Inactivation of C30A Trimethylamine Dehydrogenase by *N*-Cyclopropyl-α-methylbenzylamine, 1-Phenylcyclopropylamine, and Phenylhydrazine†

Deanna J. Mitchell,‡ Dejan Nikolic,§ Mei-Huei Jang,|| Richard B. van Breemen,§ Russ Hille,|| and Richard B. Silverman*,‡

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3113, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois, Chicago, Illinois 60612-7231, and Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210-1218

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ABSTRACT: Trimethylamine dehydrogenase (TMADH) from the bacterium *Methylophilus methylotrophus* (sp. W_3A_1) and its C30A mutant were inactivated with three known inactivators of monoamine oxidase, namely, phenylhydrazine, *N*-cyclopropyl-α-methylbenzylamine, and 1-phenylcyclopropylamine. All three compounds irreversibly inactivated both the wild-type and C30A mutant enzymes, although phenylhydrazine was 10 times more potent than *N*-cyclopropyl-α-methylbenzylamine, which was much more potent than 1-phenylcyclopropylamine. The change in the UV−visible absorption spectra upon modification indicated that the flavin was modified. In the case of the C30A mutant, the absence of a covalent attachment of the flavin to the polypeptide has permitted LC-electrospray mass spectrometry of the reaction product to be undertaken, demonstrating new mass peaks corresponding to various chemically modified forms of the flavin cofactor. In the case of *N*-cyclopropyl-α-methylbenzylamine, masses corresponding to hydroxy-FMN and hydroxyriboflavin were detected. 1-Phenylcyclopropylamine inactivation of the C30A mutant produced three modified flavins, as evidenced by the electrospray mass spectrum: hydroxy-FMN, FMN plus $C_6H_5COCH_2CH_2$, and hydroxy-FMN plus $C_6H_5COCH_2CH_2$. Phenylhydrazine inactivation of the C30A mutant gave at least seven different modified flavins: hydroxyriboflavin, hydroxy-FMN, two apparently isomeric compounds corresponding to hydroxy-FMN plus one phenyl group, two apparently isomeric compounds corresponding to FMN plus one phenyl group, and FMN plus two phenyl groups. Covalent flavin adduct formation appears to be the only modification because dialysis of the inactive enzyme followed by reconstitution with FMN restores the enzyme activity to that of a noninactivated control.

Trimethylamine dehydrogenase (TMADH;[1] EC 1.5.99.7) from the bacterium *Methylophilus methylotrophus* (sp. W_3A_1) is an iron−sulfur flavoenzyme responsible for the oxidative demethylation of trimethylamine, generating dimethylamine and formaldehyde (*1*). TMADH is a homodimer with each of the 83 000 Da subunits containing 1 equiv of a covalently linked flavin mononucleotide (FMN) and a bacterial ferredoxin-type [4Fe-4S] cluster; the enzyme also possesses 1 equiv of tightly bound ADP, which is not located near the active site and apparently serves a structural role (*2*−*7*). The transformation catalyzed by this enzyme can be separated into two half-reactions, one reductive and one oxidative (*8*−*10*). The reductive half-reaction, involving reduction of TMADH by substrate, is a multistep process that involves substrate C−H bond cleavage and electron transfer to the flavin, followed by electron transfer to the iron−sulfur center. Several mechanisms have been proposed to account for the reduction of TMADH by substrate, including a carbanion mechanism (*11*), a direct hydride-ion transfer to the flavin (*10*), and a radical mechanism (*12*). In an effort to study the mechanism of the reductive half-reaction, several mutants have been prepared and their reactions with substrate characterized. Results of the mutagenesis studies indirectly support the mechanism involving radical cleavage of the C−H substrate bond (*10*), a mechanism similar to that proposed for monoamine oxidase (MAO) (*13*).

As with flavoenzymes catalyzing the oxidation of amines [e.g., monoamine oxidase (MAO) and sarcosine oxidase], the flavin of TMADH is covalently attached to the protein. In the case of TMADH, this attachment is not at the usual 8α-position of the isoalloxazine ring (*14*). Early work by Steenkamp and co-workers established that TMADH con-

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* Correspondence should be addressed to this author at the Department of Chemistry, Northwestern University, Evanston, IL 60208-3113. Phone: 847-491-5653. FAX: 847-491-7713. Email: Agman@chem.northwestern.edu.

‡ Northwestern University.

§ University of Illinois at Chicago.

|| The Ohio State University.

[1] Abbreviations: TMADH, trimethylamine dehydrogenase; FMN, flavin mononucleotide; MAO, monoamine oxidase; NCαMBA, *N*-cyclopropyl-α-methylbenzylamine; 1-PCPA, 1-phenylcyclopropylamine; RP-HPLC, reverse-phase high-performance liquid chromatography; ADP, adenosine diphosphate; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; TIC, total ion chromatogram.

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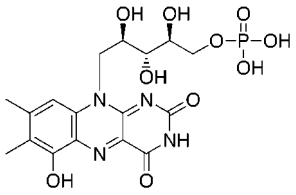
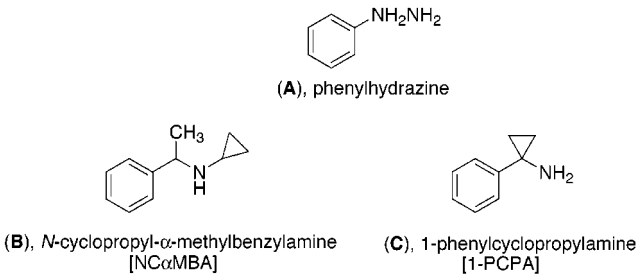


FIGURE 1: Structure of 6-hydroxy-FMN.

tained a 6-*S*-cysteinyl linkage to the flavin mononucleotide cofactor (*2, 15, 16*). The subsequent synthesis of 6-*S*-cysteinylriboflavin allowed for the comparison of physical properties and provided further evidence for the C^6 flavin linkage in TMADH (*17*).

