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In crystallo posttranslational modification within a MauG/pre-methylamine dehydrogenase complex

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Abstract

MauG is a di-heme enzyme responsible for the posttranslational modification of two tryptophan residues to form the tryptophan tryptophylquinone cofactor (TTQ) of methylamine dehydrogenase (MADH). MauG converts preMADH, containing monohydroxylated- β Trp57, to fully functional MADH by catalyzing the insertion of a second oxygen atom into the indole ring and covalently linking β Trp57 to β Trp108. Here we report the 2.1 Å resolution X-ray crystal structure of MauG complexed with preMADH. The *c*-type heme irons and the nascent TTQ site are separated by long distances over which electron transfer must occur to achieve catalysis. In addition one of the hemes has an atypical His-Tyr axial ligation. The crystalline protein complex is catalytically competent, as on addition of hydrogen peroxide MauG-dependent TTQ synthesis occurs.

Diversity of the cellular proteome is created by genetically encoded polypeptides and their subsequent chemical modification. Most posttranslational modifications, such as phosphorylation, glycosylation and ubiquitination, occur at the protein surface enabling direct contact between the processing enzyme and site of modification. In contrast, protein cofactors essential for function often require in situ modification of amino acids buried within a protein. The formation of some of these is auto-catalytic, such as the fluorophore in green fluorescent protein, but others require external enzymes (1). MauG is a di-heme enzyme (2) that completes synthesis of the catalytic cofactor tryptophan tryptophylquinone (TTQ) (3) from two Trp residues in the β -polypeptide chain of methylamine dehydrogenase (MADH), a metabolic enzyme of methylotrophic and autotrophic bacteria (4). In MADH from *Paracoccus denitrificans*, two oxygen atoms are incorporated into the indole ring of β Trp57 and a covalent bond is formed between the indole rings of β Trp57 and β Trp108 (Scheme 1) (5).

The natural substrate for MauG (preMADH) is a 119 kDa protein precursor of MADH with monohydroxylated β Trp57 and no cross-link (6,7). PreMADH can be generated by expression of recombinant MADH in the background of a *mauG* deletion (6). MauG catalyzes a six-electron oxidation to complete TTQ biosynthesis (8). Oxidizing equivalents may be provided by three moles of either O₂ (plus an electron donor) or H₂O₂ (8,9). As such, the overall reaction can be viewed as three 2-electron oxidations (of unknown order) to catalyze (i) insertion of –OH at C6 of β Trp57, (ii) formation of the cross-link between β Trp57 and β Trp108, and (iii) oxidation of the quinol to the quinone. MauG contains two *c*-type hemes, with the diferric form being the resting state. The hemes are covalently bound to the protein and have a His ligand to the iron. *c*-Type hemes typically function as electron

transfer (ET) mediators, or catalytically in certain peroxidases, and indeed there is a sequence relationship between MauG and bacterial di-heme cytochrome *c* peroxidases (di-heme CCPs) (10). However, unlike di-heme CCPs or other *c*-type hemes, MauG can activate molecular oxygen and forms an unprecedented di-heme *bis*-Fe(IV) intermediate that is catalytically competent (11). This intermediate is composed of an Fe(IV)=O (ferryl) heme with the second oxidizing equivalent stored as Fe(IV) at the second heme. It is an intriguing alternative to Compound I, an Fe(IV)=O heme/porphyrin cation-radical, which is the activated oxygen species required for cytochrome P450 and heme-dependent peroxidase-catalyzed chemistry (12,13).

The X-ray crystal structure of the MauG-preMADH complex of *P. denitrificans* has been determined to 2.1 Å resolution (Fig. S1 and Table S1) (14). The 203.6 kDa complex consists of two MauG and one preMADH, and comprises the asymmetric unit (Fig. 1A). MADH is an $\alpha_2\beta_2$ heterotetramer that contains two active sites and consequently two sites of posttranslational modification. MauG is monomeric and binds in an identical orientation to each $\alpha\beta$ half of preMADH, with 2870 Å² surface area buried at each interface. The preMADH structure is essentially identical to mature MADH (5) except at β Trp57 and β Trp108, the residues that are modified by MauG. As predicted by ¹⁸O isotope labeling and mass spectrometry studies, β Trp57 of preMADH is monohydroxylated at the C7 carbon of the indole ring with no crosslink between the Trp residues (Fig. 2A) (7). Significantly the two Trps are in buried positions comparable to their positions in the mature enzyme. They have no direct contact to any part of MauG, with the edge of the β Trp108 indole ring being closest to the interface (Fig. 1B). There is remarkable separation between the MauG hemes and the nascent TTQ site, with a distance of 40.1 Å between the most distant heme iron and β Trp108 (Table S2).

