Structural basis for methylphosphonate biosynthesis

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Methylphosphonate synthase (MPnS) produces methylphosphonate, a metabolic precursor to methane in the upper ocean. Here, we determine a 2.35-angstrom resolution structure of MPnS and discover that it has an unusual 2-histidine-1-glutamate iron-coordinating motif. We further solve the structure of a related enzyme, hydroxyethylphosphonate dioxygenase from *Streptomyces albus* (SaHEPD), and find that it displays the same motif. SaHEPD can be converted into an MPnS by mutation of glutamine-adjacent residues, identifying the molecular requirements for methylphosphonate synthesis. Using these sequence markers, we find numerous putative MPnSs in marine microbiomes and confirm that MPnS is present in the abundant *Pelagibacter ubique*. The ubiquity of MPnS-containing microbes supports the proposal that methylphosphonate is a source of methane in the upper, aerobic ocean, where phosphorus-starved microbes catabolize methylphosphonate for its phosphorus.

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Structural Biology

Methane concentrations in oxygenated surface ocean waters are supersaturated relative to the atmosphere (1). This methane has been shown to derive from a biological source (2); however, canonical methanogenesis only occurs in obligate anaerobic archaea (3). Although anoxic microenvironments could provide habitats for methanogenic archaea (4), in situ methanogenesis has not been directly observed. Thus, the source of biological methane from the aerobic upper ocean is unknown, a co-paradox (5). Indeed, MPnS was recently shown to be present in one marine microbe, the abundant archaeon *Nitrosopumilus maritimus* (10). The key enzyme discovered, MPn synthase (MPnS), uses molecular oxygen and a nonheme mononuclear Fe(II) center to convert 2-hydroxymethylphosphonate (2-HEP) into MPn and CO₂ (10) (Fig. 1). MPnS is similar in sequence to hydroxyethylphosphonate dioxygenase (HEPD) (11) and hydroxypyrophosphonate epoxidase (HppE) (10, 12), which catalyze the formation of hydroxymethylphosphonate (HMP) from 2-HEP (Fig. 1) (11) and the formation of the antibiotic fosfomycin from 2-S-hydroxypropylphosphonate (13), respectively.

To establish the molecular determinants of MPnS activity and thereby establish sequence markers to use in the search for additional MPnSs, we determined x-ray structures of MPnSs from *N. maritimus* in both the substrate-free and the Fe(II)- and substrate-bound states to 2.37- and 2.35-Å resolution, respectively (table S1). As expected based on sequence (fig. S1), the overall structure of MPnS is highly similar to the HEPD from *Streptomyces viridochromogenes* (St-HEPD) (12). Both enzymes are dimeric and contain the same domain structure: two β-sheet domains (β1 and β2) which together comprise a bimicron fold and two entirely α-helical domains (α1 and α2) (fig. S2A). The active site is situated in the first of the two cupin folds and completed by contributions from the N-terminal α-helical domain and the C-terminal tail of the second protomer. As noted previously (12), the dimeric architectures of St-HEPD, and now MPnS, are analogous to the tetramer structure of HppE from *Streptomyces wadsworthensis* (12), with each protomer of HppE corresponding to a single α-helical domain and a single β-strand fold.

Mononuclear nonheme iron enzymes are traditionally characterized by a three-residue iron-binding motif composed of two His residues and one carboxylate-bearing residue (Asp or Glu), termed the facial triad (14). In the active site of MPnS, a Gln residue (Q152) coordinates the Fe(II) along with two His residues (H148 and H190), forming an unusual 2-His-1-Gln facial triad (Fig. 2A and fig. S3A). The position of Gln152 in MPnS is distinct from that of the iron coordinating Glu (E176) in St-HEPD (12), originating from an adjacent β-strand at the analogous position of the iron-coordinating Glu in HppE (12) (fig. S2B). The substrate 2-HEP coordinates Fe(II) in the active site of MPnS through its hydroxyl oxygen and one phosphoryl oxygen (Fig. 2A and figs. S3A and S4A). The phosphonate moiety is further stabilized by interaction with Arg192, Tyr180, and Asn147 from the cupin barrel; Lys267 from the α-helical domain of the neighboring protomer; and Trp146 from the C-terminal tail of the neighboring protomer. Interestingly, the Trp146-containing C-terminal tail in MPnS is mobile; in the crystal structure of substrate-free MPnS, residues Ala442 to Ser457 have an alternative position where Trp146 is no longer in the active site and a channel is open from the active site to bulk solvent (fig. S5, A and B). Thus, movement of the C-terminal tail and Trp146 appears to open and close the active site in MPnS. Although Trp146 is conserved between MPnSs and St-HEPD, the residue has not been visualized in the St-HEPD structures (12). Three other residues of the cupin fold in MPnS (T126, I126, and F135) further contribute to substrate binding through hydrophobic interactions (fig. 2A and fig. S3A).

