Structural mechanism for bacterial oxidation of oceanic trimethylamine into trimethylamine N-oxide

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Summary

Trimethylamine (TMA) and trimethylamine N-oxide (TMAO) are widespread in the ocean and are important nitrogen source for bacteria. TMA monooxygenase (Tmm), a bacterial flavin-containing monooxygenase (FMO), is found widespread in marine bacteria and is responsible for converting TMA to TMAO. However, the molecular mechanism of TMA oxygenation by Tmm has not been explained. Here, we determined the crystal structures of two reaction intermediates of a marine bacterial Tmm (RnTmm) and elucidated the catalytic mechanism of TMA oxidation by RnTmm. The catalytic process of Tmm consists of a reductive half-reaction and an oxidative half-reaction. In the reductive half-reaction, FAD is reduced and a C4a-hydroperoxyflavin intermediate forms. In the oxidative half-reaction, this intermediate attracts TMA through electronic interactions. After TMA binding, NADP+ bends and interacts with D317, shutting off the entrance to create a protected micro-environment for catalysis and exposing C4a-hydroperoxyflavin to TMA for oxidation. Sequence analysis suggests that the proposed catalytic mechanism is common for bacterial Tmms. These findings reveal the catalytic process of TMA oxidation by marine bacterial Tmm and first show that NADP+ undergoes a conformational change in the oxidative half-reaction of FMOs.

Introduction

Methylated amines (MAs), such as trimethylamine (TMA) and trimethylamine N-oxide (TMAO), are ubiquitous in marine environments, and represent a significant pool of carbon and nitrogen in the ocean (Gibb and Hatton, 2004; Chen et al., 2011; Carpenter et al., 2012). Volatile MAs are precursors of nitrous oxide (a greenhouse gas) in marine atmospheres and marine aerosols (Quinn et al., 1988; Carpenter et al., 2012). TMAO plays important roles in many physiological processes (Seibel and Walsh, 2002). For example, TMAO acts as a potent protein stabilizer in deep-sea organisms (Ma et al., 2014; Yancey et al., 2014). TMAO can also act as an electron acceptor for anaerobic respiration (Arata et al., 1992; Gon et al., 2001).

Flavin-containing monooxygenases (FMOs) and cytochrome P450 are two effective families involved in the metabolism of xenobiotics in eukaryotes. FMOs belong to the class B of flavoprotein monooxygenases (FMOs), is found widespread in marine bacteria and is responsible for converting TMA to TMAO. However, the molecular mechanism of TMA oxygenation by Tmm has not been explained. Here, we determined the crystal structures of two reaction intermediates of a marine bacterial Tmm (RnTmm) and elucidated the catalytic mechanism of TMA oxidation by RnTmm. The catalytic process of Tmm consists of a reductive half-reaction and an oxidative half-reaction. In the reductive half-reaction, FAD is reduced and a C4a-hydroperoxyflavin intermediate forms. In the oxidative half-reaction, this intermediate attracts TMA through electronic interactions. After TMA binding, NADP+ bends and interacts with D317, shutting off the entrance to create a protected micro-environment for catalysis and exposing C4a-hydroperoxyflavin to TMA for oxidation. Sequence analysis suggests that the proposed catalytic mechanism is common for bacterial Tmms. These findings reveal the catalytic process of TMA oxidation by marine bacterial Tmm and first show that NADP+ undergoes a conformational change in the oxidative half-reaction of FMOs.
Reductive half-reaction:
\[ \text{FAD} + \text{NADPH} + \text{O}_2 \rightarrow \text{C4a-hydroperoxyflavin} + \text{NADP}^+ \]

Oxidative half-reaction:
\[ \text{C4a-hydroperoxyflavin} + \text{TMA} + \text{NADP}^+ \rightarrow \text{FAD} + \text{TMAO} + \text{H}_2\text{O} + \text{NADP}^+ \]

Fig. 1. The reaction scheme for TMA oxidation by Tmm. The catalytic process of Tmm can be divided into a reductive half-reaction and a followed oxidative half-reaction. In the oxidative half-reaction, NADP$^+$ is colored in gray because its function is controversial.

