PCuAC domains from methane-oxidizing bacteria use a histidine brace to bind copper

Oriana S. Fisher‡,1, Madison R. Sendzik‡, Matthew O. Ross‡, Thomas J. Lawton‡,2, Brian M. Hoffman‡, and Amy C. Rosenzweig‡,3

From the ‡Departments of Molecular Biosciences and of Chemistry, Northwestern University, Evanston IL 60208

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1Present address: Department of Chemistry, Lehigh University, Bethlehem PA 18015
2Present address: Johns Hopkins Applied Physics Laboratory, Laurel MD 20723
3To whom correspondence should be addressed: Amy C. Rosenzweig, Departments of Molecular Biosciences and of Chemistry, Northwestern University, Evanston IL 60208. Tel.: 847-467-5301; Fax: 847-467-6489; E-mail: amyr@northwestern.edu.

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Data Deposition: The coordinates of the structures presented here have been deposited in the Protein Data Bank: apo SMpMoF1-ΔC (6P17), copper-bound PmoF1-ΔC (6P16, 6P1E), apo PmoF2-ΔC (6P1F), copper-bound SMpMoF2-ΔC (6P1G).

ABSTRACT

Copper is critically important for methanotrophic bacteria because their primary metabolic enzyme, particulate methane monooxygenase (pMMO), is copper-dependent. In addition to pMMO, many other copper proteins are encoded in the genomes of methanotrophs, including proteins that contain periplasmic copper-A chaperone (PCuAC) domains. Using bioinformatics analyses, we identified three distinct classes of PCuAC domain-containing proteins in methanotrophs, termed PmoF1, PmoF2, and PmoF3. PCuAC domains from other types of bacteria bind a single Cu(I) ion via an Hx₁₀Mx₂₁/₂₂HxM motif, which is also present in PmoF3, but PmoF1 and PmoF2 lack this motif entirely. Instead, the PCuAC domains of PmoF1 and PmoF2 bind only Cu(II), and PmoF1 binds additional Cu(II) ions in a His-rich extension to its PCuAC domain. Crystal structures of the PmoF1 and PmoF2 PCuAC domains reveal that Cu(II) is coordinated by an N-terminal histidine brace Hx₁₀H motif. This binding site is distinct from those of previously characterized PCuAC domains, but resembles copper centers in CopC proteins and lytic polysaccharide monooxygenase (LPMO) enzymes. Bioinformatics analysis of the entire PCuAC family revealed previously unappreciated diversity, including sequences that contain both the Hx₉Mx₂₁/₂₂HxM and Hx₁₀H motifs, and sequences that lack either set of copper-binding ligands. These findings provide the first characterization of an additional class of copper proteins from methanotrophs, further expand the PCuAC family, and afford new insight into the biological significance of histidine brace-mediated copper coordination.

Copper enzymes, including cytochrome c oxidase, nitrous oxide reductase, nitrite reductase, superoxide dismutase, and particulate methane monooxygenase (pMMO), play important roles in bacterial metabolism (1). In gram negative bacteria, these enzymes are located in the inner membrane and/or periplasm, where copper loading likely takes place either via periplasmic chaperone proteins or directly from extracytoplasmic pools (2). One family of periplasmic copper-binding proteins implicated in assembly of the cytochrome c oxidase copper centers is the periplasmic copper-A chaperone (PCuAc) proteins. These proteins have been
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proposed to play a role in loading the cytochrome c oxidase CuA and/or CuB sites (3-8). Genes encoding PCuACs are often found neighboring genes encoding Sco1 proteins (3,9), and transfer of Cu(I) from PCuAC to Sco1 has been demonstrated for PCuACs from Streptomyces lividans (7) and Rhodobacter capsulatus (10).

However, it remains unclear whether PCuAC is absolutely required for metalation of cytochrome c oxidase since multiple gene disruption studies indicate that it is not critical for cytochrome c oxidase activity (5,7,11). In addition, there is evidence that some PCuAC homologs have other functions. For example, the PCuAC homolog AccA is essential for copper nitrite reductase activity in pathogenic Neisseria strains (12). Consistent with a broader physiological role, PCuAC-encoding genes are not always found in proximity to genes encoding Sco1s, cytochrome c oxidases, or nitrite reductases (2,6,13), and some bacterial genomes contain multiple PCuAC paralogs (11). Moreover, only a few PCuAC proteins have been studied in vitro, and their properties might not be universally applicable to the entire family. Of the three structurally-characterized PCuAC proteins, all bind a single Cu(I) ion (3,4,6,14) using a conserved \textit{Hx}_n\textit{Mx}_{21/22}\textit{HxM} motif, and biochemical data indicate that the \textit{R. capsulatus} PCuAC homolog not only binds Cu(I), but also has a Cu(II) binding site (10).

Many other bacteria also encode PCuAC homologs in their genomes, but little is known about these proteins. Strikingly, in methanotrophs, bacteria that utilize methane as their sole source of carbon and energy (15), \textit{pCuA} genes are frequently contained within the same operon that encodes the copper-dependent, particulate methane monooxygenase (pMMO) enzyme. pMMO, the central metabolic enzyme in methanotrophs, comprises three subunits, PmoC, PmoA, and PmoB, arranged in a trimer of PmoCAB protomers (16-19). In most alpha-proteobacterial methanotrophs, the \textit{pmo} operons encoding these three subunits contain up to four additional genes encoding the proteins PmoD, CopC, CopD, and a PCuA domain (9,20,21). In the methanotroph \textit{Methylosinus (Ms.) trichosporium} OB3b, these four genes are coregulated with the \textit{pmoC}, \textit{pmoA}, and \textit{pmoB} genes, exhibiting mild upregulation in response to copper (21). Previous studies have shown that PmoD (21) and CopC (9) are both copper-binding proteins, and that PmoD is needed for copper-dependent growth (21). Notably, the periplasmic domain of PmoD has been demonstrated to form an unusual CuA site in vitro (21,22), but how it is loaded with copper in vivo remains unknown.

In addition to the \textit{pcuAC} genes in some \textit{pmo} operons, many methanotrophs also encode other PCuAC proteins that are differentially regulated in response to copper (21,23). A number of methanotroph \textit{pcuAC} genes do not have a \textit{sco1} neighbor so their functions remain unknown and are likely distinct from those of PCuAC proteins that have been studied previously. Also of interest, methanotrophic PCuACs encoded within \textit{pmo} operons frequently have an additional C-terminal extension that includes a cluster of histidine residues.

To further understand the diversity of the PCuAC family, we have performed detailed bioinformatics analyses and have biochemically and structurally characterized two selected methanotroph PCuAC proteins, PmoF1 and PmoF2. These two homologs lack the \textit{Hx}_n\textit{Mx}_{21/22}\textit{HxM} Cu(I)-binding motif present in other family members (3,4,6) and in the third methanotrophic PCuAC homolog, PmoF3. Instead, both PmoF1 and PmoF2 bind Cu(II) using a ligand set similar to the histidine-brace coordination observed in CopCs (9,24), and lytic polysaccharide monooxygenase (LPMO) enzymes (25,26). Furthermore, PmoF1 contains a His-rich extension that binds additional copper. Bioinformatics analyses indicate that divergent copper binding sites in PCuAC domains are more prevalent in, but not unique to, methanotrophs. Thus, the PCuAC family is more diverse than appreciated previously, and the different types of copper binding sites coupled with distinct genomic neighborhoods may correlate with distinct biological functions, including, in the cases of PmoF1 and PmoF2, ones specific to methanotroph copper homeostasis.

