Involvement of a Flavin Iminoquinone Methide in the Formation of 6-Hydroxyflavin Mononucleotide in Trimethylamine Dehydrogenase: A Rationale for the Existence of 8α-Methyl and C6-Linked Covalent Flavoproteins†

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ABSTRACT: In trimethylamine dehydrogenase, substrate is bound in the active site via cation−π bonding to three aromatic residues (Tyr-60, Trp-264, and Trp-355). Mutation of one of these residues (Trp-355 → Leu, mutant W355L) influences the chemistry of the flavin mononucleotide in the active site, enabling derivatization to 6-hydroxy-FMN. The W355L mutant is purified as a mixture of deflavo, natural 6-*S*-cysteinyl-FMN, and inactive 6-hydroxy-FMN forms, and the enzyme is severely compromised in its ability to oxidatively demethylate trimethylamine. Analysis of samples of the native and recombinant wild-type trimethylamine dehydrogenases also revealed the presence of 6-hydroxy-FMN, but at much reduced levels compared with that of the W355L enzyme. Unlike that for a C30A mutant of trimethylamine dehydrogenase, addition of substrate to the W355L trimethylamine dehydrogenase is not required for the production of 6-hydroxy-FMN. A mechanism is proposed for the 6-hydroxylation of FMN in trimethylamine dehydrogenase that involves an electrophilic flavin iminoquinone methide. The proposed mechanism involving the flavin iminoquinone methide could apply to the flavinylation of trimethylamine dehydrogenase at the C6 position but also to the flavinylation of enzymes via the 8α position, thus providing a rationale for the evolution of covalent flavoproteins in general. Covalent linkage at C6 or the 8α-methyl prevents 6-hydroxylation by direct modification at the C6 atom or by preventing formation of the flavin iminoquinone methide, respectively.

Trimethylamine dehydrogenase (TMADH)[1] from *Methylophilus methylotrophus* (sp. W_3A_1) is an iron−sulfur flavoprotein that catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde (Steenkamp & Mallinson, 1976)

$$(CH_3)_3N + H_2O \rightarrow (CH_3)_2NH + CH_2O + 2H^+ + 2e^-$$

The enzyme is a homodimer, and each subunit contains 1 equiv of a ferredoxin-type 4Fe-4S center (Hill et al., 1977) and FMN in the form of a 6-*S*-cysteinyl-FMN (Steenkamp et al., 1978; Kenney et al., 1978). During catalysis, the 6-*S*-cysteinyl-FMN accepts two electrons from trimethylamine which are then transferred sequentially to the 4Fe-4S center and then to an electron-transferring flavoprotein (ETF) that associates transiently with TMADH (Duplessis et al., 1994; Steenkamp & Gallup, 1978; Rohlfs & Hille, 1991, 1994; Huang et al., 1995; Rohlfs et al., 1995; Wilson et al., 1997a,b). The structure of TMADH is solved at high resolution (Lim et al., 1986; F. S. Mathews et al., unpublished). The enzyme is comprised of an N-terminal β/α barrel domain containing the active site and 6-*S*-cysteinyl-FMN. A buried loop that binds the 4Fe-4S center is located at the C-terminal end of the barrel. Two C-terminal domains rich in α/β structure are located beyond this loop; these domains bind ADP and resemble in fold the dinucleotide binding domains found for example in the disulfide oxidoreductase family of enzymes (Lim et al., 1988; Scrutton, 1994).

TMADH and the highly related dimethylamine dehydrogenase (DMADH) (Yang et al., 1995) are unique in possessing a 6-*S*-cysteinyl-FMN. In other covalent flavoproteins, the cofactor is usually linked to the 8α-methyl of the isoalloxazine ring as a 8α-histidyl, 8α-cysteinyl, or 8α-tyrosyl flavin [for a review, see Mewies et al. (1997)]. A mutant (C30A) of TMADH in which the cysteine that forms the 6-*S*-cysteinyl link is replaced by alanine has been isolated and shown to retain biological activity (Scrutton et al., 1994). Rapid-mixing stopped-flow studies have revealed that the limiting rate constant for the first of the three observed kinetic phases in the reductive half-reaction of the enzyme using diethylmethylamine as substrate is reduced by a factor of 6 (Huang et al., 1996). The two subsequent kinetic phases [representing electron transfer to the 4Fe-4S center and formation of a spin-interacting state (Rohlfs & Hille, 1991, 1994; Rohlfs et al., 1995)] are not compromised in the C30A mutant compared with those of the wild-type enzyme. The

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[1] Abbreviations: TMADH, trimethylamine dehydrogenase; FMN, flavin mononucleotide; ETF, electron-transferring flavoprotein.