In this study, we cloned a tmm gene from Roseovarius nubinhibens ISM, an MRC strain isolated from surface waters of the Caribbean Sea (Gonzalez et al., 2003). The tmm gene was over-expressed in Escherichia coli, and the recombinant Tmm (RnTmm) was characterized. The crystal structures of RnTmm/FAD/NADPH complex, a mutant Y207S with marginal activity in complex with FAD and NADPH (the Y207S/FAD/NADPH complex), the Y207S/FAD/NADPH complex soaked with TMA and the Y207S/FAD/NADPH/methimazole complex were obtained. Structural and biochemical analyses suggest that NADP$^+$ binds to RnTmm throughout the catalytic process. In addition to reducing FAD and stabilizing the C4a-hydroperoxyflavin intermediate in the reductive half-reaction, NADP$^+$ is also involved in the oxidative half-reaction of RnTmm. Our results reveal the molecular mechanism of TMA oxidation by marine bacterial Tmms and provide novel insight into the catalytic mechanism of FMOs.

Results

Expression and characterization of RnTmm

Full-length tmm was amplified from R. nubinhibens ISM and was expressed in E. coli BL21(DE3) cells. The gene tmm contains 1344 nucleotides and encodes a protein of 447 residues. The optimal temperature for RnTmm enzymatic activity was $\sim 30^\circ$C (Fig. 2A), and the optimal pH was $\sim 8.0$ (Fig. 2B). Since FMOs exhibit striking substrate promiscuity, we analyzed the substrate specificity of RnTmm (Table 1). RnTmm can oxidize TMA, methimazole, indole and dimethylamine (DMA), and TMA is the best substrate.

Overall structure of RnTmm

To study the catalytic mechanism of RnTmm to oxygenate TMA, we tried to obtain the crystal structure of RnTmm. However, X-ray analysis showed that WT RnTmm crystals suffered from severe twinning. We noticed that bacterial FMO from Methylophaga sp. strain SK1 (mFMO) also encountered the twinning problem (Alfieri et al., 2008), and that mutation of two solvent-exposed charged residues (E158A/E159A) gave mFMO
crystals free from twinning (Alfieri et al., 2008). Sequence alignment indicated that RnTmm possesses E153/D154 at the equivalent position to E158/E159 of mFMO. We, therefore, generated an E153A/D154A double mutant of RnTmm. This mutation enabled RnTmm crystals free from twinning, and we obtained the 1.5 Å crystal structure of this mutant in complex with FAD and NADPH (Table 2). The biochemical properties of this mutant exhibited no substantial difference from those of WT RnTmm (Table 1), indicating that the E153A/D154A mutation had little effect on the catalytic properties of RnTmm. Thus, the E153A/D154A mutant was used to create additional mutants. For clarity, the crystal structure of E153A/D154A in complex with FAD and NADPH was simply termed as the crystal structure of WT RnTmm hereafter.

The overall structure of WT RnTmm is similar to those of other reported bacterial FMOs (Eswaramoorthy et al., 2006; Alfieri et al., 2008; Orru et al., 2010; Cho et al., 2011). WT RnTmm contains a smaller NADP⁺ binding domain and a larger FAD binding domain, which are connected by two hinge regions (H164 to N169 and C271 to H276) (Fig. 3A). FAD molecule and NADP⁺ molecule can be clearly observed in the structure (Fig. 3A). To obtain the crystal structure of RnTmm in complex with TMA, we constructed a mutant Y207S, which had significantly lower activity compared with that of WT RnTmm (Fig. 3B). Although our attempt to obtain the Y207S/FAD/NADPH complex with TMA by co-crystallization was not successful, the crystal structures of Y207S/FAD/NADPH complex without ligand and of Y207S/FAD/NADPH complex soaked with TMA were determined to 1.75 Å and 1.5 Å respectively (Table 2). For brevity, the crystal structure of Y207S/FAD/NADPH complex without ligand was termed the crystal structure of Y207S and that of Y207S/FAD/NADPH complex soaked with TMA termed the crystal structure of Y207S soaking hereafter. The overall structures of Y207S and Y207S soaking are quite similar to that of WT RnTmm, with the root mean square deviation (RMSD) between WT RnTmm and Y207S of 0.42 Å, and the RMSD between WT RnTmm and Y207S soaking of 0.44 Å.