Mutagenesis of Cys-30, the cysteine that covalently binds the FMN cofactor, to Ala-30 eliminates the covalent attachment to the flavin in TMADH (*18*). This mutant was expressed from a cloned gene (*tmd*) in the heterologous host *Escherichia coli*, and was found to bind FMN noncovalently. While there was little change in the steady-state parameters for the C30A mutant relative to wild-type enzyme, it did exhibit a relatively modest 5.5-fold decrease in the limiting rate constant observed for the fast phase of the reaction (involving reduction of the flavin) at high substrate concentration. The remainder of the reductive half-reaction sequence, including the rate-limiting product dissociation process, was negligibly affected by the loss of the covalent linkage. This work demonstrated that the covalent linkage was not required for catalysis, and that the C30A mutant reacted effectively with substrate, passing through the same sequence of intermediates as was observed for the native enzyme. Significantly, however, it was found that after only approximately nine turnovers, the C30A mutant becomes inactivated via the formation of 6-hydroxy-FMN (Figure 1) (*19*). The rate of formation of 6-hydroxy-FMN is dependent on the substrate used; diethylmethylamine is slow to form the 6-hydroxy species, whereas trimethylamine is faster. The 6-hydroxy-FMN species was shown not to be the product of direct oxidation, as it was only observed to form when C30A TMADH is incubated with substrate. Therefore, it has been proposed that one function of the unusual 6-*S*-cysteinyl flavin attachment in TMADH is to prevent the formation of 6-hydroxy-FMN, which renders the enzyme inactive (*19, 20*). Crystal structures of the wild type and C30A mutant at 2.2 Å resolution reported recently showed that the active site structure and flavin conformation for the native and C30A mutant are identical (*21*). In conjunction with the earlier kinetic study, the crystal structure of the mutant demonstrates that studies of the C30A mutant are very likely to be relevant to the function of the wild-type enzyme.

Although bacterial TMADH and mammalian MAO do not share any common substrates, the reactions catalyzed are fundamentally similar, involving amine oxidation. TMADH is irreversibly inhibited by phenylhydrazine (Figure 2A), known to inactivate MAO (*22*−*25*). Because of this connection between TMADH and MAO, we have investigated whether other MAO inactivators, such as *N*-cyclopropyl-α-methylbenzylamine (*26*) (Figure 2B) and 1-phenylcyclopropylamine (*27*) (Figure 2C), whose inactivation mechanisms have been proposed previously, have an effect on TMADH. Unlike TMADH and MAO, however, C30A TMADH contains noncovalently bound flavin, so any modified flavin could be readily determined by cofactor



(**A**), phenylhydrazine

(**B**), *N*-cyclopropyl-α-methylbenzylamine [NCαMBA]

(**C**), 1-phenylcyclopropylamine [1-PCPA]

FIGURE 2: Inactivators of TMADH.

separation and mass spectral analysis. This may give some insight into the inactivator mode of action with other amine oxidases, including MAO. The results of those studies are reported here.

MATERIALS AND METHODS

Materials. All materials were of the highest grade available and used without further purification unless otherwise noted. Phenylhydrazine was purchased from Aldrich Chemical Co. *N*-Cyclopropyl-α-methylbenzylamine (NCαMBA) (*26*) and 1-phenylcyclopropylamine (1-PCPA) (*27*) were synthesized as previously described. Sephadex G-50, buffer salts, and other reagents used in the enzymological studies were purchased from Sigma Chemical Co. Poly-prep disposable columns were purchased from Bio-Rad. Pierce supplied the BSA standard and Coomassie Plus reagent.

Analytical Methods. TMADH assays and UV−visible spectra were recorded on a Perkin-Elmer Lambda 10 spectrophotometer. Measurements of pH were performed on an Orion 701-A pH meter equipped with a general combination electrode. Low molecular weight impurities were removed using Pharmacia Biotech PD-10 columns (Sephadex 25 M). An Eppendorf Microfuge, model 5415C, was used for microcentrifugations. A Savant SC100 speed vac concentrator, equipped with a Savant RT 100 refrigerated condensation trap, was used to dry the samples. Samples were incubated in an Eppendorf thermostat, model 3401. RP-HPLC was performed on a Beckman 125P solvent delivery module, equipped with a Beckman 166 detector and Nouveau Gold software. The HPLC system was equipped with a 250 × 4.6 mm Alltech Alltima C18, 100 Å, 10 μm, analytical column. The LC system coupled to the mass spectrometer consisted of either an ABS 140A syringe pump and a Rheodyne injector model 7725 equipped with a 10 μL injection loop, or a Waters Alliance 2690 separations module equipped with a Waters Alliance 2690 autosampler, and a 2 × 250 mm, 5 μm, Hewlett-Packard Hypersil BDS C18 column with a guard cartridge. Electrospray ionization mass spectra were acquired using a Micromass Quattro II mass spectrometer (Manchester, U.K.).

Enzyme and Assays. The wild-type native TMADH (obtained heterologously from the bacterium *Methylophilus methylotrophus* W_3A_1) and the recombinant mutant C30A TMADH (from *Escherichia coli* strain JM109 transformed with the plasmid pSV2tmdvegC30A) were prepared as previously reported by Scrutton and co-workers (*1, 18, 28*). The concentration of wild-type TMADH was determined using a BSA standard curve; 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, both the recombinant native and site-directed mutant of TMADH possess the full complement of iron−sulfur and ADP, but only approximately half of the flavin sites are occupied; with recombinant wild-type enzyme (not used in the present study), all of the FMN that binds to the protein becomes covalently attached. In the case of the C30A mutant, the bound FMN can be reversibly removed, but subsequent reconstitution with exogenously added FMN is never greater than the original extent of flavin incorporation (*19*). It is thus evident that a portion of the mutant enzyme is unable to effectively take up the flavin cofactor. For the purposes of the present work, however, the portion of the enzyme lacking flavin is catalytically inert, both to substrate and to the inactivators used here. Its presence in the reaction mix requires that greater amounts of enzyme be used to obtain a given amount of reaction product, but does not complicate interpretation of the results as it does not possess flavin and thus cannot yield covalent flavin adducts. SDS−PAGE electrophoresis (Coomassie Blue) was used to determine protein homogeneity. TMADH activity was monitored spectrophotometrically using the dye-linked assay of phenazine ethosulfate and 2,6-dichloroindophenol in sodium diphosphate buffer (0.1 M, pH 7.7) as previously described (*22*, *29*). The enzymes were stored at −80 °C.

