Arginine-42 and Threonine-45 Are Required for FAD Incorporation and Catalytic Activity in Human Monoamine Oxidase B†

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ABSTRACT: Monoamine oxidase B (MAO B) is an integral protein of the outer mitochondrial membrane that is involved in the deamination of vasoactive and neuroactive amines. The oxidation of these amine substrates requires the cofactor FAD, which is covalently bound to Cys-397 of human MAO B. Previously, Glu-34 and Tyr-44 of MAO B have been identified as residues which engage in noncovalent interactions with FAD that are required for subsequent covalent FAD binding and generation of catalytic activity. In this study, we have identified two additional residues, Arg-42 and Thr-45, which form noncovalent contacts with FAD that are prerequisite steps to the covalent attachment of FAD. Arg-42 and Thr-45, along with Tyr-44, comprise part of a highly conserved flavin binding sequence, RXY(T,S), that is found in other flavoproteins, several of which have well-defined X-ray crystal structures. We tested the roles of Arg-42 and Thr-45 in MAO B by constructing mutant MAO B cDNAs which encode amino acid substitutions at these residues and expressed the variant proteins in COS-7 cells. Substitution of Arg-42 or Thr-45 with alanine resulted in complete loss of MAO B activity and FAD incorporation. However, conservative substitutions of Arg-42 with lysine or Thr-45 with serine resulted in MAO B variants that retain both partial activity and partial FAD incorporation. These results indicate that Arg-42 and Thr-45 form critical noncovalent interactions with FAD that are required for the subsequent activation of MAO B by covalent coupling of FAD.

Monoamine oxidases A and B (MAO A and B;[1] EC 1.4.3.4) comprise the major amine-degrading enzymes in the mammalian central nervous system and peripheral tissues (*1*). These isozymes are integral proteins of the outer mitochondrial membrane (*2*), which can be distinguished by differences in substrate preference and inhibitor specificity (*3*), tissue and cell distribution (*4*), and immunological properties (*5*−*9*). The genes for MAO A and B are comprised of 15 exons with identical exon−intron organization (*10*−*12*). Furthermore, the nucleotide and deduced amino acid sequences of rat, bovine, and human MAO A and B show a remarkable degree of similarity between the two isozymes (*13*−*16*). Interestingly, recent studies (*17*) have shown that the substrate selectivity of MAO A and B appears to be determined by a single amino acid residue (Phe-208 in MAO A and Ile-199 in MAO B). Switching of these residues into the other isozyme establishes the preferred character of the substrate (hydrophilic for wild-type MAO A and hydrophobic for wild-type MAO B).

MAO A and B oxidize amine substrates with the concomitant reduction of the obligatory cofactor FAD. FAD is covalently attached to MAO via an 8α-methyl-*S*-cysteinyl linkage at Cys-406 and Cys-397 in human MAO A and B, respectively (*18*, *19*, *13*). The cysteine residue is required for catalytic activity, as demonstrated in site-directed mutagenesis studies in which the substitution of serine or histidine for Cys-397 yields catalytically inactive enzyme in transfected mammalian cells (*20*, *21*).

Previous studies have identified amino acid residues in MAO B which are thought to form noncovalent bonds with FAD (*22*−*24*). One such residue is Glu-34, which is contained in a putative dinucleotide binding motif (β_1-strand/α-helix/β_2-strand), located in the N-terminal portion of the protein, that comprises a noncovalent FAD binding site. Glu-34 resides at the end of the second β-strand and is proposed to bind to the 2′-hydroxyl group of ribose of FAD through a hydrogen bond to the glutamate α-carboxyl group. Replacing the glutamate at position 34 with alanine, aspartate, or glutamine results in over 90% loss of activity. The loss of activity in these variants also correlates closely with a loss of covalent FAD incorporation into MAO B (*22*, *24*).

Another putative FAD-binding region in MAO B was identified by sequence comparison with other flavoproteins (Figure 1). A highly conserved flavin-binding sequence, RXY(T,S) (*25*, *26*), was found in several flavoproteins and contains two strictly conserved residues, arginine and tyrosine (Arg-42 and Tyr-44 in MAO B). Half of the proteins in the homology comparison (Figure 1) contains a serine and the

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[1] Abbreviations: FAD, flavin adenine dinucleotide; MAO A, monoamine oxidase A; MAO B, monoamine oxidase B.; FNR, ferredoxin-NADP$^+$ reductase; PDR, phthalate dioxygenase reductase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; ELISA, enzyme-linked immunosorbance assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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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 (corn)*	59-L C M **R** A **Y** **T** P
Phthalate dioxygenase reductase (*P. cepacia*)*	52-G S R **R** T **Y** **S** L
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: Putative FAD binding site in human MAO [modified from Zhou et al. (*23*)]. The conserved residues of the consensus sequence RXY(T,S) involved in flavin binding in flavoproteins are shown in bold (*26*). Proteins with known X-ray crystal structure are indicated with an asterisk. Sequences for the individual proteins were obtained as follows: MAO A and B (*13*); ferredoxin-NADP$^+$ reductase (*27*); NADH-nitrate reductase (*29*); phthalate dioxygenase reductase (*25*); NADH-cytochrome b_5 reductase (*36*); NADPH-cytochrome P450 reductase (*37*); and NADPH-sulfite reductase (*38*).

other half contains a threonine at a position corresponding to residue 45 of MAO B.

