Flavinylation of Monoamine Oxidase B*

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Monoamine oxidase B (MAO B) catalyzes the oxidative deamination of biogenic and xenobiotic amines. The oxidative step is coupled to the reduction of an obligatory cofactor, FAD, which is covalently linked to the enzyme at Cys397. In this study, we developed a novel riboflavin-depleted (Rib−) COS-7 cell line to study the flavinylation of MAO B. ApoMAO B can be obtained by expressing MAO B cDNA in these cells. We found that MAO B is expressed equally in the presence or absence of FAD and that apoMAO B can be inserted into the outer mitochondrial membrane. Flavinylation of MAO B was achieved by introducing MAO B cDNA and different flavin derivatives simultaneously into Rib− COS-7 cells via electroporation. Since the addition of riboflavin, FMN, or FAD resulted in equal levels of MAO B activity, we conclude that the flavin which initially binds to apoMAO B is FAD. In our previous work, we used site-directed mutagenesis to show that Glu34 in the dinucleotide-binding motif of MAO B is essential for MAO B activity, and we postulated that this residue is involved in FAD binding. In this study, we tested the role of residue 34 in flavin binding by expressing wild-type or mutant MAO B cDNA in Rib− COS-7 cells with the addition of [14C]FAD. We found that Glu34 is essential for both FAD binding and catalytic activity. Thus, FAD binds to MAO B in a dual manner at Glu34 noncovalently and Cys397 covalently. We conclude that Glu34 is critical for the initial noncovalent binding of FAD and is instrumental in delivering FAD to the covalent attachment site at Cys397.

The major amine-degrading enzymes in the central nervous system and peripheral tissues of mammals are monoamine oxidase A and B (MAO1 A and B, amine:oxygen oxidoreductase (deaminating, flavin-containing), EC 1.4.3.4). These isozymes are integral proteins of the outer mitochondrial membrane (1) and can be distinguished by differences in substrate preference (2), inhibitory specificity (3), tissue and cell distribution (4–6), and immunological properties (7–9). Furthermore, comparison of their nucleotide and deduced amino acid sequences show that human MAO A and B are two distinct proteins encoded by different genes (10).

Oxidation of amines by MAO is coupled to the reduction of an obligatory cofactor, FAD, which is covalently linked to the enzyme. Five types of bonds are generally found in the covalent linkage of flavins to their respective apoproteins (11). These include a histidine residue which can be attached through its N-1 or N-3 atom to the 8α-methyl group of the isalloxazine ring to form a tertiary amine; a cysteine residue which forms a thioether linkage with either the 8α-methyl group or the C-6 of the xylene ring of the flavin molecule; or a tyrosine residue can become linked to the 8α-methyl group to form an (O)-8α-flavin bond. In MAO A and B, the 8α-methyl group of FAD is bound covalently to cysteine through a thioether linkage in the pen-tapeptide SGGCة (12, 13). Comparison of this segment with the complete deduced amino acid sequences of MAO A and B indicated that FAD is covalently bound to Cys397 in MAO A and Cys397 in MAO B, respectively (10). In addition, site-directed mutagenesis studies of MAO B, where Cys397 was substituted with serine or histidine, showed that this cysteine residue is essential for catalytic activity (14, 15).

Although the amino acid sequences surrounding the FAD covalent attachment site in different flavoproteins bear little homology, a distinct non-covalent FAD-binding site displays high sequence identity in many FAD-containing enzymes of diverse function (16, 17). This non-covalent FAD binding region is commonly referred to as the dinucleotide-binding site or motif due to its interaction with the AMP moiety of FAD. This motif consists of a β-sheet-α-helix-β-sheet beginning with a highly conserved Gly-X-Gly-X-Gly sequence between the first β-sheet and the α-helix. The second β-sheet usually ends with a glutamate residue in which the γ-carboxylate group is thought to interact through a hydrogen bond with the 2'-hydroxyl group of ribose in the AMP moiety of FAD. In MAO A and B, this motif is located at the N terminus of MAO A (residues 15–43) and MAO B (residues 6–34) and ends in Glu34 and Glu34, respectively. Site-directed mutagenesis studies, where Glu34 was replaced with aspartate, glutamine, or alanine, resulted in near complete or total loss of catalytic activity in MAO B (18).