Inactivation of Native TMADH. Native TMADH (5 μL, 355 μM) was incubated in 100 μL total volume of a solution containing 360 μM phenylhydrazine in sodium diphosphate buffer (0.1 M, pH 7.7) at 30 °C (*23*). Aliquots (10 μL) were removed and assayed for activity at various time points (every 5−10 min). A sample containing native TMADH (5 μL, 355 μM) in 95 μL of sodium diphosphate buffer (0.1 M, pH 7.7) was incubated at 30 °C and served as the control. The experiment was repeated using 70 and 175 μM phenylhydrazine. The experiment was also repeated using 10 mM 1-PCPA, or 10 mM, 1 mM, and 0.1 mM NCαMBA in place of phenylhydrazine.

UV−Visible Spectrophotometric Analysis of Inactivated Native TMADH. Inactivation of native TMADH was accomplished as described above. Aliquots (50 μL) of each of the inactivated samples (phenylhydrazine, 1-PCPA, and NCαMBA) and the control were then loaded into a microcuvette and subjected to UV−visible spectral analysis (scan range 300−700 nm).

Irreversible Inactivation of Native TMADH. To demonstrate the irreversibility of the inactivation, native TMADH (2 μL, 355 μM) was incubated in 200 μL total volume of a solution containing 0.1 mM phenylhydrazine in sodium diphosphate buffer (0.1 M, pH 7.7) at 30 °C (*23*). Aliquots (10 μL) were removed and assayed for activity at 0.5 and 120 min. The incubation mixture was applied to a Penefsky spin column (Sephadex G-50), and the enzyme was recovered by spinning in an IEC Clinical centrifuge (2 min) (*30*). An aliquot (10 μL) was removed and assayed for enzyme activity. A sample containing native TMADH (2 μL, 355 μM) in 198 μL of sodium diphosphate buffer (0.1 M, pH 7.7) was incubated at 30 °C and served as the control. The experiment was repeated using 1 mM NCαMBA and 10 mM 1-PCPA in place of phenylhydrazine.

Inactivation of C30A TMADH. To remove the excess flavin in the enzyme preparation, C30A TMADH was applied to a PD-10 column, equilibrated with sodium diphosphate buffer, 0.1 M, pH 7.7, and eluted with the same buffer. Beer's Law was used to calculate the enzyme concentration ($\epsilon = 197.1\ mM^{-1}\ cm^{-1}$ at 280 nm). The total volume of enzyme (3 mL) was divided into three Eppendorf tubes of 1 mL each. To each Eppendorf tube was added NCαMBA or phenylhydrazine, for a final concentration of 2 mM in a total volume of 1.3 mL of sodium diphosphate buffer, 0.1 M, pH 7.7. To the third tube was added only buffer (control, no inactivator). The tubes were incubated for 135 min at 25 °C. Aliquots (2 μL) were removed and assayed for enzyme activity at time 0, 75 min, and 135 min.

The experiment was repeated using 1-PCPA (2 mM final concentration in 1.3 mL), with the incubation time extended to 4.5 h. Aliquots (2 μL) were removed and assayed for enzyme activity at time 0, 45 min, 2 h, and 4.5 h. The incubation mixture was stored at −80 °C.

C30A TMADH Adduct Isolation. After inactivation, the enzyme was denatured and precipitated by adding cold acetone/cold water (1:1) and incubating on ice (30 min). The cloudy solution was then sonicated (5 min) and microfuged [14 000 rpm (16000*g*), 15 min]. The yellow supernatant was collected and concentrated by speed vac.

UV−Visible Spectrophotometric Analysis of Inactivated C30A TMADH. Isolation of C30A TMADH flavin−inactivator adducts was accomplished as described above. Aliquots (50 μL) of each of the inactivated samples (phenylhydrazine, NCαMBA, and 1-PCPA) and the control were loaded into a microcuvette and analyzed by UV−visible spectrophotometry (scan range 200−700 nm).

RP-HPLC Analysis of the C30A TMADH Adducts. Aliquots (15−20 μL) of the inactivated samples and the control were injected onto an Alltech Alltima C18 column (4.6 × 250 mm, 10 μm) and eluted using the following system: mobile phase A, 100% water; mobile phase B, 100% methanol; gradient, 5% B to 25% B over 4 min, 25% B to 50% B over 8 min, remain at 50% B for 10 min, 50% B to 5% B over 2 min. The flow rate was 1.0 mL/min. The visible tungsten lamp detector was set at 442 nm for the FMN standard and the control TMADH, at 361 nm for the phenylhydrazine adduct, and at 422 nm for the NCαMBA adduct.

Electrospray Ionization Mass Spectrometry of C30A TMADH Adducts. The adducts were isolated as described above. Aliquots not subjected to RP-HPLC were diluted with water and used for mass spectral analysis. Aliquots of 5 μL each were loop-injected onto a Hypersil BDS C18 column (2 × 250 mm, 5 μm), connected to the mass spectrometer. The adducts were eluted with the following system: mobile phase A, 100% water; mobile phase B, 100% methanol; gradient, 5% B to 25% B over 4 min, 25% B to 50% B over 8 min, remain at 50% B for 10 min, 50% B to 5% B over 2 min. The flow rate was 0.190 mL/min. Triethylamine in methanol was added postcolumn to facilitate the formation of deprotonated molecules. A delay time of 3.5 min was used to prevent the entry of salts into the mass detector. Negative ion electrospray mass spectra were acquired using the following operational parameters: capillary 3.04 kV; cone voltage 26 V; source temperature 100 °C. For the 1-PCPA adduct: capillary 2.90 kV; cone voltage 40 V; source temperature 140 °C; 5 min delay. The mass range was set at *m*/*z* 350−850, with a scan rate of 2 s/scan. Tandem mass spectra were acquired with a collision energy of 26 eV and argon gas pressure of 3×10^{-3} mbar.

