suspensions were first added to 3 ml KP-glucose and the 4 ml RCS pre-incubated with 25 µM ATU for 30 min. Butane gas was injected immediately after ATU addition such that the 30 min gas-equilibration step occurred during the same 30 min time period as the RCS-ATU pre-incubation step. For acetylene inhibition, the 4 ml RCS was incubated in a sealed vial with acetylene (10% headspace volume) for 30 min (with shaking), and then removed by purging with N₂ gas for 2 min. Vials were then resealed; butane was added and equilibrated for 30 min before sampling the headspace for the r=0 sample.

Homology modelling. Predicted NBB4 HMO structure was conducted by homology modelling via the SWISS-MODEL suite (http://swissmodel.expasy.org/) (Arnold et al., 2006; Guex & Peitsch, 1997; Schwede et al., 2003) using the Methylosinus OB3b pMMO structure as template (Data Bank ID, 3CHX) (see Fig. 1). Protein model images were created using the UCSF Chimera molecular visualization application (Petterson et al., 2004).

RESULTS

Confirmation of HMO-mutant constructs and expression

The full-length sequence for each hmoCAB mutant was determined after cloning into pMycoFos. All intended sequence alterations from the wild-type were observed and were the only amino acid changes for all three polypeptides. The hmoA gene in construct B-H155V contained one additional nucleotide change (nt position 39, T→C), which was a sense mutation and had no effect on the predicted protein sequence (Arg codon CGT→Arg codon CGC). After validation, all seven forms of the HMO constructs were purified from E. coli and transformed into Mycobacterium smegmatis mc²-155 for expression.

To confirm the expression of recombinant HMOs in mc²-155 we performed SDS-PAGE of crude membrane preparations extracted from cells grown under inducing conditions (0.2% acetamide). Relative to the pMycoFos control (vector with no HMO insert), all recombinant HMO strains contained comparable levels of three new proteins with the expected molecular masses that are inferred to represent the HMO proteins (Fig. 2). The presence of NBB4 HmoB and HmoA in bands 1 and 2, respectively, was confirmed by peptide mass spectrometric fingerprinting (Fig. S3). These data suggest that each recombinant construct the SDM regime did not impair expression and all three HMO proteins were found at comparable stoichiometry in the membrane fraction of each strain.

Characterization of HMO activity

Whole cells expressing wild-type HMO (HMOWT) showed activity towards butane and ethane of approximately 6 and 9 nmol min⁻¹ mg⁻¹, respectively (Figs 3 and S2). No activity was detected towards either ethane or butane for the three HMO forms in which the predicted subunit-C site ligands had been mutated (C-D139A, C-H143V and C-H156A), nor for the C-D149A mutant. All four of these mutants showed no significant difference in activity from cells containing empty pMycoFos (C-D139A, P=0.83, n=3; C-H143V, P=0.59, n=3; C-D149A, P=0.31, n=4; C-H156A, P=0.79, n=3). Together with the SDS-PAGE results (Fig. 2), these data suggest the absence of activity was due to the specific effects of the mutation, and not due to reduced protein expression or failure of proteins to associate with the membranes. In contrast, mutation of the predicted dicopper subunit-B site did not abolish activity. HMOB-H155V had significant activity (approximately 1 nmol min⁻¹ mg⁻¹) towards butane and ethane compared with the vector control (P<0.009, n=3). However, activity was significantly reduced compared with the wild-type (P<0.0001, effect size approximately 81%, n=3).

We also examined the importance of the HmoC residue Ala151. Comparison across representative CuMMOs shows correlation between the physiological role of the enzyme and the amino acid at this site (Fig. 1). For example, aspartate is strongly conserved in all pMMOs and alanine in HMOs. Mutation of the HmoC residue to the equivalent results (Fig. 2), these data suggest the absence of activity was due to the specific effects of the mutation, and not due to reduced protein expression or failure of proteins to associate with the membranes. In contrast, mutation of the predicted dicopper subunit-B site did not abolish activity. HMOB-H155V had significant activity (approximately 1 nmol min⁻¹ mg⁻¹) towards butane and ethane compared with the vector control (P<0.009, n=3). However, activity was significantly reduced compared with the wild-type (P<0.0001, effect size approximately 81%, n=3).