A fundamental process in the intracellular generation of functional flavoenzymes is the molecular mechanism which generates holoenzyme from apoenzyme and its cofactor. Following the discovery of the first known enzyme with covalently linked FAD (succinate dehydrogenase, 19), extensive research in many laboratories has been conducted to elucidate how FAD is coupled to its respective proteins. The precise steps involved remain unknown. In this study, we developed a novel riboflavin-depleted (Rib−) COS-7 cell line to investigate the flavinylation of MAO B. ApoMAO B was obtained by expressing MAO B cDNA in these cells. We show that the expression of MAO B apoenzyme is independent of FAD and that apoMAO B can be inserted into the outer mitochondrial membrane. Coupling of flavin to the apoenzyme was studied using FAD, flavin derivatives, or [14C]FAD. We also examined the role of a critical glutamate residue (Glu34) in flavinylation of MAO B using site-directed mutants. We find that Glu34 plays an essential role in flavinylation.
role in flavin coupling to the apoenzyme. We propose that the dinucleotide-binding site at the N terminus of MAO B provides a topological dock for the initial binding of FAD, and then FAD is delivered to the covalent attachment site at Cys397.

MATERIALS AND METHODS

Synthesis of [14C]FAD—[14C]FAD was prepared by a modified method of Manstein and Pai (20). The reaction mixture (530 μl) contained 15 mM MgCl2, 6.5 mM ATP, 0.12 mM [14C]riboflavin (Amersham Corp., 50 mCi/mmol) and 200 μg of FAD synthetase (purified from Brevibacterium ammoniagenes). After incubation at 37°C for 20 h, the mixture was filtered through a 100,000 molecular weight cut-off spin filter (Millipore) to remove the insoluble components. The clear yellow solution was loaded on a C-18 Semi-Prep HPLC column (Beckman), and eluted with a linear gradient from 100% A/0% B (A = 10 mM (NH4)2HPO4, pH 6.8, B = acetonitrile) to 60% A/40% B in 20 min at a flow rate of 4 ml/min using a Beckman HPLC (System Gold). The peak corresponding to [14C]FAD eluted at a retention time identical to a FAD standard (Sigma). [14C]FAD was collected in sterilized silicone-coated glass tubes, dried in a Beckman speedvac, and stored at -20°C in powder form.

Synthesis of 8a-Hydroxyriboflavin—Synthesis of 8a-hydroxyriboflavin was carried out by the method of McCormik (21). Briefly, riboflavin was added to a solution of acetic acid/acetic anhydride (1:1) and the yellow product was determined by thin-layer chromatography. 8a-Hydroxyriboflavin (TAR) was extracted from the aqueous reaction mixture with CHCl3, followed by extraction with water and evaporation to give a yellow residue of essentially pure TAR. Dibenzoyl peroxide and dioxygen dibromide in dioxane were added to a solution of TAR in dioxane, and the solution was refluxed. The crude bromo-TAR was separated from the reaction mixture on a C-18 Semi-Prep HPLC column (Beckman). The bromo-TAR was hydrolyzed to yield 8a-hydroxyriboflavin, which was separated by HPLC (System Gold, Beckman) through a linear gradient from 0.5% trifluoroacetic acid in water to 0.5% trifluoroacetic acid in acetonitrile for 50 min at a flow rate of 4 ml/min.

Cell Culture—Mammalian COS-7 cells were selected for transient expression of MAO B cDNA because they were found to contain no endogenous MAO B, as determined by ELISA, Western blot, and radiometric activity assays in initial experiments. Mammalian COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37°C with 5% CO2. Since this medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum) contains riboflavin, COS-7 cells grown in this medium were defined as riboflavin-containing COS-7 cells (Rib+ COS-7 cells). MAO B holoenzyme was obtained by expressing MAO B cDNA in Rib+ COS-7 cells. Mammalian COS-7 cells were also grown in riboflavin-free medium (riboflavin-free Dulbecco's modified Eagle's medium + 10% dialyzed fetal bovine serum, Life Technologies, Inc.) at 37°C with 5% CO2. Since this medium contains no riboflavin, COS-7 cells grown in this medium were defined as riboflavin-depleted COS-7 cells (Rib- COS-7 cells). MAO B holoenzyme was obtained by expressing MAO B cDNA in Rib- COS-7 cells. Rib foothoMAOB was obtained by expressing MAO B cDNA in Rib- COS-7 cells. COS-7 cells were grown in riboflavin-free medium for greater than 5 months without any detectable change in morphology.