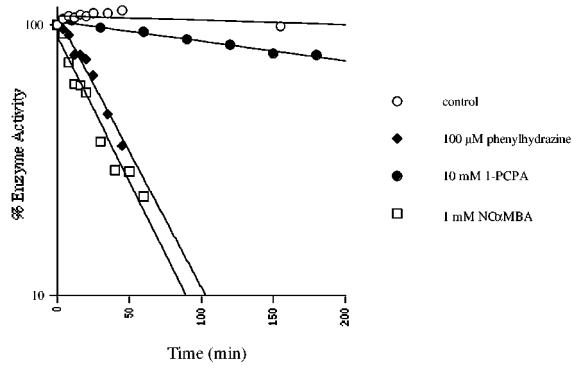


FIGURE 3: Inactivation of native TMADH with 0 mM inactivator (control, ○), 100 μM phenylhydrazine (◆), 10 mM 1-PCPA (●), and 1 mM NCαMBA (□).

Reconstitution of 1-PCPA-Inactivated C30A TMADH with FMN. C30A TMADH was applied to a PD-10 column equilibrated with 0.1 M sodium diphosphate buffer, pH 7.7, and was eluted with the same buffer to remove the excess FMN (excess FMN is added to stabilize the mutant enzyme; active-site FMN is not removed by gel filtration under these conditions). TMADH C30A (150 μL, 66 μM) was incubated with 1-PCPA (200 μL, 10 mM) and 0.1 M sodium diphosphate buffer (450 μL, pH 7.7) for 10 h, at which time the enzyme activity is about 10% of that of the control. A control, containing no 1-PCPA, was carried out in parallel. The incubation solution was dialyzed exhaustively at 4 °C versus potassium bromide (1 M) in 50 mM potassium phosphate buffer (500 mL, pH 7.5), changed 5 times over a 36 h period to remove the FMN and 1-PCPA. After dialysis, less than 10% of the enzyme activity remained in both the inactivated and noninactivated enzyme solutions. FMN (1.5 mg) was added to both enzyme solutions. After 22 min, the activity of C30A TMADH which was inactivated by 1-PCPA was about 70−75% of that prior to inactivation, and that of the control was 80−85%.

RESULTS

Inactivation of Native TMADH. As shown in Figure 3, phenylhydrazine (Figure 2A) was the most potent inactivator of native TMADH, about 10 times more potent than *N*-cyclopropyl-α-methylbenzylamine (NCαMBA, Figure 2B); 1-phenylcyclopropylamine (1-PCPA, Figure 2C) was, by far, the least potent. The inactivation of native TMADH by NCαMBA or 1-PCPA, followed by gel filtration to remove unreacted or loosely bound inactivator, did not result in the return of enzyme activity.

UV−Visible Spectrophotometric Analysis of Native TMADH Inactivated with NCαMBA and 1-PCPA. Changes in the UV−visible absorption spectra of native TMADH inactivated with NCαMBA and 1-PCPA (Figure 4) indicated an interaction between the inactivators and the flavin. After 3.5 h incubation, there was no loss of enzyme activity from the control (λ_{max} 442 nm). The NCαMBA-treated enzyme was completely inactivated with a shift in absorbance to 395 nm; the 1-PCPA-treated enzyme lost only 30% of its activity in that time period with a decreased and shifted absorption spectrum.

Inactivation of C30A TMADH. As with the native enzyme, both phenylhydrazine and NCαMBA inactivated C30A TMADH; less than 5% of enzyme activity remained after 135 min at 25 °C (Figure 5). 1-PCPA was found to inactivate C30A TMADH, but at a very slow rate. After 4.5 h at 25 °C, 49% of the enzyme activity remained.

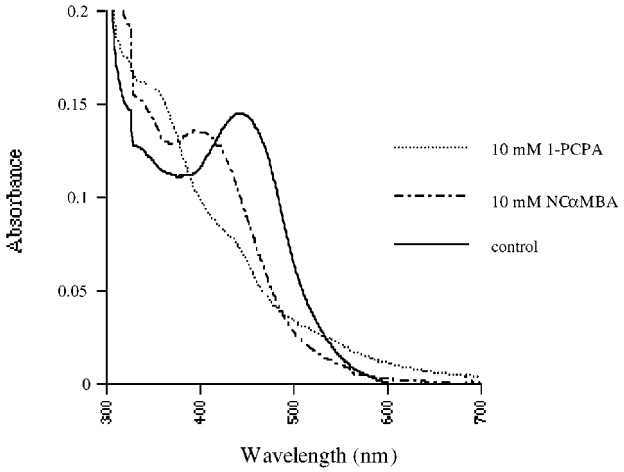


FIGURE 4: Absorption spectra of native TMADH (18 μM) with no inactivator, with 10 mM NCαMBA, and with 10 mM 1-PCPA after incubation for 3.5 h at 30 °C.