We also examined the importance of the HmoC residue Ala151. Comparison across representative CuMMOs shows correlation between the physiological role of the enzyme and the amino acid at this site (Fig. 1). For example, aspartate is strongly conserved in all pMMOs and alanine in HMOs. Mutation of the HmoC residue to the equivalent results (Fig. 2), these data suggest the absence of activity was due to the specific effects of the mutation, and not due to reduced protein expression or failure of proteins to associate with the membranes. In contrast, mutation of the predicted dicopper subunit-B site did not abolish activity. HMOB-H155V had significant activity (approximately 1 nmol min⁻¹ mg⁻¹) towards butane and ethane compared with the vector control (P<0.009, n=3). However, activity was significantly reduced compared with the wild-type (P<0.0001, effect size approximately 81%, n=3).

We also examined the importance of the HmoC residue Ala151. Comparison across representative CuMMOs shows correlation between the physiological role of the enzyme and the amino acid at this site (Fig. 1). For example, aspartate is strongly conserved in all pMMOs and alanine in HMOs. Mutation of the HmoC residue to the equivalent results (Fig. 2), these data suggest the absence of activity was due to the specific effects of the mutation, and not due to reduced protein expression or failure of proteins to associate with the membranes. In contrast, mutation of the predicted dicopper subunit-B site did not abolish activity. HMOB-H155V had significant activity (approximately 1 nmol min⁻¹ mg⁻¹) towards butane and ethane compared with the vector control (P<0.009, n=3). However, activity was significantly reduced compared with the wild-type (P<0.0001, effect size approximately 81%, n=3).
Significant difference in activity towards ethane was also observed, but in this case the effect size appeared to be different. This prompted us to examine substrate preference in more detail.

**Mutation of an ‘indicator site’ alters HMO substrate preference**

We have previously shown that recombinant NBB4 HMO<sub>WT</sub> has activity towards ethane, propane and butane, but not methane (Coleman et al., 2012). Here we investigate the substrate-velocity kinetics of mc<sup>-1</sup>-155 expressing HMO<sub>WT</sub> compared with HMO<sub>B-H155V</sub> and HMO<sub>C-A151D</sub> towards ethane, propane and butane (Fig. 4). Our data and non-linear regression analysis indicated that the HMO<sub>WT</sub> followed first-order (Michaelis–Menten) kinetics in our whole-cell expression system. Maximum apparent velocity (V<sub>max</sub>) and K<sub>m</sub> were calculated by Michaelis–Menten modelling (Y = V<sub>max</sub> × X/(K<sub>m</sub> + X)) (Table 1). Kinetic parameters did not differ substantially across the three substrates for HMO<sub>WT</sub> (V<sub>max</sub> ~15 nmol min<sup>-1</sup> mg<sup>-1</sup>, K<sub>m</sub> ~2 μmol per vial) or HMO<sub>B-H155V</sub> V<sub>max</sub> (~2 nmol min<sup>-1</sup> mg<sup>-1</sup>) but significant differences in V<sub>max</sub> were observed between substrates for HMO<sub>C-A151D</sub>.

For ease of comparing activity towards all substrates and across all enzyme forms, Fig. 5 shows the average activity of assays conducted at saturating substrate concentration (hereafter average max activity). For all enzyme forms these values were comparable to the extrapolated V<sub>max</sub> values (Table 1). HMO<sub>WT</sub> showed no significant difference between the average max activities on all alkanes (~13 nmol min<sup>-1</sup> mg<sup>-1</sup>, P>0.5, n=2–4). For HMO<sub>B-H155V</sub> these were all significantly lower than HMO<sub>WT</sub> (~1.5 nmol min<sup>-1</sup> mg<sup>-1</sup>, P<0.03, n=2–4) but also had no significant difference between substrates (P>0.3, n=2). In contrast, the V<sub>max</sub> for HMO<sub>C-A151D</sub> towards ethane was approximately double that on propane and butane. HMO<sub>C-A151D</sub> and HMO<sub>WT</sub> had comparable average max activities towards ethane (10.6 nmol min<sup>-1</sup> mg<sup>-1</sup>, P=0.19, n=2) but significantly different values for propane (P<0.007, n=2) and butane (P<0.001, n=2). Overall, our data demonstrate that the C-A151D mutation strongly shifts the substrate preference of HMO away from larger alkanes. We also tested methane as a substrate for HMO<sub>WT</sub> and HMO<sub>C-A151D</sub>, but no detectable activity was observed (data not shown).

