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A Mechanism for Substrate-Induced Formation of 6-Hydroxyflavin Mononucleotide Catalyzed by C30A Trimethylamine Dehydrogenase

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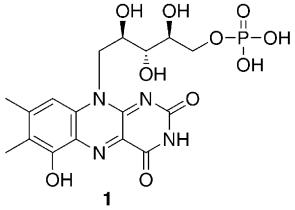
Abstract—Experiments are described to determine the origin of the 6-hydroxyl group of 6-hydroxyFMN produced by the substrate-induced transformation of FMN in the C30A mutant of trimethylamine dehydrogenase. The conversion of FMN to 6-hydroxy-FMN is carried out in the presence of $H_2^{18}O$ and $^{18}O_2$, and the results clearly show that the 6-hydroxyl group is derived from molecular oxygen and not from water.

Trimethylamine dehydrogenase (TMADH; EC 1.5.99.7) from *Methylophilus methylotrophus* (sp. W_3A_1) is a homodimer in which each subunit contains a bacterial ferredoxin-type [4Fe-4S] cluster[1] and FMN covalently bound to Cys-30 at the C-6 position of the flavin.[2] The enzyme catalyzes the oxidative demethylation of trimethylamine to give dimethylamine and formaldehyde.[3] Two electrons are transferred from the substrate to the 6*S*-cysteinyl-FMN, which then transfers them sequentially to the [4Fe-4S] cluster and then to an electron-transferring flavoprotein which associates with TMADH.[4]

The C30A mutant of TMADH has been expressed from a cloned gene (*tmd*) in the heterologous host *Escherichia coli* and is found to bind FMN noncovalently.[5] It contains all of the [4Fe-4S] center but only about 30% of FMN and retains the expected 30% of the wild-type biological activity. Removal of the cysteine-flavin bond by mutation increases the apparent K_m for trimethylamine by 100 and decreases the apparent k_{cat} by 2.[5] On the other hand, in reductive half-reaction studies, the limiting rate of flavin reduction is found to decrease by a factor of approximately 6, while the K_d for trimethylamine is essentially unchanged. Upon reoxidation of C30A TMADH following reduction with excess substrate, but not by reduction with dithionite, the flavin is converted into 6-hydroxyFMN (6-OHFMN; **1**) and is rendered inactive.[6] The 6-OHFMN species, which is naturally occurring in several enzymes,[7] is only observed to form in this case when TMADH C30A is incubated with substrate, although small amounts of 6-OHFMN have been reported to be isolated from both wild-type and W355L TMADH. This suggests that a possible role for the attachment of the FMN to Cys-30 is to prevent conversion of the cofactor to 6-OHFMN during turnover. Because the substrate is required for conversion of the FMN to 6-OHFMN, it was earlier proposed that hydroxide ion attacks a substrate-bound reduced FMN at C-6 as shown in Scheme 1.[6] An alternative mechanism for



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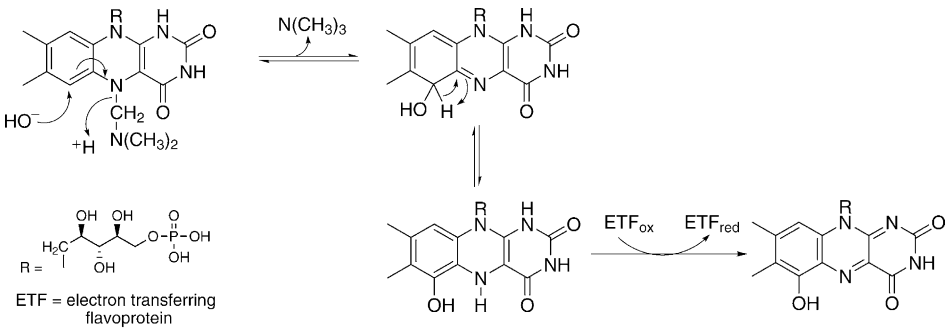
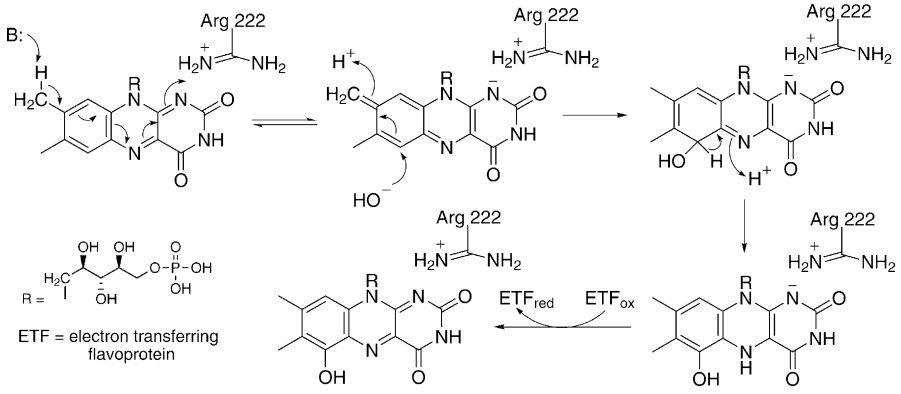


