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An oxidative N-demethylase reveals PAS transition from ubiquitous sensor to enzyme

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The universal Per-ARNT-Sim (PAS) domain functions as a signal transduction module involved in sensing diverse stimuli such as small molecules, light, redox state and gases [1-2]. The highly evolvable PAS scaffold can bind a broad range of ligands, including heme, flavins and metal ions. However, while these ligands have the inherent ability to support a catalytic activity, no enzymatic PAS domain has been found. We report characterization of the first PAS enzyme: a heme-dependent oxidative N-demethylase. Unrelated to other amine oxidases, this enzyme contains heme, FMN, 2Fe-2S and THF cofactors, and is specific in catalyzing the NAD(P)H-dependent oxidation of dimethylamine. The structure of the alpha subunit reveals it is a heme-binding PAS domain, similar in structure to PAS gas sensors [3]. The dimethylamine substrate forms part of a highly polarized oxygen binding site, and directly assists oxygen activation by acting both as electron and proton donor. Our data reveal that the ubiquitous PAS domain can make the transition from sensor to enzyme, suggesting the PAS scaffold can support the development of artificial enzymes.

The Per-ARNT-Sim (PAS) domain has been found in all kingdoms of life, functioning as a signal transducer that couples ligand binding to a cellular signaling response [1-2]. The highly divergent PAS family couples a remarkable plasticity in binding ligands to an equally wide variety of sensory stimuli. The typical PAS fold consists of a single antiparallel, five stranded beta-sheet with intervening alpha-helices that create the ligand-binding pocket on the beta-sheet surface. Despite structural similarity, PAS domains often have low sequence similarity, with no universally conserved residues. This suggests a high adaptability, which has been experimentally verified for photoactive yellow protein [4]. Despite PAS domains binding cofactors with inherent chemical reactivity, no enzymatic activity has been associated with the PAS family. In contrast, other folds have made the transition to enzyme, such as globins [5,6]. Given the remarkable plasticity and universal occurrence of PAS, this might suggest an inherent limitation to developing enzymatic activity. Understanding of such limit(s) is key to the emerging field of artificial enzyme development, which often makes use of ligand-binding scaffolds [7].

We show that an orphan enzyme previously described as a secondary amine mono-oxygenase [8,9], but here referred to as heme-dependent oxidative N-demethylase (HODM), contains a heme-dependent PAS-scaffold that serves as the site of the oxidative N-demethylase activity. HODM functions in bacterial methylated amine catabolism, linking alkylamine oxidation to the tetrahydrofolate C1-pool [10]. Methylated amines are formed through various processes [11] and the trimethylamine degradation pathway has been outlined since the 1970s (Extended Data Fig. 1a). Previous

spectroscopic characterization of HODM revealed that it combines features typically associated with heme proteins as diverse as globins, cytochromes P450 and cytochrome b_5 [8,9]. The NAD(P)H-dependent dimethylamine oxidation reaction catalysed by HODM appears similar to a typical cytochrome P450 N-dealkylation reaction [12], although HODM lacks the characteristic P450 cysteine ligation. Whether the enzyme catalyses a P450-like mono-oxygenation has not been established. Until recently, further studies have been hampered by the lack of sequence information [13].

We expressed the operon proposed to encode for HODM from the proteobacterium *Pseudomonas mendocina* in *E. coli.* This includes a gene coding for a DUF3445 family representative of unknown function (42 kDa, alpha), a phthalate dioxygenase reductase-like FMN/2Fe-2S dependent oxidoreductase (39 kDa, beta), a glycine cleavage T-protein (34 kDa, gamma) and a smaller protein of unknown function (19 kDa, delta subunit) (Fig. 1a). The purified gene products form a heterotetrameric enzyme (Ext. Data Fig 1a) with biochemical and spectral properties similar to the *Aminobacter aminovorans* HODM [8,9] (Fig. 1).

EPR spectroscopy of anaerobically isolated HODM reveals signals for all redox centres, including the presence of a NO-bound ferrous heme (Fig. 1b-f). HODM catalyses the NADPH-dependent oxidative N-demethylation of dimethylamine with a k_{cat} of 9.2 ± 0.5 s^{-1} and a K_m^{DMA} of 24 ± 6 μM, resulting in the release of one equivalent of formaldehyde under *in vitro* conditions (i.e. no THF present; Fig. 1g). The presence of the gamma subunit significantly lowers the DMA-dependent formaldehyde release *in vivo (*Extended Data Fig. 1b). This suggests substrate channeling occurs between the alpha and gamma subunits, as observed for other amine oxidases [14,15]. Binding of oxygen to reduced HODM results in globin-type spectral changes (Fig 1h), and the resulting ferrous:oxygen complex is remarkably stable (half-life of ~50 min Ext. Data Fig. 1c), likely linked to the high heme redox potential (Extended Data Fig 1d). Dimethylamine binding perturbs the heme spectrum, and DMA affinity is significantly higher for the ferrous:oxygen complex (K_d = 15 ± 3 μM) (Extended Data Fig 1e,f).

DMA binding to anaerobically purified HODM leads to the loss of superhyperfine coupling to the ^{14}N atom of the His ligand in the NO-ferroheme spectrum, Extended Data Fig. 2, a likely consequence of a change in the relative orientation of the NO and histidine ligands to the heme iron [16,17]. This suggests DMA binding affects the NO ligand, in line with the allosteric effect of oxygen binding on DMA affinity. DMA binding also leads to a change in the *g* values exhibited by the $[2Fe\text{-}2S]^{1+}$ cluster, Ext. Data Fig. 2d, suggesting communication between the beta and alpha subunits. These effects only occur with DMA and not with poor substrates (Extended Data Fig. 2e-h).

