J. Biochem. 131, 107–111 (2002)

Tyrosine Residues near the FAD Binding Site Are Critical for FAD Binding and for the Maintenance of the Stable and Active Conformation of Rat Monoamine Oxidase A

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Received July 23, 2001; accepted November 2, 2001

Monoamine oxidase is a flavin-containing enzyme which is located at the mitochondrial outer membrane and catalyzes the oxidative deamination of amines. To investigate the role of tyrosine residues near the FAD-binding site, Cys-406, of monoamine oxidase A, the tyrosine residues at positions 402, 407, and 410 were individually replaced with alanine or phenylalanine and the effects of the mutations on catalytic activity, FAD binding, and enzyme structure were examined. Half or fewer of the mutant proteins incorporated FAD. The mutation of Tyr-407 to alanine led to an almost completely loss of catalytic activity for serotonin, phenylethylamine, tyramine, and tryptamine. A substantial decrease in the catalytic activity was also observed with the enzymes mutated at Tyr-402 and Tyr-410 to alanine, although the effect of the latter mutation was much less. All these mutants were sensitive to trypsin treatment of the purified enzyme, while the wild type enzyme was resistant to treatment. On the other hand, substitution of Tyr-402 or Tyr-407 with phenylalanine had little effect on these properties. Taken together, we conclude that tyrosine residues near Cys-406 may form a pocket to facilitate FAD incorporation, the catalytic center, and a stable conformation, probably through interactions among the aromatic rings of the tyrosine residues and FAD.

Key words: covalent-bound flavin, monoamine oxidase A, tyrosines around FAD binding site.

Monoamine oxidase (MAO, EC 1.4.3.4) is a flavin-containing mitochondrial outer membrane protein (*1*). It catalyzes the oxidative deamination of primary aromatic amines and primary aliphatic, secondary, and tertiary amines. In human and animals, the enzyme plays important roles in the metabolism of neurotransmitters, such as serotonin and dopamine, in the central nervous system and peripheral tissues, and is related to some neurodegenerative diseases and psychological disorders. There are two forms of MAO, monoamine oxidase A (MAOA) and monoamine oxidase B (MAOB), and they are defined by their substrate and inhibitor specificities (*2*). MAOA has a higher affinity for catecholamines and 5-hydroxytryptamine (5-HT or serotonin) and is more sensitive to inhibition by clorgyline, whereas MAOB has a higher affinity for dietary amines such as phenylethylamine (PEA), and is selectively inhibited by deprenyl. Deduced amino acid sequences from isolated cDNA clones (*3–6*) show about 70% identity between MAOA and MAOB. Both forms of MAO contain covalently bound FAD, in an 8-α-*S*-cysteinyl-FAD linkage (*7, 8*) to Cys406 in MAOA and to Cys397 in MAOB.

The covalent bindings of FAD to a protein has been found in more than 20 flavoenzymes *via* 8-α-*N*(1)-histidyl, 8-α-*N*(3)-histidyl, 8-α-*S*-cysteinyl, 6-*S*-cysteinyl, or 8-α-*O*-tyrosyl FAD linkages (*9*). The mechanism of the covalent binding and its significance to the enzyme catalytic activity are still not known. In 6-hydroxy-nicotine oxidase and in fumarate reductase, replacing the histidine residue that links to FAD with other amino acids lead to the loss of covalently bound FAD, but the mutant enzymes retain activities (*10, 11*). This was also true for rat MAOA according our previous study (*12*). Substitution of the Cys406 of rat MAOA with alanine led to the loss of covalently bound FAD, but the activity remained due to the presence of non-covalently bound FAD, although at a lower level and decreased quickly *in vitro*. Further, the addition of FAD to the apo-enzyme can partially protect against inactivation, but does not lead to to the recovery of an active enzyme. All these findings suggest that FAD may not necessarily bind to the enzyme in a covalent form for catalytic activity, but may function as a structural core of the active conformation as well.