The crystal structures of three of the enzymes in the homology comparison, spinach ferredoxin-NADP$^+$ reductase (FNR), *Pseudomonas cepacia* phthalate dioxygenase reductase (PDR), and corn nitrate reductase, have been solved and their flavin-binding domains characterized (*25*,*27*−*29*). Two of the flavin-dependent enzymes, FNR and nitrate reductase, utilize noncovalently bound FAD, whereas PDR utilizes noncovalently bound FMN. These three proteins show similar domain structures consisting of a C-terminal pyridine nucleotide-binding domain and an N-terminal flavin-binding region composed of a six-stranded antiparallel β-barrel. The isoalloxazine ring of the noncovalently bound flavin is sandwiched between the two domains. The conserved Arg, Tyr, and Ser/Thr residues, which correspond to human MAO B residues Arg-42, Tyr-44, and Thr-45 in the homology diagram in Figure 1, are contained in the fourth β-strand of the β-barrel. The conserved Arg hydrogen bonds to the phosphate moiety in the flavin. The aromatic ring of Tyr makes extensive van der Waals contacts with the isoalloxazine ring of the flavin, and the hydroxyl group forms a hydrogen bond with the ribityl 4′-hydroxyl group of the flavin. The conserved Ser/Thr is proposed to form hydrogen bonds to the redox active N5 nitrogen and/or O4 of the flavin (*25*, *27*−*29*).

Tyr-44 of MAO B has been proposed to play a role in FAD binding (*23*) similar to that proposed for the conserved Tyr of FNR, PDR, and nitrate reductase. Mutation of Tyr-44 to serine or alanine produces variant proteins essentially devoid of activity, whereas replacement with phenylalanine causes only a 7% decrease in activity, suggesting that the aromatic ring of Tyr-44 is required for activity. Further characterization of these variants with respect to their ability to covalently bind FAD showed that the losses of activity in the variants correlate to a loss of covalent FAD binding, suggesting that noncovalent interactions of Tyr-44 with FAD are required for covalent coupling of the cofactor (*23*).

In this study, we investigated the roles of Arg-42 and Thr-45 of MAO B in enzymatic activity and FAD binding. Using site-directed mutagenesis, the arginine at position 42 was substituted with alanine or lysine and the threonine at position 45 was substituted with alanine or serine. Substitution of an alanine at either position resulted in proteins devoid of activity, whereas changes of Arg-42 to Lys and Thr-45 to Ser gave variants that retained partial activity. Covalent FAD incorporation experiments on these variants suggest that losses in activity are due to a loss of covalent FAD binding. We propose that Arg-42 and Thr-45 form important noncovalent interactions with the diphosphate and isoalloxazine moieties of FAD, respectively, which are required for subsequent covalent FAD incorporation of MAO B.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutagenesis was performed by the method of Deng and Nickoloff (*30*), using the Transformer Site-directed Mutagenesis kit (Clontech) as previously described (*22*). The mutagenic primers and the corresponding amino acid changes are shown in Figure 2. Arg at position 42 was replaced by either Ala or Lys in the R42A and R42K mutants, respectively. Thr at position 45 was replaced by either Ala or Ser in the T45A and T45S mutants, respectively. The construction of the L46V mutant as a positive control, in which Leu at this position was replaced with Val, has been previously described (*23*). All mutagenic oligonucleotides were designed to create a new restriction site or to remove an existing restriction site for the purposes of screening and/or selection without changing the coding sequence of any other amino acid. All oligonucleotides were custom-synthesized by National Biosciences.