**Entrance for NADPH and substrates**

The electron density map of RnTmm shows the location of the FAD molecule and the NADP⁺ molecule. The FAD molecule is bound deeply in the protein (Fig. 4A). Residues N73 and T321 form hydrogen bonds with the isooxazine ring, the backbone carbonyl of V126 forms interaction with the adenine moiety, residues E38 and Q40 form hydrogen bonds with the ribose moiety, and residues S13, L46, W47, H63 and Q318 interact with the other parts of FAD (Fig. 4B). For the NADP⁺ molecule, only the nicotinamide ring is located inside RnTmm, forming pi–pi stacking interaction with the isooxazine ring of FAD, and the other parts nest the surface of RnTmm (Fig. 4A). Residues W71, D211, and R413 form hydrogen bonds with the nicotinamide ring, residues Y173 and R229 form interactions with the adenine moiety, and residues N73, A205 and S208 interact with the other parts of NADP⁺ (Fig. 4C).

Analysis of WT RnTmm structure indicated that the entrance is still partially solvent accessible after the NADP⁺ molecule binds to RnTmm, and that the entrance is obviously divided into two zones, the basic

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**Table 1.** Kinetic parameters for recombinant RnTmm with substrates.

| Substrate   | $K_m$ (µM) | $k_{cat}$ (s⁻¹) |
|-------------|------------|-----------------|
| TMA         | 110.5 ± 14.5 | 0.53 ± 0.04     |
| TMA (E153A/D154A) | 85.1 ± 11.3 | 0.45 ± 0.03     |
| methimazole | 123.3 ± 44.6 | 0.22 ± 0.03     |
| indole      | 244.0 ± 36.0 | 0.15 ± 0.01     |
| DMA         | 164.9 ± 36.5 | 0.17 ± 0.02     |

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zone and the acidic zone (Fig. 4A). The basic zone is formed mainly by the basic side-chain of R229 and the nitrogen atoms from the main-chain of residues G204, A205 and G273; the acidic zone contains several acidic residues, such as D317, E360, E361 and D364. The NADP$^+$ molecule nests in the basic zone of the entrance by forming hydrogen bonds with RnTmm residues. Among Tmm’s substrates, most contain a basic amine group, such as TMA, methimazole, N,N-dimethylaniline and indole. Therefore, the acidic zone can attract these basic substrates, bringing an appropriate substrate into the catalytic site.

![Figure 3](image_url)  
**Fig. 3.** Overall structure of WT RnTmm.  
A. The overall structure of WT RnTmm. The RnTmm molecule contains a smaller NADP$^+$ binding domain (colored in cyan) and a larger FAD binding domain (colored in blue-white). The NADP$^+$ molecule (colored in purple) and the FAD molecule (colored in yellow) can be clearly identified in the structure. Residue Y207 is colored in green.  
B. The effect of mutation Y207S on the enzymatic activity of RnTmm. The activity of WT RnTmm was defined as 100%.