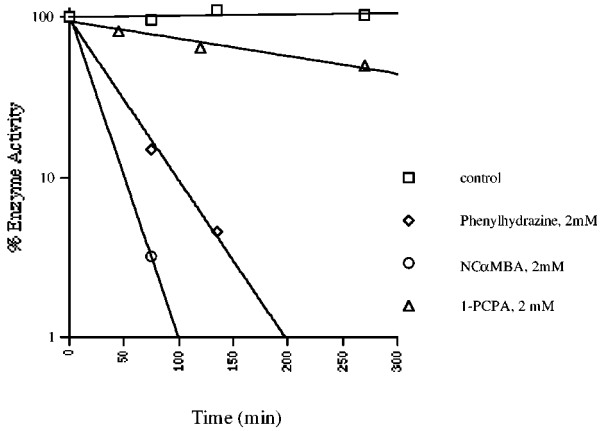


FIGURE 5: Inactivation of C30A TMADH by 2 mM phenylhydrazine, 2 mM NCαMBA, and 2 mM 1-PCPA.

UV−Visible Spectrophotometric Analysis of C30A TMADH Inactivated with Phenylhydrazine, NCαMBA, and 1-PCPA. Acetone precipitation of inactivated C30A TMADH released the flavin, which was isolated and the absorption spectrum taken. The control (incubation without any inactivator) showed the expected FMN absorbance bands at 375 and 442 nm (Figure 6). Bleaching of the flavin band at 442 nm was accompanied by a simultaneous appearance of a new peak at 344 nm for the phenylhydrazine adduct. C30A TMADH inactivated with NCαMBA showed absorbances at 381 and 422 nm. With 50% enzyme activity remaining, the adduct resulting from the incubation of C30A TMADH with 1-PCPA exhibited a shift in the A band of flavin from 442 to 428 nm (Figure 7).

LC/ESI-MS Characterization of the Flavin Adducts. Analytical RP-HPLC was used to optimize the separation conditions for each of the flavin−inactivator adducts. On-line LC/ESI-MS (negative mode) was then used to examine the FMN adducts that resulted from the inactivation of C30A TMADH with NCαMBA, 1-PCPA, and phenylhydrazine.

(A) Control. A peak with a retention time of 8.3 min was detected. The *m*/*z* of the 455 peak corresponded to unmodi-

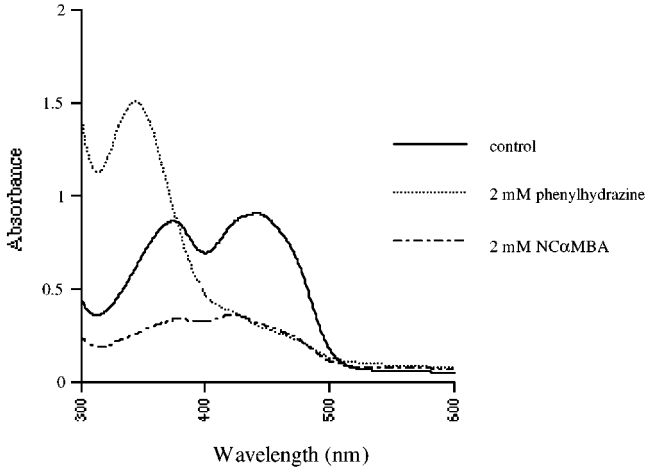


FIGURE 6: 6: UV−vis spectra of the flavin cofactor recovered after C30A TMADH (7 μM) was incubated with 0 mM inactivator (control), 2 mM phenylhydrazine, or 2 mM NCαMBA for 135 min at 25 °C.

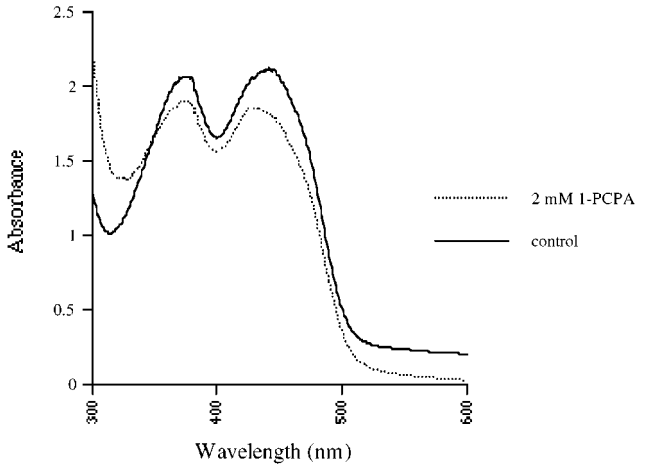


FIGURE 7: UV−vis spectra of the flavin cofactor recovered after C30A TMADH (7 μM) was incubated with 0 mM inactivator (control) or 2 mM 1-PCPA for 4.5 h at 25 °C.

fied FMN (Figure 8). Ions of *m*/*z* 453 and 451 were also observed in some spectra, which is speculated to arise from the oxidation of FMN in the source of the mass detector (see Discussion). Ions of *m*/*z* 455, 453, and 451 were also observed when the FMN standard was injected under identical conditions.

(B) Inactivation Products from NCαMBA Treatment. Three main peaks were observed in the total ion chromatogram (TIC), at 7.9, 8.4, and 9.9 min. The peak at 8.4 min was identified as unmodified FMN with an *m*/*z* of 455. The peak at 9.9 min had an *m*/*z* of 471, which corresponds to hydroxylated FMN (Figure 9). The peak at 7.9 min, *m*/*z* of 391, resulted from the loss of a phosphate group (*m*/*z* 80) from the hydroxylated FMN (*m*/*z* 471 − 80 = 391), i.e., hydroxylated riboflavin. This *m*/*z* value also was observed as a fragment ion in the spectrum of the 9.9 min peak (Figure 9).