Construction of all mutants was carried out directly in a pSVK3 expression vector (LKB-Pharmacia) which contained the human MAO B cDNA cloned into the *Eco*RI site. For construction of the R42A mutant, a 29-mer oligonucleotide was used as a selection primer, where the unique *Kpn*I site (5′-GAATTCGAGCTCGGTACCCGGGGATCCTC-3′) was changed to a *Hpa*I site (5′-GAATTCGAGCTCGtTAaC-CGGGGATCCTC-3′) in the plasmid vector. In addition, a 33-mer mutagenic primer for the MAO B sequence, in which a unique *Nar*I/*Kas* restriction site was created, was used in the initial screening of positive mutant clones. The R42A plasmid was then used as a template for the subsequent construction of the R42K, T45A, and T45S mutants. This allowed the use of a single mutagenic/selection oligonucleotide which introduces the desired codon changes and simultaneously removes the unique *Nar*I/*Kas*I site, permitting the use of this site for selection/enrichment for mutant clones. The use of a single mutagenic/selection primer theoretically increases the efficiency of the mutagenesis reaction, resulting in higher yields of mutant clones. The mutagenic/selection primer for the R42K mutant converts the Ala codon at position 42 of the R42A template to a Lys codon and removes the *Nar*I/*Kas*I site. The mutagenic/selection primers for the T45A and T45S mutants convert the Ala codon at position 42 of the R42A template back to the wild-type Arg codon and at the same time convert the wild-type Thr codon at position 45 to an Ala (for T45A) or a Ser (for T45S) codon. These oligonucleotides also remove the unique *Nar*I/*Kas*I site. The T45A primer creates a Bsi WI site used in the initial screening of clones. The relevant portions of all four mutant cDNAs were sequenced using dideoxy DNA sequencing (*31*) to confirm the presence of only the desired mutations. Wild-type and mutant cDNAs were purified through CsCl gradients prior to transfection studies. A control variant (L46V) previously constructed (*22*) was used for comparison.

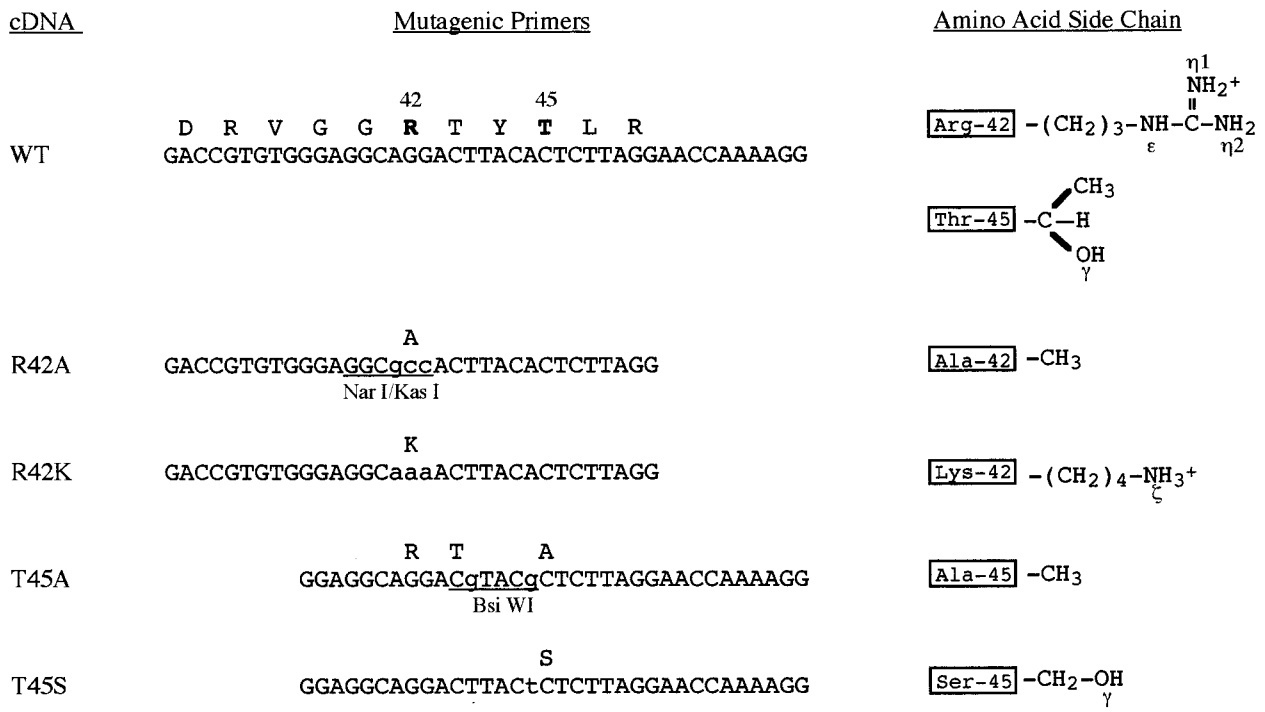

FIGURE 2: Mutagenic primers for site-directed mutagenesis studies. The wild-type coding sequence along with the corresponding amino acids is given in the top line with the wild-type amino acids at positions 42 and 45 in bold. Lowercase letters in the nucleotide sequence indicate base substitutions, and the corresponding amino acid change is indicated above the sequence. Restriction sites introduced for the purpose of mutagenesis and/or screening are underlined. Corresponding amino acid side chains, along with the Greek letter designation of potential side chain hydrogen bond donors, are shown to the right.

