Mutagenesis at a Highly Conserved Tyrosine in Monoamine Oxidase B Affects FAD Incorporation and Catalytic Activity†

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ABSTRACT: Monoamine oxidase B (MAO B), an integral protein of the outer mitochondrial membrane, catalyzes the oxidative deamination of various neuroactive and vasoactive amines. A covalently bound FAD cofactor at Cys-397 of human MAO B is required for the oxidation of the amine substrates. In addition to the covalent binding site, MAO B also contains a noncovalent FAD binding region (residues 6−34) known as the dinucleotide binding motif. Previously, we have shown that Glu-34 is required for catalytic activity, presumably by forming a hydrogen bond between the carboxylate group of glutamate and the 2′-hydroxyl group of ribose in the AMP moiety of FAD. In this work, we have identified a third FAD binding site in MAO B (residues 39−46) by sequence comparisons to other flavoenzymes. The conserved sequence contains a tyrosine residue (Tyr-44) which, based on the X-ray crystal structure of ferredoxin−$NADP^+$ reductase, is postulated to participate in FAD binding through van der Waals contact with the isoalloxazine ring and a hydrogen bond to the 3′-hydroxy of the ribityl moiety. To test the postulated role of this tyrosine residue, site-directed mutants that encode substitutions at Tyr-44 were prepared and expressed in mammalian COS-7 cells. Variant MAO B enzymes were then characterized with respect to enzymatic activity and [^{14}C]FAD incorporation. Substitution of tyrosine with phenylalanine had no effect on MAO B activity or the level of [^{14}C]FAD incorporation compared to the wild-type enzyme, indicating that the hydroxyl group of the tyrosine residue was not essential at residue 44. Substitution of tyrosine with serine or alanine, however, which do not have an aromatic ring, resulted in a dramatic decrease in enzymatic activity and FAD incorporation. We conclude that the aromatic ring of the tyrosine residue at position 44 is required for FAD binding and catalytic activity of MAO B.

Monoamine oxidases A and B (MAO A and B,[1] EC 1.4.3.4) are the major enzymes that catalyze the oxidative deamination of neuroactive and vasoactive amines in the central nervous system and peripheral tissues of mammals (Von Korff, 1979). These isozymes are located in the outer mitochondrial membrane (Greenawalt & Schnaitman, 1970) and can be distinguished by differences in substrate preference and inhibitor specificity (Dostert et al., 1989), tissue and cell distribution (Weyler et al., 1990), and immunological properties (Levitt et al., 1982; Westlund et al., 1985, 1988; Denney et al., 1982; Kochersperger et al., 1985). Although not identical, the nucleotide and deduced amino acid sequences of human MAO A and B are strikingly similar (Bach et al., 1988; Hsu et al., 1988; Chen et al., 1993), and their genes contain identical exon−intron organization (Grimsby et al., 1991; Chen et al., 1991; Kwan et al., 1992).

Oxidation of amines by MAO B is coupled to the reduction of an obligatory FAD cofactor. MAO B contains one FAD molecule per subunit (Weyler, 1989), covalently linked at Cys-397 (Kearney et al., 1971; Bach et al., 1988). FAD is attached through an 8α-methyl-*S*-cysteinyl bond in MAO B and in flavocytochromes *c*-552 and *c*-553 (Kenney & Singer, 1977; Kenney et al., 1977). All other proteins with covalently bound FAD are linked through the 8α-methyl-N-1 or -N-3 of histidine (Edmondson & Kenney, 1976; Mohler et al., 1972) or to a tyrosine residue (McIntire et al., 1980). The sequence of events required for covalent attachment of FAD to these enzymes remains unknown.

A second site of FAD binding, known as the dinucleotide binding domain, is located in the N-terminal region of MAO B, spanning residues 6−34. The dinucleotide binding motif is thought to consist of a β_1-sheet−α-helix−β_2-sheet (β_1−α−β_2) beginning at Asp-6 with a highly conserved Gly-x-Gly-x-x-Gly sequence in the turn between the first β-sheet and the α-helix. The second β-sheet ends with Glu-34 in which the γ-carboxylate group is postulated to bind through a hydrogen bond to the 2′-hydroxy group of ribose in the AMP moiety of FAD. Mutagenesis at residue 34, which replaced glutamate with alanine, aspartate, or glutamine, resulted in a dramatic loss of MAO B catalytic activity (Kwan et al., 1995).