Results

Bioinformatics analysis of methanotrophic PCuAC proteins

To identify PCuAC domain-containing proteins in methanotrophs, we searched all methanotroph genomes deposited in the IMG-JGI
database for genes encoding proteins containing a PCuAC (pfam04314) domain. PCuAC domains are found primarily in the alpha-proteobacterial methanotroph strains, with only the Methyllococcus genus represented among gamma-proteobacteria (Fig. 1A). Many alpha-proteobacterial methanotroph genomes contain up to three PCuAC-encoding genes. The majority of these are found in one of three different genome neighborhoods: within a pmo operon immediately downstream of pmoCAB, pmoD, copC, and copD (which we denote pmoF1); neighboring a lone pmoD gene (pmoF2); or approximately 9 kb away from the operon encoding components of the soluble methane monooxygenase (sMMO) and the direct neighbor of a sco1 gene (pmoF3) (Fig. 1B, C). All three pmoF genes are present and exhibit copper-dependent regulation in Ms. trichosporium OB3b. Specifically, pmoF1 exhibits slight upregulation in response to copper similar to pmoCAB and pmoF2 is also upregulated in response to copper, while pmoF3 is markedly downregulated (21). Both the PmoF1 and PmoF2 homologs are exclusively found in alpha-proteobacterial strains, suggesting that these proteins may be linked to pMMO function in these organisms, but not in other methanotrophs.

All three PmoF proteins contain signal peptides at their N-termini preceding their PCuAC domains (Fig. 1D). In addition, PmoF1 and PmoF2 homologs include C-terminal extensions while PmoF3 homologs do not (Fig. 1D, S1A). Previously studied PCuAC proteins contain a Cu(I) binding HxnMx21/22HxM motif (3,4,6,7) and/or have been implicated in copper transfer to Sco1 (3,7,8,10,14). While the PmoF3 proteins share the HxnMx21/22HxM motif and also frequently neighbor Sco1 proteins, the conserved metal binding motif is notably absent in the PmoF1 and PmoF2 proteins (Fig. 1D, S1). The C-terminal extension of PmoF1 proteins (~60 residues), which has no predicted secondary structure, includes a cluster of 5-11 histidines within its most C-terminal half (Fig 1E, S1). By contrast, the ~60 residue C-terminal extensions of PmoF2 proteins lack the concentrated cluster of histidine residues (Fig. 1E, S1). Despite the absence of the canonical PCuAC copper-binding motif in PmoF1 and PmoF2, their genomic neighborhoods and previously observed copper-dependent regulation patterns suggest that they may also bind copper.

PmoF1 and PmoF2 are Cu(II)-binding proteins

To test for copper binding experimentally, we expressed full-length PmoF1 (locus tag Met49242_1449, residues 33-202, PmoF1-FL) from Methylcocystis sp. Rockwell and full-length PmoF2 (locus tag MettrDRAFT_3935, residues 29-193, PmoF2-FL) from Ms. trichosporium OB3b in E. coli as N-terminally SUMO-tagged constructs. These proteins were purified to homogeneity, and the SUMO tags were cleaved using SUMO protease to yield proteins with the authentic amino terminus that would be produced upon signal peptide cleavage. Given the preference for Cu(I) exhibited by other members of the PCuAC family, we first investigated Cu(I) binding by anaerobically loading PmoF1-FL and PmoF2-FL with 2-5 molar equivalents of tetrakis(acetonitrile) Cu(I) hexafluorophosphate. After removing unbound copper using a desalting column, no bound copper could be detected by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Thus, neither PmoF1-FL nor PmoF2-FL binds Cu(I), unlike previously characterized PCuAC domain-containing proteins that all reportedly bind 1 equivalent of Cu(I) (3-5).

We then assessed Cu(II) binding in a similar manner, adding 0-10 Cu(II) equivalents. PmoF1-FL binds 2.2 ± 0.6 molar equivalents of Cu(II) (Fig. 2A). To localize the copper binding sites, we generated an additional construct lacking the C-terminal extension (residues 33-153, PmoF1-ΔC). Addition of 0-5 Cu(II) equivalents indicates PmoF1-ΔC binds 0.75 ± 0.3 Cu(II) ions (Fig. 2A). Together, these data suggest that the His-rich extension, which in Ms. trichosporium OB3b PmoF1 includes 7 histidine residues (Fig. S2), contains additional copper binding sites. The variability in copper-binding stoichiometry of PmoF1-FL may be related to changing accessibility of the presumably unstructured C-terminal extension. Other members of the PCuAC family, including the AccA protein from Neisseria species (12) and PcuC from Bradyrhizobium diazoefficiens (14), also contain C-terminal His- or Met-rich extensions. In the case of B. diazoefficiens PcuC, this extension delivers copper to Sco1 and to cytochrome c.
oxidase (14). In other types of proteins, such extensions have also been implicated in copper binding. For example, some Cu/Zn superoxide dismutases include an N-terminal His-rich region that binds copper and has been proposed to either directly insert the metal into a neighboring protein molecule or to increase the local copper concentration near the active site (27). Similarly, the N-terminal metal-binding domain of the Cu(I)-specific CopB P1in-ATPase contains a large number histidine residues and binds up to 8 additional copper ions (28). It is possible that the His-rich extension in PmoF1-FL binds periplasmic copper and delivers it to the PCuAC domain or to other protein targets. Alternatively, it may serve a regulatory function.

In contrast to PmoF1-FL, addition of excess Cu(II) to PmoF2-FL results in 0.90 ± 0.4 Cu(II) ions bound per protein molecule (Fig. 2B). PmoF2 homologs also contain a C-terminal extension to the PCuAC domain. Many of these extensions include two histidine residues in the equivalent part of the sequence to the PmoF1 His-rich region (Fig. S1D). Ms. trichosporium OB3b PmoF2, however, does not have these two histidine residues, but does have three other histidine residues within its C-terminal region, His152, His 154, and His175 (Fig. S2). The PmoF2 PCuAC domain alone (residues 29-159, PmoF2-ΔC), which includes His152 and His154, but not His175, binds as many Cu(II) equivalents as PmoF1-FL, 0.94 ± 0.2 Cu(II) per domain (Fig. 2B), indicating that unlike PmoF1, this full-length PmoF2 does not bind significantly more copper than the PCuAC domain alone.