(C) 1-PCPA Adduct. Several peaks appeared in the TIC (10.3, 14.7, and 14.9 min). Unmodified FMN was identified with an *m*/*z* of 455 (identical to the mass found in the control). The peak at 10.3 min had an *m*/*z* of 469, which corresponds to hydroxylated FMN (the difference in *m*/*z* for 6-hydroxy-FMN in this experiment relative to that in the

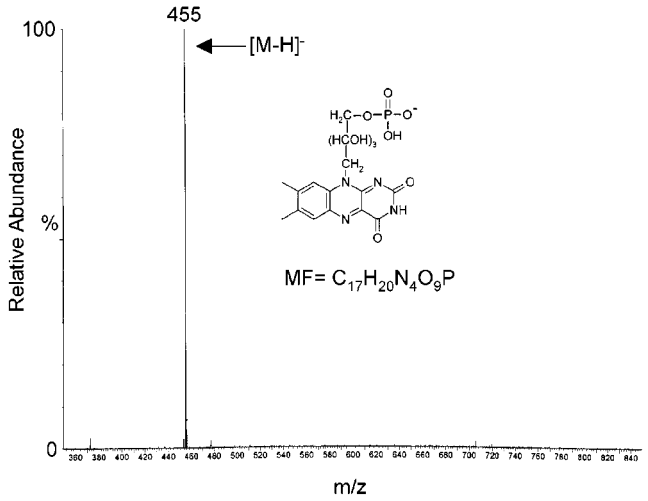


FIGURE 8: Negative mode electrospray ionization mass spectrum of the flavin isolated from the C30A TMADH control (0 mM inactivator).

above experiment is explained under Discussion). The peak at 14.7 min, *m*/*z* of 588, corresponds to FMN + $C_6H_5COCH_2$-CH_2. This adduct readily fragmented to an *m*/*z* of 455 (unmodified FMN) under the conditions used for mass spectral analysis (Figure 10). The peak at 14.9 min, with an *m*/*z* of 602, is proposed to be hydroxylated FMN + C_6H_5-$COCH_2CH_2$ (see Discussion for explanation of the *m*/*z* value).

(D) Phenylhydrazine Adducts. Multiple peaks were observed in the TIC (Figure 11) and were characterized as shown in Table 1.

Reconstitution of 1-PCPA-Inactivated C30A TMADH with FMN. Following inactivation of C30A TMADH by 1-PCPA, the enzyme was dialyzed. The inactive enzyme was incubated with FMN, and the enzyme activity returned to about the same level as the noninactivated control carried through the same procedures (data not shown).

DISCUSSION

Early work by Colby and Zatman (*22*) established that TMADH was inactivated by monoamine oxidase inhibitors such as alkyl- and arylhydrazines, a propargylamine, and *trans*-2-phenylcyclopropylamine. In each case, inhibition was found to be time-dependent and irreversible, and could be prevented by the presence of substrate and competitive inhibitors (*22, 23*). With the use of [^{14}C]phenylhydrazine, Nagy, Kenney, and Singer later demonstrated the formation of a covalent flavin adduct containing 1 equiv of phenyl group at the C^{4a} position of the flavin (*23*). This result was the basis for our investigation into the inactivation of the C30A mutant with the cyclopropyl MAO inactivators NCαMBA and 1-PCPA, as well as with phenylhydrazine (Figure 2).

Phenylhydrazine, NCαMBA, and 1-PCPA all inactivated TMADH irreversibly (Figure 3); phenylhydrazine was 10 times more potent than NCαMBA, which in turn was much more potent than 1-PCPA. Concomitant changes in the UV−visible spectra (Figure 4), as noted previously for phenylhydrazine (*22, 23*), indicate that reaction of enzyme with either reagent results in covalent modification of the enzyme flavin. A similar experiment using 1-PCPA to inactivate C30A TMADH showed a much smaller effect on the UV−

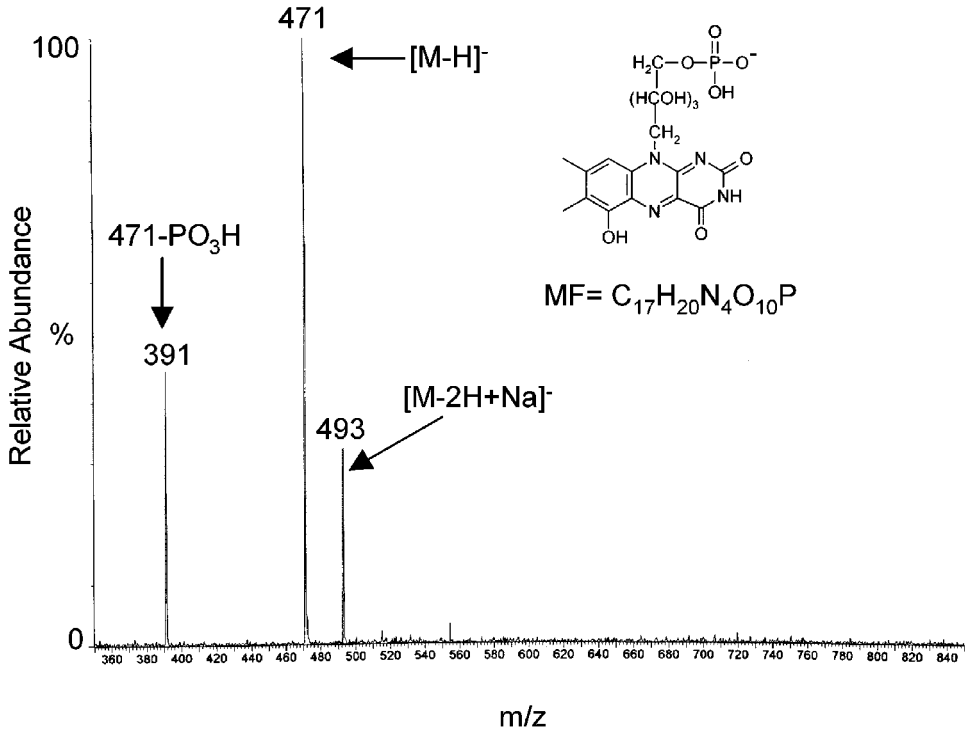


FIGURE 9: Negative mode electrospray ionization mass spectrum of the modified flavin isolated from C30A TMADH incubated with 2 mM NCαMBA.