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; from Gibco-BRL) with 10% fetal bovine serum (FBS; from Gibco-BRL) and 5% CO_2 at 37 °C. Riboflavin-depleted COS-7 cells were produced by maintaining these cells in riboflavin-depleted DMEM/dialyzed FBS (Gibco-BRL) for greater than 100 days as described by Zhou et al. (*24*).

Wild-type or mutant MAO B cDNAs were transfected into COS-7 cells by electroporation (*32*) for transient expression as described previously (*22*). Briefly, cells were harvested during late log-phase growth and resuspended to a concentration of 3.125×10^6 cells/mL in DMEM supplemented with 10% FBS. Fifteen micrograms of normal or mutant cDNA was added to 0.8 mL (2.5×10^6 cells) of cell suspension. In experiments where covalent FAD incorporation of wild-type or mutant MAO B was studied, 20 μL of 0.8 mM [^{14}C]riboflavin 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). Either riboflavin, FMN, or FAD can be used to obtain approximately equal levels of holoenzyme (*24*). Electroporations were carried out in a Bio-Rad gene pulser with a setting of 250 V and 500 μF. The cells were incubated on ice for 10 min following the electroporation pulse, then resuspended in 15 mL of DMEM/FBS (or riboflavin-free DMEM/FBS), and incubated at 37 °C with 5% CO_2 for 48 h.

Transfected COS-7 cells were harvested at 48 h and homogenized in a lysis solution (500 μL) containing 50 mM potassium phosphate, 0.5 mM EDTA, and 0.5 mM PMSF (phenylmethanesulfonyl fluoride), pH 7.4, using a glass mortar and pestle (Wheaton). MAO B was extracted by adding Ultrapure Triton X-100 (Pierce) to each sample to a final concentration of 0.25% and stirring the samples for 50 min at 4 °C. The samples were then centrifuged at 735*g* for 5 min, and the resulting supernatant was used for assays.

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. Quantitation of MAO B was performed by ELISA, using a modification of the method of Yeomanson and Billet (*33*) as previously described, with a goat polyclonal antibody to MAO B (*22*). Expression levels of wild-type or mutant MAO B were determined in duplicate for three separate experiments using transfected cells from three separate electroporation experiments.

Enzyme Activity Determination. MAO B activity was assayed by a modification of the method of Wurtman and Axelrod (*34*) as previously described (*22*). Briefly, 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 μmol of 55 mCi/mmol [^{14}C]benzylamine hydrochloride (Amersham), and 10 nmol of unlabeled benzylamine. For each experiment, controls of nontransfected COS-7 cell lysate were assayed along with tranfected COS-7 cell lysates. All samples and controls were run in duplicate. After incubation at 37 °C for 9 min, all reactions were terminated with 6 N HCl (25 μL). The reaction product was extracted with 500 μL of toluene, and 200 μL of the organic phase was counted in liquid scintillation fluid in a Beckman LB3801 model liquid scintillation counter. The activity of wild-type and variant

MAO B was determined in duplicate using transfected cells from three separate electroporation experiments.

Immunoprecipitation of Wild-Type and Variant MAO B. Transfected COS-7 cells were homogenized in 200 μL of lysis buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, pH 8.0), and MAO B was extracted with 0.25% Triton X-100 by stirring for 45 min at 4 °C. After centrifugation at 735*g* for 5 min, the supernatant was saved and the pellet was re-extracted with 100 μL of the lysis buffer and Triton X-100. Samples were centrifuged again, and the supernatants from the two extractions were combined. The extracts were adjusted to equal MAO B concentration following ELISA quantitation; then equal volumes of adjusted extract were incubated with 10 μg (25 μg for fluorography experiments) of goat polyclonal anti-MAO B antibody (*22*) overnight at 4 °C. Protein-G Sepharose beads were added (50 μL for Western blot, 100 μL for fluorography) and incubated overnight. The protein-G Sepharose/goat antibody/MAO B immunocomplex was collected by centrifugation at 10000*g* for 20 s and washed six times with lysis buffer. Bound proteins were 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 (*22*). MAO B was positively identified with mouse MAO B-1C2 monoclonal antibody. Western blots were performed on proteins expressed in both riboflavin-containing COS-7 cells (as described above for use in activity assays) and riboflavin-depleted COS-7 cells transfected in the presence of [^{14}C]-riboflavin (as described above for use in covalent FAD incorporation assays).

Fluorography. Immunoprecipitated wild-type and mutant MAO B (obtained as described above) were subjected to electrophoresis in a 10% SDS−polyacrylamide gel. The gel was fixed in a solution of 7% acetic acid/10% methanol for 1 h and processed for fluorography as described by Bonner and Laskey (*35*). The dried gel was exposed to Kodak X-OMAT AR film at −80 °C.