**Crystal structures reveal that PmoF proteins bind copper using a histidine brace**

Given the absence of the Hx₅Mₓ₁₂HₓM motif in both PmoF1 and PmoF2 and the unusual preference for Cu(II) rather than Cu(I), we pursued structural studies of both apo and copper-loaded PCuAC domains from each protein. We obtained crystals of the apo SM²PmoF1-ΔC (a construct identical to PmoF1-ΔC but with two additional N-terminal vector-derived residues, a serine and a methionine) and PmoF2-ΔC, and solved the structures to 1.85 and 1.65 Å resolutions, respectively (Table 1). Both structures comprise primarily two β sheets in a Greek key topology (Fig. 3A, B), similar to the overall fold of other structurally characterized members of the PCuAC family. Superpositions of the SM²PmoF1-ΔC and PmoF2-ΔC structures with that of the ECuC protein from S. lividans yield rmsd values of 1.42 Å over 106 Ca atoms and 1.82 Å over 108 Ca atoms, respectively (Fig. 4A). The SM²PmoF1-ΔC and PmoF2-ΔC structures are also highly similar to one another with an rmsd of 1.70 Å over 119 Ca atoms (Fig. 3). One notable divergence between previously characterized PCuAC domains and the current structures is the presence of an additional β strand at the N-termini of PmoF1 and PmoF2 (termed β0) that extends the β-sheet formed by strands β1, β3, and β5 (Fig. 4). We also crystallized Cu(II)-loaded forms of each protein, and determined structures of copper-loaded PmoF1-ΔC and copper-loaded SM²PmoF2-ΔC to 1.91 Å and 2.05 Å resolutions, respectively (Table 1). The overall fold is similar in both cases (Fig. 3), with rmsd values of 0.63 Å and 1.36 Å for superposition of 120 Ca atoms from the apo and copper-loaded forms of PmoF1 and PmoF2, respectively. Thus, copper binding does not induce significant conformational changes.

The copper-loaded PmoF1-ΔC structure contains a single copper ion in square pyramidal geometry (Fig. 3A). This copper ion is coordinated by the δ nitrogen atoms of two histidine residues at the N-terminus of the protein (bond lengths ~ 2 Å). These two histidine ligands derive from a Hx₅MₓH motif. The amino terminus itself provides a third nitrogen ligand in the same plane, forming a T-shaped histidine brace coordination motif (Fig. 3A, Table 2). An axial water molecule and a glutamate side chain oxygen from the second molecule in the asymmetric unit are located 2.7/3.1 Å and 2.2 Å from the copper ion, respectively. Additionally, size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis of the copper-loaded PmoF1-ΔC revealed a mix of species with an average molar mass of 17,930 Da ± 6%, which is between the expected molar masses of a monomer and a dimer (Fig. S3A). However, SEC-MALS analysis of copper-loaded PmoF1-FL indicates a single species with the expected mass of a monomer (Fig. S3B). Since the glutamate coordination observed in the crystal structure can only occur in a dimeric species, this coordination most likely represents a
crystallographic artifact. Copper coordination by a glutamate from a neighboring molecule in the crystal lattice is also observed in one of the LPMO structures (29).

The copper-loaded SM-PmoF2-ΔC structure similarly reveals a single copper ion coordinated by an Hx10H motif (Fig. 3B). Although the construct used to obtain this structure included two additional vector-derived residues upstream of the authentic N-terminal histidine (we did not obtain crystals of the PmoF2-ΔC construct), the copper coordination is similar to that in the PmoF1-ΔC structure, includes the δ nitrogen atoms from two histidine side chains and the amino terminus of the peptide. The peptide backbone from the vector-derived residues provides two additional deprotonated amide ligands, oriented similarly to the water molecule and glutamate side chain observed in the PmoF1-ΔC structure (Fig. 3B).

Consistent with the crystallographic observations, copper-loaded PmoF2-ΔC (as well as copper-loaded PmoF2-FL) is a single, monomeric species by SEC-MALS (Fig. S3C,D). For both PmoF1-ΔC and SM-PmoF2-ΔC, there is a large conformational change in the side chains of the two coordinating histidine residues between the apo and copper-loaded forms (Fig. 3).

Despite the similar tertiary structure (Fig. 4A), the copper binding sites observed in the PmoF1-ΔC and SM-PmoF2-ΔC structures are completely different from those in the other structurally characterized PCu4C domains. In the other PCu4C domains, the copper ion is coordinated by one histidine residue and one methionine residue from strand β5 (e.g. His127 and Met129 in ECuC), a second histidine from the β4 strand antiparallel to it (e.g. His98 in ECuC), and a second methionine in a short extension of β4 termed β4′ (e.g. Met105 in ECuC). Not only do PmoF1 and PmoF2 lack both methionine ligands (Methylocystis sp. Rockwell PmoF1 has an arginine and an alanine while Ms. trichosporium OB3b PmoF2 has a leucine and a glutamate), but in both structures, β4 is significantly shortened and β4′ is absent (Fig. 4B). PmoF1 also has a tyrosine residue in the equivalent position to ECuC His98. Likewise, the N-terminal regions of the proteins diverge significantly. In other previously characterized PCu4C domains, neither of the coordinating histidine residues found in PmoF1 and PmoF2 is conserved, and the β0 strand observed is absent, precluding formation of a histidine brace copper coordination site (Fig. 4).

In the copper-loaded SM-PmoF2-ΔC structure, we also observed strong electron density that could not be modeled as a water molecule in a similar position to the Cu(I) site observed in other PCu4C proteins (7) (Fig. S4A,B). However, anomalous difference Fourier maps calculated using data collected at the copper absorption edge do not exhibit peaks at this position, indicating that it is not a copper ion. Due to the presence of low levels of zinc in this particular sample (0.2 eq per protein molecule as determined by ICP-OES), we have modeled a Zn(II) ion in this position, coordinated by His111, His83, and Glu113 (Fig. S4C). In support of this model, Zn(II) binding experiments indicate that PmoF2-ΔC and PmoF2-FL bind 0.78 ± 0.4 and 1.16 ± 0.1 equivalents, respectively (Fig. S5). In one of the two molecules in the asymmetric unit, His87 provides an additional nitrogen ligand, but is disordered in the second molecule (Fig. S4D). Whereas His83 is completely conserved, His 87 is 33% conserved, and both His111 and Glu113 are only 22% conserved among PmoF2 sequences. Because some samples of PmoF2-FL contained >1 equivalent of Cu(II) (Fig. 2B), we cannot rule out the possibility that there is a second metal binding site in PmoF2-FL (presumably featuring histidine ligation), although the lack of conservation of these additional histidine residues suggests that it is an artifact.

Electron paramagnetic resonance (EPR) characterization of the PmoF1 and PmoF2 Cu(II) sites

We further characterized the Cu(II) sites of PmoF1 and PmoF2 by X-band continuous wave (CW) EPR spectroscopy. PmoF1-ΔC loaded with 1.0 Cu(II) equivalent exhibits a mononuclear Cu(II) EPR spectrum (g = [2.25, 2.07, 2.02], 63Cu

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A_1 = 560 \text{ MHz or } 187 \times 10^4 \text{ cm}^{-1}
\]

(Fig. 5A). This Cu(II) signal is assigned to the only copper site observed in the PmoF1-ΔC crystal structure, the square pyramidal N-terminal histidine brace site (Fig. 3A). Consistent with this assignment, the ratio of \( g_{1}/A_1 \), which corresponds to the tetrahedral distortion of a Cu(II) center (in which a larger number indicates greater distortion), is...
120 cm for the PmoF1-ΔC Cu(II) signal, a value indicative of square planar equatorial ligation (30,31). Indeed, all PmoF1 and PmoF2 Cu(II) species reported herein fall within the range for square planar equatorially-ligated Cu(II) sites (31), \( g_1/A_1 = 105-135 \) cm (except for one Cu(II) species with \( g_1/A_1 = 136 \) cm).