**Effect of CuMMO inhibitors on HMO mutant activity**

We next examined whether the two mutants and HMO<sub>WT</sub> responded differently to the CuMMO inhibitors ATU and acetylene. The spmoB model indicates that the subunit-B metal site is sufficient for activity, whereas our mutagenic analysis indicates that the subunit-C site is essential. Is the residual activity for mutants in each subunit equally susceptible to CuMMO inhibitors? For these experiments resting cell suspensions were initially incubated for 30 min with the relevant inhibitor (acetylene in headspace, ATU in solution) before addition of butane and testing for activity. Owing to overlap in GC elution times for butane (substrate) and acetylene (inhibitor), detection of butane degradation required removal of acetylene by sparging. Therefore, it is important to note that acetylene was only present during this 30 min pre-incubation and only ATU remained present during the assay.

The data show that in both mutant forms the residual HMO activity is strongly inhibited by acetylene and ATU. Low levels of HMO<sub>WT</sub> activity were observed after acetylene treatment in these assays over long time periods (data not shown). We attribute this to the transient presence of acetylene meaning no impact on newly synthesized protein. Activity was very significantly reduced compared with uninhibited activity (HMO<sub>B-H155V</sub>, P<0.001, n=3; HMO<sub>C-A151D</sub>, P<0.0004, n=3) and there was no significant difference between the two inhibitors for HMO<sub>C-A151D</sub> (P=0.14, n=3) and HMO<sub>B-H155V</sub> (P=0.69, n=3) (Fig. 6). This suggests that neither mutation differentially impacted the effect of the enzyme inhibitors. ATU-inhibited HMO<sub>C-A151D</sub> activity was also not significantly different from ATU-inhibited HMO<sub>WT</sub> activity (P=0.07, n=3) or ATU-inhibited HMO<sub>B-H155V</sub> activity (P=0.13, n=3).

**DISCUSSION**

To date, only one recombinant SDM experiment has been published for a CuMMO (Balasubramanian et al., 2010).
These workers constructed a synthetic protein in which the two soluble cupredoxin domains of PmoB were tethered by a short linker sequence to produce spmoB. This protein lacks the membrane-spanning helices of the native PmoB, but includes all ligands of the crystallographically observed PmoB metal centres. Expression of this spmoB protein was sufficient for activity towards methane and propylene at levels that were very low relative to the whole cell system, but comparable to purified pMMO. Importantly, the spmoB activity was shown to be dependent on the presence of copper and the histidine ligands. By extension, these authors concluded that the PmoB metal site was required for methane oxidation, that it contained a dicopper centre coordinated by the three histidines and that the PmoC/PmoA site containing the variable-metal centres in the structures could not be the active site of pMMO. Here we explore the extent to which the spmoB-based model can be considered generally applicable to other CuMMOs. In our system the CuMMO was expressed as the membrane-bound holoenzyme where the potential for interaction between HmoA, B and C subunits exists.