While NADPH flavin reduction occurs rapidly (k = 304 ± 25 s^{-1} under anaerobic conditions; Fig. 2a), heme reduction is extremely slow in absence of both DMA and oxygen (k = 0.059 ± 0.005 min^{-1}; Fig. 2b). Heme reduction remains slow in the presence of either oxygen (leading to the ferrous:O_2 complex) or DMA alone (leading to the ferrous:DMA complex), but rapid mixing of the HODM ferrous:oxygen:DMA complex (containing oxidised FMN/[2Fe-2S]) with NADPH under anaerobic conditions reveals that the presence of substrate enhances the initial beta reduction rate by > 1000 fold, and the ferrous:oxygen:DMA complex decay rate by 2.5 fold (Fig. 2c-e). A rate enhancement for the beta subunit reduction and intramolecular electron transfer to the ferrous:oxygen:DMA (as opposed to the ferrous:oxygen) complex is likely linked to the observed effects of DMA binding on the $[2Fe\text{-}2S]^{1+}$ cluster. However, the ferric to ferrous heme iron transition under anaerobic conditions remains ~100 times slower (both with and without DMA) compared to the k_{cat} of 9.2 ± 0.5 s^{-1}.

Following the aerobic mixing of HODM with excess DMA and NADPH, steady-state conditions are established, during which the enzyme remains predominantly in the ferric:DMA-bound state (Fig. 2e). This finding suggests intermolecular electron transfer is rate-limiting, supported by the fact that no significant kinetic isotope effect (KIE) on the NADPH oxidation rates could be observed using perdeuterated DMA or D_2O. The mechanism that underpins the rate enhancement observed only in the presence of both DMA and O_2 remains unclear at this stage.

As observed for holo-HODM, reduction of the isolated alpha subunit is a slow process. Mixing of the ferrous:O_2:DMA alpha subunit with NADH and phenazine methosulfate under anaerobic conditions leads to accumulation of the ferrous:DMA enzyme via transient formation of the ferric:DMA species (Fig. 2f). Low nM (sub-stoichiometric) levels of formaldehyde can be detected following this experiment, confirming that the alpha subunit is catalytically active. Although certain heme enzymes can make use of peroxide to bypass the initial oxygen reduction step, no spectroscopic evidence for higher Fe oxidation species could be obtained in the presence of DMA and peroxide, nor was formaldehyde formation observed under these conditions (Extended Data Fig. 3).

Unfortunately, we were unable to determine the structure of the HODM holoenzyme. However, the isolated alpha subunit responsible for heme, oxygen and dimethylamine binding has spectral and ligand binding properties nearly identical to those of the holoenzyme (Extended Data Fig. 4), is catalytically competent and amenable to crystallization. The 1.7 Å alpha subunit structure reveals that it consists of an N- and C-terminally extended PAS heme binding domain (Fig. 3), most similar in structure to the Aer2 PAS domain, a gas-sensing receptor protein controlling chemotaxis in *Pseudomonas aeruginosa* ([18] PDB code 4HI4; 2.25 Å rmsd over 85 C alpha atoms, Z score 5.6, 12% homology; Fig. 3b). The HODM PAS domain (residues 145-231) contains the canonical Aβ, Bβ, Gβ, Hβ, and Iβ PAS β-sheet as well as the core Dα, Eα and Fα elements. Similar to Aer2, HODM includes an extended Hβ-Iβ loop and a shifted Fα helix not seen in other heme-binding PAS domains. The HODM Eη1 His194 acts as the proximal heme ligand, comparable to Aer2 Eη His234. However, HODM lacks the core PAS-domain Cα helix that encompasses the Aer2 heme pocket, and features extended β-strands as well as a longer Gβ-Hβ loop. The N- and C-terminal extensions to the central HODM PAS domain cover the PAS beta-sheet on the side opposite to the heme binding pocket.

Although the stoichiometry of the overall reaction catalysed by HODM bears similarity to the cytochromes P450 [12], HODM does not contain a Cys heme-thiolate ligand that assists with oxygen-oxygen heterolytic bond cleavage to form compound I (the so-called 'push'-effect [19]). In contrast, many heme peroxidases also contain a His ligand and have been shown to contain a highly polarised distal active site that achieves the same feat (referred to as the 'pull' mechanism [19]). We determined the structures of HODMalpha in complex with DMA (to 1.75 Å, Fig. 3d) and with both DMA and NO (a mimic for the DMA:O_2 complex; to 1.9 Å, Fig. 3e). DMA binding displaces two water molecules and occurs in close proximity of the distal heme ligand (Fig. 3d). Direct electrostatic interactions between the DMA substrate and the enzyme are made with the conserved Hβ Glu266, in addition to van der Waals interactions between the substrate methyl groups, Trp180 and the heme porphyrin. NO binding to the heme iron occurs at a distance of 1.95 Å (Fig. 3e). The oxygen atom of NO (mimicking the distal β-oxygen of O_2) is within hydrogen bonding distance of the conserved Gβ Arg224 and Hβ Gln268, as well as the DMA amine group (Fig. 3e). This is consistent with the ~90-fold higher DMA affinity of the ferrous-oxy complex compared with ferrous HODM. Substitution of Trp180, Arg224 or Glu266 all lead to enzyme inactivation (data not shown). Although oxygen binding is detected for W180A, E266Q and R224A variants, no DMA binding is

detected for E266Q, while direct heme ligation by DMA occurs in the R224A variant (Extended Data Fig. 4c-e).