PmoF1-FL loaded with 0.9 equivalents of Cu(II) exhibits an EPR spectrum (\( g = [2.24, 2.07, 2.02] \)), \( ^{63}\text{Cu} A_1 = 535 \text{ MHz} \) or \( 178 \times 10^{-4} \text{ cm}^{-1} \), \( g_1/A_1 = 126 \) cm) resembling that of PmoF1-ΔC (Fig. 5A). One notable difference between the two spectra is the well-resolved \(^{14}\text{N} \) hyperfine splitting along \( g_2 \) in the PmoF1-FL, but not PmoF1-ΔC, spectrum. Unlike PmoF1-ΔC, PmoF1-FL contains the ligands for both the N-terminal histidine brace Cu(II) site as well as a His-rich region (Figs. 1D, S1D) which binds Cu(II) (Fig. 2A). The simplest interpretation of these results is that in PmoF1-FL, the His-rich region binds Cu(II) before the histidine brace site, and binds with a similar ligation and geometry to the histidine brace site, resulting in a similar but not identical EPR spectrum. Consequently, the EPR spectrum of PmoF1-FL loaded with 0.9 equivalents of Cu(II) corresponds to Cu(II) bound in the His-rich region.

Addition of 3 equivalents of Cu(II) to PmoF1-FL results in additional Cu(II) binding (Fig. 2A) but minimal changes to the EPR spectrum (\( g = [2.24, 2.07, 2.02] \)), \( ^{63}\text{Cu} A_1 = 545 \text{ MHz} \) or \( 182 \times 10^{-4} \text{ cm}^{-1} \), \( g_1/A_1 = 123 \) cm) (Fig. 5A), the two most evident being (1) the broadened lowest-field Cu(II) hyperfine line and (2) decreased resolution of the \(^{14}\text{N} \) hyperfine splitting along \( g_2 \). These results are consistent with the superstoichiometrically Cu(II)-loaded PmoF1-FL spectra being the sum of overlapping signals observed for PmoF1-ΔC and the signal observed for PmoF1-FL loaded with 0.9 equivalents of Cu(II). Therefore, in the superstoichiometrically Cu(II)-loaded PmoF1-FL sample, both the N-terminal histidine brace site and the His-rich region have Cu(II) bound. These combined results indicate that the additional Cu(II) sites in PmoF1-FL involve similar, but not identical, ligation and geometry to that of the N-terminal histidine brace Cu(II) site.

PmoF2-ΔC loaded with 1.0 equivalent of Cu(II) exhibits a Cu(II) EPR spectrum (\( g = [2.25, 2.07, 2.03] \)), \( ^{63}\text{Cu} A_1 = 525 \text{ MHz} \) or \( 175 \times 10^{-4} \text{ cm}^{-1} \), \( g_1/A_1 = 129 \) cm) with well-resolved \(^{14}\text{N} \) hyperfine splitting along \( g_2 \) (Fig. 5B). This Cu(II) signal is assigned to the N-terminal histidine brace Cu(II) binding site, which is the only Cu(II) site observed in the crystal structure (Fig. 3B). Addition of 0.9 Cu(II) equivalents to PmoF2-FL results in a similar EPR spectrum to that of PmoF2-ΔC (\( g = [2.25, 2.07, 2.03] \)), \( ^{63}\text{Cu} A_1 = 495 \text{ MHz} \) or \( 165 \times 10^{-4} \text{ cm}^{-1} \), \( g_1/A_1 = 136 \) cm), albeit without well-resolved \(^{14}\text{N} \) hyperfine splitting along \( g_2 \) (Fig. 5B). This suggests that both PmoF2-ΔC and PmoF2-FL bind Cu(II) in the N-terminal histidine brace Cu(II) site, but in PmoF2-FL the site is not as rigidly-structured as in PmoF2-ΔC, and therefore the \(^{14}\text{N} \) hyperfine splitting along \( g_2 \) is not as well resolved in PmoF2-FL as it is in PmoF2-ΔC.

**Bioinformatics analyses of the entire PCu_4C family**

To assess the prevalence of the PmoF type of copper binding site within the larger PCu_4C family, we extended our bioinformatics analysis to all bacterial genomes. We constructed a sequence similarity network of all PCu_4C domain-containing proteins, 22,649 proteins in total (Fig. 6). The vast majority of PCu_4C proteins are encoded by bacterial genomes, particularly those of proteobacteria and actinobacteria. Although the sequence similarity network provides information regarding global sequence similarity, it does not necessarily correspond to conservation of the metal binding motif(s). Therefore, we aligned all of the bacterial sequences to the hidden Markov model (HMM) for the PCu_4C family and analyzed each sequence for the presence of either the canonical \( \text{Hx}_{10}\text{M}_{31/22}\text{HxM} \) motif, the N-terminal \( \text{Hx}_{10}\text{H} \) binding motif identified in this study, or both. While the canonical binding motif is present in 93.0% of the total sequences, it is absent in the remainder, including 0.5% of the total PCu_4C domain-containing proteins such as PmoF1 and PmoF2 that have the N-terminal \( \text{Hx}_{10}\text{H} \) binding motif (122 sequences in total, 22 of which are from methanotroph genomes) (Fig. S6A). Similar to the majority of the methanotroph genomes represented among this group, the majority of the other species represented are also alpha-proteobacteria. More functional data will be
required to ascertain whether these proteins represent a functionally distinct group. Of the total sequences (2836 sequences), 12.5% contain both the canonical Cu(I) binding motif and the histidine brace Cu(II) binding motif, while 6.5% (1468 sequences) lack both Cu-binding motifs. The latter are more frequently found in actinobacterial genomes (Fig. S6B). Interestingly, a protein from *Neisseria meningitidis* that has both sites has been reported to bind two copper ions, although the specific coordinating residues have not been identified experimentally (12).

**Discussion**

Previously characterized PCuAC domains bind Cu(I) with two histidines and two methionines from an HxnMx21/22HxM motif (3,4,6) whereas PmoF1 and PmoF2 bind Cu(II) with a histidine brace provided by an Hx10H motif. Histidine brace coordination of copper is fairly rare, but has been observed in several other proteins. Most relevant to PmoF1 and PmoF2 (Figs. 7A, B) is the nearly identical coordination observed for the Cu(II) site in the periplasmic copper binding protein CopC, which contains an aspartate in or near the fourth equatorial ligation position (9,24,32) (Fig. 7C, Table 2). The majority of CopCs, denoted C_{0-1} CopCs, have only this site, although one subset, the C_{1-1} CopCs, also contains a distinct Cu(I) site coordinated by primarily methionine residues (9). The C_{0-1} CopCs are usually found in genomic proximity or as fusions to CopDs, which are proposed to import copper, and CopC has been proposed to deliver copper to CopD, a function consistent with the solvent-exposed location of its binding site (20,33,34).

Similarly, PmoF1 and PmoF2 may play a role in copper delivery to various target proteins. The gene encoding PmoF1 is part of the *pmo* operon (Fig. 1C), and PmoF1 could interact with pMMO itself, PmoD, CopC, or CopD. Interestingly, the gene encoding PmoF2 is not found in a *pmo* operon, but is paired with a *pmoD* gene (Fig. 1C). PmoD proteins are only found in methane- and ammonia-oxidizing bacteria (21), an observation that combined with the genomic proximity to PmoF-encoding genes and the prevalence of the Hx10H motif-containing PCuAC homologs in alpha-proteobacterial methanotrophs, suggests a function specific to methanotroph copper homeostasis. One possibility is that PmoF supplies PmoD with copper, while the canonical Hx,Mx21/22HxM motif-containing PCuAC proteins function in assembly of the cytochrome *c* oxidase copper centers. Studies to address this hypothesis are underway.