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Fig. 1. The reactions of MPnS and HEPD have been proposed to proceed through a common radical intermediate. The hydrogen derived from the C2 pro-R position in 2-HEP is highlighted in red throughout. The radical recombination in MPnS or HEPD (17) is colored red or blue, respectively. For an alternative mechanism involving a cationic intermediate, see fig. S9.

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To determine whether the presence of a 2-His-1-Gln motif is predictive of MPnS activity, we selected to clone, express, and characterize a gene product from Streptomyces albus (strain NRRL B-16041) that displays a 2-His-1-Gln motif and 34% sequence identity to MPnS. Using a previously described phosphorus nuclear magnetic resonance (NMR) spectroscopy assay (17), this enzyme was shown to produce only HMP when 2-HEP is provided as substrate (fig. S6), classifying this enzyme as an HEPD. HEPDs containing a 2-His-1-Gln facial triad will hereafter be referred to as class II, whereas the previously characterized 2-His-1-Glu HEPDs will be called class I. Identification of class II HEPDs that share the same iron-ligating ligand set as MPnS demonstrates that the 2-His-1-Gln motif is not diagnostic of enzyme function, raising the question of what sequence motifs distinguish an MPnS from a class II HEPD.

To investigate the structural differences between MPnS and a class II HEPD, we determined a crystal structure of HEPD from S. albus (SaHEPD) to 1.8-Å resolution with 2-HEP and Fe(II) bound (table S1). SaHEPD has the same fold and oligomeric state as MPnS and is highly similar [root mean square deviation of 1.9 Å over 427 out of 450 Cα atoms, as calculated with PyMOL (15)] (fig. S2D). Consistent with the sequence alignment (fig. S1), all three iron-coordinating residues (H149, Q153, and H190) in SaHEPD and all residues forming the substrate-binding pocket (Y109, Y110, Y111, I127, N146, F192, K29, and W449 in SaHEPD) are conserved between SaHEPD and MPnS (figs. 2, A and B; fig. S3, A and B; and fig. S4B). The crystal lattice affords four independent views of this active site, and in two of the four active sites in the asymmetric unit, the electron density for Gln153 is consistent with an iron-coordinating conformation. However, in the other two active sites, the electron density indicates that a water molecule has replaced Gln153 as the ligand to Fe(II) and that Gln153 is now pointing away from the iron (fig. S4, D and E). This Gln is positioned such that it can hydrogen bond to Tyr163 that packs against Fe(II) ligand His190 (fig. S3A). With the side chain of Gln152 (fig. 2A and fig. S4C). Interestingly, Tyr163 is one of two residues (Gly164 is the other) that are close to the ligand triad and not identical between SaHEPD (Y163/G164) and MPnS (F162/H164). In MPnS, Phe162 and Ile164 are in van der Waals contact with each other and fill in the area under Gln153 such that movement of Gln away from Fe(II) does not appear possible (fig. 2A). In contrast, the two residues (Y163 and G164) in SaHEPD form a cavity that can accommodate a water molecule when Gln153 is a ligand to Fe(II) (fig. 2B) and can also accommodate an alternative (noncoordinating) conformation of Gln153 (fig. 2C).

To investigate the importance of the alternative conformation of Gln153 in SaHEPD, we mutated both Tyr163 and Gly164 in SaHEPD to the corresponding residues in MPnS, Phe and Ile, respectively. Our structures suggest that in this double mutant the solvent cavity beneath Gln153 would be filled, restricting Gln153 to an iron-binding configuration as observed in MPnS. Indeed, the purified SaHEPD-Y163F/G164I variant catalyzes the conversion of 2-HEP to MPn with no detectable side products and with kinetic parameters ([turnover number (kcat), 1.2 ± 0.1 s⁻¹), and Michaelis constant for HEP (Kcat/M[HEP], 15 ± 2 μM) very similar to those of purified wild-type SaHEPD (figs. S6 and S7 and table S2). The catalytic efficiency is in fact slightly better than for N. maritimus MPnS (NnMPnS) (kcat/M[HEP], 10.18 ± 0.01 s⁻¹, Kcat/M[HEP], 4.5 ± 1.1 μM) (16). These results demonstrate that switching the identity of only two noncoordinating active-site residues is sufficient to transform an HEPD into an MPnS. We then investigated which of these two residues is most important; SaHEPD-Y163F produced only HMP, whereas SaHEPD-G164I produced only MPn (fig. S8). Thus, disruption of hydrogen bonding between Tyr163 and Gln153 is insufficient to alter the reaction outcome, but preventing Gln153 from occupying the alternative conformation does result in the switch in product formation. We also replaced the unusual Gln iron-coordinating ligand with Gln or Ala in both SaHEPD and NnMPnS. SaHEPD-G164A was inactive, whereas the Q153A mutant still produced HMP, albeit with reduced efficiency (fig. S8). NnMPnS-Q152A produced HMP with a very low level of activity, whereas NnMPnS-Q152E was inactive under the same condition (fig. S8). Thus, removal of the Gln ligand is strongly deleterious for both MPnS and class II HEPD enzymes (for discussion, see fig. S8). Replacing the Gln ligand in class I HEPD with Gln had no effect on activity (17).