Sequence comparisons of MAO A and B to other flavoproteins indicate the presence of a third FAD binding site (Figure 1). In MAO B, this segment (residues 39−46) of high sequence identity is located in the N-terminal region

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[1] Abbreviations: FAD, flavin adenine dinucleotide; MAO A, monoamine oxidase A; MAO B, monoamine oxidase B; FNR, ferredoxin−NADP reductase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; ELISA, enzyme-linked immunosorbance assay.

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Monoamine oxidase B (human)	39-	V	G	G	R	T	Y	T	L
Monoamine oxidase A (human)	90-	V	G	G	R	T	Y	T	I
Ferredoxin-NADP$^+$ reductase (spinach)	90-	H	K	L	R	L	Y	S	I
NADH-nitrate reductase (*A. thaliana*)	709-	L	C	L	R	A	Y	T	P
NADH-cytochrome b5 reductase (human)	63-	L	V	V	R	P	Y	T	P
NADPH-cytochrome P450 reductase (rat)	451-	L	Q	A	R	Y	Y	S	I
NADPH-sulfite reductase (*E. coli*)	383-	L	T	P	R	L	Y	S	I

FIGURE 1: Sequence comparison of a putative FAD binding site in monoamine oxidase B. A conserved tyrosine, corresponding to residue 44 in MAO B, is enclosed in the box. The analogous tyrosine in spinach ferredoxin–NADP$^+$ reductase was observed by crystallographic data to interact with the isoalloxazine moiety of FAD. Sequences for individual proteins were obtained as follows: MAO A and B (Bach et al., 1988); ferredoxin–NADP$^+$ reductase (Karplus et al., 1984); NADH–nitrate reductase (Crawford et al., 1988); NADH–cytochrome b_5 reductase (Yubisui et al., 1984); NADPH–cytochrome P450 reductase (Porter & Kasper, 1985); and NADPH–sulfite reductase (Ostrowski et al., 1989).

of the enzyme in close proximity to the FAD dinucleotide binding motif (residues 6–34). The homologous sequence occurs in NADPH–sulfite reductase, NADH–nitrate reductase, NADPH–cytochrome P-450 oxidoreductase, ferredoxin–NADP$^+$ reductase (FNR), and NADH–cytochrome b_5 reductase and is thought to be involved in noncovalent FAD binding in these enzymes (Karplus et al., 1984; Porter & Kasper, 1986). The crystal structure of FNR has been determined, and the entire FAD binding domain was characterized (Karplus et al., 1991). This domain was shown to consist of an antiparallel β-barrel not previously observed in other flavoproteins. A short sequence containing a tyrosine residue (Figure 1) was shown to reside within a β-sheet in close proximity to FAD. The tyrosine residue is thought to make extensive van der Waals contact with the isoalloxazine moiety and to form a hydrogen bond with the 3′-hydroxy of the ribityl moiety of FAD. Since the similar sequence in MAO B is in close proximity to the known FAD dinucleotide binding region, it seems possible that the conserved tyrosine (Tyr-44) has a similar function in MAO B.

To test the hypothesis that this tyrosine interacts with FAD in MAO B, mutants that encode substitutions at Tyr-44 were prepared and expressed in mammalian COS-7 cells. Substitutions (Tyr to Phe, Ser or Ala) were selected to permit analysis of the aromatic and hydrogen bonding roles of the tyrosine residue with FAD. We show that the aromatic ring of the tyrosine residue is essential for FAD binding and catalytic activity in MAO B, but hydrogen bonding through the hydroxyl group is not required. On the basis of our findings, we have constructed a model of how FAD interacts with three binding sites in MAO B, and we have proposed a sequence of events that occur during the flavinylation process.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutagenesis was performed by the method of Deng and Nickoloff (1992) using a "Transformer Site-directed Mutagenesis" kit (Clontech) as described previously (Kwan et al., 1995). The mutagenic primers and the corresponding amino acid changes are shown in Figure 2. Tyr at position 44 was replaced with Phe in Y44F, Ser in Y44S, and Ala in Y44A. The Leu residue at position 46 was replaced with Val in L46V. All mutagenic primers were designed to create a new restriction site, without altering the coding sequence of any other amino acids, for the purpose of screening.