Besides expanding the diversity of the PCuAC family, characterization of PmoF1 and PmoF2 has implications for the role of histidine brace-ligated copper sites in biology. While PmoFs and CopCs likely use the solvent-exposed histidine site for copper transfer, similar sites are found in some copper enzymes. In particular, the defining feature of the copper active site of LPMOs is histidine brace coordination (26,35,36) (Fig. 7D). These enzymes hydroxylate C-H bonds of glycosidic linkages, which then are cleaved by elimination and are also referred to as PMOs as the lytic elimination reaction is usually not enzymatic (37). The histidine brace coordination geometry has been proposed to facilitate the formation of high-valent copper-oxygen intermediates for oxidation of an unactivated C-H bond in the substrate (26). Although the same effect has been proposed for the Cuα site in pMMO (26), which has similar coordination, albeit with a third histidine (18,19,38), recent work suggests that methane oxidation does not occur at this site (38).

Given that the CopC and PmoF proteins are not involved in cleavage of strong C-H bonds, the main advantage of their histidine brace may be to provide a versatile solvent-exposed site for copper transfer or other functions. Importantly, a solvent-exposed site is also absolutely necessary for LPMOs given that their substrate is lignocellulosic biomass (39). This requirement, rather than an ability to confer special oxidative power, may explain the presence of a histidine brace in LPMOs. Additionally, variations in the coordination such as ligation by the ε nitrogen atom of the second histidine in LPMOs, rather than the δ nitrogen atom in PmoFs and CopCs, could affect reactivity. Steric, electronic, and chemical (pKₐ) (40) differences may govern whether a histidine brace copper site is catalytic; ligation by the ε nitrogen provides more flexibility for oxygen and substrate binding (41). These considerations are important to understanding the linkage between histidine
Experimental procedures

Construct design and cloning

The full-length PmoF1 (\textsuperscript{SM}PmoF1-FL), excluding the signal peptide and comprising residues 33-202 from \textit{Methylocystis} species (sp.) Rockwell (locus tag Met49242_1449) was subcloned into the pSGC-His vector with an N-terminal TEV-cleavable His\textsubscript{6} tag, a gift from Anthony Gizzi and Steven Almo (Albert Einstein College of Medicine), using primer set 1 (Table S1). Due to the presence of two vector derived residues (Ser-Met) at the N-terminus in \textsuperscript{SM}PmoF1-FL, we also generated a construct (PmoF1-FL) in which we inserted a SUMO tag via Gibson assembly using primer sets 5 and 6 immediately N-terminal to His\textsubscript{33} in order to generate the authentic N-terminus by cleavage with SUMO protease. To produce constructs containing only the PCu\textsubscript{4}C domain (residues 33-153), residue 154 was mutated to a stop codon using primer set 2 and the QuikChange XL mutagenesis kit (Agilent) to generate His\textsubscript{6}-tagged constructs \textsuperscript{SM}PmoF1-\Delta C and PmoF1-\Delta C.

The DNA for PmoF2 from \textit{Ms. trichosporium} OB3b (locus tag MettrDRAFT_3935), encoding residues 29-193, was synthesized by Genscript with an EcoRI restriction site at the 3' end and a BamH1 restriction site at the 5' end. This sequence was subcloned into the pPR-IBA2 vector (IBA Lifesciences) using EcoRI and BamH1 restriction sites to produce Strep-tagged full-length PmoF2 (\textsuperscript{SM}PmoF2-FL). A SUMO tag was then inserted immediately upstream of His\textsubscript{29} by Gibson assembly using primer sets 5 and 6 (Table S1) to generate the \textsuperscript{SM}PmoF2-FL construct. To produce PmoF2 constructs containing only the PCu\textsubscript{4}C domain (residues 29-159), residue 160 was mutated to a stop codon using primer set 7 (Table S1) with the QuikChange XL mutagenesis kit (Agilent) to generate Strep-tagged constructs \textsuperscript{SM}PmoF2-\Delta C and PmoF2-\Delta C.

Protein expression and purification

All PmoF1 and PmoF2 constructs described above were transformed and expressed following the protocol described below. Each plasmid was inoculated into autoinduction media (42), and grown with shaking at 180 rpm at 37 °C. When cultures reached \textit{A}_{600} = 0.6, the temperature was lowered to 22 °C overnight. Cells were harvested by centrifugation at 6000 x g for 20 min and resuspended in lysis buffer (25 mM PIPES, pH 7.0, 250 mM NaCl) supplemented with 1 mM DTT, 1 mg/ml DNaseI, and 1 mM PMSF. Cells were lysed by sonication for 20 min with 1 s pulses, and the cell debris was removed by centrifugation at 24,000 x g for 1 hr.

For the His-tagged PmoF1 proteins, the clarified lysate was loaded onto NiNTA resin. The column was then washed with 25 mM PIPES, pH 7.0, 250 mM NaCl, 20 mM imidazole, and proteins were eluted with 25 mM PIPES, pH 7.0, 250 mM NaCl, 500 mM imidazole. Proteins were then incubated with His\textsubscript{6}-tagged TEV (in the case of the \textsuperscript{SM}PmoF1-\Delta C construct) or His\textsubscript{6}-tagged SUMO (in the case of the PmoF1-FL and PmoF1-\Delta C constructs) proteases overnight at 4°C with nutation (43,44). Cleavage products of the PmoF1-\Delta C construct (lacking the His-rich C terminal extension) were separated by loading the reaction onto NiNTA resin, washing with 2 column volumes of buffer containing 25mM PIPES, pH 7.0, 250 mM NaCl, 20mM imidazole, and collecting the flow-through. For the full length PmoF1 constructs, cleavage products were dialyzed into salt-free buffer (25 mM PIPES, pH 7.0) using 10 kDa MWCO SnakeSkin dialysis tubing. They were then separated using a HiTrap Q FF anion exchange column (GE Healthcare Life Sciences), from which PmoF1 eluted in the flow-through.

For the Strep-tagged PmoF2 proteins, clarified lysate was applied to a StrepTactin column, and washed with 5-10 CV of 25 mM PIPES, pH 7.0, 250 mM NaCl. The Strep-tagged protein was then eluted in 25 mM PIPES, pH 7.0, 250 mM NaCl, 2.5 mM \textit{d}-desthiobiotin. Proteins were then incubated with His-tagged TEV (in the case of \textsuperscript{SM}PmoF1-\Delta C) or SUMO (in the case of PmoF2-FL and PmoF2-\Delta C) proteases overnight at 4°C nutation (43,44). The dialyzed proteins were then loaded onto NiNTA resin to remove the His-tagged protease, and the flow-through containing the cleavage products was reapplied to the StrepTactin column to remove the Strep-tagged SUMO. The flow-through from this
column, containing the untagged PmoF2 protein, was concentrated by centrifugation to ~1-2 mL in 10 kDa MWCO concentrators (Millipore).