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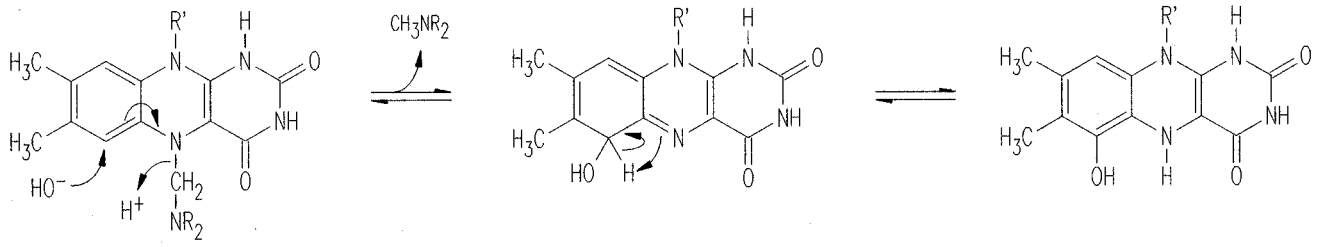
Scheme 1



flavin recovered from the C30A mutant following reaction with excess substrate is found to have been converted to the 6-hydroxy derivative, but the "as-purified" C30A enzyme contains conventional FMN. Therefore, enzyme turnover under the experimental conditions leads to inactivation of the enzyme through the formation of the C6-hydroxy derivative. The results of these studies suggested that a major role of the 6-*S*-cysteinyl-FMN in wild-type enzyme is to prevent inactivation of the enzyme by formation of a 6-hydroxy derivative, and a mechanism was proposed (Scheme 1) in the case of the C30A mutant whereby hydroxide attacks the C6 of a flavin−substrate covalent adduct in the course of steady-state turnover to yield the 6-hydroxy derivative of FMN (Huang et al., 1996).

In this paper, we describe the properties of a mutant of TMADH (W355L) in which one of the three residues in the substrate-binding aromatic bowl (Trp-355; Bellamy et al., 1989; Raine et al., 1995; Basran et al., 1997) is replaced by leucine. This mutant of TMADH is isolated with 6-hydroxy-FMN in the active site, indicating that turnover is not required for the modification of the flavin in the case of this mutant. In light of these data, it seems probable that modification of the flavin occurs through the formation of an electrophilic iminoquinone methide rather than through the previously proposed N5-substrate adduct. The implications of these results for the hydroxylation mechanisms of the W355L, C30A, and wild-type enzymes and also the flavinylation reaction previously proposed for TMADH (Scrutton et al., 1994; Mewies et al., 1996) are discussed. The potential formation of an electrophilic iminoquinone methide in the hydroxylation of flavin provides a rationale for the existence of 8α-methyl and C6-linked covalent flavoproteins in general.

EXPERIMENTAL PROCEDURES

Materials. Complex bacteriological media were prepared as described by Sambrook et al. (1989) using materials purchased from Unipath Ltd. *Escherichia coli* strain JM109 [r_k^-, m_k^+, *rec* A1, *sup* E, *end* A1, *hsd* R17, *gyr* A96, *rel* A1, *thi*, Δ(*lac-pro* AB) F′ *tra* D36, *pro* A^+B^+, *lac* I^q, *lac* Z ΔM15] was from Stratagene. Restriction enzymes *Eco*RI, *Hin*dIII, and *Kpn*I were purchased from Pharmacia Biotech Inc. Calf intestinal alkaline phosphatase was from Boehringer Mannheim. T4 DNA ligase was from Promega; T4 polynucleotide kinase was obtained from Amersham International. Timentin was from Beecham Research Laboratories. Trimethylamine, dichlorophenolindophenol (DCPIP), and phenazine methosulfate (PMS) were from Sigma. All other chemicals were of analytical quality where possible. Water was glass-distilled and further purified by the Elgastat UHP System, except for HPLC work where MilliQ-purified water was used (Millipore, Watford, U.K.).

Recombinant DNA Methods and Enzyme Purification. Bacteria were cultured in 2YT media supplemented where appropriate with timentin (Scrutton et al., 1994). Plasmid DNA was prepared using Maxi-Prep DNA kits supplied by Promega. General cloning methods were adopted from Sambrook et al. (1989). The gene encoding the W355L mutant enzyme was isolated using the Unique Site Elimination (USE) mutagenesis kit supplied by Pharmacia using the wild-type expression plasmid pSV2tmdveg as template DNA (Scrutton et al., 1994; Basran et al., 1997) and the oligonucleotide 5′-CCACCGATTTCCAAGCGGGAAATACACACG-3′ (W355L). The mutant gene was completely resequenced by the dideoxy-chain-termination method (Sanger et al., 1977) to ensure no spurious changes arose during the mutagenesis procedure.