### Table 2. Crystallographic data collection and refinement.

| Parameters                  | WT RnTmm | Y207S | TMA soaking | Y207S-Methimazole |
|-----------------------------|----------|-------|-------------|-------------------|
| **Diffraction data**        |          |       |             |                   |
| Space group                 | P$_2_1$  | P$_2_1$| P$_2_1$     | P$_2_1$           |
| a, b, c (Å)                 | 73.5, 85.4, 79.5 | 72.8, 60.9, 104.7 | 73.0, 61.4, 104.7 | 60.8, 207.6, 72.5 |
| α, β, γ (°)                 | 90.0, 113.0, 90.0 | 90.0, 93.7, 90.0 | 90.0, 94.0, 90.0 | 90.0, 90.3, 90.0 |
| Resolution range (Å)        | 50.0–1.5 | 50.0–1.5 | 50.0–1.5 | 50.0–2.2          |
| (Å)                         | (1.55–1.50)$^a$ | (1.81–1.75) | (1.53–1.50) | (2.28–2.20)       |
| Redundancy                  | 6.2 (5.9) | 6.8 (6.3) | 3.6 (3.7) | 3.1 (2.9)         |
| Completeness (%)            | 98.8 (98.3) | 98.7 (98.2) | 88.2 (98.4) | 93.2 (92.1)       |
| R$_{merge}$$^b$              | 0.1 (0.3) | 0.1 (0.5) | 0.1 (0.4) | 0.2 (0.4)         |
| ||αl                          | 31.1 (7.8) | 14.2 (2.8) | 36.8 (5.3) | 10.5 (2.7)        |
| **Refinement statistics**   |          |       |             |                   |
| R-factor                    | 0.15     | 0.18  | 0.16        | 0.20              |
| Free R-factor               | 0.17     | 0.22  | 0.21        | 0.27              |
| RMSD from ideal geometry    |          |       |             |                   |
| Bond lengths (Å)            | 0.006    | 0.015 | 0.005       | 0.008             |
| Bond angles (°)             | 1.17     | 1.10  | 1.08        | 1.163             |
| Ramachandran plot (%)       | 94.9     | 95.4  | 94.4        | 92.7              |
| Favored                     | 5.1      | 4.6   | 5.3         | 7.1               |
| Allowed                     | 0.3      | 0.3   | 0.2         |                   |
| Outliers                    | 14.1     | 23.6  | 24.3        | 33.07             |
| Overall B-factors (Å$^2$)   |          |       |             |                   |

$^a$ Numbers in parentheses refer to data in the highest resolution shell.  
$^b$ R$_{merge}$ = $\frac{\sum_{hkl} I(hk\ell) - \langle I(hk\ell) \rangle}{\sum_{hkl} I(hk\ell)}$.  

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Conformational change of NADP$^+$ molecule in the oxidative half-reaction

A quaternary complex containing Y207S, FAD, NADP$^+$ and TMA was expected in the crystal structure of Y207S soaking; however, not explicit electron density of TMA was identified during structural refinement. By superimposing the catalytic site structures of WT RnTmm, Y207S and Y207S that was soaked with TMA, we found that the FAD molecules are perfectly superimposed among these three structures (Fig. 5A). Interestingly, the NADP$^+$ molecules are perfectly superimposed in WT RnTmm and Y207S structures, but the Y207S that had been soaked with TMA exhibits a conformational change (Fig. 5B). In addition, the electron density map for the nicotinamide

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**Fig. 4.** The binding of the FAD and NADP$^+$ molecules of RnTmm. The FAD molecule is colored in yellow, the NADP$^+$ molecule is colored in blue, and the RnTmm residues are colored in cyan.

A. Electrostatic surface of the crystal structure of RnTmm. The FAD molecule is buried in RnTmm, while only the nicotinamide ring of NADP$^+$ is inserted into RnTmm. The entrance for substrates can be divided into two zones, the basic zone and the acidic zone.

B. Interactions between RnTmm residues and FAD. The possible hydrogen bonds are represented by dashed lines.

C. Interactions between RnTmm residues and NADP$^+$. The possible hydrogen bonds are represented by dashed lines.

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**Fig. 5.** Analysis of the conformational change of NADP$^+$.

A. The conformation of FAD in the crystal structures of WT RnTmm (colored in green), Y207S (colored in cyan) and Y207S soaking (colored in purple).

B. The conformation of NADP$^+$ in the crystal structures of WT RnTmm, Y207S and Y207S soaking. The colors are same to (A).