The untagged PmoF1 and PmoF2 proteins were each subjected to a final purification step on a HiLoad 16/600 Superdex 75 column (GE). Peak fractions were pooled and concentrated by centrifugation. The protein concentration was measured by absorbance at \( A_{280} \) using a Nanodrop. The following extinction coefficients were used as determined using the ExPASy ProtParam tool: PmoF1-ΔC, 15470 M\(^{-1}\) cm\(^{-1}\); PmoF1-FL, 20970 M\(^{-1}\) cm\(^{-1}\); PmoF2-ΔC, 15470 M\(^{-1}\) cm\(^{-1}\); PmoF2-FL, 20970 M\(^{-1}\) cm\(^{-1}\). For long-term storage, protein samples were flash-frozen in liquid nitrogen and stored at -80 °C.

The homogeneity of all protein samples was assessed by SDS-PAGE (Fig. S7).

Copper- and zinc- loading experiments

To test for Cu(I) binding, a 10 mM tetrakis(acetonitrile)Cu(I) hexafluorophosphate solution was prepared in 100% acetonitrile in a Coy anaerobic chamber. The buffer solution was deoxygenated on a Schlenk line by completion of 3 purge cycles with high purity argon gas. After cycling, the buffer was left under argon for 30-45 min before taking into the anaerobic chamber. To ensure removal of all dissolved oxygen, the buffer was opened and stirred overnight in the chamber. The buffer was supplemented with 5 mM 2-mercaptoethanol (βME) prior to copper-loading experiments. Then 1-5 molar equivalents of the Cu(I) solution were added to 20 µM protein in deoxygenated buffer in the anaerobic chamber. The solution was incubated at room temperature for 1-2 hr and desalted on a PD10 column equilibrated with deoxygenated buffer containing βME. To test for Cu(I) binding, 1-10 molar equivalents CuSO\(_4\) were slowly added to 20 µM protein in 25 mM PIPES, pH 7.0, 250 mM NaCl. This solution was incubated on ice for 1-2 hr prior to removing unbound metal using a PD10 desalting column. Proteins were eluted and concentrated as described above, and concentrated and desalted proteins loaded with 0, 1, and 5 equivalents Zn(II) were used to prepare samples for ICP-OES.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES)

To determine the copper content of all proteins and zinc content of PmoF2-ΔC and PmoF2-FL, the metal-loaded, desalted samples were digested in a 3% nitric acid solution in metal-free tubes (VWR). A standard curve was generated from a serial dilution of a custom multi-element standard (Inorganic Ventures) that was also digested in 3% nitric acid. The metal contents of the standards and experimental samples were determined using a Thermo iCAP 7600 ICP-OES instrument (Quantitative Bio-element Imaging Center core facility at Northwestern University). Metal concentrations and stoichiometry was calculated based on the standard curve.

EPR spectroscopy

Prior to EPR measurements, each sample was Cu(II) loaded and desalted as described above and then concentrated using 10 kDa MWCO concentrators (Millipore) via centrifugation. ~180 µL of protein solution (protein concentrations ranging from 200-450 µM in buffer containing 25 mM PIPES, pH 7.0 and 250 mM NaCl) was aliquoted into a Wilmad quartz X-band EPR tube (Sigma-Aldrich). This tube was then flash-frozen in liquid nitrogen; the sample was stored in liquid nitrogen until EPR measurements were conducted. CW X-band EPR measurements were conducted at 77 K utilizing a modified Varian E-4 spectrometer, equipped with a finger-dewar filled with liquid nitrogen. Simulation of EPR spectra was performed using EasySpin (45).

Crystallization and structure determination
An initial crystal structure was obtained using copper-loaded \textsuperscript{SM}PmoF1-ΔC. The protein crystallized at 3.5 mg/ml in 1 μL drops at a 1:1 ratio with a precipitant composed of 0.2 M potassium thiocyanate and 20% PEG 3350 (PEGs Suite, Qiagen), and was cryo-cooled in liquid nitrogen prior to data collection. A dataset was collected at 1.362 Å on this crystal at beamline 21-ID-D (Life Sciences Collaborative Access Team (LS-CAT), Advanced Photon Source). The data were processed using HKL2000 \cite{46} to 1.6 Å resolution in space group \textit{P}2\textit{1}2\textit{1}2\textit{1}. The structure was solved using the program CRANK2 \cite{47} as implemented in CCP4i2 \cite{48}. This program identified two Cu sites and built an initial model comprised of 235 residues with \( \text{R}_{\text{work}}/\text{R}_{\text{free}} = 26.0\%/29.2\% \). The structure was further refined using iterative rounds of refinement in phenix.refine \cite{49} and model-building in Coot \cite{50}. The final model has \( \text{R}_{\text{work}}/\text{R}_{\text{free}} = 17.3\%/19.2\% \). In this structure, two copper ions are coordinated at a crystallographic interface between two protein chains with an additional ligand provided by what we have modeled as a thiocyanate ion from the crystallization condition (Fig. S8, Table S2). This crystal structure, though not biologically relevant due to the thiocyanate-mediated dimer, was used as a search model to solve all subsequent crystal structures by molecular replacement.

Apo \textsuperscript{SM}PmoF1-ΔC initially crystallized at 5 mg/ml in 0.1 M sodium acetate, pH 4.6, 0.2 M ammonium acetate, and 30% PEG 4000 (Classics Suite I, Qiagen) using a 96-well screen and 1 μl drops in a 1:1 ratio of protein: precipitant. Conditions were optimized to 0.1 M sodium acetate, pH 4.6, 0.2 M ammonium acetate, and 34% PEG 4000. A dataset was collected at LS-CAT beamline 21-ID-D and was processed to 1.85 Å resolution using XDS \cite{51} in space group \textit{P}2\textit{1}2\textit{1}2\textit{1}. An initial solution was obtained using molecular replacement in phenix.refine \cite{49} with chain A of the Cu-bound \textsuperscript{SM}PmoF1-ΔC structure as a search model, followed by autobuilding resulting in a model including 102 residues and \( \text{R}_{\text{work}}/\text{R}_{\text{free}} \) of 24.7 \%/27.5 \%. Further rounds of refinement and model building were performed using phenix.refine \cite{49} and Coot \cite{50}, respectively.

For the copper-bound structure of \textsuperscript{SM}PmoF2-ΔC, the protein crystallized in 0.2 M magnesium acetate, 0.1M sodium cacodylate, pH 6.5, and 20 % PEG 8000, in 1 μL drops with a 1:1 protein: precipitant ratio and protein concentration of 10 mg/mL. A single crystal was cryocooled in liquid nitrogen prior to data collection at LS-CAT beamline 21-ID-D. A dataset was collected and processed to 2.05 Å resolution in space group \textit{P}3\textit{1}2\textit{1} using XDS \cite{51}. A partial model was obtained using phenix.phaser \cite{49} with the same search model as described above, which was then input into autosol to obtain an initial solution by MR-SAD that resulted in building 234 residues with \( \text{R}_{\text{work}}/\text{R}_{\text{free}} \) of 22.0\%/26.4 \%. Further rounds of refinement and model building were performed using phenix.refine \cite{49} and Coot \cite{50}, respectively.