Recombinant wild-type TMADH and the W355L mutant were prepared from *E. coli* strain JM109 transformed with the appropriate plasmid expression vector as described previously (Scrutton et al., 1994). Native TMADH was prepared from *M. methylotrophus* (sp. W_3A_1) using the method of Steenkamp and Mallinson (1976) incorporating the modifications of Wilson et al. (1995). The ADP and 4Fe-4S cluster stoichiometries of the mutant enzyme were determined spectrophotometrically as described previously (Scrutton et al., 1994; Mewies et al., 1996).

Matrix-Assisted Laser-Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. The mass of the flavin bound by the W355L mutant enzyme was determined by MALDI-TOF mass spectrometry. Approximately 50 nmol of protein was exchanged into 0.1 M ammonium bicarbonate buffer by gel filtration (Sephadex G25). The sample was then boiled for 3 min and the precipitated protein removed by centrifugation. The supernatant was freeze-dried, the resulting pellet redissolved in 1 mL of water, and the solution freeze-dried again. MALDI-MS analysis of this material, redissolved in 50 μL of water, was hampered by residual contaminating salts. The sample was therefore purified further using reverse phase HPLC on a Brownlee Aquapore C18 column (2.1 × 30 mm) equilibrated in 0.1% TFA and developed with a linear gradient (30 min) of 0 to 10% acetonitrile in the same solvent. The flow rate was 0.2 mL/min, and detection was by diode array using a Hewlett-Packard 1090M liquid chromatograph. The main fraction containing a spectral signature corresponding to flavin was analyzed by MALDI-MS. As a control, FMN (Sigma) was prepared by the same procedure. Samples from the HPLC run were mixed with an equal volume (1 μL) of matrix (10 mg/mL gentisic acid in 50% aqueous acetonitrile and 0.1% TFA) containing 0.1 pmol of substance P (mass of 1347.7 Da) as calibrant and allowed to dry on the sample slide at room temperature. Spectra were collected in negative ion reflectron mode on a Kratos Kompact MALDI III

instrument. Using this procedure, FMN had the correct mass (456.3 Da), thus demonstrating that the workup procedure did not affect the integrity of the flavin.

Steady-State and Stopped-Flow Kinetic Analyses. Steady-state kinetic measurements were performed with a 1 cm light path in a final volume of 1 mL. The desired concentrations of trimethylamine, phenazine methosulfate (PMS), and 2,6-dichlorophenolindophenol (DCPIP) were obtained by making microliter additions from stock solutions to the assay mix. Assays for the determination of kinetic parameters were performed in 100 mM sodium pyrophosphate buffer at pH 8.5. Reactions were started by the addition of substrate, and the decrease in absorption at 600 nm due to reduction of DCPIP (ϵ = 21 900 M^{-1} cm^{-1}) was measured using a Hewlett-Packard 8452A single-beam diode array spectrophotometer. All data were collected at 30 °C. Data were fitted to the appropriate rate equation (see Results and Discussion) using the fitting program Kaleidograph (Abelbeck Software). Rapid reaction kinetic experiments were performed using an Applied Photophysics SF.17MV stopped-flow spectrophotometer. Time-dependent aerobic reductions of TMADH with trimethylamine at pH 7.5 were performed by rapid-scanning stopped-flow spectroscopy using a photodiode array detector and X-SCAN software (Applied Photophysics) as described previously (Basran et al., 1997). Previously, we established that the reoxidation of substrate-reduced TMADH by molecular oxygen occurs slowly (half-life of about 50 min; Wilson et al., 1995), obviating the need for anaerobic conditions in the stopped-flow experiments. Time-dependent reduction of the W355L TMADH with 2 M TMA was performed under anaerobic conditions over a period of 30 min. The W355L TMADH was placed in a tonometer equipped with a side arm cuvette and a male Luer connector. TMA was placed into a small round-bottom flask attached to the side arm, and the enzyme solution was made anaerobic by alternately evacuating and flushing with oxygen-free argon. The enzyme was then mixed with the TMA in the side arm and then returned to the cuvette. Time-dependent absorbance changes were measured by repeated scanning using a Hewlett-Packard 8452A single-beam diode array spectrophotometer.