Preparation of Mutant MAO B cDNA—Mutagenesis was carried out by the method of Dong and Nickoloff (22) using a Transformet Site-directed Mutagenesis kit (Clontech). Glu in position 34 was replaced with Asp (E34D), Gln (E34Q), or Ala (E34A), and Val in position 10 with Leu (V10I) as described by Kwan et al. (18). FAD Coupling in Intact Cells—Wild-type or mutant MAO B cDNAs were transiently transfected into COS-7 cells by electroporation (23) as described previously (18). Briefly, Rib+ or Rib- COS-7 cells were harvested during late log phase growth and resuspended to a concentration of 3.1 × 106 cells/ml in either riboflavin-containing or riboflavin-free medium, respectively. Wild-type or mutant MAO B cDNAs (15 μg) were added to 0.8 ml of cell suspension. In experiments where flavinization of wild-type and variant MAO B enzymes were studied, 20 μl of 0.8 mM unlabeled FAD was added to each tube. After 30 min of incubation, the Rib- COS-7 cell suspension was removed. Electroporation was carried out in a Bio-Rad GenePulser with a setting of 250 V and 500 microfarads. Cells were resuspended in 15 ml of riboflavin-containing or riboflavin-free medium and incubated at 37°C with 5% CO2. Transfected COS-7 cells were harvested at 48 h and homogenized in a lysis solution containing 20 mM Tris-HCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0. The homogenate was diluted with an equal volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. The homogenate was centrifuged at 500 revolutions/min for 5 min. The cell lysates were then determined (Fig. 1A) and eluted at a retention time identical to a FAD standard (Sigma). Triton X-100 (Pierce) was added to the lysate to give a final concentration of 0.25%, and the samples were allowed to stir for 50 min at 4°C to extract MAO B from the outer mitochondrial membrane. After Triton extraction, the lysate was centrifuged at 1300 × g for 5 min at 4°C to remove insoluble cell debris. The supernatant was then analyzed for protein concentration, MAO B concentration, enzymatic activity, and FAD coupling.

FAD Coupling in Vitro—apoMAO B was obtained by expressing MAO B cDNA in Rib+ COS-7 cells. The cells were then harvested and homogenized as described above. Half of the lysate was stirred in the presence of 0.25% Triton X-100 at 4°C for 50 min to extract apoMAO B from the outer mitochondrial membrane. The second half of the lysate was not extracted with Triton X-100 to permit MAO B to remain in the membrane. FAD-coupled apoMAO B was carried out for both fractions in reaction vials (200 μl) containing 10 μl of cell lysate, 50 mM phosphate buffer, and FAD (1.5 mM). Assays were also carried out in the presence of an energy mixture (10 mM ATP, 32 mM P-endopyruvate, and 2.4 μg of pyruvate kinase) and 25% glyceral in the reaction vials. Each sample was run in triplicate. After 1, 2, or 3 h of incubation at 30°C, each sample was assayed for MAO B activity as described below.

Subcellular Fractionation of COS-7 Cells—COS-7 subcellular fractionation was carried out with a modified method of Clark and Waterman (24). Transfected Rib- or Rib+ COS-7 cells were harvested, washed twice with ice-cold phosphate-buffered saline, and pelleted by microcentrifugation at 500 × g for 5 min. The cells were then homogenized in a Dounce homogenizer. Greater than 95% of the cells were lysed, as determined by trypan blue staining. The homogenate was diluted with an equal volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, to obtain a 0.25 mM final sucrose concentration and layered over 0.5 volume of 0.5 M sucrose pad (0.5 M sucrose in 1 mM Tris-HCl, 1 mM EDTA, pH 8.0). The solution was centrifuged at 5,000 revolutions/min for 3 min using a swinging bucket rotor (TL.55, Beckman TL-100) to remove cell debris and nuclei (P1 fraction). The supernatant plus the interface of the 0.5 M sucrose pad was again layered over another 0.5 M sucrose pad and centrifuged as above at 17,000 revolutions/min for 20 min to isolate mitochondria (P2 fraction). The resulting supernatant was centrifuged at 100,000 revolutions/min (TL-100.3, Beckman TL-100) for 30 min to sediment microsomes (P3 fraction). The final supernatant was referred to as the cytosol (P3 fraction). After subcellular fractionation, P1, P2, and P3 were determined by a MicroBCA kit (Pierce). All samples were then adjusted to equal protein concentrations and assayed for MAO B protein by ELISA using a modification of the method of Yeomanson and Billett (25) as described previously (18).

Enzyme Activity Determination—MAO B activity was assayed radiometrically by a modification of the method of Wurtman and Axelrod (26) as described below.

Immunoprecipitation of Holo-, Apo- or Variant MAO B—Transfected Rib- or Rib+ COS-7 cells were homogenized and extracted with 0.25% Triton X-100 for 50 min at 4°C. The cell lysates were centrifuged at 1300 × g for 5 min, and an aliquot of each supernatant was analyzed for MAO B concentration by ELISA. All supernatants were then adjusted to equal MAO B concentration, and immunoprecipitation was carried out using polyclonal goat anti-MAO B antibody (18). The immunocomplex was then dissolved in SDS-PAGE sample buffer and analyzed by Western blot or fluorography.