\textit{Size exclusion chromatography with multi-angle light scattering (SEC-MALS)}
PmoF1-ΔC and PmoF2-ΔC were prepared for analysis by incubating each protein with 1 molar equivalent CuSO₄ on ice for 1-2 hr prior to desalting on a PD10 column. The desalted, copper-loaded proteins were then concentrated to 3.5 mg/mL in 10 kDa MWCO concentrators. The protein samples were filtered and 300 µL and applied to Superdex 75 10/300 GL column (GE) column in 250 mM NaCl, 25 mM PIPES, pH 7.0 at room temperature. Analysis was performed using an Agilent 1260 series high-performance liquid chromatography (HPLC) system equipped with diode-array detection absorbance in-line with a DAWN HELEOS II multi-angle static light scattering detector (Wyatt Technology), a QELS dynamic light scattering detector (Wyatt Technology), and a T-rEx differential refractive index detector (Wyatt Technology). Each sample was run at 0.4 mL/min for 60 min and data were processed using Astra software version 5.3.4 (Wyatt Technology).

**Bioinformatics analyses**

Methanotroph genomes were searched for genes encoding PCu₄C domain-containing proteins. These sequences and their associated data were identified in the IMG-JGI database by searching all methanotroph genuses (as identified in (52)) for pfam04314 (Supplemental File 1). The sequences were then used to generate a sequence similarity network by submitting the FASTA file generated above (Supplemental Files 2, 3) to EFI-EST using an alignment score of 40 and an E-value of 5 (53). The resulting networks were visualized using Cytoscape (54). To identify metal binding sites, the sequences were aligned to the PCu₄C HMM using Clustal Omega (55) (Supplemental File 4).

To generate the sequence similarity network for the full complement of PCu₄C domain-containing proteins, sequences were identified in IMG-JGI by searching all bacterial genomes using the HMM for pfam04314, yielding a total of 22,649 sequences in total (Supplemental File 5). A sequence similarity network was generated using the user-supplied FASTA file generated in this manner using EFI-EST as described above for the methanotroph sequences. Additional parameters included using an alignment score cut-off of 30, an E-value of 5, restricting the sequence length to 100-350 amino acids, and representing sequences with >90 % sequence identity by a single node (Supplemental Files 6, 7). These sequences were similarly analyzed for the presence of the metal binding ligands through alignment using Clustal Omega (55) (Supplemental File 8).

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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**FOOTNOTES**

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The abbreviations used are: PCuAC, periplasmic copper A chaperone; pMMO, particulate methane monooxygenase; LPMO, lytic polysaccharide monooxygenase; PMO, polysaccharide monooxygenase; sMMO, soluble methane monooxygenase; SUMO, small ubiquitin-like modifier; SP, signal peptide; ICP-OES, inductively coupled plasma-optical emission spectroscopy, SEC-MALS, size exclusion chromatography with multi-angle light scattering; HMM, hidden Markov model; TEV, tobacco etch virus; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MWCO, molecular weight cutoff, NiNTA, nickel nitrilotriacetic acid; CV, column volume; PEG, polyethylene glycol; LS-CAT, Life Sciences Collaborative Access Team; MES, 2-ethanesulfonic acid; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; QELS, quasi-elastic light scattering.
Table 1. X-ray structure determination and refinement statistics for PmoF1 and PmoF2 PCu₄C domains

| Crystal       | Apo SM PmoF1-ΔC | Apo PmoF2-ΔC | Cu PmoF1-ΔC | Cu SM PmoF2-ΔC |
|---------------|-----------------|--------------|-------------|---------------|
| PDB accession code | 6P17            | 6P1F         | 6P16        | 6P1G          |
| Data collection |                 |              |             |               |
| Wavelength (Å) | 1.37            | 1.37         | 1.37        | 1.37          |
| Space group   | P₂₁,₂,₂₁        | P₄₁,₂        | P₂₁,₂,₂₁    | P₃,2₁         |
| Cell dimensions |               |              |             |               |
| a, b, c (Å)   | 47.7, 60.6, 85.1 | 51.6, 51.6, 105.5 | 54.3, 65.8, 78.0 | 89.3, 89.3, 60.0 |
| α, β, γ (°)   | 90, 90, 90      | 90, 90, 90   | 90, 90, 90  | 90, 90, 120   |
| Resolution (Å) | 40.0-1.85 (1.90-1.85)* | 29.0-1.65 (1.71-1.65)* | 40.0-1.91 (1.98-1.91)* | 40.0-2.05 (2.10-2.05)* |
| R_sym         | 14.4 (62.0)*    | 7.8 (74.4)*  | 11.6 (166.5)* | 9.4 (92.7)*   |
| I / σI             | 8.71 (2.7)*     | 15.0 (3.5)*  | 10.4 (1.0)*  | 10.5 (1.9)*   |
| Completeness (%) | 97.8 (93.4)*    | 99.9 (99.4)* | 98.5 (97.3)* | 99.5 (97.3)*  |
| Redundancy     | 2.0 (2.0)*      | 12.4 (12.6)* | 2.0 (2.0)*  | 2.0 (2.0)*    |

**Refinement**

| Resolution (Å) | 37.45-1.85 (1.90-1.85)* | 29.0-1.65 (1.70-1.65)* | 33.5-1.9 (1.94-1.91)* | 35.8-2.05 (2.08-2.05)* |
| No. reflections | 21135                 | 17744                  | 21854                  | 33482                  |
| R_work / R_free | 22.7/27.7 (35.6/41.2)* | 18.3/20.9 (22.8/27.4)* | 19.7/23.9 (40.1/43.2)* | 19.1/22.9 (29.1/32.9)* |
| Protein residues in ASU | Chain A: 33-153 | Chain A: 29-153 | Chain A: 33-152 | Chain A: 28-154 |
| Ligand/ion        | 0 | 2 Cu | 2 Cu, 2 Zn |
| Water             | 271 | 106 | 133 | 80 |
| B-factors (Å²)    | 30.1 | 23.4 | 47.5 | 46.4 |
| Protein           | 35.9 | 36.5 | 48.8 | 48.0 |
| Ramachandran favored/ outliers (%) | 97.0 / 0 | 98.4 / 0 | 96.6 / 0 | 97.9 / 0 |

*Indicates data for highest resolution shell. †Residue 28 is vector-derived.
Table 2. Copper coordination geometry in proteins with a histidine brace

| Protein and resolution (PDB code) | # of chains in ASU | Average Cu(II)-ligand distance (Å) |  |
|----------------------------------|--------------------|-----------------------------------|---|
| PmoF-1ΔC 1.91 Å (6P16)           | 2                  | 2.1 (NH2-His33) 2.0 (6N-His33) 2.1 (6N-His44) 2.2 ± 0.1 (6O-Glu43') 2.8 ± 0.2 (O-H2O) |  |
| SM-PmoF2-ΔC 2.02 Å (6P1G)        | 2                  | 2.2 (NH-His29) 2.0 (6N-His29) 2.1 (6N-His40) |  |
| Mst-CopC 1.46 Å (5ICU)           | 1                  | 2.0 (NH2-His23) 2.1 (6N-His23) 2.0 (6N-His107) 2.3 (6O-Asp105) 2.4 (O-H2O) |  |
| Pf-CopC 2.00 Å (6NFQ)            | 3                  | 2.2 (NH2-His25) 2.2 ± 0.2 (6N-His25) 2.1 ± 0.2 (6N-His109) 2.4 ± 0.2 (6O-Asp107) |  |
| Tp-CopC 2.60 Å (5N1T)            | 1                  | 2.0 (NH2-His1) 2.0 (6N-His1) 2.0 (6N-His112) 1.9 (6O-Asp110) 3.2 (O-H2O) |  |
| Ba-LPMO 1.70 Å (5IJU)            | 2                  | 2.2 (NH2-His28) 2.0 (6N-His28) 2.0 (6N-His125) 2.1 ± 0.1 (6O-Asp125) 2.3 ± 0.1 (O-H2O) |  |
| Cv-LPMO 1.90 Å (5NLT)            | 6                  | 2.0 (NH2-His1) 2.0 (6N-His1) 2.0 (6N-His79) 2.7 ± 0.1 (6O-Tyr169) |  |
| Np-LPMO 1.20 Å (5TKG)            | 2                  | 2.1 (NH2-His1) 2.0 (6N-His1) 2.0 (6N-His84) 2.0 (O-H2O) 2.4 (O-H2O) |  |