RESULTS AND DISCUSSION

Trp-355 and General Properties of the W355L TMADH. Trp-355 is one of three residues (Tyr-60, Trp-264, and Trp-355) that comprise the substrate-binding aromatic bowl of TMADH (Bellamy et al., 1989; Raine et al., 1995; Basran et al., 1997). The residue was initially targetted for site-directed mutagenesis as part of a wider study (to be reported elsewhere) of cation$-\pi$ bonding in TMADH. As part of this wider study, each of the aromatic residues involved in substrate binding has been individually exchanged for leucine by site-directed mutagenesis. Both tryptophan residues of the aromatic bowl (Trp-264 and Trp-355) are conserved in the homologous DMADH, but Tyr-60 of TMADH is replaced by glutamine in DMADH (Yang et al., 1995; Raine et al., 1995; Basran et al., 1997). This single residue change in the substrate-binding aromatic bowl is thought to be responsible for the exclusivity in specificity for tertiary and secondary substrates in TMADH and DMADH, respectively (Raine et al., 1995; Basran et al., 1997). In the crystal structure of TMADH, Trp-355 is in close proximity to the 6-*S*-cysteinyl-FMN bond (Figure 1), but modeling suggests

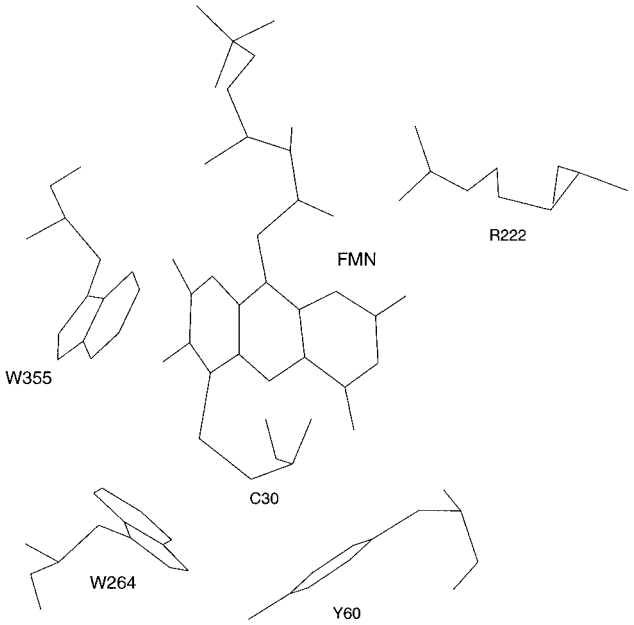


FIGURE 1: Active site of trimethylamine dehydrogenase showing the residues that comprise the substrate-binding aromatic bowl, residue Arg-222, and 6-*S*-cysteinyl-FMN. The coordinates are of the refined crystallographic structure of TMADH (F. S. Mathews et al., unpublished; PDB code 1TMD).

that an engineered leucine at this position should not result in there being bad contacts either with protein or with the active site flavin.

The W355L mutant enzyme was constructed and purified to homogeneity as described above. During purification, no TMADH activity was detected for the W355L enzyme, and only a very low level of activity was found for pure preparations of the enzyme in concentrated form under standard assay conditions (100 μM TMA, pH 8.5, and 30 °C). As seen for the native wild-type and recombinant wild-type enzymes (Scrutton et al., 1994), chemical analysis for iron released after precipitating the W355L mutant with trichloroacetic acid indicated that the mutant was assembled stoichiometrically with the 4Fe-4S center. Likewise, analysis for ADP (Scrutton et al., 1994) also revealed stoichiometric association with the W355L mutant.

Identification of the Flavin in the W355L TMADH. The spectrum of the as-isolated W355L mutant TMADH was found to be different from that of the native and recombinant wild-type enzymes (Figure 2). However, the spectrum is similar to that of a C30A mutant TMADH following treatment of the latter enzyme with substrate (Huang et al., 1996). Following about nine turnovers with TMA, the flavin in the C30A mutant is converted from FMN to 6-hydroxy-FMN. It is important to emphasize that the flavin derivative in the C30A enzyme is only formed during turnover; the as-isolated form of the enzyme possesses conventional FMN in the active site. The similarity in the absorption spectrum of the as-purified W355L TMADH and the C30A TMADH (after treatment with substrate) suggests that the flavin in the former mutant is in the 6-hydroxy form.

The chemical identity of the flavin in the W355L enzyme was confirmed by analysis of the cofactor released from the enzyme by treatment with perchloric acid. The spectrum of the released flavin (Figure 3) is as expected for a 6-hydroxyflavin, and titration of the absorption at 600 nm revealed a single ionization with a pK_a of ~7.0, which is

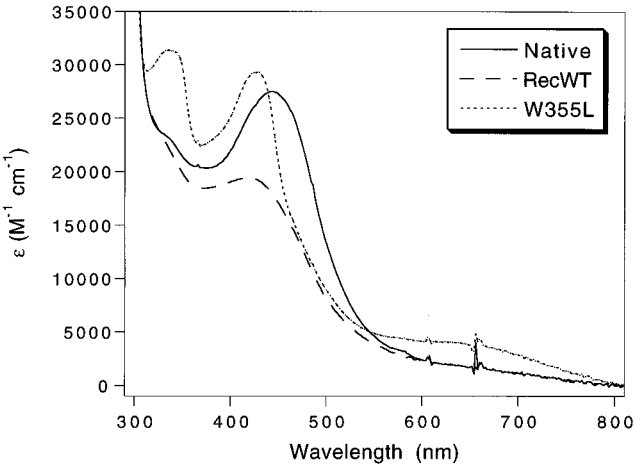


FIGURE 2: Absorption spectra of native, recombinant wild-type, and the W355L trimethylamine dehydrogenase. Molar absorption coefficients refer to a single active site. Spectra were recorded for enzyme samples dissolved in 0.1 M potassium phosphate buffer at pH 7.5. RecWT is recombinant wild type. Absorbance is due to the presence of both flavin and the 4Fe-4S center of TMADH.