C. The 2Fo–Fc densities for NADP$^+$ and D317 from the structure of Y207S soaking are contoured in blue at 1.5σ.

D. The conformation of NADP$^+$ in the crystal structures of Y207S (colored in green), Y207S soaking (colored in purple) and Y207S-methimazole (colored in cyan).
ring of NADP⁺ in the structure of Y207S that had been soaked with TMA is rather poor under the 1.5 Å resolution, whereas the electron density map for the other parts of NADP⁺ is clear (Fig. 5C), indicating that the nicotinamide ring of NADP⁺ becomes mobile during soaking. Notably, the electron density map strongly indicates an interaction between NADP⁺ and D317 in Y207S soaking (Fig. 5C). Compared with those in WT RnTmm and Y207S, the ribose ring connecting to the nicotinamide ring of NADP⁺ in Y207S soaking moves approximate 2.7 Å toward D317, and forms a hydrogen bond with the side-chain of D317 (Fig. 5B). Because D317 is one of the residues constituting the acidic zone of the substrate entrance, the formation of the hydrogen bond between NADP⁺ and D317 can "shut off" the substrate entrance of RnTmm and make the catalytic site solvent inaccessible, thereby forming a relatively enclosed micro-environment for the catalytic reaction. In addition, a mutation of D317 to alanine decreased the activity of RnTmm significantly (Fig. 6), further indicating the importance of the interaction between NADP⁺ and D317 in the catalytic cycle of RnTmm.

Among substrates RnTmm can catalyze, TMA and DMA are relatively small molecules, whereas methimazole and indole both possess a ring structure. To find out whether substrates possessing a ring structure also induce a conformational change of NADP⁺, we solved the crystal structure of Y207S/FAD/NADPH/ methimazole complex (Y207S-methimazole). The overall structure of Y207S-methimazole is similar to that of WT RnTmm, with the RMSD of 0.22 Å between these two structures. In the crystal structure of Y207S-methimazole, electron density map indicates that the NADP⁺ molecule also undergoes a conformational change during methimazole soaking, and that the ribose ring connecting to the nicotinamide ring of NADP⁺ also forms a hydrogen bond with D317 (Fig. 5D). These results indicate that RnTmm adopts similar strategies to catalyze different substrates.
of WT RnTmm and Y207S, the nicotinamide ring of NADP⁺ forms stacking interactions with the isoalloxazine ring of FAD, and the nitrogen atom of the nicotinamide ring of NADP⁺ forms hydrogen bonds with the nitrogen atom or the oxygen atom of the isoalloxazine ring of FAD (Fig. 7A). The interactions between the two rings are essential for stabilization of the C4a-hydroperoxyflavin intermediate by NADP⁺ (Alfieri et al., 2008; Orru et al., 2010). In the structure of Y207S soaking, the electron density of the nicotinamide ring is poor (Fig. 5C), suggesting that there may be no direct interactions between NADP⁺ and FAD (Fig. 7B). However, the NADP⁺ does seem to close off the entrance to protect the C4a-hydroperoxyflavin.

**The catalytic cycle of RnTmm with TMA as a substrate**

Based on previous studies on the reductive half-reaction of Tmm catalysis (Alfieri et al., 2008; Orru et al., 2010) and our results on the oxidative half-reaction, we propose a relatively complete catalytic cycle of RnTmm with TMA as a substrate (Fig. 8). In the reductive half-reaction, NADPH reduces FAD and the C4a-hydroperoxyflavin intermediate forms. It is believed that Tmm spends most of the time in this intermediate state in vivo (Alfieri et al., 2008). In the intermediate state, the NADP⁺ molecule nests the basic zone of the entrance for substrates, and the catalytic pocket of RnTmm is still partially solvent accessible. The nicotinamide ring of NADP⁺ inserted in RnTmm protects the C4a-hydroperoxyflavin intermediate from solvent attack (Beaty and Ballou, 1981b). The acidic zone of RnTmm attracts a TMA molecule and directs it into the catalytic pocket. Once TMA enters the catalytic pocket, it occupies the catalytic site and makes NADP⁺ bend to start the oxidative half-reaction. NADP⁺ bending would result in two consequences: Firstly, the ribose ring connecting to the nicotinamide ring of NADP⁺ forms a hydrogen bond with D317, shutting off the entrance and exposing the C4a-hydroperoxyflavin to TMA. After the reaction, NADP⁺ and the produced TMAO are released, and the FAD is regenerated. FAD intermediate indicates the C4a-hydroperoxyflavin.