We next considered why restricting Gln movement would alter product formation. An iron-bound MPnS-based radical and an iron-bound formate are the proposed reaction intermediates for both enzymes (fig. 1 and fig. S9) (18); in MPnS, the MPn-based radical abstracts the formate hydrogen atom to yield MPn and CO₂, and in HEPD the MPn-based radical reacts with the iron-bound hydroxyl, yielding HMP and CO₂ (16, 17). Therefore, the fate of the intermediate appears to depend directly on the geometric arrangement of formate relative to the MPn-based radical. MPnS requires the formate hydrogen atom to be in close proximity to the radical (fig. 1). Inspection of the substrate-bound MPnS active site reveals that the positioning of an iron-bound formate intermediate would be most directly affected by the side chain of Gln153 (fig. 2A) and fig. S3A). With the side-chain oxygen of the Gln coordinating the iron, the side-chain amide nitrogen would be positioned appropriately to interact with the iron-bound formate, securing its position near the iron-bound MPn-radical (fig. 3A). Thus, residues that fix the position of Gln, like a juxtaposed Ile164, favor MPn production. In contrast, residues that permit Gln to adopt an alternative (non–iron-ligating) position, like a juxtaposed Gly164, should support HMP production; Gln movement allows formate movement, potentially increasing the distance between the formyl hydrogen atom and the iron-bound MPn radical, disfavoring hydrogen-atom abstraction (fig. 3B). The same arguments can be made if the reaction were to proceed via anionic mechanism (fig. S9), except that formate would be appropriately positioned by the Gln for...
MPnS proteins are colored based on the amino acid at position 184, either Ile (blue) or Val (green), from its iron-coordinating position in SaHEPD, preventing H-atom abstraction by the MPn-based radical and promoting reaction with the iron-bound hydroxide.

REFERENCES AND NOTES

Fig. 3. Proposed intermediates for MPnS and SaHEPD. (A) Gln$^{152}$ in MPnS coordinates the formate intermediate, enabling H-atom abstraction by the MPn radical. (B) Formate displaces Gln$^{153}$ from its iron-coordinating position in SaHEPD, preventing H-atom abstraction by the MPn-based radical and promoting reaction with the iron-bound hydroxide.

Fig. 4. Phylogeny of MPnS, HEPD, and HppE-like proteins. HEPD proteins are colored based on the iron-ligating facial triad: class I HEPD, 2-His-1-Glu (green); or class II HEPD, 2-His-1-Gln (brown). MPnS proteins are colored based on the amino acid at position 184, either Ile (blue) or Val (magenta). Complete labeling of sequence names is provided in fig. S10.

Extending the MPnS sequence signature to a phylogenetic analysis of genomes, metagenomes, and transcriptome shotgun assemblies reveals additional candidate MPnS-coding genes in un-cultivated marine microbes and even eukaryotic marine dinoflagellates, including dinoflagellates of the genera *Symbiodinium* (Fig. 4). In contrast to HEPD-coding genes, which are present in microbial strains and metagenomes of both marine and terrestrial origin, all candidate MPnS-coding genes identified to date derive from marine environments, suggesting a relevant role for MPn in marine ecosystems. Importantly, *P. ubique* belongs to the SAR11 clade of o-proteobacteria, and the SAR11 clade, along with Thaumarcheota (e.g., *N. maritimus*), are two of the most abundant marine microorganisms (19, 20). Thus, the identification of MPnS enzymes in *P. ubique* and *N. maritimus* strongly supports a widespread role of MPn in marine carbon and phosphorus cycling, further supporting the MPn hypothesis for oceanic methane production and potentially resolving the oceanic methane paradox.

SUPPLEMENTARY MATERIALS

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A source of methane in the upper ocean
Methane concentrations are high in oxygenated surface waters. Methylphosphonate (MPn) is a suggested source, but an enzyme that synthesizes Mpn (MPnS) has so far only been identified in one ocean microbe, albeit an abundant one: the archaeon *Nitrosopumilus maritimus*. Born *et al.* describe the crystal structure of MPnS and of a related enzyme that acts on the same substrate but makes a different product. By comparing the structures, they determined sequence markers that allowed them to identify MPnS in other ocean microbes, including the abundant microbe *Pelagibacter ubique*. These findings support the proposal that MPn is a source of both methane and phosphorus in the upper aerobic ocean.

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