Construction of mutants Y44F and L46V was carried out in the pBluescript SK vector (Stratagene) with human MAO B cDNA inserted at the *Eco*RI site. A *Hpa*I restriction site was introduced into a 30-mer selection primer to replace the only *Kpn*I site in the plasmid vector. For initial screening of mutants, clones were picked from an NZCYM/ampicillin plate and inoculated into a microcentrifuge tube containing 0.5 mL of NZCYM/ampicillin and incubated with shaking for 4 h at 37 °C. An aliquot (50 μL) of the miniculture was stored at 4 °C for future propagation. The remaining 450 μL was sedimented and processed by the alkaline lysis method in a total volume of 45 μL. The supernatant was ethanol-precipitated and the DNA screened by restriction analysis for the presence of a new restriction site created by the mutagenic primer. The mutant cDNAs were then subcloned into the pSVK3 expression vector (LKB–Pharmacia) at the *Eco*RI site and screened for the sense orientation.

Construction of mutants Y44A and Y44S was carried out directly in the expression vector pSVK3 with human MAO B cDNA inserted into the *Eco*RI site. A *Hpa*I restriction site was introduced into a 29-mer selection primer to replace the only *Kpn*I site in the pSVK3 vector. The mutant clones were screened for the presence of the new restriction site created by the mutagenic primer as described above. The presence of the correct mutations in all mutant cDNAs was confirmed by double-stranded dideoxy DNA sequencing (Sanger et al., 1977). Both wild-type and mutant plasmid DNAs were purified through CsCl gradients prior to transfection studies.

Expression of Wild-Type or Mutant MAO B cDNAs. Mammalian COS-7 cells used for MAO B expression were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C. Riboflavin-depleted COS-7 cells were generated by maintaining these cells in riboflavin-free DMEM–FBS (Gibco) for greater than 100 days. Transient transfection by electroporation (Zimmerman & Vienken, 1982) of wild-type or mutant MAO B cDNAs into COS-7 cells was carried out as described previously (Kwan et al., 1995). Briefly, cells were harvested during late log phase growth and resuspended in DMEM supplemented with 10% FBS. Wild-type or mutant cDNA (15 μg) was then electroporated into the cells (2.5×10^6 cells/0.8 mL of DMEM–FBS). In experiments where flavinylation of wild-type or variant MAO B was studied, 20 μL of 0.8 mM [^{14}C]FAD and 15 μg of MAO B cDNA were simultaneously electroporated into riboflavin-depleted COS-7 cells in riboflavin-free medium (2.5×10^6 cells/0.8 mL). Transfected cells were resuspended in 15 mL of DMEM/FBS (or riboflavin-free DMEM–FBS) and incubated at 37 °C with 5% CO_2. Cells were harvested at 48 h and homogenized in a lysis solution (500 μL) containing 20 mM Tris-HCl, 1.0 mM EDTA, and 0.5 mM PMSF (phenylmethanesulfonyl fluoride), pH 8.0. Extraction of MAO B from each sample was carried out by addition of Triton X-100 to a concentration of 0.25% and stirring for 30 min at RT.