The structural arrangement of the HODM heme subunit active site shares the polarised nature of the heme peroxidases [20]: the conserved Arg224, the amine substrate (presumably charged when bound to conserved Hβ Glu266) and the conserved Gln268 each contribute a (partial) positive charge to the distal oxygen. On the other hand, the HODM proximal ligand environment is similar to that observed in globins, and a peroxidase-like interaction with an Asp to enhance electron-donating character is not observed. The latter is consistent with the stable O_2 complex and the positive redox potential. A key question is whether HODM achieves oxidative N-demethylation through species with higher Fe oxidation states, such as the highly reactive compound I (as typified by cytochromes P450 [12]) or compound II (as occurs in peroxidases [20]).

We propose that, following DMA binding, the beta subunit reduces the ferric heme iron to ferrous using NADPH, leading to dioxygen binding and formation of the ferrous-oxy/ferric-superoxy form (Fig. 4). Transfer of the second electron and formation of the ferric hydroperoxide complex (compound 0) is likely coupled to proton transfer from the DMA substrate. This particular step explains the strict requirement of this enzyme for amine substrates. Following deprotonation of the substrate concomitant with compound 0 formation, Arg224 is likely to act as the acid required to catalyse O-O scission. Oxidation of the substrate and O-O scission can, in principle, occur in a variety of ways (Extended Data Fig. 5), possibly including Fe(V) and/or Fe(IV) oxidation states. Both mono-oxygenase (the direct incorporation of oxygen, leading to hydroxymethylamine) as well as amine oxidase activity (leading to an imine product) appear possible, but are not readily distinguishable as both products rapidly convert to formaldehyde and mono-methylamine.

While ample spectroscopic evidence has been presented for such reaction mechanisms in other heme-dependent enzymes [19-21], we have found no evidence of either compound I or II during turnover or rapid mixing experiments with HODM. Similarly, X-ray induced photoreduction of ferrous:O_2:DMA crystals at 100 K led to formation of the ferrous species with no indication of higher Fe oxidation states (Extended Data Fig. 6). Our inability to observe transient, higher heme Fe oxidation states does not exclude the possibility that HODM operates via such species. However, the direct alignment of the substrate N-methyl group with bound oxygen also allows for direct hydride transfer from the methyl group to the compound 0 proximal oxygen. Coupled with heterolytic O-O scission and protonation of the distal oxygen by Arg 224, this mechanism does not require formation of a higher Fe oxidation state (Fig. 4). A direct hydride transfer mechanism has been proposed for the nitric oxide reductase P450nor [22], while the direct addition of Cl^- to Fe(V)=O in chloroperoxidases has some similarity [23].

Alternatively, proton transfer from Arg224 could be coupled to single electron transfer from the substrate, creating compound II and a substrate amine radical. In contrast to the hydride transfer mechanism, the radical species can result in either the N-oxide or iminium product. It is interesting to note that, unlike DMA, methylamine (MA) is oxidised without the formation of significant levels of formaldehyde (Extended Data Fig. 7). This suggests HODM is unable to form the MA derived imine product. As the MA-derived reaction intermediate(s) are similar in energy to their DMA-derived counterparts (Extended Data Fig. 8), this suggests incorrect positioning of the single methyl group of MA, leading to formation of the N-oxide product. Distinct active and

inactive methyl binding pockets have been implicated in the sequential N-demethylation process catalysed by cytochrome P450 2B4 [24].

Our data confirm that the PAS fold can accommodate enzymatic activity, which suggests other PAS enzymes are likely to exist. Furthermore, it points to the fact that existing PAS domains can be evolved to catalyse reactions: engineered variants of a PAS oxygen sensor have been shown to display heme oxygenase activity [25]. N-dealkylation is a widespread reaction, catalysed by cytochromes P450 [12], FAD/quinone amine oxidases [26,27] and α-ketoglutarate-dependent Fe oxygenases [28]. HODM represents a distinct catalyst, which we propose acts as an oxidase. Unlike the more widespread flavin amine oxidases, HODM oxidises both NADPH and the amine substrate to convert oxygen to water (as opposed to peroxide). Dimethylamine oxidation sensitive to CO has been shown to occur in yeasts [29], and this new type of amine oxidase also appears in eukaryotes, with HODMalpha homologous genes present in a range of genomes (Extended Data Fig. 9).

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Figure legends

Fig. 1. Biophysical characterisation of *P. mendocina* HODM a) Schematic overview of the HODM reaction. **b-f)** X-band continuous wave EPR spectra of: **b**) anaerobically purified HODM **c**) HODM exposed to air **d**) the difference spectrum formed by the subtraction of c) from b) showing rhombic $[2Fe\text{-}2S]^{1+}$ and flavin semiquinone contributions [30] **e**) anaerobically purified HODM spectrum around g = 2.00 run under conditions designed to emphasise the neutral flavin semiquinone signal at g = 2.00 in D) **f**) potassium ferricyanide oxidised HODM. **g**) DMA-dependent NADPH oxidation (k_{cat} = 9.2 ± 0.5 s^{-1} and K_M^{DMA} = 24 ± 6 μM). The inset reveals stoichiometric conversion of DMA to MA and formaldehyde (errors bars are *s.e.m.*, n = 3). **h**) UV-visible absorption features

of 8.5 μM HODM in the ferric (thick solid line), ferrous (thin solid line), Fe^{2+}-O_2 (dotted line) and Fe^{2+}-CO (dashed line) states.