The EPR spectrum of diferric MauG identified two distinct *c*-type hemes: one high-spin and one low-spin (2). Addition of H₂O₂ to diferric MauG resulted in formation of the unusual di-heme Fe(IV)=O/Fe(IV) reactive intermediate, as evidenced by Mössbauer and EPR spectroscopies (11). The Mössbauer spectrum of the intermediate was modeled as an Fe(IV)=O heme and a 6-coordinate Fe(IV) heme. The crystal structure reveals that the MauG heme closest to preMADH is 6-coordinate, and exhibits a rare His-Tyr axial ligation (Fig. 3A). The axial Tyr294 ligand is likely responsible for the ability of this heme to stabilize Fe(IV) without requiring an exogenous ligand. Sequence alignments of known MauGs show that the Tyr is strictly conserved (Fig. S2) (2). In contrast the di-heme CCPs, which do not stabilize the *bis*-Fe(IV) state, have either a Met or His in this position (Fig. S2) (2,10).

Spectroscopic data indicate that O₂ binding and activation, or direct reaction with H₂O₂, occurs at the high-spin 5-coordinate heme observed in the crystal structure (11). This is the heme furthest from preMADH in the complex, and thus demands a catalytic mechanism involving long-range interprotein electron and radical transfer (Fig. 1B). There is no evidence of solvent ligation at the coordination site *trans* to the proximal His35 at this heme (Fig. 3B, C). It was previously demonstrated that the initial 2-electron oxidation of preMADH by MauG exhibits a random-binding kinetic mechanism in which the presence of preMADH neither stimulates nor impedes the reaction of MauG with H₂O₂, O₂ or CO (15). The structure of the MauG-preMADH complex is consistent with that finding because it shows that the sites of H₂O₂/O₂ and preMADH binding are well-separated. This kinetic mechanism is in contrast to that of other enzymes that generate high-valent iron species as intermediates, such as the cytochromes P450, which have ordered mechanisms (16). In the distal pocket of the 5-coordinate high-spin heme, the ring of Pro107 is 4.3 Å from the heme iron (Fig. 3C). This is similar to the distance between the organic substrate and iron in ordered-mechanism oxygenases, such as cytochrome P450_{cam} in complex with camphane

(4.3 Å) (17). The rigidity of Pro107 may afford it the structural role of the substrate in an ordered mechanism by creating a stable O₂ binding site and helping to keep the reactive oxygen species localized at the iron (16). As such, the distal pocket of the 5-coordinate high-spin heme of MauG may be regarded as constitutively “on” with respect to its ability to activate O₂.

The other characterized *bis*-Fe(IV) enzyme intermediate is the non-heme Fe(IV)₂O₂ (Intermediate Q) of methane monooxygenase (18). It has a diamond core structure with an Fe···Fe interatomic distance of 2.46 Å. In contrast, the separation of the two heme irons in MauG is 21.1 Å. Despite this distance, the Fe(IV)=O/Fe(IV) intermediate forms very rapidly (>300 s⁻¹) and has remarkable stability, decaying at a rate of 2×10⁻⁴ s⁻¹ in the absence of preMADH (15). Trp93 of MauG is positioned between the two hemes such that the propionates of the high-spin and low-spin hemes are 3.3 Å and 3.8 Å respectively from the Trp indole ring (Fig. 1B). This Trp is conserved in both MauGs and di-heme CCPs (2). It has been suggested to play a role in ET between the hemes in the latter. Its position in MauG also suggests a role in mediating ET between the hemes following H₂O₂/O₂ binding and then generation of the *bis*-Fe(IV) species. MauG exhibits negative redox cooperativity between the *c*-type hemes in cycling between the diferric and diferrous forms upon successive one-electron redox events. The hemes have similar intrinsic *E_m* values with facile ET between them. Thus, while the determined *E_m* values are distinct (−159mV and −244mV), MauG acts as a single di-heme 2-electron cofactor, going through a valence-delocalized Fe(III)/Fe(II) state (19). In contrast, the *E_m* values of the hemes of di-heme CCPs are separated by more than 600 mV (20), and their reactive state is a mixed-valence Fe(III)/Fe(II) species (10). In comparing the structures of MauG and di-heme CCP, the two hemes and intervening Trp overlay well (Fig. S3). A Ca²⁺ ion is bound in an identical position in both enzymes (Fig. S3B). Therefore, a structural change altering the spatial relationship between the hemes is not responsible for the different redox and catalytic activities of the two enzymes. The key difference in the di-heme unit of MauG is the distal Tyr294 ligand to the low-spin heme, which is a Met or His in di-heme CCPs. Therefore, Tyr294 appears to be the major determinant in the redox properties and ability of MauG to stabilize the *bis*-Fe(IV) state.