Enzyme-Linked Immunosorbance Assay (ELISA). Protein concentrations of samples containing wild-type or variant MAO B were determined by a Micro-BCA kit (Pierce). All samples were then adjusted to equal protein concentration

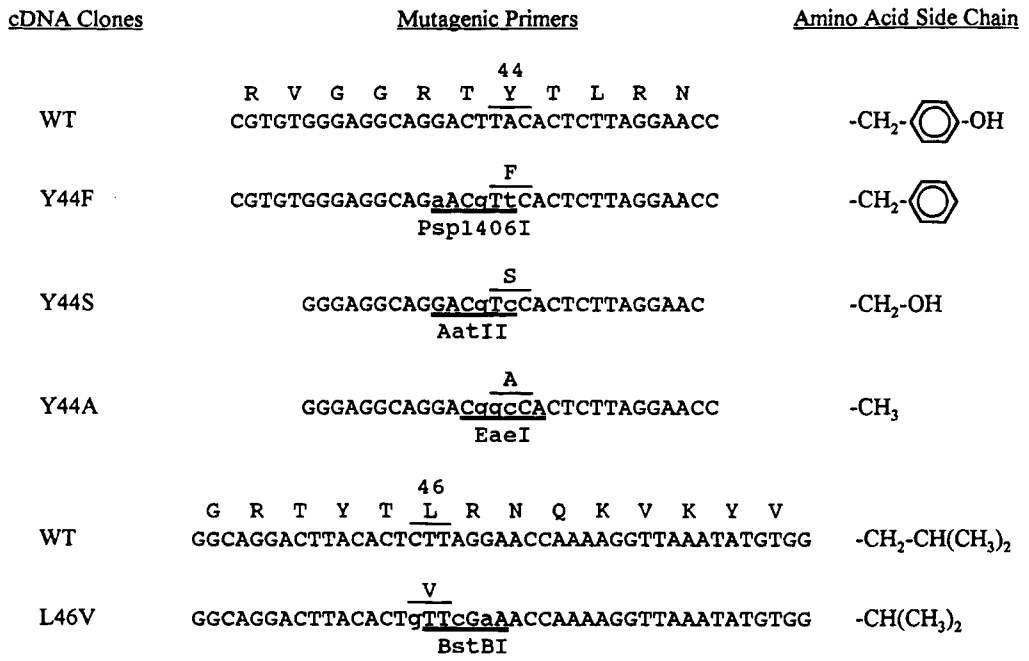

FIGURE 2: Nucleotide sequences of mutagenic primers in site-directed mutagenesis studies. Lowercase letters indicate base substitutions. The codons for wild-type and mutants at positions 44 and 46 are indicated by a single line above the nucleotides. Base substitutions which do not alter the amino acid coding sequence were also included in each mutagenic primer to create a new restriction site (double underline) for the purpose of screening. Side chains corresponding to amino acid substitutions are also shown.

prior to quantitation of MAO B by ELISA using a modification of the method of Yeomanson and Billett (1992) as previously described (Kwan et al., 1995). Expression levels of wild-type or variant MAO B were determined in duplicate for three separate experiments.

Enzyme Activity Determination. MAO B activity was measured by a modification of the method of Wurtman and Axelrod (1963). This modification allows accurate activity measurements in small amounts of MAO B (as low as 5 ng) expressed in cultured cells. For quantitative comparisons of wild-type and variant MAO B, all samples were adjusted to equal protein concentration, and 10 μL of cell lysate (containing wild-type or variant MAO B) was incubated in an assay mixture (200 μL) containing 0.05 M sodium phosphate buffer, pH 7.4, 3.6 nmol of 55 mCi/mmol [^{14}C]-benzylamine hydrochloride, and 10 nmol of unlabeled benzylamine. For each experiment, controls were assayed concurrently, including either assay buffer or lysate from nontransfected COS-7 cells. After incubation at 37 °C for 9 min, all reactions were terminated with 6 N HCl (25 μL) and placed on ice for 2 min. The reaction product was extracted with toluene (500 μL) and centrifuged at 10000*g* for 5 min. The organic phase was counted in liquid scintillation fluid (Bio-Safe) in a Beckman LB3801 model liquid scintillation counter. The activities of wild-type and variant MAO B enzymes were determined in duplicate in three separate experiments.