**Universality of the catalytic cycle of RnTmm to oxidize TMA into TMAO in bacteria**

The tmm gene is widespread in many marine bacteria, especially in MRC and the SAR11 clade. Most bacterial strains containing tmm homologs can grow on TMA as a sole nitrogen source (Chen et al., 2011), implying the importance of these bacterial strains in metabolizing TMA in the ocean. In addition, many soil bacteria also contain tmm homologs, for example, *Rhizobium* and *Mesorhizobium*. To analyze the universality of the catalytic mechanism of RnTmm to oxidize TMA into TMAO in bacteria, we performed sequence alignment of...
Fig. 9. Sequence alignment of bacterial Tmm proteins. Black dots indicate residues involved in NADPH binding, black triangles indicate residues involved in FAD binding, and residue D317 is marked by a black star. Sequences 1–8 are Tmm’s from marine bacteria, including 1–4 from MRC and 5–6 from the SAR11 clade. Sequences 9–10 are Tmm’s from soil bacteria. 1, *Roseovarius nubinhibens* ISM, EAP78254.1; 2, *Ruegeria pomeroyi* DSS3, WP_011047288.1; 3, *Roseobacter denitrificans*, WP_044032905.1; 4, *Roseovarius* sp. 217, WP_009818593.1; 5, *Pelagibacter ubique* HTCC1002, WP_006997992.1; 6, *Pelagibacter ubiquit* HTCC7211, WP_008544347.1; 7, *Marinobacterium stanieri*, WP_029511274.1; 8, *Marinobacterium litorale*, WP_027855190.1; 9, *Rhizobium leguminosarum*, WP_025397603.1; and 10, *Mesorhizobium* sp. IM-11, WP_054310147.1. The alignment was done with ClustalW (Chenna et al., 2003) and ESPript (Robert and Gouet, 2014).
bacterial Tmms. The result showed that residue D317 and most residues involved in binding FAD and NADP⁺ in RnTmm are highly conserved in Tmms from both marine bacteria and soil bacteria (Fig. 9). This indicates that the proposed catalytic cycle of RnTmm to oxidize TMA into TMAO is likely adopted by most, if not all, bacterial Tmms.

Discussion

Many studies on the catalytic mechanisms of both eukaryotic and bacterial FMOs have been reported and it is regarded as a general characteristic of FMOs that the coenzyme NADP⁺ remains bound to the enzyme throughout catalysis (Van Berkel et al., 2006; Alfieri et al., 2008; Orru et al., 2010; Crozier-Reabe and Moran, 2012). However, a structural study by Cho et al. suggested that for the catalysis of indole by Tmm from Methylphaga aminosulfidovorans MPᵀ, indole competes with NADP⁺ for binding to the catalytic site and NADP⁺ is released before the oxidative half-reaction (Cho et al., 2011). After the departure of NADP⁺, the C4a-hydroperoxyflavin intermediate might be transitorily stabilized by residues of the enzyme (Cho et al., 2011). Here, our data showed that TMA only competes the nicotinamide ring of NADP⁺, and the NADP⁺ molecule remains bound to Tmm until the catalytic reaction finishes, which is in accordance with the general characteristics of FMOs (Van Berkel et al., 2006; Crozier-Reabe and Moran, 2012). This is the first structural evidence that NADP⁺ binds to Tmm throughout the catalysis of TMA.

In addition to reducing FAD, it has been reported that NADP(H) also exhibited a moonlighting activity to protect the C4a-hydroperoxyflavin intermediate during the reductive half-reaction of the catalysis (Alfieri et al., 2008; Orru et al., 2010). Although studies indicated that NADP⁺ binds to FMOs throughout the catalysis (Van Berkel et al., 2006; Alfieri et al., 2008; Orru et al., 2010; Crozier-Reabe and Moran, 2012), the role of NADP⁺ in the oxidative half-reaction is not explicit yet. Our results indicate that the NADP⁺ molecule undergoes a conformational change in the oxidative half-reaction, which exposes the catalytic C4a-hydroperoxyflavin to TMA, and promotes a protected micro-environment for the catalytic reaction of Tmm by forming a hydrogen bond with a conserved aspartic acid residue. Therefore, in addition to functioning in the reductive half-reaction, NADPH/NADP⁺ also plays an important role in the oxidative half-reaction of Tmm for TMA oxidation. NADP⁺ bending was also identified in Baeyer-Villiger monoxygenases (BVMOs) (Yachnin et al., 2012), another subclass of the class B flavoprotein monoxygenases (Riebel et al., 2014). For BVMOs, intramolecular hydrogen bonds are important in stabilizing the rotated conformation of NADP⁺ (Yachnin et al., 2012). Here for FMOs, we highlight the importance of interactions between NADP⁺ and Tmm. In both cases, NADP⁺ bending exposes the catalytic site and promotes the oxidative reaction (Yachnin et al., 2012). Therefore, our study on FMOs should enrich our understanding of the catalytic cycle of flavoprotein monoxygenases.