Fig. 2. NADPH-dependent oxidation of DMA by HODM A) UV-vis spectral evolution (over 1 s) from the anaerobic mixing of ferric HODM (5.3 μM) with NADPH. Difference spectra (inset) reveal the gamma subunit flavin is rapidly reduced. No heme reduction is observed. **b**) As in A, but monitored over 3 min. Arrows indicate absorbance changes from ferric (409 nm, thick line) to ferrous (429 nm, thick line). The A429-A409 signal (inset) is fitted to an a→*b* model; $k = 0.059 \pm 0.005$ min^{-1} (errors bars are *s.e.m.*, n = 3). **c**. UV-vis spectral evolution (over 20 s) from the anaerobic mixing of ferrous-oxy HODM (4 μM) with 200 μM NADPH (in the presence of 2 mM DMA). An *a→b→c* model was fitted with $k_1 = 2.58 \pm 0.05$ s^{-1} for ferrous:oxy:DMA (black line) to ferric (grey line) and $k_2 = 0.087 \pm 0.007$ s^{-1} for the ferric:DMA to ferrous:DMA (dotted line) transition. Similar experiments over a 1 s period revealed these steps are preceded by flavin reduction ($k = 149 \pm 20$ s^{-1}). **d.** UV-vis spectral evolution (over 100 s) from the anaerobic mixing of ferrous-oxy HODM (4 μM) with 200 μM NADPH (in the absence of DMA). Fitted to an *a→b→c→d* model with $k_1 = 0.09 \pm 0.02$ s^{-1} (initial beta subunit reduction;black line to grey line), $k_2 = 1.0 \pm 0.2$ s^{-1} for the ferrous:oxy:DMA to ferric (dotted line) and $k_3 = 0.03 \pm 0.01$ s^{-1} for ferric to ferrous (dashed line) transition. **e.** An overlay of the HODM spectrum under steady-state conditions (enzyme in the presence of excess NADPH, O_2 and DMA; black line) with the ferric DMA-bound resting state (grey line). **f**. UV-vis spectral evolution (over 1.6 s) from the anaerobic mixing of ferrous-oxy HODM alpha subunit (7.4 μM) with NADH in the presence of 75 μM PMS and 2 mM DMA. Fitted to an *a→b→c* model with $k_1 = 0.24 \pm 0.07$ s^{-1} (ferrous:oxy:DMA to ferric) and $k_2 = 0.09 \pm 0.02$ s^{-1} (ferric:DMA to ferrous:DMA).

Fig. 3. Crystal structure of the *P. mendocina* HODM alpha subunit. a) Cartoon representation of HODMalpha, with the N-terminal and the C-terminal extensions coloured in green and pink respectively. The central PAS domain is coloured according to secondary structure. The heme cofactor is shown in atom coloured sticks. **b**) A side-by-side comparison of the HODM PAS domain (left panel) with the PAS oxygen sensor Aer2 (right panel). Colour coding as in Fig. 3A. **c)** A view of the HODM alpha subunit active site in the ferric state, with key residues shown in atom coloured sticks. Sequence alignment reveals H194, R224 and F281 are strictly conserved, while other positions contain conservative mutations: W or F occur at 180, E or D at 266 and Q or E at 268. The $2F_oF_c$ electron density map is contoured at 1 sigma (blue mesh). **d**) HODMalpha active site in complex with the DMA substrate. The F_oF_c omit density corresponding to DMA is contoured at 4 sigma (green mesh). **E**) HODMalpha active site in complex with the DMA substrate and NO. The F_oF_c omit density corresponding to DMA and NO is contoured at 4 sigma (green mesh).

Fig. 4. HODM is a heme-dependent amine oxidase. Following formation of compound 0, DMA oxidation can occur through a radical mechanism (red arrows) involving transient formation of the Fe(IV) oxidation state (Compound II) or directly through H-transfer (blue arrows), bypassing the need for higher Fe oxidation states.

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Contributions
M.O. carried out molecular biology, biophysical and structural biology studies on the *P. mendocina* HODM. M.O. and L.D. carried out purification and characterisation of variant heme domain forms. P.L., B.M. and T.T. were involved in initial screening and solution characterisation of HODM homologues. T.T. carried out *in vivo* formaldehyde detection. K.F. and S.E.J.R. performed and analysed EPR experiments. S.H. performed DFT calculations and kinetic data analysis. All authors discussed the results and participated in writing the manuscript. D.L. initiated and directed this research.

Competing financial interests
The authors declare no competing financial interests.

MATERIAL AND METHODS

Materials All materials were from Sigma-Aldrich unless otherwise stated.

Cloning, mutagenesis and heterologous expression in *E. coli* For expression of an active HODM module, the gene encoding the heme alpha subunit (Pmen_3455 in the *Pseudomonas mendocina ymp* genome) and delta subunits (Pmen_3453) were synthesised and co-expressed from the pCOLADUET-1 plasmid (Novagen). The gamma (Pmen_3456 [glycine cleavage T protein – aminomethyl transferase) and beta subunits (Pmen_3454 [ferredoxin) were synthesised and expressed from the pETDuet-1 plasmid for a two-plasmid expression system for all four subunits (alpha, beta, gamma and delta). The alpha subunit bears a C-terminal His_6 tag, the delta subunit bears a N-terminal His_6 tag, whereas the gamma and beta subunits are untagged. For heme domain isolation, the alpha subunit was expressed from the pET28a plasmid with a C-terminal His_6 tag (Novagen). Site directed mutagenesis of the alpha subunit within the pCOLADUET-1 vector was performed to include a Trp180Ala mutation (538-540 bp, TGG to **GC**G), a Arg224Ala mutation (670-672 bp, **CG**T to **GC**T) and a Glu266Gln mutation (796-678 bp, **G**AA to **C**AA). HODM variants were expressed in *E. coli* BL21 (DE3) cells (Novagen). *E. coli* transformants were grown in 1 L 2YT supplemented with 25 μg/mL kanamycin and 100 μg/mL ampicillin) and incubated at 37°C 180 r.p.m. until the culture reached an OD600 of 0.6. Protein expression was then induced with addition of 0.5 mM IPTG. Cultures were grown overnight at 30°C, 180 r.p.m. and then harvested by centrifugation (7,000g for 10 min). Cell pellets were resuspended in buffer A (100 mM Tris, 200 mM KCl pH 7.5) supplemented with DNase, lysozyme (Sigma-Aldrich) and a complete EDTA-free protease inhibitor cocktail tablet (Roche). Cells were lysed using a French press at 1,500 psi and the lysate clarified by centrifugation at 125,000g for 90 min. The supernatant was applied to a Ni-NTA agarose column (Qiagen). The column was washed with three column volumes of buffer A supplemented with 10 mM imidazole and the protein was eluted in 1 ml fractions with buffer A supplemented with 300 mM imidazole. Imidazole was removed using a 10-DG desalting column (Bio-Rad) equilibrated with buffer A. The HODM heme domain was further purified using Q-Sepharose resin at pH 8 (GE Healthcare) with a 100-500 mM elution gradient (Rz ~1.2). For crystallisation studies, the purified protein was subjected to size exclusion chromatography using a Superdex 200 column (GE Healthcare) in 50 mM KPi, 250 mM KCl (pH 7.5). Protein was concentrated as required using a Vivaspin centrifugal device (Sartorius).