Western Blot Analysis—The immunoprecipitated proteins (obtained as described above) were subjected to electrophoresis in a 10% SDS-polyacrylamide gel and examined by Western blotting as described previously (18).

Fluorography—The immunoprecipitated proteins (obtained as described above) were subjected to electrophoresis in a 10% SDS-polyacrylamide gel and examined by Western blotting as described previously (18).

RESULTS

Synthesis of [14C]FAD and 8a-Hydroxyriboflavin—Retention times of riboflavin, FMN, FAD, and ATP standards (Sigma) were determined (Fig. 1A). FAD was observed to have a retention time of 10.0 min. Synthetic [14C]FAD had an identical retention time of 10.0 min (Fig. 1B) and eluted as a large sharp
A single peak. The radioactivity of the $[^{14}C]$FAD-containing fraction was determined in a scintillation counter, and the sample was dried to obtain a fine yellow powder.

Synthesis of 8α-hydroxyriboflavin was carried out by the method of McCormick (21). Synthetic 8α-hydroxyriboflavin was isolated from the reaction mixture (see "Materials and Methods") and rerun on HPLC using the same elution conditions as above. 8α-Hydroxyriboflavin gave a single sharp peak shown on the chromatogram. The authenticity of 8α-hydroxyriboflavin was confirmed by spectroscopic analysis (UV and mass spectrometry).

MAO B Expression Is Independent of FAD Cofactor—In order to study the covalent binding of FAD to human MAO B, it was necessary to develop a method for obtaining apoMAO B. To accomplish this, mammalian COS-7 (Rib⁺) cells were grown in riboflavin-free medium to deplete the endogenous riboflavin. MAO B cDNA was expressed sequentially at different time intervals in these cells during this riboflavin depletion process (Fig. 2). Each data point in Fig. 2 represents an individual expression assay. For each assay, a sample of COS-7 cells grown in riboflavin-free medium was transfected with MAO B cDNA via electroporation. Concurrently, Rib⁺ COS-7 cells were transfected with MAO B cDNA to serve as a control. Following incubation for 48 h, the cells were homogenized and assays were performed to determine protein concentration, MAO B concentration by ELISA using polyclonal antibodies, and MAO B activity using $[^{14}C]$benzylamine. Table I shows one set of analyses performed on these COS-7 cells that had been grown in riboflavin-free medium for 76 days (point 4 in Fig. 2). The enzymatic activity of MAO B expressed in these cells was 12.7% of the control, while the level of expression was essentially identical to the control (0.90 μg MAO B/mg protein versus 0.86 μg MAO B/mg protein). As seen in Fig. 2, the percentage of MAO B activity obtained in cells grown in riboflavin-free medium, as compared to MAO B activity obtained in Rib⁺ COS-7 cells, decreased with time in sequential experiments. Moreover, MAO B expression levels (0.95 ± 0.04 μg/mg protein) remained unchanged regardless of the extent of time the cells were grown in riboflavin-free medium. At a time interval of 100 days, the MAO B expressed in these cells had an activity of less than 5% of the MAO B holoenzyme activity obtained in transfected Rib⁺ COS-7 cells. Thus, cells grown in riboflavin-free medium for greater than 100 days were defined as riboflavin-depleted COS-7 cells (Rib⁺ COS-7 cells). Since MAO B was expressed in the absence of riboflavin and mammalian cells are incapable of synthesizing riboflavin, the MAO B expressed in Rib⁺ COS-7 cells represents apoMAO B. Rib⁺ COS-7 cells were subsequently used for expression of apoMAO B to study flavin coupling.

ApoMAO B Can Be Inserted into the Mitochondria—The distribution of protein, MAO B (apo- or holoenzyme) and MAO B activity in different subcellular compartments was studied in transfected Rib⁺- and Rib⁻ COS-7 cells by subcellular fractionation (Table II). The distribution of total protein in Rib⁺- or Rib⁻ COS-7 cells was essentially identical, with the largest amount of protein found in the cytosolic fraction. Approximately 80% of the holo- or apoMAO B enzymes were found in the mitochondrial fraction of the Rib⁺- or Rib⁻ COS-7 cells, respectively. The activity of expressed holo- or apoMAO B in various fractions was also determined. The activity distribution of holo-MAO B corresponded closely with the distribution of the enzyme with the majority of activity (about 80%) located in the mitochondrial fraction. Although the total activity of apoMAO B expressed in Rib⁻ COS-7 cells was dramatically reduced, the small amount of remaining activity was also found mainly in the mitochondrial fraction (about 83%).