Immunoprecipitation of Wild-Type and Variant MAO B. Transfected COS-7 cells were homogenized in 300 μL of 20 mM Tris-HCl, 1 mM EDTA, and 0.5 mM PMSF, pH 8.0, and MAO B was extracted with 0.25% Triton X-100 for 30 min at 4 °C. After centrifugation at 1300*g* for 5 min, an aliquot of each supernatant was assayed by ELISA, and all supernatants were adjusted to equal MAO B concentrations. The supernatants (300 μL) were then incubated with 10 μg of goat polyclonal anti-MAO B antibody overnight at 4 °C. Protein G−Sepharose beads were added (50 μL), and the samples were further incubated for 3 h. The protein G−Sepharose/goat antibody/MAO B immunocomplex was collected by centrifugation at 10000*g* for 20 s, and washed 6 times with 20 mM Tris buffer, pH 8.0. The immunocomplex was eluted with SDS−PAGE sample buffer and subsequently 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 analyzed by Western blotting as previously described (Kwan et al., 1995).

Fluorography. Immunoprecipitated wild-type and variant MAO B (obtained as described above) were subjected to electrophoresis in a 10% SDS−polyacrylamide gel. The gel was fixed in 7% acetic acid, 10% methanol, and 83% H_2O for 1 h and processed for fluorography as described by Bonner and Laskey (1974). The dried gel was exposed to Kodak X-OMAT AR film at −80 °C.

RESULTS

All expression studies were carried out in mammalian COS-7 cells, since they do not contain any detectable endogenous MAO B, as determined by ELISA, Western blot, and radiometric activity assays (Kwan et al., 1995). Expression parameters were optimized for wild-type MAO B prior to studying variant MAO B or apo-MAO B. Optimal transient expression was achieved by electroporating 15 μg of cDNA into COS-7 cells at log phase growth (2.5×10^6 cells/800 μL of DMEM−FBS). Maximum expression of MAO B was obtained 48 h after transfection, yielding approximately 1.0 μg of MAO B/mg of cellular protein.

Expression levels of wild-type and variant MAO B enzymes are shown in Table 1. The amount of MAO B expressed for all variants shows no significant difference from that of wild-type MAO B (897 ± 17 ng of MAO B/mg of protein). The average protein concentration in the 500

Table 1: Comparison of Expression Levels and Activity of Wild-Type and Variant MAO B[a]

enzyme	MAO B concn of (ng of MAO B/mg of protein)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]	enzymatic act. [μmol min^{-1}(mg of MAO B)$^{-1}$]
wild-type	897 ± 17 (100)[b]	1.01 ± 0.067 (100)[b]	1.13 ± 0.078 (100)[b]
Y44F	873 ± 56 (97)	0.92 ± 0.080 (91)	1.05 ± 0.036 (93)
Y44S	891 ± 36 (99)	0.02 ± 0.003 (2)	0.02 ± 0.003 (2)
Y44A	864 ± 19 (96)	0.01 ± 0.007 (1)	0.01 ± 0.008 (1)
L46V	896 ± 26 (100)	0.84 ± 0.065 (83)	0.94 ± 0.090 (83)

[a] Samples were run in duplicate in each experiment. Each value represents the mean ± SE from three separate experiments. [b] Percent of wild-type values is shown in parentheses.

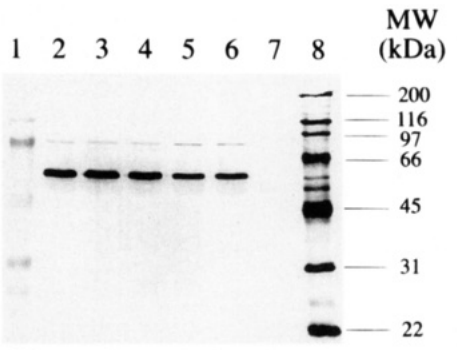

FIGURE 3: Western blot analysis. Wild-type and mutant MAO B cDNAs were transfected in COS-7 cells. Expressed wild-type and variant MAO B enzymes were adjusted to equal concentrations based on ELISA results before immunoprecipitation. The immunoprecipitated enzymes were separated on 10% SDS−PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting using the MAO B specific monoclonal antibody MAO B-1C2. Lane 1, prestained MW markers; lane 2, wild-type MAO B; lane 3, Y44F MAO B; lane 4, L46V MAO B; lane 5, Y44A MAO B; lane 6, Y44S MAO B; lane 7, untransfected COS-7 cells; lane 8, biotinylated MW marker.