RESULTS

Four variant proteins, R42A, R42K, T45A, and T45S, were constructed by site-directed mutagenesis to determine the roles of Arg-42 and Thr-45 in MAO B catalytic activity and covalent FAD coupling. These variant proteins, along with wild-type MAO B and a control variant L46V, were expressed in a COS-7 cell line, since these cells do not contain any detectable MAO B activity. Optimal transient expression was obtained using conditions previously described (*22, 23*).

The expression levels for wild-type and variant MAO B (Table 1) were determined by ELISA using a goat polyclonal antibody to MAO B and are shown as ng of MAO B (determined by ELISA) per mg of total cell protein (determined by BCA protein assay.) The variant proteins were expressed at levels greater than 75% of wild-type, except for T45A, which shows 56% of wild-type expression.

Equal amounts of wild-type and variant proteins (determined by ELISA) expressed in riboflavin-containing COS-7 cells were immunoprecipitated using goat polyclonal antibody to MAO B and subjected to Western blot analysis. The MAO B proteins were positively identified using the monoclonal antibody MAO B-1C2, which is specific for MAO B. All variant proteins were expressed as full length MAO B (59 kDa), whereas untransfected COS-7 cells show no MAO band, as shown in Figure 3. Both variant and wild-type MAO B proteins were immunoprecipitated with equal efficiency, as shown by the bands of similar intensity in the Western blot analysis (Figure 3). Western blots of wild-type and variant proteins expressed in riboflavin-depleted COS-7 cells electroporated in the presence of [^{14}C]riboflavin gave identical results (data not shown) to those shown in Figure 3.

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

enzyme	MAO B concn (ng of MAO B/mg of protein)	specific act (nmol/min/mg of protein)	enzyme act (μmol/min/mg of MAO B)
WT	464 ± 91 (100)[b]	0.450 ± 0.050 (100)[b]	1.14 ± 0.16 (100)[b]
R42A	358 ± 75 (77)	ND	ND
R42K	410 ± 8 (88)	0.056 ± 0.013 (12)	0.157 ± 0.059 (14)
T45A	260 ± 35 (56)	ND	ND
T45S	357 ± 99 (77)	0.040 ± 0.012 (9)	0.136 ± 0.045 (12)

[a] Samples were run in duplicate for each experiment. Enzyme activity measurements were performed using benzylamine as the substrate. Each value represents the mean ± SEM from three separate experiments. [b] Percent of wild-type values is shown in parentheses. ND, = not detected

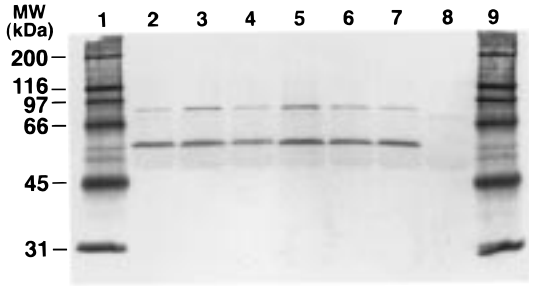

FIGURE 3: Western blot analysis of wild-type and variant MAO B. MAO B proteins expressed in riboflavin-containing COS-7 cells were adjusted to equal concentrations using ELISA prior to immunoprecipitation. Immunoprecipitated proteins were resolved on 10% SDS−PAGE and transferred to a nitrocellulose membrane, and MAO B enzymes were positively identified by the MAO B-specific monoclonal antibody MAO B-1C2. Lanes 1 and 9, biotinylated molecular weight markers; lane 2, wild-type MAO B; lane 3, R42A MAO B; lane 4, R42K MAO B; lane 5, T45A MAO B; lane 6, T45S MAO B; lane 7, L46V MAO B; lane 8, untransfected COS-7 cells.

The activities of wild-type and variant proteins were determined by a radiometric assay using benzylamine as the substrate (Table 1). These results are expressed both as specific activity, which represents the amount of activity per mg of total protein (determined by BCA assay), and enzymatic activity, which is the amount of activity per mg of MAO B (determined by ELISA). The enzymatic activity of wild-type was 1.14 μmol/min/mg of MAO B, which is similar to previously reported values (*22*−*24*). Substitution of alanine for Arg-42 resulted in a complete loss of detectable MAO B activity, whereas the more conservative substitution of lysine for Arg-42 resulted in a protein with 14% of wild-type enzymatic activity. Likewise, the T45A variant showed