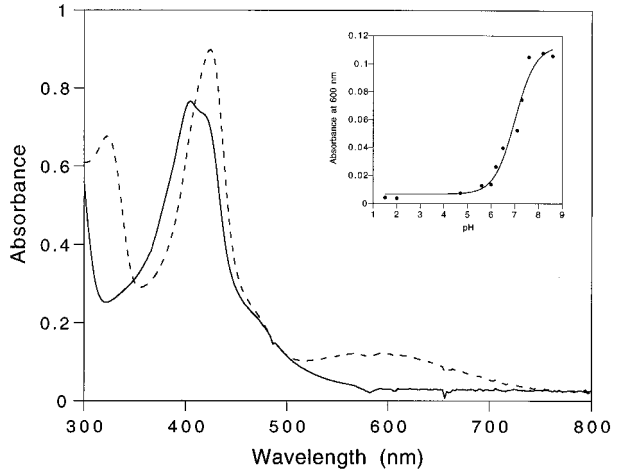


FIGURE 3: Spectra of the neutral and anionic forms of 6-hydroxyflavin released from W355L TMADH. The flavin was released from the enzyme by precipitation with perchloric acid. The spectrum at pH 2 (solid line) is that for neutral 6-hydroxy-FMN and at pH 9.1 (hatched line) that of the anionic 6-hydroxy-FMN. (Inset) Plot of absorbance at 600 nm as a function of pH due to changes in the distribution of the different tautomeric/mesomeric forms of 6-hydroxyflavin (Massey & Hemmerich, 1980). A fit of the data to the equation describing a single ionization yields a pK_a of 7.0 ± 0.14, which compares favorably with that reported previously (pK_a = 7.1) for 6-hydroxy-FMN (Mayhew et al., 1974).

again consistent with the flavin being in the 6-hydroxy form. Further evidence for a 6-hydroxyflavin was obtained by MALDI-MS. A mass of 472.2 ± 0.1 Da (standard deviation from nine determinations) was found for the released and purified flavin, which is within experimental error of the 472.3 Da mass expected for 6-hydroxy-FMN.

In previous work (Scrutton et al., 1994; Mewies et al., 1996; Packman et al., 1995), we demonstrated that the presence of 6-*S*-cysteinyl-FMN in native and recombinant wild-type TMADH can be detected readily by precipitating the protein with perchloric acid. Following microcentrifugation and resolubilization of the precipitated protein using phosphate-buffered guanidine, an absorbance peak at about 440 nm is indicative of the presence of the 6-*S*-cysteinyl-FMN. Treatment of the W355L mutant in this way revealed the presence of 6-*S*-cysteinyl-FMN in addition to the liberated

Table 1: Flavin Content of the Wild-Type and Mutant Forms of TMADH[a]

enzyme	6-*S*-cysteinyl-FMN	6-hydroxy-FMN	FMN	deflavo
native	96	1.5	0	2.5
rWT	25−30	3	0	72−67
C30A	0	0	100	0
C30A turnover	0	100	0	0
W355L	8−21	38−65	0	54−14

[a] Figures are expressed as a percentage of the total enzyme sample.

6-hydroxy-FMN discussed above. The relative proportions of 6-hydroxy-FMN and 6-*S*-cysteinyl-FMN varied slightly in two different preparations of the enzyme, the lower level of 6-*S*-cysteinyl-FMN being 8% (preparation 1) and the higher 21% (preparation 2). On the basis of the extinction coefficient for 6-hydroxy-FMN (21.3 mM^{-1} cm^{-1} at 400 nm and pH 2[2]), the 6-hydroxy content of the enzyme samples was found to be 38% (preparation 1) and 65% (preparation 2). The remaining portions of the W355L mutant enzyme samples were the deflavo forms of the enzyme (Table 1).