μL of cell lysates was 2.23 ± 0.24 mg/mL. Thus, each expression resulted in a yield of approximately 1 μg of MAO B.

Expressed MAO B was further identified by Western blotting. Wild-type and variant MAO B enzymes were adjusted to equal concentrations based on ELISA results, and immunoprecipitated with goat anti-MAO B polyclonal antibodies. The immunoprecipitated wild-type and variant MAO B enzymes were positively identified using a monoclonal antibody specific to MAO B (MAO B-1C2). As shown in Figure 3, wild-type and variant MAO Bs have a band of equal intensity corresponding to 59 kDa, whereas the untransfected COS-7 cells show no MAO B band.

The activity of expressed wild-type and variant MAO B was determined in duplicate for three separate experiments by a radiometric assay using benzylamine as substrate (see Experimental Procedures). Substitution of Tyr-44 with phenylalanine in Y44F resulted in only a slight decrease (93% of wild-type) in enzymatic activity (Table 1). However, substitutions of tyrosine to serine and alanine in Y44S and Y44A, respectively, resulted in a dramatic loss of MAO B activity. A fourth mutant, L46V, was constructed to serve as a control. This mutant cDNA encodes a variant protein containing Val-46 in place of Leu-46 at a site near the critical tyrosine residue. Because Val-46 was not highly conserved among the other flavoenzymes (Figure 1) and has not been postulated to play a role in FAD binding, a mutation at this site was not expected to have a dramatic effect on MAO B activity. In fact, the L46V variant showed only a slight loss of enzymatic activity (83% of wild-type). Furthermore, the enzymatic activities (micromoles per minute per milligram of MAO B) of all the variants closely correlated with their specific activities (micromoles per minute per milligram of protein). MAO B variants retaining the aromatic moiety at position 44 retained enzymatic activity, whereas those without the aromatic moiety (Y44S and Y44A) were nearly devoid of enzymatic activity.

To study the flavinylation of wild-type and variant MAO B enzymes, riboflavin-depleted COS-7 cells were produced by maintaining cells in riboflavin-free medium for greater than 100 days. Wild-type MAO B cDNA was transfected into these cells at 1 week intervals during the process of riboflavin depletion to monitor the effect on MAO B expression and activity. Expression levels of MAO B remained constant (approximately 1 μg/mg of protein) throughout the process of riboflavin depletion. MAO B activity in sequential transfections, however, decreased rapidly as the endogenous riboflavin was depleted from the COS-7 cells. After 100 days of riboflavin depletion, transiently expressed apo-MAO B was completely devoid of activity. Cells grown continuously in riboflavin-free medium for greater than 5 months showed no detectable change in morphology. Furthermore, trypan blue staining did not detect the presence of damaged cells.

Riboflavin-depleted COS-7 cells cannot synthesize FAD. Consequently, expressed MAO B is devoid of an FAD cofactor. Flavinylation of wild-type and variant MAO B enzymes was studied in these cells by simultaneous electroporation of [^{14}C]FAD with MAO B cDNA. The expressed enzymes were first adjusted to equal MAO B concentrations based on ELISA, followed by immunoprecipitation using goat anti-MAO B polyclonal antibodies. The immunoprecipitated MAO B was then subjected to SDS−PAGE and analyzed by fluorography. The amount of [^{14}C]FAD incorporated into wild-type or variant MAO B was determined by the intensity of banding on the fluorogram. As seen in Figure 4, the wild-type and variants L46V and Y44F were capable of incorporating [^{14}C]FAD, as observed by dark bands of equal intensity at a molecular mass of about 59 kDa. However, Y44S and Y44A, which do not contain an aromatic moiety at position 44, showed only very faint bands.