FAD Coupling in Intact Cells—When exogenous FAD was added simultaneously with MAO B cDNA in Rib⁻ COS-7 cells during electroporation, restoration of MAO B activity was observed (Table III). Transfection of MAO B cDNA with exogenous FAD resulted in the recovery of 75% of MAO B holoenzyme activity, where MAO B holoenzyme activity refers to the enzymatic activity of MAO B holoenzyme expressed in Rib⁻ COS-7 cells. Assuming that 1 mol of FAD binds to 1 mol of MAO B subunit, the amount of FAD (16 nmol) used in the transfection was more than 500-fold the molar amount of expressed MAO B. A higher restoration of MAO B enzymatic activity could not be achieved by adding more exogenous FAD during electroporation. FMN or riboflavin were also capable of restoring approximately 75% of MAO B holoenzyme activity.

Fig. 1. A, elution chromatogram of standards. Pure ATP, FAD, FMN, and riboflavin (Sigma) were eluted on a C-18 Semi-Prep column with a linear gradient from 100% A/0% B (A = 10 mM (NH₄)₂HPO₄, pH 6.8; B = acetonitrile) to 60%/40% B in 20 min at a flow rate of 4 ml/min. The FAD standard was eluted at a retention time of 10 min. B, elution chromatogram of $[^{14}C]$FAD. The $[^{14}C]$FAD peak eluted at a retention time of 10 min using the same elution profile as above. C, elution chromatogram of 8α-hydroxyriboflavin. Synthetic 8α-hydroxyriboflavin was isolated from the reaction mixture (see "Materials and Methods") and rerun on HPLC using the same elution conditions as above. 8α-Hydroxyriboflavin gave a single sharp peak shown on the chromatogram. The authenticity of 8α-hydroxyriboflavin was confirmed by spectroscopic analysis (UV and mass spectrometry).
However, only 40% of MAO B holoenzyme activity was obtained by the addition of 8α-hydroxyriboflavin. As expected, the addition of NAD⁺ along with MAO B cDNA during electroporation did not yield active MAO B. Expressed MAO B enzymes, which were further analyzed by Western blot using our MAO B-specific monoclonal antibody MAO B-1C2 (Fig. 3). A band at approximately 59 kDa was observed in all lanes that contained apo- or holoMAO B.

FAD Coupling in Vitro—Expressed MAO B holoenzyme, which served as a control, remained fully active in a cell lysate for up to 3 h at 30°C. (Fig. 4). When exogenous FAD was added to Triton-extracted or non-extracted lysates, which contained mitochondrial membrane-free or mitochondrial membrane-bound apoMAO B, respectively, no MAO B catalytic activity was observed. Flavinylation of apoMAO B in vitro was also attempted in the presence of an energy mixture and glycerol, but no MAO B activity was obtained.

Glu³⁴ in the Dinucleotide-binding Site of MAO B Is Required for FAD Covalent Binding—To test the role of Glu³⁴ in FAD binding, we constructed and transiently expressed several mutant cDNAs to human MAO B in Rib⁺ COS-7 cells (18). Mutation of the glutamate residue at position 34 in MAO B to glutamine (E³⁴Q) or alanine (E³⁴A) resulted in a complete loss of activity, and a mutation to aspartate (E³⁴D) resulted in a 93% reduction in activity. The loss of activity was thought to be due to a loss of contact of the Glu³⁴ side chain with the ribose moiety of FAD. Whether the mutation resulted in misalign-

**Table I**

| Protein concentration (mg/ml) | Rib⁺ COS-7 (76 days) | Rib⁻ COS-7 (76 days) |
|-------------------------------|----------------------|----------------------|
| MAO B concentration (μg/ml)   | 3.50                 | 3.70                 |
| Expression (μg MAO B/mg protein) | 0.86                 | 0.90                 |
| Enzymatic activity (μmol/min/mg MAO B) | 1.10                 | 0.14                 |
| % of holoMAO B enzymatic activity | 100                  | 12.7                 |

*Enzymatic activity was expressed as μmol benzylamine/min/mg MAO B.

However, only 40% of MAO B holoenzyme activity was obtained by the addition of 8α-hydroxyriboflavin. As expected, the addition of NAD⁺ along with MAO B cDNA during electroporation did not yield active MAO B. Expressed MAO B enzymes (with or without cofactor additions) were further analyzed by Western blot using our MAO B-specific monoclonal antibody MAO B-1C2 (Fig. 3). A band at approximately 59 kDa was observed in all lanes that contained apo- or holoMAO B.