The presence of noncovalently bound 6-hydroxy-FMN in the W355L enzyme prompted us to take a closer look at the flavin content of the native and recombinant wild-type enzymes. Precipitation of concentrated samples of native and recombinant wild-type enzyme (1 mL of a 10 μM solution with respect to the TMADH subunit) indicated that small amounts of 6-hydroxy-FMN were also present in both enzyme samples. On a mole-to-mole basis, about 1.5% of the native and 3% of the recombinant wild-type protein contain 6-hydroxy-FMN (Table 1). In previous work, we demonstrated by electrospray ionization mass spectrometry (ESMS) that about 4% of a native enzyme sample had a mass expected for the deflavo enzyme (Packman et al., 1995). The C30A mutant, in which the flavin is necessarily noncovalently attached to the protein as a result of the loss of the cysteine, has a mass expected for the deflavo enzyme, thereby indicating that noncovalently bound flavin is lost during ESMS analysis. By analogy, therefore, that portion of the native enzyme sample with the deflavo mass most likely represents enzyme containing 6-hydroxy-FMN or a mixture of deflavo- and 6-hydroxy-FMN-containing enzyme. In contrast, recombinant wild-type enzyme contains a mixture of three distinct enzyme species comprising 25−30% of the sample in the 6-*S*-cysteinyl-FMN form (Scrutton et al., 1994), about 3% in the 6-hydroxy-FMN form (this work), and the remainder in the deflavo form (Scrutton et al., 1994; Packman et al., 1995).

Kinetic Characterization of the W355L TMADH. Activity was detected for the W355L mutant TMADH at elevated concentrations of TMA (>500 μM). A steady-state kinetic analysis of the W355L mutant revealed a simple hyperbolic dependence on substrate concentration; this contrasts with the wild-type enzyme which shows complex behavior with respect to substrate concentration (Falzon & Davidson, 1996). The Michaelis constant for the W355L TMADH is elevated (2.5 ± 0.3 mM) compared with the same parameter determined for the native wild-type enzyme (10 ± 2 μM), and this elevation in K_m is expected given that Trp-355 is

[2] The extinction at pH 2 was determined empirically from the known extinction of 6-hydroxy-FMN at pH 9.1 (Mayhew et al., 1974) and the measured differences in absorption at the two pH values.