DISCUSSION

We have examined the role of one amino acid (Tyr-44) in MAO B which is highly conserved among several flavoenzymes (Figure 1). One of these enzymes, spinach ferredoxin−$NADP^+$ reductase (FNR), has been crystallized, and the three-dimensional structure of the FAD binding region was elucidated (Karplus et al., 1991). The conserved tyrosine residue in FNR was found to make extensive van der Waals contact with the isoalloxazine moiety of the FAD

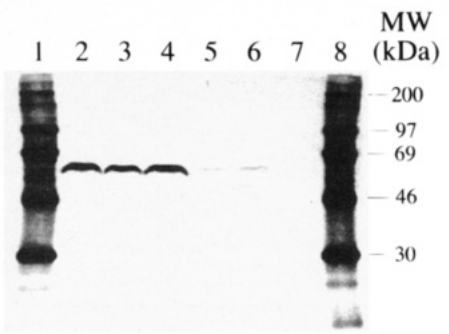

FIGURE 4: Fluorogram. Wild-type and mutant cDNAs were transfected in riboflavin-depleted COS-7 cells with the addition of exogenous [^{14}C]FAD during electroporation. Expressed wild-type and variant MAO B enzymes were adjusted to equal concentrations based on ELISA before immunoprecipitation. The immunoprecipitated enzymes were separated on 10% SDS−PAGE and analyzed by fluorography. Lane 1, ^{14}C-methylated MW marker; lane 2, wild-type MAO B; lane 3, Y44F MAO B; lane 4, L46V MAO B; lane 5, Y44A MAO B; lane 6, Y44S MAO B; lane 7, untransfected riboflavin-depleted COS-7 cells; lane 8, ^{14}C-methylated MW marker.

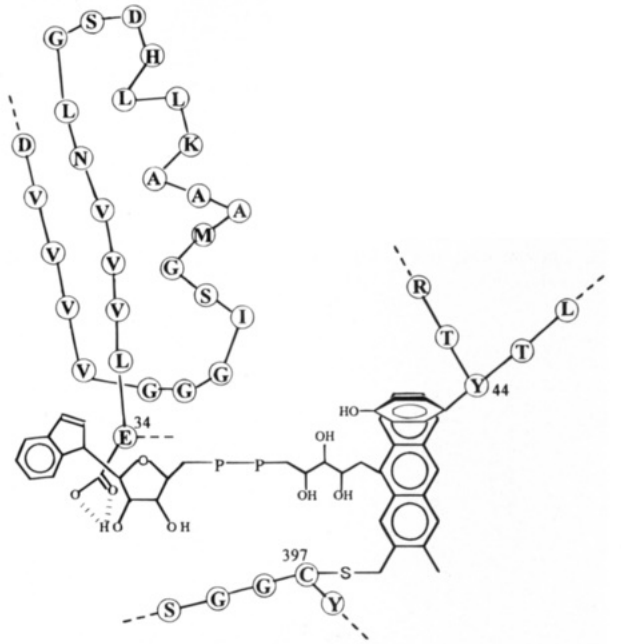

FIGURE 5: Model of FAD interacting with three distinct binding sites of MAO B. Amino acids 6−34 comprise the dinucleotide binding motif in MAO B. The glutamate residue of MAO B at position 34 forms a hydrogen bond with the 2′-hydroxy group of ribose in the AMP moiety of FAD. Cys-397 forms a covalent linkage with the flavin through the 8α-methyl group of FAD. A third FAD binding site is shown in which the aromatic moiety of Tyr-44 forms an essential contact with the isoalloxazine moiety of FAD. Variants that lack an aromatic ring at residue 44 show a dramatic decrease in their ability to incorporate FAD and in catalytic activity.

cofactor and to form a hydrogen bond to the ribityl 3′-hydroxy (Karplus et al., 1991). To examine the role of Tyr-44 in MAO B, the tyrosine residue was replaced with phenylalanine (Y44F), serine (Y44S), or alanine (Y44A). Interestingly, substitution of tyrosine with phenylalanine (Y44F), which does not have a hydroxyl group, resulted in only a 7% decrease in enzymatic activity as compared to the wild-type. If the hydroxyl group of Tyr-44 forms a hydrogen bond to the ribityl 3′-hydroxy of FAD, as in FNR to align FAD for catalysis, we would expect a significant decrease in enzymatic activity in variants that cannot participate in hydrogen bonding at position 44. Since little activity was lost with the Y44F variant, we conclude that the hydroxyl group of Tyr-44 is not critical for MAO B activity. To assess the role of the aromatic ring in Tyr-44, this amino acid was also replaced with either serine or alanine, both of which do not have the aromatic group. These two variants showed a dramatic loss in activity, retaining only 2% and 1% of wild-type enzymatic activity, respectively. Thus, the aromatic ring of Tyr-44 is essential for MAO B catalytic activity.