Scheme 1.



Scheme 2.

Table 1. Conversion of C30A TMADH FMN to 6-hydroxyFMN

Experiment[a]	*m*/*z* observed	Conclusion (%)
(1) C30A TMADH[b] + Me_3N in air + H_2O	455	FMN (18)
	471	6-OHFMN (82)
(2) C30A TMADH + Me_3N in air + $H_2^{18}O$[c,d]	455	FMN (9)
	471	6-OHFMN (91)
	473	6-^{18}OHFMN (0)
(3) C30A TMADH + Me_3N $^{18}O_2$[c,e] + H_2O	455	FMN (8)
	471	6-OHFMN (70)
	473	6-^{18}OHFMN (22)
(4) C30A TMADH + Me_3N $^{18}O_2$ + H_2O + catalase (0.2 mg)	455	FMN (20)
	471	6-OHFMN (44)
	473	6-^{18}OHFMN (36)

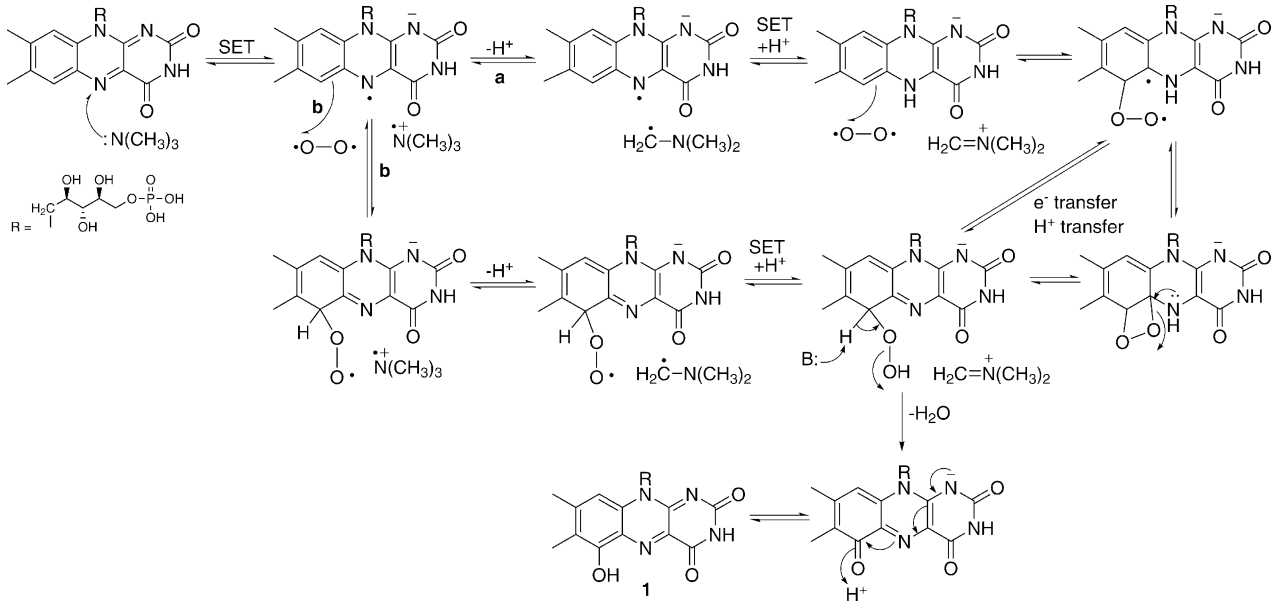
[a]After incubation of the TMADH C30A (100 μL, 66 μM) in sodium pyrophosphate buffer (200 μL, 0.1 M, pH 7.7) with trimethylamine (100 μL, 2 mM) for 5 h, the enzyme was denatured and precipitated by injection of cold acetone/water (1:1) and was incubated on ice for 30 min. The cloudy solution was sonicated for 5 min and microfuged (16,000*g*) for 15 min. The supernatant was collected and concentrated by speed vac. The flavin products were analyzed by LC/electrospray ionization mass spectrometry in the negative ion mode. Instrument tuning parameters (ES^-) were used along with the following operational parameters: capillary 3.04 kV; cone voltage 26 V; source temperature 100 °C. The mass range was set at 350–850 amu, with a scan rate of 2 s/scan.