The abbreviations above correspond to the following organisms: Mst, Methylosinus trichosporium OB3b; Pf, Pseudomonas fluorescens SBW25; Tp, Thioalkalivibrio paradoxxus ARh 1; Ba, Bacillus amyloliquefaciens; Cv, Collariella virescens; Np, Neurospora crassa
Figure 1. PCu₄C domain-containing proteins in methanotrophs. (A) Sequence similarity network of all PCu₄C proteins in methanotrophs colored by taxonomy. Each colored node represents a single PCu₄C domain sequence, and the gray edges connecting the nodes represent the degree of sequence identity between nodes, with shorter edges denoting more closely related proteins. (B) Same sequence similarity network as in (A) colored by PmoF type as defined by genome neighborhood. (C) Typical gene neighborhoods in which pmoF₁, pmoF₂, and pmoF₃ are found. (D) Domain organization of PmoF₁, PmoF₂ and PmoF₃. The locations of signal peptides (SP), PCu₄C domains, and the His-rich region are indicated. (E) Sequence logos (generated by Skylign (56)) for PmoF₁, PmoF₂, and PmoF₃ homologs in the region of the canonical PCu₄C Cu(I) binding motif. The locations of the canonical Hₓ₅Mₓ₃₁₋₂₂Hₓ₂M residues are indicated by boxes.
**Figure 2.** Copper binding stoichiometry for (A) PmoF1 (PmoF1-ΔC in light teal; PmoF1-FL in dark teal) and (B) PmoF2 (PmoF2-ΔC in light purple; PmoF2-FL in dark purple). Protein samples were loaded with the number of molar equivalents of copper indicated on the x-axis prior to desalting. All data are representative of n=3-5 independent measurements.
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Figure 3. Crystal structures of PmoF1 and PmoF2. Superpositions of (A) apo SM-PmoF1-ΔC (green) and copper-loaded PmoF1-ΔC (cyan) and (B) apo PmoF2-ΔC (purple) and copper-loaded PmoF2-ΔC (pink) with the copper ions depicted as blue spheres. The top inset in each panel highlights the conformational changes of the histidine ligands observed upon copper binding. The bottom inset in each panel shows a close up view of the electron density at the copper site. The blue mesh represents the 2Fₒ-Fᵡ map contoured to 1.5σ and the anomalous difference map is shown in orange mesh contoured to 5σ.
Figure 4. Comparison of PmoF1 and PmoF2 to a canonical PCuAC domain. (A) Superposition of PmoF1 and PmoF2 with the homolog ECuC from *Streptomyces lividans* (PDB: 3ZJA). (B) Sequence alignment showing secondary structure and metal binding ligands.
Figure 5. X-band CW EPR spectra and simulations of PmoF1 and PmoF2. (A) PmoF1 and (B) PmoF2 EPR spectra, with heights of resonances normalized to unity: amplitudes in the figure are multiplied by the inverse of the ICP-determined Cu-bound equivalents. For all spectra, collection conditions were 9.135 GHz microwave frequency, 300 ms time constant, 6.3 G modulation amplitude, and 2 min scan rate. All spectra were corrected for cavity/finger-dewar impurity resonance by subtraction of a spectrum of 50 mM Tris pH 8.0, 150 mM NaCl measured under the same conditions. Simulation parameters were as follows. PmoF1-FL + 3.0 Cu(II) Eq.: $g = [2.244, 2.072, 2.020]$, $^{63}\text{Cu} A = [545, 29.5, 14.0]$ MHz, anisotropic $g$-strain (EasySpin $g$-strain parameters 0.03, 0.03, 0 for $g_3$, $g_2$, $g_1$, respectively) with additional 55 G Gaussian isotropic linewidth broadening. PmoF1-FL + 0.9 Cu(II) Eq.: $g = [2.243, 2.068, 2.020]$, $^{63}\text{Cu} A = [535, 29.5, 14.0]$ MHz, anisotropic $g$-strain (EasySpin $g$-strain parameters 0.03, 0.03, 0 for $g_3$, $g_2$, $g_1$, respectively) with additional 55 G Gaussian isotropic linewidth broadening. PmoF1-∆C + 1.0 Cu(II) Eq.: $g = [2.246, 2.072, 2.020]$, $^{63}\text{Cu} A = [560, 29.5, 14.0]$ MHz, anisotropic $g$-strain (EasySpin $g$-strain parameters 0.03, 0.03, 0 for $g_3$, $g_2$, $g_1$, respectively) with additional 50 G Gaussian isotropic linewidth broadening. PmoF2-FL + 0.9 Cu(II) Eq.: $g = [2.253, 2.068, 2.032]$, $^{63}\text{Cu} A = [495, 29.5, 14.0]$ MHz, anisotropic $g$-strain (EasySpin $g$-strain parameters 0.03, 0.03, 0 for $g_3$, $g_2$, $g_1$, respectively) with additional 60 G Gaussian isotropic linewidth broadening. PmoF2-∆C + 1.0 Cu(II) Eq.: $g = [2.247, 2.068, 2.030]$, $^{63}\text{Cu} A = [525, 29.5, 14.0]$ MHz, anisotropic $g$-strain (EasySpin $g$-strain parameters 0.03, 0.03, 0 for $g_3$, $g_2$, $g_1$, respectively) with additional 60 G Gaussian isotropic linewidth broadening.
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Figure 6. Sequence similarity network for the PCuAC family. Sequence similarity network for all bacterial PCuAC domain-containing proteins, colored by putative copper binding motif. Each colored node represents a set of sequences that share ≥ 90% sequence identity, with the length of the gray edges connecting the nodes indicative of the relative sequence similarity between them. The clusters that contain a sequence corresponding to proteins that have been structurally characterized are indicated by colored boxes.
Figure 7. Histidine brace coordination sites. The histidine brace copper coordination site in PmoF1 (A) and PmoF2 (B) in comparison to histidine brace sites in other proteins. (C) CopC from *Ms. trichosporium* OB3b (PDB ID: 5ICU), and (D) LPMO from *Bacillus amyloliquefaciens* CBM33 (PDB ID: 2YOX).
PCu\textsubscript{A}C domains from methane-oxidizing bacteria use a histidine brace to bind copper

Oriana S. Fisher, Madison R. Sendzik, Matthew O. Ross, Thomas J. Lawton, Brian M. Hoffman and Amy C. Rosenzweig

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