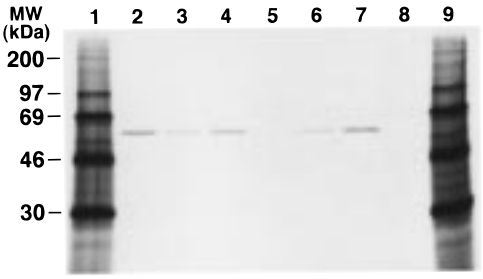

FIGURE 4: Fluorography. Wild-type and mutant MAO B cDNAs were transfected in riboflavin-depleted COS-7 cells with the addition of exogenous [^{14}C]riboflavin. Expressed wild-type and variant MAO B proteins were adjusted to equal concentrations (based on ELISA) and immunoprecipitated. The immunoprecipitated proteins were then electrophoresed on 10% SDS−PAGE and analyzed by fluorography: lanes 1 and 9, ^{14}C-methylated molecular weight markers; lane 2, wild-type MAO B; lane 3, R42A MAO B; lane 4, R42K MAO B; lane 5, T45A MAO B; lane 6, T45S MAO B; lane 7, L46V; lane 8, untransfected riboflavin-depleted COS-7 cells.

no detectable activity, but the conservatively substituted variant T45S gave 12% of wild-type activity. A control variant, L46V, which represents a nonconserved residue close to Arg 42 and Thr 45, did not show any decrease in activity compared to the wild-type (data not shown).

The ability of wild-type and variant MAO B to bind FAD covalently was determined using riboflavin-depleted COS-7 cells (*23*, *24*), which cannot synthesize FAD in the absence of exogenous riboflavin. Covalent FAD incorporation was studied by coelectroporating [^{14}C]riboflavin along with wild-type or mutant MAO B cDNAs. The concentration of expressed MAO B in these cells was determined by ELISA, and equal amounts of wild-type and variant MAO B were immunoprecipitated. The immunoprecipitated proteins were electrophoresed on SDS−PAGE and subjected to fluorography. In this semiquantitative method, the amount of covalent FAD incorporation is proportional to the intensity of the resulting band on the fluorogram. As shown in Figure 4, both the wild-type and the control variant L46V (lanes 2 and 7, respectively) have similar band intensities at approximately 59 kDa, indicating that they can covalently incorporate FAD. In contrast, R42K shows reduced incorporation, as indicated by a lesser band intensity (lane 4), and R42A shows even less incorporation (lane 3). A faint band for T45S indicates little FAD incorporation (lane 6), and no detectable band is seen for T45A (lane 5). The degree of FAD incorporation in the wild-type and variant MAO B approximates the amount of catalytic activity observed in these proteins.

DISCUSSION

In this paper, we have examined the role of two residues, Arg-42 and Thr-45, in a putative FAD binding site of human MAO B. Previously, we described the role of Tyr-44 in this FAD binding site (*23*). These three residues, Arg-42, Tyr-44, and Thr-45, comprise part of a highly conserved sequence, RXY(T,S) (*25*, *26*), common to several flavoprotein reductases (Figure 1).

From X-ray crystallographic studies, the highly conserved Arg, Tyr, and Thr (or Ser) residues in flavoproteins have been proposed to play an important role in noncovalent FAD binding. Of the proteins whose sequences are represented in the homology alignment in Figure 1, crystal structures have been solved for spinach FNR (*27*, *28*), corn nitrate reductase (*29*), and *P. cepacia* PDR (*25*). The conserved Arg, Tyr, and Thr/Ser residues are thought to play similar roles in these three enzymes. The conserved Arg is proposed to hydrogen bond to the diphosphate moiety of FAD in FNR and nitrate reductase and to the phosphate group of FMN in PDR. The aromatic ring of the conserved Tyr packs against the *si* face of the isoalloxazine ring of the flavin, and the Tyr hydroxyl group hydrogen bonds to the 4′-hydroxyl group of the ribityl moiety of the flavin. Both the peptide nitrogen and the side chain hydroxyl of the Thr/Ser residue are in close proximity to N5 and O4 of the isoalloxazine ring and can potentially form hydrogen bonds to these atoms (*25*,*27*−*29*).

Previously, we investigated the role of Tyr-44 of MAO B through site-directed mutagenesis (*23*). Tyr-44 was proposed to play a role in FAD binding similar to that proposed for the corresponding Tyr-95 of FNR. Mutation of Tyr-44 to serine or alanine produces variant proteins essentially devoid of activity, whereas replacement with phenylalanine yields a variant with 93% of the activity of the wild-type. Further characterization of these variants with respect to the ability to covalently bind FAD showed that the losses of activity in the variants correlate to a loss of covalent FAD incorporation, suggesting that the noncovalent interactions of Tyr-44 with FAD are prerequisite for covalent coupling of the cofactor. Interestingly, these results suggest that the hydroxyl group of Tyr-44 in MAO B does not play an important role in noncovalent FAD binding and subsequent covalent incorporation. This is in contrast to FNR, PDR, and nitrate reductase, in which the hydroxyl of the corresponding Tyr is thought to form a hydrogen bond with the 3′-hydroxyl of the ribityl moiety (*25*,*27*−*29*).