[b]Recombinant mutant C30A TMADH from *E. coli* strain JM109 was prepared as previously reported.[5] The concentration of C30A TMADH was determined using an extinction coefficient of 197.1 mM^{-1} cm^{-1} at 280 nm. In the expression system presently available, C30A TMADH possesses the full complement of iron-sulfur and ADP, but only approximately half of the flavin sites are occupied.

[c]Purchased from Isotec, Inc.

[d]The enzyme solution was lyophilized, and the residue was taken up in $H_2^{18}O$; lyophilization does not affect the enzyme.

[e]After removing oxygen from the vessel by a series of four freeze–pump–thaw cycles, the vessel was filled with $^{18}O_2$, then the enzyme and substrate were combined, and the incubation was carried out under $^{18}O_2$.



Scheme 3.

conversion of oxidized FMN to 6-OHFMN in the absence of substrate reduction, which appears to occur in the W355L mutant, was proposed earlier that involves hydroxide attack on the quinone methide tautomeric form of the oxidized flavin (Scheme 2).[8]

Given the rich oxidative chemistry of molecular oxygen, it is reasonable that the 6-hydroxyl group incorporated into FMN might be derived from O_2 rather than from H_2O as shown in Schemes 1 and 2. In this letter we describe experiments in which the substrate-induced transformation of FMN of the C30A mutant of TMADH to 6-OHFMN is carried out in the presence of $H_2^{18}O$ and $^{18}O_2$ to determine the origin of the 6-hydroxyl group. The results clearly show that the 6-hydroxyl group is derived from molecular oxygen and not from water.

The results of several experiments are summarized in Table 1. Incubation of C30A TMADH with trimethylamine in air (Experiment 1) gave two flavin products, as determined by LC-electrospray ionization mass spectrometry (LC-ESIMS), one FMN (m/z 455) and the other 6-OHFMN (m/z 471) in the ratio 18:82. The same experiment carried out in $H_2^{18}O$ in air gave the same two peaks in a similar ratio (9:91) as was observed in H_2O; no peak corresponding to [^{18}O]-6-OHFMN (m/z 473) could be detected in the mass spectrum. When the experiment was carried out under $^{18}O_2$ in H_2O, three peaks were observed in the LC-ESIMS at m/z 455 (FMN), 471 (6-OHFMN), and 473 ([^{18}O]-6-OHFMN) in the ratio 8:70:22. These results clearly preclude the mechanisms previously proposed for formation of 6-OHFMN (Schemes 1 and 2).[6,8] The oxygen atom in the 6-hydroxyl group of 6-OHFMN is, apparently, derived from molecular oxygen, not from water. When the experiment with $^{18}O_2$ was repeated in the presence of catalase (0.2 mg), these same three peaks were observed in the ratio 20:44:36. Therefore, catalase does not block the formation of 6-OHFMN, indicating that either hydrogen peroxide is not relevant to the oxygenation mechanism or hydrogen peroxide that forms does not escape the active site prior to oxygenation of FMN. Mechanisms that are consistent with these findings are depicted in Scheme 3. Evidence for a dioxetane intermediate, such as proposed in pathway a, was reported for an oxygenation reaction in the methionine salvage pathway.[9] Both pathways would need a mechanism for spin compatibility because a singlet organic molecule reacts with triplet oxygen; it is possible that the [4Fe-4S] cluster is involved in this electron transfer process because it is located only about 4 Å from the flavin.[10] Excess substrate is known to induce formation of the spin-interacting state[11] with the anionic flavin semiquinone;[12] this also could explain why 6-OHFMN is formed much more readily upon substrate reduction than with the chemical reductant dithionite.[6]

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