**Importance of the subunit-B site**

Our data from HMO corroborate the importance of the PmoB metal site, but also highlight a number of important differences from the spmoB model. His155 in HMO is not essential for activity. This site is homologous to the His139 specific activity (nmol mg<sup>-1</sup> min<sup>-1</sup>)

<table>
<thead>
<tr>
<th>pHMO activity on ethane</th>
<th>pHMO activity on propane</th>
<th>pHMO activity on butane</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 5 15 20 25 30</td>
<td>0.0 5 10.0</td>
<td>0.0 5 10.0</td>
</tr>
</tbody>
</table>

**Subunit-C metal centre is essential for CuMMO activity**

Fig. 4. Activity saturation curves for HMO<sub>WT</sub>, HMO<sub>B-H155V</sub> and HMO<sub>C-A151D</sub> in mc<sup>2</sup>-155 towards ethane, propane and butane. Each data point represents the apparent specific activity observed for that starting substrate concentration. Apparent specific activities were calculated from individual rates of substrate depletion by mc<sup>2</sup>-155 RCS. Curves were modelled to Michaelis–Menten kinetics using the following equation: \( Y = V_{\text{max}} \times X/(K_m + X) \). \( R^2 \) values indicate goodness-of-fit for each curve. Dotted line indicates the 5 µmol alkane per vial concentration, and values above this were deemed to be above substrate saturation concentration. For HMO<sub>B-H155V</sub>, the dotted line is at 3 µmol alkane per vial to calculate \( t \)-test significance in Fig. 5 and Table 1.
of the *Methylococcus capsulatus* Bath pMMO and is predicted to be essential from the spmoB model. The activity observed for the HMOB-H155V mutants cannot be attributed to background activity from other cellular enzymes as mc2-155 transformed with vector (pMycoFos) or four other mutant HMOs (HMOC-D139A, HMOC-H143V, HMOC-D149A and HMOC-H156A) all exhibited no hydroxylation activity towards the substrates tested. We only mutated one residue at the subunit-B site, whereas double mutants were constructed in the spmoB system (Balasubramanian et al., 2010). Although available data implicate this residue as essential for dicopper coordination, the resolution is limited and thus it is possible that the natural metal centre may be different and/or that our single mutant does not completely disrupt it. Whilst we cannot exclude the possibility that targeting other residues or double mutants may eliminate activity, our demonstration that His155 is not essential for HMO activity is consistent with the absence of this site from verrucomicrobial pMMOs.

**Essentiality of the subunit-C site**

A second, more striking, distinction from the spmoB model is that four mutated residues of HmoC were found to be essential for activity. The residues included the three consistently observed ligands in the pMMO structures and Asp149, which is universally conserved across all sequenced CuMMOs and has been postulated to also play a role in coordination of a metal centre (Martinho et al., 2007; Semrau et al., 2010). All four mutants showed no activity. This is not consistent with the prediction of the spmoB model that a subunit-B metal centre is sufficient for activity. One difference between the spmoB and HMO models is the potential for subunit interactions. We considered that the HmoB metal centre could be disrupted by interaction with the mutant HmoC proteins. However, this would imply

Table 1. *K*<sub>m</sub> and *V*<sub>max</sub> values calculated by Michaelis–Menten analysis and comparison with the average activity observed at substrate saturation for HMO<sub>WT</sub>, HMO<sub>B-H155V</sub> and HMO<sub>C-A151D</sub> towards ethane, propane and butane

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th><em>K</em>&lt;sub&gt;m&lt;/sub&gt; (μmol per vial)</th>
<th><em>V</em>&lt;sub&gt;max&lt;/sub&gt; (nmol mg&lt;sup&gt;-1&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Activity at substrate saturation (nmol mg&lt;sup&gt;-1&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Ethane</td>
<td>1.6 ± 0.39</td>
<td>15.4 ± 1.4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Propane</td>
<td>2.6 ± 0.57</td>
<td>17.5 ± 1.4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Butane</td>
<td>2.1 ± 0.29</td>
<td>13.9 ± 0.8</td>
<td>12.2</td>
</tr>
<tr>
<td>C-A151D</td>
<td>Ethane</td>
<td>2.3 ± 0.38</td>
<td>11.9 ± 0.8</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>Propane</td>
<td>0.6 ± 0.23</td>
<td>5.18 ± 0.44</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>Butane</td>
<td>0.7 ± 0.25</td>
<td>5.95 ± 0.59</td>
<td>5.01</td>
</tr>
<tr>
<td>B-H155V</td>
<td>Ethane</td>
<td>3.6 ± 1.7</td>
<td>2.37 ± 0.55</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>Propane</td>
<td>3.6 ± 2.1</td>
<td>2.30 ± 0.62</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Butane</td>
<td>0.5 ± 0.4</td>
<td>1.24 ± 0.23</td>
<td>1.08</td>
</tr>
</tbody>
</table>