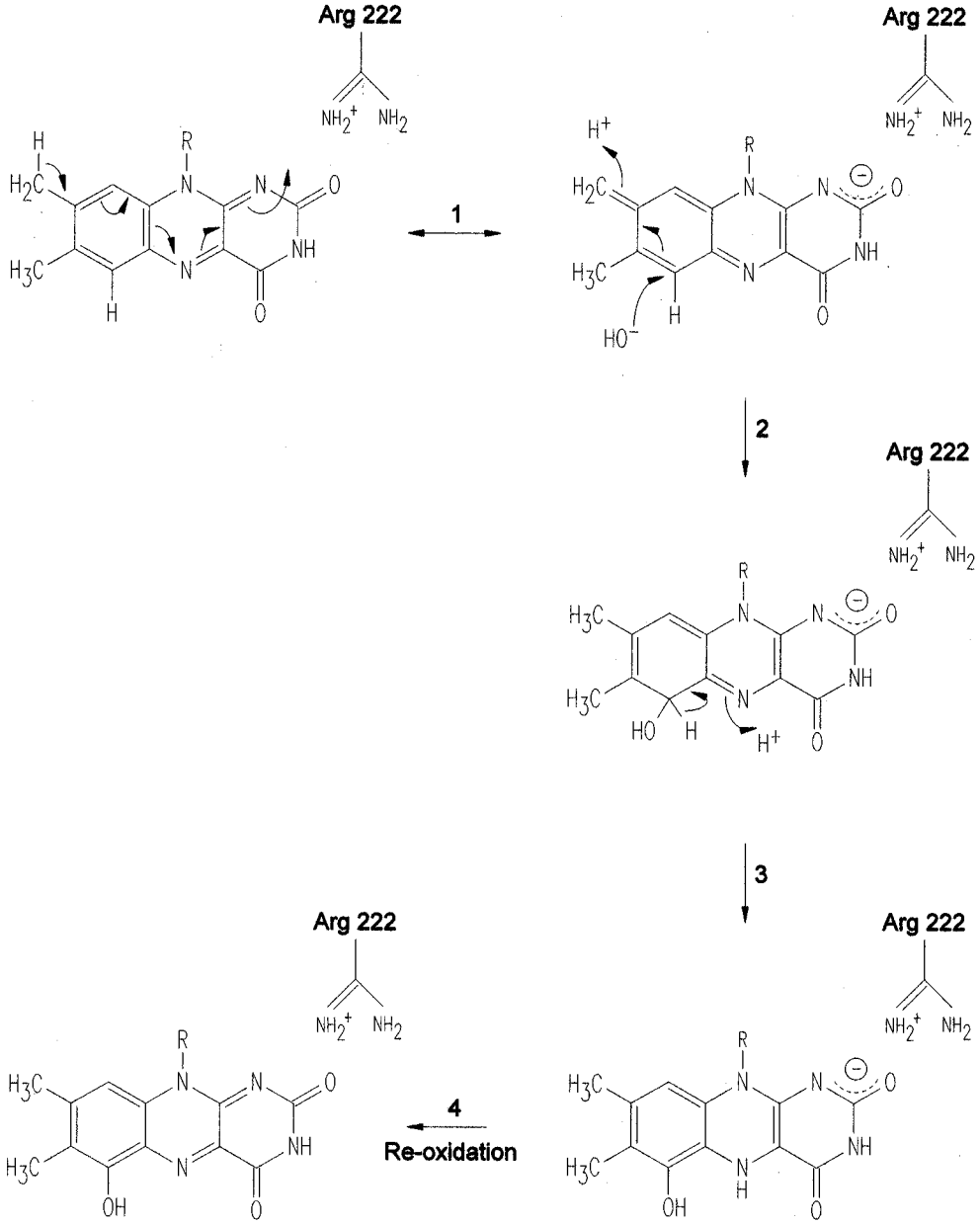


FIGURE 4: Proposed mechanism for the formation of 6-hydroxy-FMN in TMADH.

one of three aromatic residues that binds substrate by cation$-\pi$ bonding. The turnover number for W355L TMADH (k_{cat}, 0.05 $\pm$ 0.002 s^{-1} compared with 12 $\pm$ 0.8 s^{-1} for the native wild-type enzyme) was calculated on the basis of the concentration of 6-*S*-cysteinyl-FMN in the enzyme preparation, since in previous work (Huang et al., 1996) we demonstrated that the 6-hydroxy-FMN form of the C30A enzyme was catalytically inactive. The k_{cat} values for the first and second preparations of the W355L mutant calculated using the content of 6-*S*-cysteinyl-FMN in the enzyme preparations were identical. Given the fact that the ratio of 6-hydroxy-FMN and 6-*S*-cysteinyl-FMN in preparations 1 and 2 is 1:1.7 and 1:2.6, respectively, the data are consistent with the 6-hydroxy-FMN form of W355L being catalytically inert.

No detectable spectral changes were observed in rapid-scanning stopped-flow spectroscopy measurements of the reaction between W355L (60 μM) and high concentrations of TMA (250 mM), even over extended times (50 s) or in single-wavelength studies at 443 nm (the wavelength used to monitor flavin reduction in TMADH). However, in static titration experiments under anaerobic conditions using 2 M TMA and 60 μM W355L TMADH, up to 30 min were required to effect an absorbance change at 443 nm of 0.02 absorbance unit, which corresponds closely to the expected change in absorbance for the reduction of that portion of the W355L TMADH in the 6-*S*-cysteinyl-FMN form. In the static titration, the time course for full reduction is longer than might be expected from the turnover number measured in the steady state. The most likely explanation for this disparity is the presence of residual oxygen in the static titration due to attainment of partial anaerobosis. Consequently, full reduction will only be achieved after residual oxygen has been removed by turnover in the earlier stages of the titration. Clearly, the W355L TMADH is extremely compromised in its ability to oxidize TMA even when the flavin is in the 6-*S*-cysteinyl-FMN form. Unlike that for the wild-type enzyme, the rate of flavin reduction is rate-limiting in the W355L TMADH, and this accounts for the very poor turnover number (0.05 s^{-1}) seen in the steady state.

Mechanism for 6-Hydroxylation of FMN in TMADH. The W355L mutant of TMADH is the only form of the enzyme examined to date with substantial quantities of 6-hydroxy-FMN in the as-isolated active site. Previously, we have shown that the flavin of the C30A mutant can be converted to 6-hydroxy-FMN, but only after treatment of the enzyme with substrate; i.e. the as-purified mutant is isolated with conventional FMN bound in the active site. Our findings reported here indicate that the W355L mutant is isolated as a mixture of enzyme forms containing 6-*S*-cysteinyl-FMN, 6-hydroxy-FMN, and the deflavo enzyme, and these observations caused us to re-evaluate the flavin content of the native and recombinant wild-type enzymes. On close inspection, these latter enzyme preparations were found to contain very small quantities of 6-hydroxy-FMN. In this regard, our observations are similar to those made by Mayhew and co-workers, who reported small amounts of 6-hydroxy-FMN and 6-hydroxy-FAD in *Peptostreptococcus elsdenii* electron-transferring flavoprotein and pig liver glycolate oxidase, respectively (Mayhew et al., 1974). The fact that a small quantity of 6-hydroxy-FMN is observed in as-isolated native TMADH indicates that the capacity to derivatize the flavin is contained within the protein itself, and indeed, heterologous expression of the W355L TMADH in *E. coli* leads to much higher levels of 6-hydroxy-FMN. Our data indicate that the intrinsic susceptibility of the isoalloxazine ring to 6-hydroxylation in the wild-type enzymes can be substantially increased either by modifying the protein environment (e.g. mutation of Trp-355 to leucine) or by treating selected forms of the enzyme (mutant C30A) with substrate.