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The effect of adding different cofactors with MAO B cDNA into Rib\(^+\) COS-7 cells via electroporation during the transfection process

| Cofactor | MAO B expressed | Enzymatic activity\(^a\) | Percent of holdMAO B activity |
|----------|-----------------|--------------------------|-----------------------------|
|          | \(\mu g/\text{mg total protein}\) | \(\mu \text{mol/min/mg MAO B}\) |                           |
| HoloMAO B\(^b\) | 1.05 ± 0.25 | 1.12 ± 0.14 | 100 |
| ApoMAO B\(^c\) | 1.12 ± 0.04 | 0.02 ± 0.00 | 1.8 |
| Riboflavin | 0.90 ± 0.13 | 0.86 ± 0.06 | 76.1 |
| FMN | 0.81 ± 0.17 | 0.85 ± 0.03 | 75.2 |
| FAD | 0.85 ± 0.03 | 0.85 ± 0.01 | 75.2 |
| 5\(^b\)-OH Rib | 0.95 ± 0.08 | 0.45 ± 0.01 | 39.8 |
| NAD\(^+\) | 1.14 ± 0.06 | 0.02 ± 0.00 | 1.8 |

\(^a\) Enzymatic activity was expressed as \(\mu \text{mol benzylamine/min/mg MAO B}\).

\(^b\) HoloMAO B was obtained by expressing MAO B cDNA in Rib\(^+\) COS-7 cells, which served as a positive control.

\(^c\) ApoMAO B was obtained by expressing MAO B cDNA in Rib\(^-\) COS-7 cells without adding any cofactor.

Fig. 3. Western blot analysis. MAO B cDNA was transfected in Rib\(^-\) COS-7 cells with the addition of different cofactors via electroporation. Expressed MAO B enzymes were adjusted to equal concentrations using ELISA before immunoprecipitation. Immunoprecipitated enzymes were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting using the MAO family-specific monoclonal antibody MAO B-1C2. Lane 1, prestained molecular mass marker; lane 2, MAO B obtained from transfected Rib\(^+\) COS-7 cells, which served as a positive control. Lanes 3–8 contain MAO B obtained from transfected Rib\(^-\) COS-7 cells with or without the addition of different cofactors. Lane 3, riboflavin; lane 4, FMN; lane 5, FAD; lane 6, 5\(^b\)-hydroxyriboflavin; lane 7, NAD\(^+\); lane 8, no cofactor addition; lane 9, untransfected Rib\(^-\) COS-7 cells; lane 10, biotinylated molecular mass marker.

Enzymatic activity was determined by the intensity of the bands at a molecular mass of about 59 kDa on the fluorogram (Fig. 5). The wild-type and the control variant (V10I) were each observed to have a dark band, which indicated that \(^{14}\text{C}\)FAD was incorporated into the enzyme. Variants E34A and E34Q did not exhibit bands, indicating that FAD was absent. A faint band was observed using a densitometer with variant E34D, indicating that a small amount of \(^{14}\text{C}\)FAD was incorporated (about 10% of wild-type). Thus, all variants of MAO B at residue 34 showed either a dramatic decrease or total loss of \(^{14}\text{C}\)FAD incorporation and a corresponding loss of enzymatic activity.

**DISCUSSION**

Flavinylation of MAO B has been difficult to study in the past because FAD is covalently attached to Cys\(^{397}\), and this cofactor cannot be removed without sacrificing MAO B activity (28). For mammalian flavoproteins, the conventional approach has been to study flavinylation in animals. Rabbits or mice were fed riboflavin-free diets to deplete the endogenous riboflavin, and the animals were sacrificed to obtain the organs or tissues for analysis (29). This method is time-consuming, tedious, and subject to variation due to individual differences in animals. We have now developed a convenient and rapid method to study flavinylation of eucaryotic proteins in Rib\(^-\) COS-7 cells. Since COS-7 cells are not capable of synthesizing riboflavin, enzymes expressed in these cells lack flavin cofactors. Rib\(^+\) COS-7 cells were used to produce apoMAO B to study the steps involved in flavinylation. In other related studies, Nishikimi et al. (30) produced the apoenzyme of \(\gamma\)-gulono-\(\gamma\)-lactone oxidase in a baculovirus expression system in which riboflavin levels were reduced. Enzymatic activity was observed upon addition of FAD, but no covalently bound FAD could be obtained using this system.