**Fig. 5.** Average activity observed for HMO<sub>WT</sub>, HMO<sub>B-H155V</sub> and HMO<sub>C-A151D</sub> in mc<sup>2</sup>-155 at substrate saturating concentrations of ethane, propane and butane. Averages were calculated from data points (n=2–4 independent velocities) at substrate concentrations past the dotted line, as shown in Fig. 4. Error bars indicate SEM. Significant differences are shown (n.s.=P>0.1, **P<0.001).
that all four single amino acid substitutions in HmoC caused sufficient structural disruption to HmoB to abrogate activity, and seems unlikely in four independent mutants. All three HMO proteins were still present in membrane preparations so any alteration to tertiary structure did not prevent comparable levels of membrane association across the three proteins. We conclude that in native HMO a metal centre at the subunit-B site is not sufficient for activity.

Our data do not exclude the possibility that a soluble Hm0B equivalent of spmoB may be sufficient for activity. It is possible that the membrane domain of Hm0B and interaction with Hm0C and Hm0A impair substrate access to the subunit-B site. Although the physiological relevance of this is questionable, it raises an interesting mechanistic question. Our HmoB\textsubscript{H155V} mutant and the recombinant spmoB both show activity towards their alkane substrates and this implies two distinct centres in CuMMOs with this capability. With this possibility in mind we looked for different responses to inhibitors of CuMMOs. Acetylene and ATU are both inhibitors of all known CuMMOs. Prior work has shown that acetylene inhibits HMO, AMO and pMMO and in each case results in covalent binding to subunit-A (Gilch \textit{et al.}, 2009; Hamamura \textit{et al.}, 1999; Hyman & Wood, 1985; Zahn & DiSpiro, 1996). The mechanism of ATU inhibition is not clear, but it is apparently specific to CuMMOs (Bédard & Knowles, 1989; Hubley \textit{et al.}, 1975; Juliette \textit{et al.}, 1993), whereas acetylene is broadly inhibitory against monooxygenases. Here we observed comparable inhibition of activity of HMO\textsubscript{WT}, HMO\textsubscript{H155V} and HMO\textsubscript{C-A151D} by both acetylene and ATU. These data further emphasize that the activity observed in the two mutants is CuMMO activity. In summary, the spmoB model predicts that the subunit-B site is necessary and sufficient for CuMMO activity, but neither prediction is supported in the HMO model. Although pMMO and HMO are distinct enzymes, these observations represent a surprising level of mechanistic divergence predicted by the model systems.

**Residue 151 of subunit-C influences substrate preference**

The region surrounding the subunit-C site forms a strongly conserved pocket lying at the interface of the three subunits (Fig. 1). One site within this region (site HmoC\textsubscript{151}) is associated with limited sequence variation (Fig. S4). Across the diversity of characterized CuMMO-containing bacteria, five amino acid types (alanine, aspartate, glutamate, serine or proline) are seen at this position. Notably, the distribution of these five variants is strongly associated with phylo-physiological groups. This led us to hypothesize that the substrate preferences for all CuMMOs will be strongly influenced by the amino acid at this site. Our experimental observation that the mutation of the alanine in HmoC to aspartate significantly shifted the preference of HMO towards smaller substrates is consistent with this hypothesis.