Hemoprotein concentration estimations The pyridine hemochromogen method was used to quantify HODM heme and to determine an extinction coefficient at the Soret maximum (ε_{423} = 94 mM^{-1} cm^{-1}) according to the method of Berry and Trumpower (1987) [31].

UV-visible absorbance studies UV-visible absorbance analysis was carried out using a Cary UV-50 UV-visible scanning spectrophotometer (Varian) using a 1 cm pathlength quartz cuvette, recording spectra between 250 and 700 nm, and typically with HODM at 8 μM in buffer A. Potassium ferricyanide was used to chemically oxidise HODM and O_2 binding experiments were performed in an anaerobic glove-box (Belle Technology, Weymouth UK) under a nitrogen atmosphere with O_2 levels maintained at less than 2 ppm. All solutions were deoxygenated by sparging with nitrogen gas. To obtain the ferrous dioxygen complex, HODM (holoenzyme or the heme subunit in isolation) was reduced by sodium dithionite, followed by slow bubbling of air into the reaction mixture. The rate of decay of the ferrous-O_2 complex was determined by continual scanning every ~2 min over ~6 hours. Half-life values were derived by plotting the induced optical change against time and fitting using an exponential decay equation using Origin software (OriginLab, Northampton, MA). Rate of decay values were derived using a first order rate equation.

Ligand binding titrations Optical titrations to determine K_d values for dimethylamine were performed at 25 °C using HODM at ~8 μM in buffer A. Dimethylamine stocks were made up in the same buffer and added volumes did not exceed 0.1% of the total volume. K_d values were determined by plotting the induced optical change against ligand concentration and by fitting using a hyperbolic equation within Origin software, or (for tight-binding ligands) using the Morrison (quadratic) equation [32] (OriginLab, Northampton, MA).

NADPH spectrophotometric activity assays At each concentration of dimethylamine (0-600 μM), 200 μM NADPH was added to initiate the reaction and the rate of change in absorbance at 340 nm monitored over 1 min at room temperature to follow substrate-dependent NADPH oxidation. Reactions were performed in triplicate to produce a mean rate calculated as moles of NADPH oxidised/mol HODM/second. These data were plotted versus the relevant DMA concentration and fitted using the Michaelis-Menten hyperbolic function within Origin software. Similar experiments were conducted using perdeuterated DMA or in D_2O, revealing no significant differences in NADPH oxidation rates.

***In vitro* formaldehyde detection using Purpald** For the detection of substrate-dependent formaldehyde production, the Zurek & Karst method [33] was used, however with a Purpald solution of 1 mg/mL in 1 M NaOH. HODM activity reactions were set up as described above with an enzyme concentration of 200 nM with 250 μM DMA and allowed to react for 5 min at RT. Microtitre plate assays were performed using a 96-well plate reader (BioTek Synergy HT, Biotek). 100 μL of Purpald solution was mixed with 100 μL of sample and absorbance measurements were carried out after a reaction time of 60 min at a wavelength of 550 nm. A formaldehyde standard curve was calculated at known concentrations of formaldehyde. Reactions without HODM or NADPH result in the loss of formaldehyde formation. The assay underestimated this loss due to background absorbance from the standard curve and so data were normalised accordingly.

***In vivo* formaldehyde detection** Enzymatically catalysed formaldehyde production was assayed *in vivo* as reported previously [34]. Briefly, the respective assay makes use of a UV-light sensitive GFP ($excitation_{max}$ = 395 nm, $emission_{max}$ = 509 nm) fused to FrmR as sensor and is hosted in *E. coli* W3GM as a formaldehyde-sensitive host. Following transformation with expression constructs of formaldehyde-producing enzymes, this reporter strain allows quantitative measurement of cytosolic formaldehyde levels *in vivo*. The corresponding assays were performed using a 96-well

microtitre plate reader (BioTek Synergy HT, Biotek, USA) equipped with a $360_{Ex}/528_{Em}$ nm filter set with readings taken in the early stationary phase (22 to 24 h after inoculation). The data were corrected for background fluorescence of uninduced controls and adjusted for cell density and enzyme activity as necessary. All assays were done as 6-fold biological replicates at least, and the results were found to be statistically significant within a confidence interval of 3 %. Low levels of formaldehyde were measured colorimetrically with acetylacetone/ammonium acetate as described (39). Aliquots were taken (0-1 mL) and made up to a final volume of 1 mL with water and combined with 0.03 mL 7.5 M HCl and 0.25 mL of a 4X Nash reagent solution, prior to incubation at 38°C for 90 min. Samples were centrifuged for 5 min at 14,000 rpm in a microfuge and the A_{414} determined in a 1-cm cell.