The expression level (0.95 ± 0.04 \(\mu g/\text{mg protein}\)) of MAO B in transfected Rib\(^-\) COS-7 cells remained unchanged in sequential transfections during the process of riboflavin depletion (Fig. 2). This observation indicates that MAO B expression is not dependent upon riboflavin or FAD concentrations in the cell. The level of expressed MAO B was determined by ELISA, which is based upon epitope recognition by antibodies and is susceptible to major conformational changes. Both MAO B-1C2 monoclonal antibody and goat anti-MAO B polyclonal antibodies were capable of recognizing apoMAO B. In another study, the apoenzyme of bacterial 6-hydroxy-\(\alpha\)-nicotine oxidase, which contains covalently bound FAD in its holoenzyme, is not recognized by a molecular chaperone as aberrant (31). We suggest that the conformation of the apoMAO B, like apo-6-hydroxy-\(\alpha\)-nicotine oxidase, may be similar to that of the native holoenzyme.

Mitoma and Ito (32) found that the mitochondria targeting sequence of MAO B is located on the C terminus of the molecule. Deletion of the C-terminal 28 amino acids of MAO B abolished transfer of the enzyme to the mitochondria, while deletion of the N-terminal 55 amino acids had no effect on mitochondrial targeting. Furthermore, an expressed hybrid protein, in which the C-terminal 29 amino acids of MAO B was fused to the hydrophilic portion of cytochrome \(b_6\), was localized in the mitochondria. In our work, we found that apoMAO B expressed in Rib\(^-\) COS-7 cells was localized in the mitochondrial fraction of cell lysates (Table I), indicating that bound FAD is not necessary for MAO B insertion into the mitochondria membrane. These results are consistent with those of Mitoma and Ito (32) and support the notion that the target C-terminal sequence alone is sufficient for insertion into the membrane.

One advantage of using Rib\(^-\) COS-7 cells to study flavinylation is that exogenous FAD or its derivatives can be introduced with MAO B cDNA into the cells during the transfection process. The enzymatic activity of MAO B with the addition of
different flavins can be determined and compared with MAO B holoenzyme expressed in Rib<sup>-1</sup> COS-7 cells (Table III). Addition of FAD resulted in the restoration of about 75% of holoMAO B activity. Interestingly, approximately 75% of holoMAO B activity was also achieved by the addition of riboflavin or FMN to transfected Rib<sup>-</sup> COS-7 cells, suggesting the presence of abundant levels of cellular FAD synthetase. The addition of 8α-hydroxyriboflavin gave an enzyme with 40% activity of the control, which raises the possibility that this flavin may represent an intermediate in the activation of FAD (discussed below). Full recovery of MAO B enzymatic activity obtained in Rib<sup>-1</sup> COS-7 cells was not achieved for reasons that remain unknown. In related studies, however, Brandsch and Bichler (33) found that the covalent flavinylation of 6-hydroxy-D-nicotine oxidase in vitro required specific effectors (phosphorylated three carbon compounds), such as glycerol 3-phosphate, glyceraldehyde 3-phosphate, or glycerate 3-phosphate. Effectors that could enhance the activity of MAO B have not been identified. We speculate that the achievement of only 75% of activity may be due to a slight change in metabolism of Rib<sup>-</sup> COS-7 cells which have been adapted to grow in riboflavin-free medium for more than 100 days.

Although it is known that FAD is covalently attached to active MAO B molecules, the form of the flavin which initially binds to MAO B in vivo has not previously been established. Theoretically, riboflavin or FMN could first bind to apoMAO B followed by phosphorylation and adenylation, respectively, to form FAD. If riboflavin or FMN is the form that initially binds to apoMAO B, we would expect FAD binding to be much less effective than riboflavin or FMN. Since MAO B activity was recovered to approximately the same extent (75%) using FAD, FMN, or riboflavin, we conclude that the flavin moiety which initially binds to apoMAO B is FAD. Apparently, FAD synthetase in these cells rapidly converted riboflavin and FMN to FAD by phosphorylation and adenylation, respectively, prior to incorporation. The presence of FAD was confirmed by measuring the covalent binding of [14C]FAD to MAO B.