The general pattern of association between HmoC\textsubscript{151} variants and physiological groups is summarized in Table S3 and briefly described below. An aspartate is associated with pMMOs, an alanine with HMOs and there is no consistent association with all AMOs (Fig. 1). The AMO variants do, however, show strong association within the three phylogenetic subgroups. All archaeal ammonia-oxidizers have an AMO with alanine, all \textit{Betaproteobacteria} ammonia-oxidizing bacteria (βAOB) have an AMO with serine, and all \textit{Gammaproteobacteria} AOB (γAOB) have an AMO with aspartate. Thus, each phylo-physiological group of CuMMO-containing organisms appears to have one characteristic amino acid at this site. In addition to these broad group differences, allelic variation at the HmoC\textsubscript{151} site is also seen in at least three groups. These include alternative alleles with proline in many gammaproteobacterial methanotrophs, with glutamate in verrucomicrobial methanotrophs and with serine in gammmaproteobacterial hydrocarbon degraders (Table S3). We postulate that these patterns reflect biochemical differences between HmoC\textsubscript{151} variants (in either substrate kinetics or susceptibility to competitive inhibition) that contribute to the ecological separation of CuMMO-dependent organisms.

This hypothesis requires experimental testing but we note that available data are consistent with the model. Methanotrophs and nitrifiers commonly contain multiple alleles of CuMMO genes and additional copies of the gene encoding subunit-C appear to be essential in some cases (Berube & Stahl, 2012; Berube \textit{et al.}, 2007; Stolyar \textit{et al.}, 1999). The extent of sequence divergence between operons varies, but in at least one case this allelic variation represents kinetically distinct forms. One allele of \textit{Methylocystis} SC2 (pmoCAB1) is a high-rate pMMO and another (pmoCAB2) is a high-affinity pMMO. These pMMO forms are differentially regulated in response to concentration of methane, the physiological substrate and ammonia, a co-metabolic substrate (Bann & Liesack, 2008; Dam \textit{et al.}, 2014). These studies illustrate that kinetic parameters of CuMMO activity towards either physiological or co-metabolic substrates can drive ecological fitness. Although the activity of site HmoC\textsubscript{151} allelic variants in the same organism has not been reported, comparative studies of methane and ammonia oxidation have been reported for \textit{Nitrosomonas} and \textit{Nitrosococcus} strains (Jones & Morita, 1983; Ward, 1987). These members of the βAOB and γAOB contain distinct site HmoC\textsubscript{151} variants and differ in their affinity for methane. Although both groups are obligate autotrophic ammonia-oxidizers they have distinct biogeographical distribution (Campbell \textit{et al.}, 2011). It is notable that the aspartate-AMO-containing γAOB are more susceptible to competitive inhibition by methane (Ward, 1987) and are primarily found in marine environments where methane is rarely at high concentrations. The serine-AMO-containing βAOB are predominantly from freshwater and terrestrial habitats, where methane is more likely to co-occur.

In conclusion, the recombinant HMO system is a physiologically relevant model for studying CuMMO
biochemistry. These data show that the modelled dicopper site of subunit-B is not sufficient for activity in HMO and that a metal centre involving conserved charged residues in subunit-C is essential for activity. We predict that these observations are generalizable to other bacterial CuMMOs. The CuMMOs have two substrates, dioxygen and either an alkane or ammonia. The C-A151D residue is located within a conserved pocket adjacent to the subunit-C site and this site strongly influences substrate preference in the HMO. We predict that allelic divergence at this site reflects ecological specialization that could be based on either alternative substrates or mechanisms to avoid competitive inhibition. CuMMOs are widely used as biomarkers in environmental science and this site may serve as a useful marker for interpretation of such datasets (Hatzenpichler, 2012; Henckel et al., 1999; Reim et al., 2012; Singh et al., 2010).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Research Council to N.V.C and A.J.H. We thank Ben Crossett for his assistance with MS experiments via the Sydney University Proteome Research Unit. We also thank Mai-Anh Ly and Nga Le for their assistance with MS experiments via the Sydney University Proteome Research Unit. We also thank Mai-Anh Ly and Nga Le for their assistance with MS experiments via the Sydney University Proteome Research Unit. We also thank Mai-Anh Ly and Nga Le for their

REFERENCES


Evidence of microbial regulation of...