EPR Analysis Continuous wave EPR spectra for HODM were recorded at X-band (~9.4 GHz) using a Bruker ELEXSYS E500/E580 EPR spectrometer (Bruker GmbH, Rheinstetten, Germany). Temperature was maintained using an Oxford Instruments ESR900 helium flow cryostat coupled to an ITC 503 controller from the same manufacturer. The experimental conditions employed to obtain individual spectra are given in the figure captions. EPR sample tubes were 4 mm Suprasil quartz supplied by Wilmad (Vineland, NJ).

Redox potentiometry To determine the midpoint potential for the HODM heme domain Fe^{III}/Fe^{II} couple, a spectroelectrochemical titration was performed in an anaerobic glove box (Belle Technology, Weymouth UK) under a nitrogen gas atmosphere. All solutions were degassed under vacuum with nitrogen prior to use in the glove-box. Oxygen levels were maintained at less than 5 ppm. The concentrated HODM heme domain protein sample was passed through a Sephadex G25 column (1 x 20 cm) (10DG column, Bio-Rad, Hemel Hempstead UK) immediately on admission to the glove box to remove all traces of oxygen. This column was pre-equilibrated and proteins were buffer-exchanged into anaerobic redox buffer (100 mM Tris, 200 mM KCl, pH 7.0). The HODM heme domain solution (~ 10-15 μM enzyme in 5 mL buffer), was titrated electrochemically according to the method of Dutton [35], using sodium dithionite as reductant and potassium ferricyanide as oxidant. Mediators were added to expedite electronic equilibration in the system (2 μM phenazine methosulfate [PMS], 5 μM 2-hydroxy-1,4-naphthoquinone [HNQ], 5 μM methyl viologen [MV], and 1 μM benzyl viologen [BV]) and to mediate in the range between approximately +100 mV and -480 mV *versus* the normal hydrogen electrode (NHE) [36]. 10-15 minutes were allowed to elapse between each addition of reductant/oxidant to ensure equilibration and stabilization of the electrode. Spectra (250-750 nm) were recorded using a Cary UV-50 Bio UV-Visible scanning spectrophotometer coupled to a fibre optic probe immersed in the HODM heme domain solution. The potential was measured using a SevenEasy S20-K meter (Mettler Toledo, Leicester UK) coupled to a Calomel electrode (ThermoRussell, Cupar UK) at 25 °C. The calibration of the electrode was done by using the Fe^{III}/Fe^{II} EDTA couple as a standard (+108 mV). The electrode reading was corrected by +244 mV relative to the NHE. Absorbance data at wavelengths reporting on the transition of the heme Soret band between oxidized (Fe^{III}) and reduced (Fe^{II}) forms of the HODM heme domain were plotted *versus* applied potential, and the data were fitted using the Nernst function in Origin 8.5 (OriginLab, Northampton MA).

Stopped-flow spectroscopy Stopped-flow absorbance measurements were made using an Applied Photophysics SX18 MVR stopped-flow spectrophotometer in an anaerobic glove box (Belle Technology Weymouth, UK). The drive syringes were loaded with separate solutions of ferric or ferrous-oxy protein (generation of this complex with aerobic buffer is described above) and varying concentrations of reductant in buffer A in the presence and absence of DMA (260 μM). Multiple wavelength data were collected at

room temperature using a photodiode array (PDA) detector and XSCAN software. The resulting spectra were subjected to global analysis using SpecFit/32 software (Bio-Logic Science Instruments, Grenoble, France). Singular value decomposition of the dataset provides assessment of the number of spectrally distinct components present in the reaction. The data were fitted using irreversible models.

Crystallisation, refinement and model building The HODM-heme subunit was crystallised at 3 mg ml^{-1} in 50 mM KPi, 100 mM KCl, pH 7.5. Initial crystallisation conditions were identified using the JCSG+ matrix screen (Molecular dimensions). Crystals suitable for diffraction experiments were obtained by sitting drop vapour diffusion at 4 °C in 400 nL drops containing equal volumes of protein and a solution containing 30% PEG 2K MME and 0.1 M potassium thiocyanate. The crystals were derivatised by soaking with the same solution supplemented with 1 mM mercury (II) acetate for 10 min. For the dimethylamine-bound structures, native crystals were soaked with 1 mM ligand for 10 min. For ferrous structures, the crystals were soaked with sodium dithionite followed by 1 mM $NaNO_3$ and 1 mM DMA for ferrous-DMA-nitric oxide bound structures. The crystals were cryoprotected by the addition of 10% PEG 200 to the mother liquor and flash cooled in liquid nitrogen. Data were collected on beamline I24 (wavelength 0.9173/ 1.0118 Å) at the Diamond Light Source Facility and reduced and scaled with the X-ray Detector Software suite (XDS [37]). The ligand-free HODM heme subunit crystal structure was determined by SIRAS using the MLPHARE program in the CCP4 suite [38]. In brief, the SIRAS signal extending to 1.86 Å from the comparison of a platinum derivative with a mercury derivative with a figure of merit of 0.85, phasing power of $1.04^{centric}/1.19^{acentric}$ and R_{cullis} of $0.7^{centric}/0.8^{acentric}$ and $1^{anomalous}$ was used for initial map calculation following density modification using the DM program in the CCP4 suite. A non-isomorphic native data set with higher resolution (1.65 Å) was used for refinement. Automated model building with BUCCANEER in the CCP4 suite successfully constructed the entire model of the ligand-free HODM heme subunit structure. The model was completed by iterative cycles of manual model building and real space refinement using the program Coot and crystallographic refinement using Refmac5. The processing and final refinement statistics are presented in Extended Data Table 1.