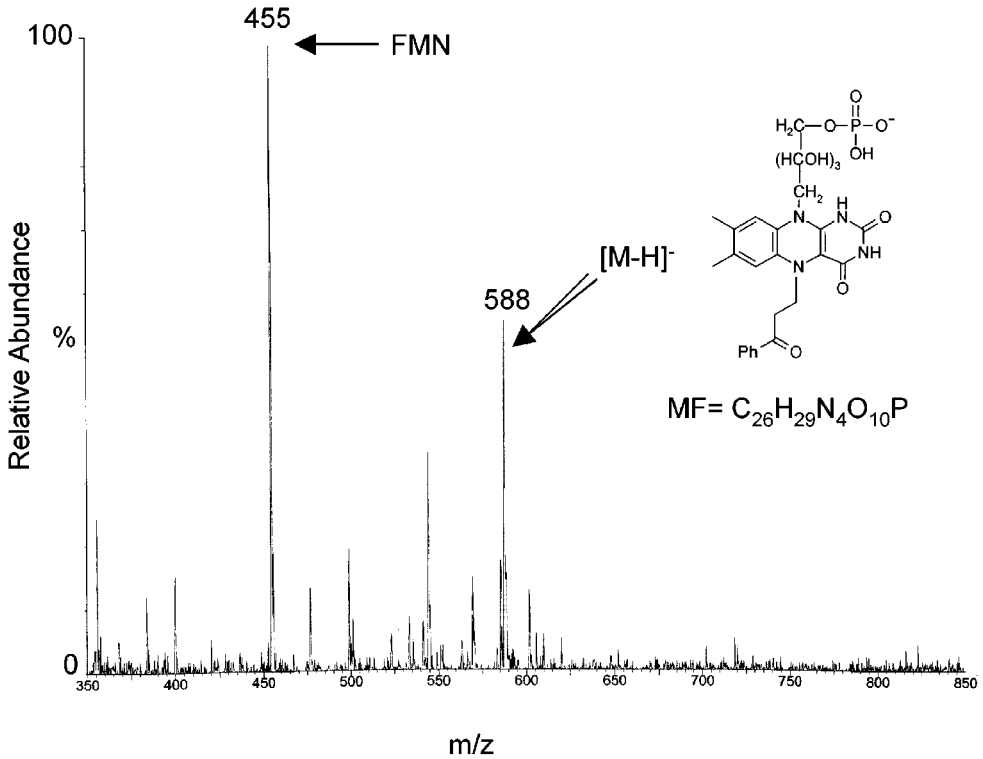


FIGURE 10: Negative mode electrospray ionization mass spectrum of the modified flavin isolated from C30A TMADH incubated with 2 mM 1-PCPA.

visible spectrum (Figure 7). The difference in the absorption change for TMADH versus the C30A TMADH may be because the chromophore is different in the two enzymes: FMN in the mutant, but 6-cysteinyl-FMN in the WT. 6-Cysteinyl-FMN has considerably different absorption features than FMN (*24*), so there is no reason the two spectral changes should be the same.

Likewise, the mutant enzyme exhibits a rank order of reactivity of NCαMBA > $PhNHNH_2$ > 1-PCPA (Figure 5), and the WT order of reactivity is $PhNHNH_2$ ≫ NCαMBA ≫ 1-PCPA. This difference in reactivity also could be attributed to the difference in the cofactors for the two enzymes. It is unlikely, however, that a conformational change due to the mutation is responsible for the differences

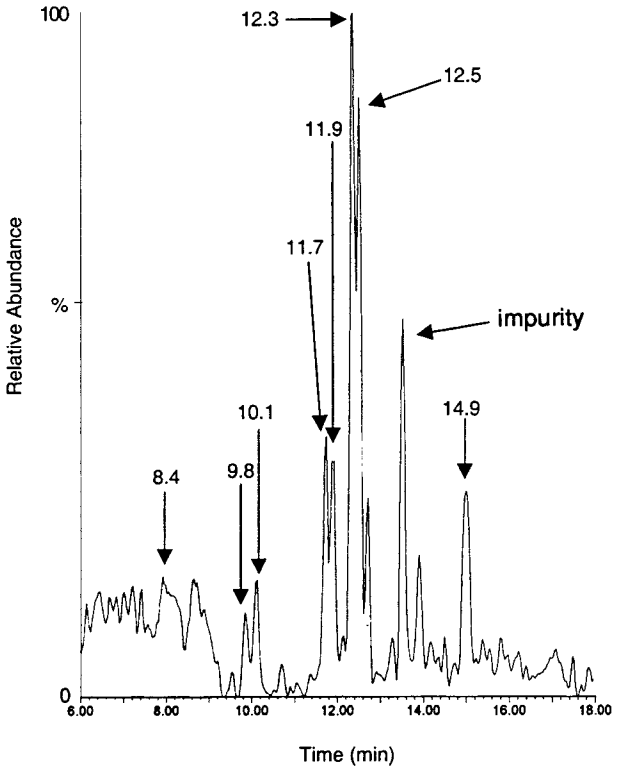


FIGURE 11: Total ion chromatogram of the products resulting from incubation of C30A TMADH with 2 mM phenylhydrazine.

Table 1: Identification of the Peaks Observed in the TIC of Flavins Isolated from C30A TMADH Incubated with 2 mM Phenylhydrazine for 135 min at 25 °C

retention time (min)	mass (amu)	proposed structure
8.4	455, 453	unmodified FMN
9.8	391	hydroxylated riboflavin
10.1	471	hydroxylated FMN
11.7, 11.9	549	hydroxylated FMN + 1 phenyl ring
12.3, 12.5	533	FMN + 1 phenyl ring
14.9	609	FMN + 2 phenyl rings

in reactivity, as the C30A enzyme has essentially the identical structure as WT (*21*).