Our previous observations with the C30A TMADH in which flavin modification occurred only in the course of turnover led us to propose that hydroxylation proceeds by OH^- attack of a flavin−substrate covalent adduct thought to form in the course of catalysis (Huang et al., 1996). In this mechanism (Scheme 1), derivatization of the flavin does not occur as a result of turnover since the TMA that is bound at the flavin N5 is displaced on attack by hydroxide at the C6 position and TMA is regenerated. Hydroxylation at C6 can therefore be termed "substrate-assisted"; i.e. substrate acts catalytically. The potential involvement of a substrate−N5 adduct was invoked because formation of the adduct would render the C6 atom susceptible to nucleophilic attack by hydroxide, and the mechanism also includes a role for substrate in hydroxylation. However, a substrate-assisted mechanism cannot explain hydroxylation at the flavin C6 in the W355L and wild-type forms of TMADH, which are derivatized in the absence of TMA.

To account for derivatization of the W355L TMADH in the absence of substrate, we propose that hydroxylation proceeds via the flavin iminoquinone methide tautomeric form (Figure 4). The presence of a positively charged residue, Arg-222, in the vicinity of the N1 atom and C2 carbonyl should favor formation of the methide by stabilizing negative charge in the N1/C2 region of the isoalloxazine ring. In the methide form, the electrophilicity of the C6 atom of the flavin will be substantially increased, making it susceptible to attack by hydroxide derived from solvent. Following addition of hydroxide at C6, a simple tautomerization and reoxidation of the flavin gives rise to the 6-hydroxy-FMN. The methide form of the flavin is likely to be the reactive species of the flavin since in the non-methide form the electrophilicity of C6 is unlikely to be sufficient to support the reaction. It is worth emphasizing that formation of the flavin methide is likely to be important in the formation of the natural 6-*S*-cysteinyl-FMN of TMADH which is formed by the nucleophilic attack of the Cys-30 nucleophile at the C6 position of the flavin. A mechanism involving the formation of the methide form is a refinement of the mechanism we have proposed for flavinylation of TMADH (Scrutton et al., 1994; Mewies et al., 1996); the basic mechanism remains unchanged, but initial attack by the Cys-30 thiolate is aided (by enhancement of C6 electrophilicity) by flavin tautomerization. Our previous mutagenesis studies are consistent with the role of Arg-222 in stabilizing the flavin iminoquinone methide; a mutant of TMADH in which Arg-222 is replaced by lysine retains some (albeit compromised) ability to form the 6-*S*-cysteinyl-FMN link. Removal of the positively charged side group in the N1/C2 region (by mutation of Arg-222 to valine or glutamate), however, prevents flavinylation of the enzyme (Mewies et al., 1996). The involvement of an iminoquinone methide has also been postulated in the 8α-methyl flavinylation reactions of 6-hydroxy-D-nicotine oxidase (Brandsch & Bichler, 1991) and *p*-cresol methylhydroxylase (Kim et al., 1995), and it may be a general feature of flavinylation mechanisms at the 8α-methyl and C6 positions of flavin (Mewies et al., 1997). In the case of TMADH, the flavinylation and hydroxylation reactions compete for the noncovalently bound flavin, and the amount of 6-hydroxy-FMN present in the active site depends on the relative magnitudes of the rate constants for these two reactions.

Substrate-assisted hydroxylation of the C30A TMADH is likely to proceed via the same mechanism proposed for the wild-type and W355L forms of TMADH. In this case, however, minor structural changes probably occur in the active site of C30A upon binding TMA or during catalysis that are able to "switch on" the hydroxylation activity in this mutant (it is likely that the Trp 355 → Leu mutation makes the C6 position of the flavin more accessible to solvent). In the case of C30A TMADH, minor structural changes on binding TMA are likely also to expose the C6 atom of the isoalloxazine ring to solvent. On the basis of our results with TMADH, an attractive explanation for the existence of covalent flavoproteins is that flavin 8α and C6 covalent links to protein have evolved to prevent inactivation of the flavin redox center through hydroxylation. Covalent linkage at C6 prevents modification at C6 by hydroxide. Similarly, linkage at the 8α-methyl would prevent formation of the iminoquinone methide, which is proposed to be central to the hydroxylation mechanism. Consequently, covalent flavoproteins likely have evolved to suppress hydroxylation in those cases where the electronic structure of the isoalloxazine ring, as dictated by its protein environment, renders C6 susceptible to nucleophilic attack.

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