Single crystal microspectrophotometry Changes in visible absorbance spectra during X-ray exposure were monitored at the European Synchrotron Radiation Facility (ESRF) in Grenoble on beamline ID14-1 using an on-line spectrophotometer (OCEAN OPTICS DH 2000 light source and HR 2000 detector). A HODM alpha subunit crystal was soaked with sodium dithionite and DMA followed by transfer to mother liquor supplemented with DMA in the absence of dithionite to allow for oxygen complex formation. Initial spectra prior to X-ray illumination revealed formation of a species consistent with the ferrous:O_2:DMA complex.

Data availability Coordinates and structure factors have been deposited under accession numbers 5LTE, 5LTH and 5LTI.

Material and Methods References

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Extended Data Figure Legends

Extended Data Table 1: Data collection and refinement statistics for *P. mendocina* HODM alpha subunit.

Extended Data Fig. 1. Catabolic pathway featuring HODM and additional HODM characterization *in vivo* and *in vitro*. a) Trimethylamine degradation pathway with links to C1 metabolism in proteobacteria. 1, trimethylamine monooxygenase; 2, trimethylamine N-oxide demethylase; 3, heme-dependent oxidative demethylase (HODM), also known as secondary amine monooxygenase (SAMO); 4, methylamine dehydrogenase (EC 1.4.99.3); 5, methylamine oxidase (EC 1.4.3.21); 6, N-methylglutamate synthase (EC 2.1.1.21); 7, N-methylglutamate dehydrogenase (EC 1.5.99.5); 8, γ-glutamylmethylamide synthetase (EC 6.3.4.12); 9, γ-glutamylmethylamide-dissimilating enzyme; 10, methylene-H4F dehydrogenase/cyclohydrolase (EC 1.5.1.15); 11, formyl-H4F deformylase/ formyl-H4F synthetase (EC 3.5.1.10); 12, formate dehydrogenase (EC 1.2.1.2). Formaldehyde can be assimilated via the ribulose monophosphate (RuMP) pathway or, via methylene-tetrahydrofolate, through formation of serine. The inset shows an SDS-PAGE gel of purified recombinant HODM, revealing bands corresponding to all four subunits. Additional bands are visible that appear to correspond to multimeric forms or proteolytic fragments of HODM subunits as verified by MS of tryptic digests. **b.** Formaldehyde-leakage from HODM *in vivo***.** Efficient detoxification of formaldehyde requires the gamma subunit as well as THF. Cells containing *wt* and mutant HODM enzyme (Δgamma = deletion of subunit gamma) from *Methylobacillus flagellatus* KT were grown in the presence of DMA (grey bars) or DMA and glycine (shaded bars), respectively. Fluorescence readings were subsequently corrected for background activity and scaled with the mutant enzyme set as 100 %. The data show that glycine as well as the gamma subunit have strong effects on enzymatic formaldehyde production. While the first increases intracellular levels of THF, the latter is required for THF binding. **c)** The HODM ferrous-oxy complex is long-lived at room temperature. The ferrous-oxy decay to the ferric state was monitored at 409 nm and plotted as a function of time. The decay curve observed was fitted using the exponential decay equation to derive a half life of 50 ± 10 min with a decay rate of k= 0.014 ± 0.001 min^{-1}. **d)** Determination of the redox potential of the HODM heme subunit. The main panel shows UV-visible spectra for the HODM heme domain during a redox titration. The oxidized enzyme (thick black line, spectrum recorded at -76 mV vs. NHE) has its Soret maximum at 409 nm. The fully reduced enzyme (thick line, spectrum recorded at -317 mV vs. NHE) has its Soret maximum at 422 nm. Intermediate spectra are indicated in thin lines, and there is an isosbestic point at ~415 nm. The HODM heme domain also displays

increased absorbance in the Q-band region (~520-620 nm) in the reduced (ferrous) state. The inset shows a plot of absorbance change (ΔA_{422} minus ΔA_{409}) vs. applied potential (vs. NHE) fitted using the Nernst equation. This provides a midpoint reduction potential value for the HDOM heme domain Fe^{III}/Fe^{II} couple of 128 ± 4 mV vs. NHE. **e**) DMA binding to ferrous HODM and **f**) DMA binding to ferrous:oxy HODM. Absorbance changes (as shown in the inset) as a function of DMA concentration are fitted using a hyperbolic function/Morrison equation, leading to a K_d = 1.7 ± 0.2 mM for ferrous HODM (ferric HODM has a K_d of 1.5 ± 0.1 mM, data not shown) and a K_d = 15 ± 3 μM for ferrous-oxy HODM (errors bars are *s.e.m.*, n = 3).

Extended Data Fig. 2. X-band continuous wave EPR spectra of anaerobically purified HODM. a) Anaerobically purified HODM exposed to air for two minutes; **b**) the HODM enzyme of a) with the addition of excess dimethylamine; **c**) anaerobically purified HODM; **d**) the HODM enzyme of c) with the addition of excess dimethylamine. Experimental parameters: microwave power 0.5 mW, modulation amplitude 3 G, temperature 30 K. **e)** HODM alone; **f)** HODM plus excess dimethylamine; **g)** HODM plus excess diethylamine; **h)** HODM plus excess piperidine. Experimental parameters: microwave power 0.5 mW, modulation amplitude 3 G, temperature 30 K. Only dimethylamine (b) shows the loss of the ^{14}N His superhyperfine coupling in the ferroheme-NO component of the spectrum and a significant change in the g values exhibited by the $[2Fe\text{-}2S]^{1+}$ component of the spectrum.