The covalent attachment of FAD to Cys<sup>397</sup> could be autocatalytic or catalyzed by an as yet uncharacterized enzyme. In either case, one of the participants, the 8α-methyl group of the flavin moiety or Cys<sup>397</sup> of MAO B, must be activated prior to coupling. Although the nucleophilicity of the Cys<sup>397</sup> residue may be influenced by surrounding amino acid residues, it is difficult to envision that a cysteine derivative would react with the inert 8α-methyl group of the flavin moiety. From a chemical point of view, activation of the 8α-methyl group appears essential for coupling of FAD to apoMAO B. An enzymatically facilitated pathway for the incorporation of FAD into flavoproteins has been proposed by Decker (11) in which a flavin cofactor may be enzymatically activated by hydroxylation of the 8α-methyl group, followed by (pyro)phosphorylation (Fig. 6). Since the (pyro)phosphosphate is a good leaving group, a simple S<sub>N</sub>2 reaction could facilitate the formation of the thioether between the flavin moiety and MAO B. To test this hypothesis, we synthesized the putative activated intermediate 8α-hydroxyriboflavin and determined in Rib<sup>-1</sup> COS-7 cells its ability to generate MAO B enzymatic activity (synthesis of 8α-phosphate-riboflavin was also attempted, but was unsuccessful because the highly reactive hydroxyl groups on the ribityl moiety were also phosphorylated). If the flavin derivative is truly an intermediate, we assumed that it would be capable of entering the flavinylation pathway to produce active MAO B. MAO B activity was obtained, but the level was only about half of that obtained with the addition of riboflavin (Table III). One possible explanation for the low activity is that a flavinylation enzyme binds the flavin substrate and catalyzes hydroxylation and phosphorylation sequentially without release of the 8α-hydroxy intermediate. Thus, the 8α-hydroxy intermediate may not be recognized as efficiently as riboflavin during the initial binding step. Alternatively, the covalent flavinylation of MAO B may be autocatalytic, since the unactivated form of the flavins (riboflavin, FMN, and FAD) has higher efficiency of incorporation into apoMAO B than the putative activated form.
When FAD and MAO B cDNA were added simultaneously to the translation process, flavinylating enzymes which could have catalyzed the coupling reaction in MAO. However, when FAD was added to apoMAOB, attempts to regenerate active flavinylated MAO B were unsuccessful, even in the presence of various energy mixtures and glycerol (Fig. 4). Furthermore, when apoMAOB was extracted from the mitochondrial membrane, attempts to regenerate active flavinylated MAO B were unsuccessful, even in the presence of various energy mixtures and glycerol (Fig. 4). The inability to couple FAD to apoMAOB in vitro may indicate that flavinylation occurs as a cotranslational process during elongation of nascent chains to form functionally competent MAO B molecules.

Our previous work demonstrated that Glu34 in the dinucleotide binding motif was critical for MAO B catalytic activity (18). Two variants at Glu34 (E34A and E34Q) were devoid of enzymatic activity, and another conservative variant, E34D, had only 7% of the wild-type activity. It was not known, however, whether the role of Glu34 is confined to alignment of FAD for participation in the oxidation-reduction cycle of catalysis, or is involved in FAD incorporation. In this study, we show that the loss of activity in Glu34 variants is linked to the inability to bind FAD covalently (Fig. 5).

Since FAD binds to two regions of MAO B (noncovalently at Glu34 and covalently at Cys397), the absence or low levels of FAD incorporation into Glu34 variants reveals an important feature of the flavinylation process. If FAD coupling occurred by initial covalent attachment to Cys397, Glu34 variants would contain covalently bound FAD, but would be inactive because FAD could not interact properly at the dinucleotide-binding site. Since we find little or no covalent binding of FAD in the Glu34 variants, we conclude that FAD binds to Glu34 first. We propose that the dinucleotide-binding site (including Glu34) provides a topological dock for the initial binding of FAD and is instrumental in the delivery of FAD to Cys397 in MAO B. The incoming flavin cofactor, which is initially bound to the dinucleotide-binding site of MAO B, could be held for a finite time in a position which places the 8α-methyl group of FAD in exact and close proximity to Cys397 to facilitate covalent flavinylation.

The dinucleotide-binding sites in various flavoproteins contain high sequence identity (17). However, the location within the primary structure varies from protein to protein, indicating that this site performs an autonomous function of cofactor binding within a heterologous group of flavoproteins. Furthermore, in many flavoproteins containing dinucleotide-binding sites, FAD is not covalently bound (17). Our finding that the dinucleotide-binding site in MAO B plays a role in initial FAD binding indicates that this site alone is sufficient for a flavoprotein to bind a flavin cofactor. The significance of covalent linkage between FAD and its flavoenzyme remains unresolved, but covalent binding could play a role in enzyme integrity and stability, substrate stereospecificity, cofactor economy, or redox potentials. Understanding the MAO flavinylation process may lead to the design of MAO enzymes with high redox potentials for better catalysis and to the rational design of MAO inhibitors. Since MAO inhibitors have long been used for the treatment of various psychiatric and neurological disorders, including depression (35) and Parkinson's disease (36), our studies on flavinylation may lead to the development of therapeutic drugs (analogs of FAD) for these disorders.

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