Structural analysis demonstrated that there is no direct interaction between the substrate and the residues of Tmm (Eswaramoorthy et al., 2006; Cho et al., 2011). Substrates containing a ring structure, such as indole and methimazole, can form stacking interactions with the isoalloxazine ring of FAD (Eswaramoorthy et al., 2006; Cho et al., 2011). Because TMA does not possess a ring structure, there may be no effective interaction to stabilize its conformation when TMA enters the catalytic pocket, and the positive charge on TMA probably repels the positive charge on the pyridinium ring of NADP⁺ to drive the movement of the nicotinamide during the reaction. In the catalytic cycle of Tmm, when TMA enters the catalytic site, it should be directed to the C4a-hydroperoxyflavin, triggering the oxidative half-reaction. Because TMA molecule cannot form stacking interactions with FAD, we suggest that the catalytic reaction of Tmm proceeds quickly, and a prolonged steady-conformation of TMA is not necessary. This may be the reason why we have observed the conformational change of NADP⁺, but cannot identify the location of TMA in the structure of Y207S soaked with TMA. The aromatic ring of Y207 residue forms a pi-pi stacking with the isoalloxazine ring of FAD in the Tmm without NADPH (Orru et al., 2010; Cho et al., 2011). When NADPH binds to the catalytic site, it competes for the isoalloxazine ring and frees Y207 (Orru et al., 2010; Cho et al., 2011). Thus, the isoalloxazine ring moves between the aromatic ring of Y207 and the nicotinamide ring of NADP⁺, and Y207 plays a role in releasing NADP⁺ after each catalytic cycle during TMA oxidation.

In conclusion, this study illustrated the catalytic cycle of TMA oxidation by marine RnTmm and showed the first structural evidence of NADP⁺ binding to a Tmm throughout the catalytic reaction and its involvement in the oxidative half-reaction by a conformational bending. The proposed mechanism of TMA oxidation by RnTmm may have universal significance among bacteria containing Tmm. Our results provide novel insights into the catalytic mechanism of FMOs and promote a better understanding of TMA-involved marine carbon and nitrogen cycling.
Experimental procedures

Bacterial strains and growth conditions

*R. nubinhibens* ISM was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures and was cultured in the 974 medium at 30°C for 2 days according to the provided protocol (http://www.dsmz.de/). *E. coli* strains DH5α and BL21 (DE3) were grown in Lysogeny Broth (LB) medium at 37°C.

Gene cloning, point mutation and protein expression and purification

The *tm* gene was amplified from the genomic DNA of *R. nubinhibens* ISM using PCR and was subcloned into the pET22b (Novagen) vector with a C-terminal His tag. All of the point mutations (E153A/D154A, Y207S and D317A) in *RnTmm* were introduced using PCR-based methods and were verified by DNA sequencing. Y207S and D317A were constructed base on mutant E153A/D154A. The *RnTmm* protein and all of its mutants were expressed in *E. coli* strain BL21 (DE3). The cells were cultured at 37°C in LB medium to an *OD* of 0.8–1.0 and then induced at 20°C for 16 h with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The proteins were purified first with Ni²⁺-NTA resin (Qiagen) and then fractionated by gel filtration on a Superdex-200 column (GE Healthcare).