In this study, we examined the role of Arg-42 in MAO B activity and covalent FAD coupling by replacing the arginine with lysine (R42K) or alanine (R42A) by site-directed mutagenesis. R42A shows a complete loss of activity, whereas the more conservative substitution in the R42K variant retains 14% of the wild-type activity (Table 1).

We also studied the ability of these variants to covalently incorporate FAD using COS-7 cells depleted of riboflavin. Previous studies have shown that wild-type MAO B expressed in these cells in the absence of exogenous flavin is devoid of activity, due to the lack of covalently bound FAD (*24*). However, the lack of flavin cofactor in these cells does not affect the level of expression of MAO B or MAO B insertion into the mitochondria. MAO B covalent FAD incorporation and activity can be recovered by coelectroporating FAD along with MAO B cDNA. The FAD precursors, riboflavin and FMN, are equally as effective as FAD in recovering MAO B activity, presumably due to the ability of endogenous FAD synthetase to rapidly convert these forms to FAD. These previous studies also suggest that the riboflavin to FAD conversion occurs prior to FAD binding to MAO B; i.e., FAD is the flavin form which initially binds to MAO B (*24*).

In this study, mutant and wild-type MAO B cDNAs were electroporated into the riboflavin-depleted COS-7 cells along with [^{14}C]riboflavin. MAO B expressed in these cells was immunoprecipitated with MAO B-specific polyclonal antibody, run on SDS−PAGE, and subjected to fluorography. Since these proteins are analyzed under denaturing conditions

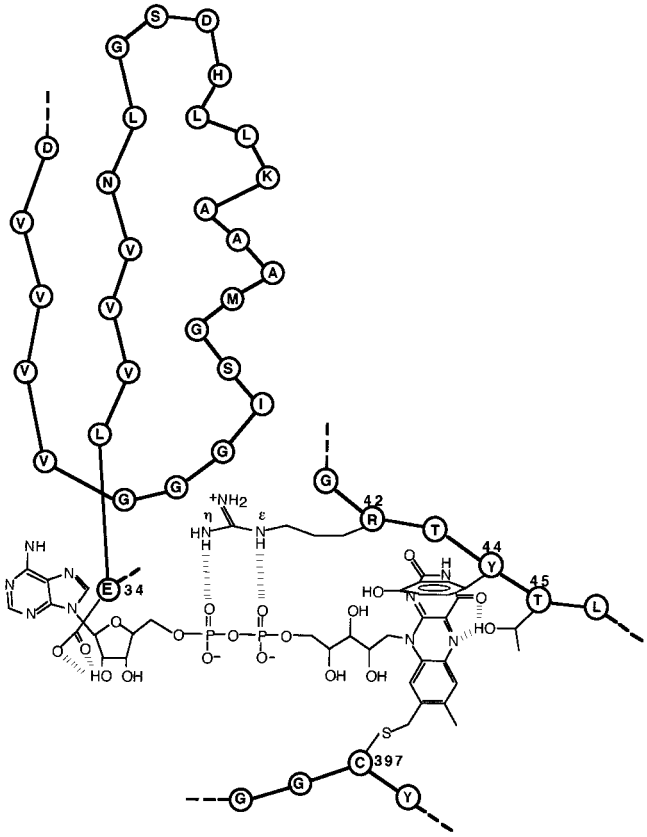

FIGURE 5: Model of FAD interaction with three distinct sites of MAO B. The model is a composite based upon published X-ray crystal structures of FNR (*27*, *28*), PDR (*25*), and nitrate reductase (*29*) in addition to previously proposed models (*22*, *23*). Cys-397 forms a covalent linkage to the FAD via the 8α-methyl group of the isoalloxazine ring. Glu-34 is part of the $\beta_1-\alpha-\beta_2$ motif (residues 6−34) and forms hydrogen bonds to the 2′-hydroxyl of ribose in the AMP moiety of FAD. Arg-42, Tyr-44, and Thr-45 are part of the third binding site of MAO B. Arg-42 forms hydrogen bonds to the diphosphate moiety of FAD. Tyr-44 forms an aromatic−aromatic interaction with the isoalloxazine ring of FAD. Thr-45 forms hydrogen bonds to the O4 and/or N5 of the isoalloxazine ring of FAD.

prior to fluorography, only proteins with *covalently* bound [^{14}C]FAD will produce a 59-kDa band on the fluorogram. The results of this assay (Figure 4) suggest that losses in the amount of covalent coupling in these variants correlate with the loss of activity. As expected, both wild-type and the control variant L46V show similar band intensities, indicative of the ability of these two proteins to covalently incorporate [^{14}C]FAD. In comparison, R42K and R42A show substantially less FAD incorporation, as indicated by their decreased and faint band intensities, respectively.