Extended Data Fig. 3. HODM cannot make use of peroxide. Spectral changes observed following mixing of 10μM ferric HODM with 200 μM H_2O_2. The heme Soret peak (black line) decays upon H_2O_2 addition (200 μM**)** (thin grey lines each after 30 s) with further decay at 5 min incubation (thin black line) and 60 min (black dotted line). The inset shows a similar experiment conducted in the presence of 2 mM DMA.

Extended Data Fig. 4. The HODM heme subunit has similar properties to the HODM holoenzyme. a) UV-visible absorption features of alpha HODM (8.5 μM). Ferric (thick solid line), ferrous (thin solid line) $Fe^{2+}\text{-}O_2$ (dotted line) and Fe^{2+}-CO (dashed line). The major (Soret) absorption band is centered at 409, 423, 418 and 421 nm, respectively. The α/β bands are expanded for *Inset*. **b)** Titration of ferric and ferrous-oxy HODM alpha subunit with DMA. Absorbance changes associated with the heme Soret peak are plotted as a function of DMA concentration. The data are fitted using a hyperbolic function or the Morrison equation. *Top panel*. Ferric with K_d = 12.6 ± 0.2 mM, *Lower panel*. Ferrous-oxy with K_d = 41.0 ± 5.0 μM (errors bars are *s.e.m.*, n = 3). ***Insets.*** UV-vis spectra of the titration of DMA (0-50 mM) against ferric and ferrous-oxy HODM alpha subunit (7.5/4.8 μM). The UV-vis spectrum of alpha in buffer A was recorded initially and then after each addition of DMA. The direction of the absorbance changes of the ferric and ferrous-oxy Soret peak on substrate addition is measured from 409/ 415 nm (thick line) to 416/414 nm (dotted line), respectively. **c-e**) Spectral properties of HODM heme subunit variants, respectively E266Q, W180A and R224A. Ferric (thick black line), ferric in presence of 1 mM DMA (thick grey line), ferrous (thin black line), ferrous in presence of 1 mM DMA (thin grey line) and $Fe^{2+}\text{-}O_2$ (dotted line). The α/β bands are expanded for *Inset*. While the E226Q variant lacks spectroscopic features associated with DMA binding, the R224A variant appears to bind DMA as a 6^{th} ligand to the heme iron in the ferrous state. Oxygen binding can be detected for all variants.

Extended Data Fig 5. Possible routes for DMA oxidation in HODM. Following formation of compound 0, various possibilities exist for O-O bond cleavage in principle. A direct hydride transfer from the substrate is shown in A, bypassing the need for higher oxidation states of the heme Fe. Route B depicts the homolytic bond cleavage with formation of compound II and an amine radical. Route C depicts a P450-like mechanism,

with formation of the highly oxidising compound I. Both the B and C routes can give rise to various products. The N-oxide product is not observed with DMA as a substrate, which results in stoichiometric production of formaldehyde *in vitro*. This can be derived from either the iminium or hydroxylamine products.

Extended Data Fig 6. X-ray induced photoreduction of the HODM heme subunit in complex with DMA and O_2. The initial spectrum recorded before X-ray illumination (ESRF beamline ID14-1 (wavelength 0.934 Å)) was subtracted from the post illumination spectrum. A shift in the α/β bands is recorded with a decrease at 575 nm and an increase at 556 nm. This corresponds to formation of the ferrous species (merged α/β band at 556 nm).

Extended Data Fig. 7. Methylamine-dependent NADPH oxidation by HODM in absence of formaldehyde production. The kinetic curve (mean values of initial rate plotted as a function of MA concentration) was fitted using the Michaelis-Menten function k_{cat} = 7.0 ± 0.1 s^{-1} and K_M = 0.55 ± 0.04 mM. *Inset* shows Purpald colourimetric assay for detection of substrate-dependent formaldehyde production. Data are represented as a percentage of expected formaldehyde produced for 1:1 conversion of DMA/MA to formaldehyde. Error bars represent standard deviation.

Extended Data Fig. 8. Gas phase density functional theory and *ab initio* comparison of the energies of DMA and MA-based reaction intermediates. Quoted energies in the Table are the sum of electronic and thermal enthalpies for structures in the gas phase at 298.15K with units of hartrees. Values in parenthesis are the difference relative to the neutral amine. The relative energies of the DMA species are lower than those of the MA species by 10 – 65 kJ mol^{-1} (0.04 – 0.025 Ha). The p*K*a of dimethylamine (DMA; R = CH_3) and methylamine (MA; R = H) **(1)** are very similar (10.64 and 10.62 respectively). Calculations were performed at both the (U)B3LYP/6-311++G(d,p) and (U)MP2/6-311++G(d,p) level using the 'Freq' keyword in Gaussian 09 rev. B.01 (Frisch, X.M.J. *et al*. Gaussian 09 (Gaussian, Wallingford, Connecticut, revision B.01, 2010).

Extended Data Fig. 9. Multiple sequence alignment of SAO including Aer2 PAS domain secondary structure information Alignment of HODM proteins from the DUF3445 family from the bacteria; *Pseudomonas mendocina* (Pmen), *Rhizobium etli* (Retl), *Candidatus Puniceispirillum marinum* (Cpun) and *Gordonia bronchialis* (Gbro) and from the fungi *Candida dubliniensis* (Cdub) and *Nectria haematococca* (Nhae). The catalytic Arg, Glu and Gln residues and the substrate discriminating Trp are marked with black dots. The conserved heme binding His in Pmen and Aer2 are marked with a grey and a black asterisk, respectively. Secondary structural elements are highlighted, PAS domain specific alpha helices in blue and beta sheets in pink, and HODM specific in alpha helices in pale grey and beta sheets in dark grey.