Although there is little homology among the amino acid sequences around the FAD binding sites of known flavoenzymes, a distinct non-covalent FAD binding site shows high identity in many FAD-containing enzymes (*13*). This region is commonly referred to as the dinucleotide-binding site due to its interaction with the AMP moiety of FAD. It comprises a β1-sheet-α-helix-β2-sheet beginning with a highly conserved sequence of Gly-X-Gly-X-X-Gly between the β1-sheet and α-helix. The β2-sheet usually ends with a glutamate residue in which the δ-carboxylate group is thought to interact with the 2′-hydroxyl group of ribose in the AMP

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Abbreviations: MAO, monoamine oxidase; PEA, phenylethylamine.

moiety of FAD. This structure has been suggested to act as a "nucleation center." That is, during biosynthesis this domain will form first and the rest of the protein structure forms around it. This region is located at the N terminus, residues 15–43 in MAOA and 6–34 in MAOB. Site-directed mutagenesis studies of human MAOB have shown that Glu34 is essential for enzyme activity and the covalent binding of FAD (*14, 15*). Tyr-44 was found to be essential for the covalent binding of FAD (*16*). It is thought that this residue interacts with FAD through a π-π interaction of their aromatic rings, forming a non-covalent bond, and then is transferred to Cys406 to form a covalent bond during synthesis of the enzyme.

Since the three-dimensional structure of MAO has not been solved, we still do not know its folding patterns, substrate recognition, and catalytic mechanism. In this study, we report, in addition to the residues in the FAD binding site described above, the importance of tyrosine residues near the FAD covalently-binding site, Cys406 in rat MAOA, enzyme activity, and conformation stability using a purified his-tagged recombinant enzyme.

MATERIALS AND METHODS

Materials—Yeast cell *Saccharomyces cerevisiae* strain BJ2168 (*a prc1-407 prb1-1122 prp4-3 leu2 trp1 ura3-52*) was purchased from Wako (Osaka). Yeast expression vector YEp51 was a kind gift from Dr. Y. Jigami of the Institute of Molecular and Cell Biology, National Institute of Advanced Industrial Science and Technology. Restriction endonucleases were purchased from New England Biolab (Beverly, USA). Primer DNA was synthesized by a Hokkaido System Science Work Station (Sapporo). The QuickChange kit for making site-directed mutations and Pfu DNA polymerase were from STRATAGENE (USA). Bacto-Yeast extract, Bacto-Peptone, Bacto-Yeast extract without amino acids were purchased form DIFCO (Detroit, USA). Zymolyase 100T was from Seikagaku Kogyo (Tokyo). Kynuramine and clorgyline were from Sigma. The radioactive-chemicals, 5-[2-^{14}C]hydroxyltryptamine bioxalate (serotonin), [1-^{14}C]phenylethylamine hydrochloride (PEA), and [1-^{14}C]tyramine hydrochloride, and [Side chain-2-^{14}C]tryptamine bisuccinate were from New England Nuclear (Boston, USA). HiTrap Chelating column was from Pharmacia (Uppsala, Sweden).

Construction of Expression Plasmid and Site-Directed Mutagenesis—We constructed a plasmid containing the full-length cDNA of rat MAOA (*6, 12*) with 6 histidine residues tagged at the amino terminal by a PCR method. A yeast expression plasmid was constructed based on the above plasmid. The his-MAOA cDNA was amplified by PCR and subcloned into YEp51 vector at the *Sal*I and *Bam*HI sites, and named plasmid YEp51-hisMAOA.

A QuickChange kit was used to prepare the mutant enzymes, Y402A, Y402F, Y407A, Y407F, and Y410A. The method used was essentially according to the manufacturer's instructions, using YEp51-hisMAOA plasmid as a template. All mutated sites were confirmed by sequencing on a Hitachi 5500 sequencer with Texas Red labeled primers.

Expression of Rat MAOA in Yeast—The method of transformation of yeast was basically according to Ito *et al.* (*17*). The transformed cells were first cultured in 200 ml of CM leucine drop-out medium to let the cells grow for 30 h. Then the medium was changed to replace the glucose with 2% galactose and the cells were cultured for another 30 h. The cells were then harvested for further treatment.