Enzyme assays

The enzymatic properties of *RnTmm* were measured by following the decrease of absorbance at 340 nm (ε₃₄₀ = 6.22 mM⁻¹ cm⁻¹ for NADPH) (Alfieri et al., 2008). The reaction mixture contains 1 µM *RnTmm*, 0.25 mM NADPH and 1 mM TMA. For the measurement of *Kₘ* of *RnTmm*, substrate (TMA, methimazole, indole or DMA) of different concentrations was added into the reaction system containing 1 µM *RnTmm* and 0.25 mM NADPH. The optimal pH and the optimal temperature of *RnTmm* were determined using TMA as the substrate. For measurement of the optimal temperature of *RnTmm*, a buffer containing 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl was pre-incubated at different temperatures for 30 min, and then 1 µM *RnTmm*, 0.25 mM NADPH and 1 mM TMA were added into the buffer and the mixture was incubated at different temperatures for 3 min before detection by V550 UV/VIS spectrophotometer (Jasco). The optimal pH of *RnTmm* was measured using Britton-Robinson buffer over a pH range from 7.0 to 9.5. Britton-Robinson buffer is a mixture of 0.04 M H₃BO₃, 0.04 M H₃PO₄ and 0.04 M CH₃COOH (Barek et al., 1999).

The enzymatic activities of *RnTmm* mutants were determined by detecting the fluorescence of indoxyl on a FP-6500 spectrofluorometer (Jasco) (Woo et al., 2000). The reaction mixture contained 2 µM *RnTmm* or a mutant, 0.15 mM NADPH and 0.15 mM indole. The mixture was incubated for 10 min before detection. Fluorescence spectra were collected from 450 to 490 nm at a scan speed of 1000 nm min⁻¹ with the excitation wavelength of 365 nm (Woo et al., 2000).

Crystallography and data collection

The purified *RnTmm* protein was concentrated to approximately 10 mg ml⁻¹ in 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl. To obtain crystals of WT *RnTmm*, NADPH was added into the protein solution to a final concentration of 5 mM before crystallization. Initial crystalization trials for WT *RnTmm* were performed at 20°C using the sitting drop vapor diffusion method. Diffraction-quality crystals of WT *RnTmm* were obtained in hanging drops containing 0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate (pH 6.5) and 20% (w/v) polyethylene glycol (PEG) 8000 at 20°C after 2-weeks incubation.

Diffraction-quality crystals of Y207S were obtained in hanging drops containing 0.2 M sodium acetate, 0.1 M Bis-Tris propane (pH 6.5) and 20% (w/v) PEG 3350 at 20°C. The crystals of Y207S soaking were obtained using crystals of Y207S soaked with 15 mM TMA for 15 min. The crystals of Y207S-methimazole were obtained using crystals of Y207S soaked with 15 mM methimazole for 15 min. X-ray diffraction data were collected on the BL19U1 beamline at the Shanghai Synchrotron Radiation Facility. The initial diffraction data sets were processed by the HKL3000 program (Minor et al., 2006).

Structure determination and refinement

The crystals of WT *RnTmm*, Y207S, Y207S soaking and Y207S-methimazole all belong to the P2₁ space group. The crystal structure of WT *RnTmm* was determined by molecular replacement using the CCP4 program Phaser (Potterton et al., 2003) with the crystal structure of bacterial FMO (PDB code: 2XVE) as the search model. The structures of Y207S, Y207S soaking and Y207S-methimazole were determined using WT *RnTmm* as the search model. The refinements of these structures were performed using Coot (Emsley and Cowtan, 2004) and Phenix (Adams et al., 2002). All of the structure figures were made using the program PyMOL (http://www.pymol.org/).

Circular dichroism (CD) spectroscopic assay

WT *RnTmm* and its mutant were subjected to CD spectroscopic assay at 25°C on a J-810 spectropolarimeter (Jasco). CD spectra of the samples at a final concentration of approximately 10 µM were collected from 250 to 200 nm at a scan speed of 500 nm min⁻¹ with a bandwidth of 2 nm. All of the samples were in the buffer containing 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl.

Accession numbers

The structures of WT *RnTmm*, Y207S, Y207S soaking and Y207S-methimazole have been deposited in the Protein Data Bank (PDB) under the accession codes 5IPY, 5IQ1, 5IQ4 and 5GSN respectively.
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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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