The dissociable FMN of the C30A mutant of TMADH, which lacks the 6-*S*-cysteinyl covalent attachment to the FMN cofactor, offered the opportunity to study the structure of the modified flavin cofactor without possible adduct degradation associated with exhaustive hydrolysis of the protein to isolate the 6-cysteinyl-FMN, as was the case with the native enzyme (*23*). After inactivation (Figure 5) and acetone precipitation of the protein, the modified flavins could be conveniently isolated from the supernatant and analyzed by LC/ESI-MS.

Three peaks in the total ion chromatogram (TIC) were observed from the inactivation of C30A TMADH by NCαMBA. The one at 8.3 min corresponded to unmodified FMN (Figure 8), which was routinely observed because the enzyme was not completely inactivated. The other two peaks appeared to come from the same modified flavin, one corresponding to a hydroxylated FMN (*m*/*z* 471; Figure 9) and the other (*m*/*z* 391), also shown as a fragment in the hydroxylated FMN spectrum (Figure 9), to a dephosphorylated hydroxylated FMN. Given that C30A TMADH is

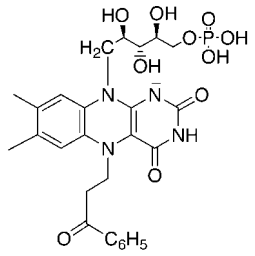


FIGURE 12: Proposed structure for FMN modified by 1-PCPA.

known to undergo substrate-induced auto-inactivation via formation of 6-hydroxy-FMN (*19*; see also Figure 1), it is likely that these modified flavins are derived from the same species. Unlike $PhNHNH_2$ and 1-PCPA (vide infra), NCαMBA does not form a covalent adduct with the flavin. This inactivator also is different from the other two with MAO: whereas $PhNHNH_2$ (*23*) and 1-PCPA (*27*) form covalent adducts with the flavin, NCαMBA forms a covalent adduct only with an active-site cysteine (*26*).

Four peaks were observed in the TIC of the flavins from inactivation by 1-PCPA. Again, one was identified as FMN (*m*/*z* 455) and one as hydroxylated (presumably, 6-hydroxy) FMN (*m*/*z* 469). As noted above, inactivation with NCαMBA produced 6-hydroxy-FMN with *m*/*z* 471 (as predicted for this compound). This difference of two mass units (and sometimes four mass units) was a common problem in the electrospray mass spectral analyses for all of the species detected. For example, the *m*/*z* 455 peak corresponds to unmodified FMN (Figure 8). However, ions of *m*/*z* 453 and 451 also were observed in some spectra, which appears to arise from oxidation of FMN in the source of the mass detector. Ions of *m*/*z* 455, 453, and 451 were observed when the FMN standard was injected under identical conditions. Because the abundances of the dehydrogenated ions were always lower than the expected ions and because their abundances were highly variable (even for the same sample), we conclude that they are artifacts formed in the ion source. Electrospray ionization is known to produce artifactual oxidation (*31*). The same situation arises for 6-hydroxy-FMN, which can be detected at *m*/*z* 471 or 469, as well as inactivator adducts of FMN (see below).

A third peak (14.7 min) has an *m*/*z* of 588, corresponding to FMN + 133, which we attribute to FMN modified with a $C_6H_5COCH_2CH_2$ group (Figure 10). By analogy to previous studies with MAO N (*32*) and MAO B (*33*) with 1-PCPA, it is likely that the flavin is covalently modified at N-5 (Figure 12). The fourth product, with *m*/*z* of 602, corresponds to FMN that is both hydroxylated and substituted with C_6H_5-$COCH_2CH_2$. As with 6-hydroxy-FMN, this *m*/*z* value is two mass units lower than expected (*m*/*z* 604), presumably because of oxidation in the electrospray mass spectrometer. Again, on the basis of earlier work demonstrating that hydroxylation occurs at C-6, it is most likely that the flavin is hydroxylated at the 6-position and covalently modified by the inhibitor at N-5. Reaction with the protein does not appear to be occurring with 1-PCPA. Following inactivation by 1-PCPA and dialysis to remove the modified flavins, reconstitution with FMN led to reactivation of the enzyme to the same level of the noninactivated control carried through the same procedures. It is interesting, although possibly irrelevant, that NCαMBA inactivates MAO exclusively by attachment to the protein, not to the flavin (*26*),

whereas 1-PCPA forms adducts with both the protein and the flavin of MAO (*27*). This may account for the difference observed in the modified flavins of TMADH by these compounds.

Surprisingly, phenylhydrazine inactivation of C30A TMADH gave the most complex mixture. Whereas inactivation of the native enzyme was reported to give just one modified flavin product, C^{4a}-phenyl-FMNH (*23*), the flavin from C30A TMADH is modified in at least seven different ways (Table 1, Figure 11). In addition to unmodified FMN, hydroxyriboflavin, and hydroxy-FMN, five different phenyl-substituted FMN derivatives appear to form. The TIC (Figure 11) shows two unresolved peaks with very close retention times (11.7 and 11.9 min) for a mass corresponding to hydroxylated FMN + 1 phenyl ring (*m*/*z* 549). Two unresolved peaks with very close retention times (12.3 and 12.5 min) also were observed with a mass corresponding to FMN + 1 phenyl ring (*m*/*z* 533). Peaks that have identical masses and slightly different retention times are typically indicative of the presence of isomers. Possibly, phenylation occurs at both the C^{4a}- and the N^5-positions in the mutant. There is only one peak corresponding to FMN + 2 phenyl rings (14.9 min; *m*/*z* 609), as would be expected if both the C^{4a}- and N^5-positions were modified.

It is quite apparent from the number of reaction products that inactivation of the C30A mutant by phenylhydrazine involves a variety of reactions at different sites. Possibly because of a less rigid active site in the mutant arising from a noncovalently bound flavin, radicals generated may have more mobility to attack different sites on the flavin. The mechanism for formation of 6-hydroxy-FMN is being investigated further, which may provide evidence for the inactivation chemistry by these mechanism-based inactivators of TMADH.

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