The expression of wild-type and variant MAO B enzymes was evaluated by ELISA using polyclonal anti-MAO B antibodies. Since quantitation is dependent upon recognition by polyclonal antibodies at multiple epitopes under nondenaturing conditions, major conformational changes could affect quantitation. Furthermore, major conformational changes could result in the formation of insoluble inclusion bodies. Our expression assays show that the concentration of all MAO B variants was within 4% of the wild-type level, indicating that activity losses in the mutants were not due to anomalies in expression or conformational changes in the proteins.

In studies on flavinylation of these enzymes, wild-type MAO B was found to incorporate [^{14}C]FAD, observed as a single dark band on the fluorogram (Figure 4). Also, Y44F, which retains the aromatic ring at position 44, incorporated covalently bound [^{14}C]FAD, as seen by a band of equal intensity to the wild-type. However, Y44S and Y44A, which lack the aromatic ring, showed only very faint bands, indicating that very little [^{14}C]FAD was incorporated. Thus, the loss of activity observed for these two variants can be attributed to their inability to bind FAD. We conclude that an aromatic residue is required at position 44 for flavinylation to occur.

The inability of MAO B variants that do not contain an aromatic residue at position 44 to incorporate FAD reveals important information about the process of flavinylation. If FAD first forms a covalent link to the apo-MAO B at Cys-397 and then binds to Tyr-44, we would expect all variants at position 44 to contain covalently bound FAD. Since variants Y44S and Y44A did not contain a significant amount of covalently bound FAD, we conclude that FAD binds initially to Tyr-44 instead of Cys-397. Thus, the N-terminal region including Tyr-44 and Glu-34 forms topological prerequisites which are necessary for initial FAD binding. Once bound, the FAD cofactor is subsequently delivered to Cys-397 to form a stable thioether linkage. Our results suggest that the aromatic ring of Tyr-44 plays a crucial role in binding to the aromatic isoalloxazine ring of FAD during the initial steps of flavinylation.

Binding of Tyr-44 to the isoalloxazine ring requires an aromatic−aromatic interaction. The analogous tyrosine in FNR appears on the *si*-face of the isoalloxazine ring tilted at an angle of approximately 53° (Karplus, et al., 1991). Such a geometric arrangement allows the hydrogen atoms on the edge of the aromatic ring to approach the π-electron cloud of the isoalloxazine ring. Burley and Petsko (1988) found that such edge-to-face interactions are approximately −1.5 kcal/mol of stabilization energy, which is enthalpically favored over face-to-face aromatic stacking. Furthermore, they demonstrated that an interplanar angle of 55° is an enthalpically optimal geometric arrangement. While the bond angles of Tyr-44 in MAO B remain unknown without

resolution of the three-dimensional structure, the conservation of sequence and function within this short segment of amino acids may indicate a similar alignment.

In a previous study, we showed that Glu-34 is a crucial amino acid for noncovalent binding to the AMP moiety of FAD (Kwan et al., 1995). Kearney et al. (1971) and Bach et al. (1988) showed that FAD is covalently bound to Cys-397. Collectively, these and our current study indicate that FAD is positioned in MAO B through noncovalent binding at Glu-34 and Tyr-44 and covalent linkage at Cys-397 (Figure 5). Since FAD forms part of the active site in MAO B by functioning as an electron acceptor in the oxidation of amines, it is likely that these three FAD binding sites together form part of the catalytic pocket in MAO B. Future studies of the FAD binding regions of MAO B and other flavoenzymes may reveal important insights into their substrate specificities and enzymatic mechanisms.

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