The catalytic competence of the MauG-preMADH complex was examined in crystallo by solving the crystal structure following treatment with excess H₂O₂ (Table S1) (14). The initial electron density (to 2.1 Å resolution) clearly showed that the second oxygen atom had been incorporated at C6 of βTrp57, and that a cross-link had been formed between βTrp57 and βTrp108 (Fig. 2B). It has not been possible to crystallize preMADH alone, so the catalytic requirement for MauG in H₂O₂-dependent TTQ synthesis was confirmed in solution under conditions that matched those of crystallization (Fig. S4) (9). The structures before and after H₂O₂ addition are superimposable (rmsd 0.158 Å), except for the posttranslational modifications. The TTQ cofactor of mature MADH purified from source (PDB code, 2BBK) overlays well with that generated in the H₂O₂-treated MauG-preMADH crystals (5). Thus the complex is catalytically competent, and no major conformational rearrangements of the two proteins are required. At the center of the interface half-way between the MauG low-spin heme and preMADH βTrp108 is MauG residue Trp199 (Fig. 1B). This residue may facilitate ET across the interface through stabilization of a transient radical, though it is not conserved in other MauG sequences (Fig. S2). The catalytic competence of the crystals also indicates a processive mechanism in which MauG and preMADH do not dissociate between each of the three 2-electron oxidations. Furthermore, solvent must provide the second oxygen incorporated at βTrp57 C6, as this carbon is 43.7 Å from the Fe(IV)=O. The distal side of the high-spin heme of H₂O₂-treated crystals has residual electron density not present in the MauG-preMADH structure. The low occupancy and complexity of the electron density prevent it from being modeled, but it is consistent

with a mixture of species that could include diatomics, such as hydroperoxo, and is evident in both copies in the asymmetric unit, confirming that reaction with H₂O₂ occurs at this site (Fig. S5).

This structure reveals that the purpose of the high-valent intermediate in MauG is to provide an extremely high potential oxidant to extract electrons from the preMADH substrate, generating reactive radical intermediates that then acquire the oxygen atom from solvent and form the covalent crosslink between the Trp side-chains. As such the preMADH structure contributes significantly to the catalytic reaction through positioning the reacting portions of the Trps and creating the required chemical environment for TTQ synthesis. Thus the posttranslational biosynthetic reaction requires both substrate-assisted and long-range catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Overall ribbon representation of the MauG-preMADH complex. (B) Spatial layout of potential redox groups. Color scheme is MauG (pink); preMADH α (blue) and β (green). The hemes, Trp93, Trp199 of MauG and β Trp108 and mono-hydroxylated β Trp57 of preMADH are drawn explicitly in a stick representation. Figure produced using PyMOL (<http://www.pymol.org/>).

Figure 2.

Site of TTQ formation in MADH. (A) 2Fo-Fc electron density for the MauG-preMADH complex (resolution 2.1 Å). (B) The first 2Fo-Fc electron density calculated with MauG-preMADH + H₂O₂ structure factors (resolution 2.1 Å) and MauG-preMADH model phases with the preMADH βTrp57 and βTrp108 side-chains omitted. Electron densities were contoured at 1 σ. Carbon coloring: preMADH, light green; preMADH + H₂O₂, dark green. Figure produced using PyMOL (<http://www.pymol.org/>).

Figure 3. MauG hemes. (A) 6-coordinate low-spin heme (CHE600), (B) 5-coordinate high-spin heme (CHE500), and (C) residues that line the distal pocket. 2Fo-Fc electron density contoured at 1 σ . Figure produced using PyMOL (<http://www.pymol.org/>).

Scheme 1.
Overall reaction catalyzed by MauG