These results are consistent with our proposed role for Arg-42 in which the arginine side chain forms hydrogen bonds to FAD, interactions which are prerequisite for covalent FAD coupling. The arginine side chain has three amino groups, designated ϵ, $\eta 1$, and $\eta 2$, which can potentially act as hydrogen bond donors (Figures 2 and 5). In the role proposed above, a complete loss of hydrogen bond donors of these side chain amino groups should result in a total loss of FAD incorporation and MAO B enzymatic activity, as is the case for the R42A variant. On the other hand, an amino acid side chain containing only one amino group hydrogen bond donor might result in only a partial retention of FAD incorporation and MAO B activity, an effect seen by the arginine to lysine substitution in the R42K variant. From the X-ray crystallographic structures of FNR, PDR, and nitrate reductase (*25*,*27*−*29*), the arginine residue contained in the consensus sequence RXY(T,S) corresponding to Arg-42 is proposed to form hydrogen bonds from the ϵ and/or η side chain amino groups to the phosphate(s) of the flavin. On the basis of our mutagenesis studies and the homology comparison with the above structures, we propose that Arg-42 of MAO B forms one or more hydrogen bonds to the diphosphate moiety of FAD (Figure 5). Formation of these noncovalent bonds to FAD is required for subsequent covalent FAD coupling to MAO B and enzymatic activity.

The role of Thr-45 of MAO B was also investigated by the substitution of alanine or serine for threonine. The T45A variant shows a complete loss of activity, whereas the more conservative amino acid substitution in the T45S variant results in 12% of wild-type activity (Table 1). The results of the covalent FAD incorporation assays (Figure 4) suggest that the loss in activity is due to an inability of these variants to covalently incorporate FAD. The threonine side chain appears to form noncovalent interactions with FAD which are required for subsequent covalent FAD incorporation into MAO B. The γ-hydroxyl of threonine can potentially act as a hydrogen bond donor in such noncovalent interactions. In this scenario, loss of the hydroxyl group should result in a loss of FAD incorporation and enzymatic activity, an effect observed in the T45A variant. However, retention of the γ-hydroxyl group should result in at least partial activity and FAD incorporation, as is the case for the T45S variant. It is somewhat surprising that the T45S variant retained only 12% activity, given that four of the seven flavoproteins in the homology alignment in Figure 1 show the conservative substitution of serine for threonine.

The specific noncovalent interactions of the conserved Thr/Ser in FNR, nitrate reductase, and PDR with the flavin are less well-defined than that of the conserved Arg residue in the RXY(T,S) sequence. The side chain hydroxyl of Thr-65 in nitrate reductase forms a hydrogen bond indirectly via a water molecule to the O4 oxygen in the isoalloxazine ring of FAD. In the crystal structure of the oxidized form of FNR, the main chain amino group of Ser-96 hydrogen bonds to the N5 nitrogen, and the side chain hydroxyl forms an indirect hydrogen bond to the O4 oxygen of the isoalloxazine ring of FAD. On the other hand, in oxidized PDR, the main chain amino group of Ser-58 hydrogen bonds to O4 of the FMN isoalloxazine ring.

Crystallographic studies of FNR and PDR suggest that the conserved Ser is involved in protonation of the N5 nitrogen of the isoalloxazine ring in the reduction of the flavin. Comparisons of the oxidized and reduced structures of FNR and PDR have suggested that the side chains of Ser-96 of FNR and Ser-58 of PDR swing into position to hydrogen bond to the N5 nitrogen of the reduced flavin. Thus, one role proposed for the conserved Ser is to protonate the N5 nitrogen of the flavin (*28*, *26*).

The results of our mutagenesis study tend to rule out the possibility that the *only* role of Thr-45 of MAO B is to protonate the redox active N5 nitrogen in the reduction of FAD. If this were the case, we would expect the Thr-45 to Ala substitution to cause a decrease in enzymatic activity without a corresponding decrease in covalent FAD incorporation. However, this substitution results in complete loss

of covalent FAD incorporation along with the complete loss of activity. While these results indicate that Thr-45 is critical for covalent FAD incorporation, they do not exclude the possibility that the residue also plays some role in the reduction of FAD during oxidation of the amine substrate. Thus, we propose that the hydroxyl of Thr-45 can potentially form hydrogen bonds to the O4 and/or the N5 of the isoalloxazine ring of FAD (Figure 5).

The results of this study and previous studies (*22*−*24*) provide important insight into the events of covalent FAD incorporation into MAO B. We propose a model for FAD incorporation in which Glu-34, Arg-42, Tyr-44, and Thr-45 collectively comprise part of a topological dock in apo-MAO B to which FAD must be bound noncovalently in order for latter covalent attachment at Cys-397 (*18*, *13*) to occur (Figure 5). In this model, the residues could hold FAD with the 8α-methyl group in proper juxtaposition to Cys-397 of MAO B to allow covalent binding to occur. Whether this process of covalent attachment occurs autocatalytically or enzymatically remains to be resolved.

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