Purification of His-Tagged Rat MAOA and Its Mutants—The harvested cells were washed with distilled water and then suspended in Zymolyase buffer (50 mM Tris-Cl buffer pH 7.5, 1 M sorbitol, 10 mM $MgCl_2$) containing 30 mM DTT at a ratio of 1:1 (weight of the cell pellet: volume of the homogenizing buffer), and left at room temperature for 15 min. After centrifugation at 1,500 ×*g* for 5 min, the resultant pellet was suspended in the above buffer containing 1 mM of DTT at a ratio of 1:3. Zymolyase 100 T (2 mg per gram of cell) was added to the suspended cells, and the suspension was shaken gently for 1 h at 30°C. After Zymolyase treatment, the cells were collected by centrifugation at 1,500 ×*g* for 5 min, suspended in lysis buffer (50 mM Tris-Cl pH 7.5, 0.65 M sorbitol, 10 mM potassium acetate, 2 μg/ml of aprotinin, 100 μM of chymostatin, 100 μM of leupeptin, 1 μg/ml of pepstatin, and 1 mM of PMSF), and homogenized with a glass homogenizer. The homogenate was centrifuged at 10,000 ×*g* for 30 min, and the resulting pellet was solubilized in 100 mM imidazole, pH 7.5, containing 500 mM NaCl, 30% glycerol, 1% Triton X-100 on an ice bath for 1 h. After centrifugation at 100,000 ×*g* for 30 min at 4°C, the supernatant was applied to a 1 ml HisTrap chelating column pre-loaded with $NiCl_2$ and equilibrated with 10 ml of the above buffer. The column was washed with 20 ml of the same buffer containing 0.1% of Triton X-100, and the yellow-colored MAOA was eluted with the same buffer containing 500 mM imidazole and stored at –20°C.

Assay of MAOA Activity—A radiochemical method (*18*) was employed in this study. Substrates used were 5-[2-^{14}C]hydroxytryptamine binoxalate, 5-[2-^{14}C]tryptamine bisuccinate, [side chain-2-^{14}C]tyramine hydrochloride, [1-^{14}C]-phenylethylamine hydrochloride. The reaction was at 30°C for 1 to 10 min to keep the amount of substrate catalyzed less than 10%. After stopping the reaction with 2 M HCl, the product was extracted with water–saturated ethyl acetate–toluene (1:1 v/v). The radioactivity of the extracted product was measured in a liquid scintillation spectrometer. The K_m and k_{cat} values were calculated from a Lineweaver-Burk plot.

Trypsin Treatment—Ten micrograms of purified MAOA or its mutants was treated with 10 or 100 μg/ml of trypsin in 100 μl buffer comprising 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.85 mM dodecyl-maltoside. The reaction was carried out at 30°C for 30 min.

Other Methods—Protein was determined by the BCA method (*20*) with bovine serum albumin as the standard. SDS-PAGE was carried out according to Leammli (*21*). Western Blot was done as described by Towbin *et al.* (*22*), using antibody against rat MAOA. The amount of FAD in the purified MAOA preparation was calculated from the absorbance at 456 nm using an absorption coefficient of 11,800 cm^{-1} M^{-1} (*19*).

RESULTS

Expression and Purification of His-Tagged MAOA in Yeast Cells—Saccharomyces cerevisiae strain BL2168 was transformed with YEp51-MAOA plasmid, and the enzyme

was expressed as described in "MATERIAL AND METHODS." The his-tagged protein was correctly expressed and located in the mitochondrial membrane, and all the expressed MAOA was proved to be in the active form when estimated by titration with clorgyline (data not shown, *cf. 12*). To examine whether the his-tag has any influence on enzyme properties, his-MAOA and wild type MAOA in the mitochondrial fractions were used and their K_m values for serotonin, PEA, tyramine, and tryptamine were measured. For all four substrates used, the wild type and his-tagged MAOs shared similar K_m values (data not shown). The V_{max} values were also similar when the amount of MAOA was estimated by Western Blot. This indicates that a his tag at the N-terminus does not impair the catalytic properties of MAOA. Imidazole, which was used to purify the enzyme, also did not influence the K_m values.

The his-tagged MAOA and its mutants made by site-directed mutation were expressed under the same conditions and purified by a one–step Ni-chelating column chromatography method as described in "MATERIAL AND METHODS." The yield was about 1.5 mg per liter of yeast culture, and purification about 90 fold was achieved. The purified his-tagged MAOA appeared nearly homogenous in SDS-PAGE (Fig. 1, lane 1).

Effect of Mutation of Tyrosine Residues Near the FAD Binding Site on Catalytic Properties—Several tyrosine residues are located near the FAD-binding site in both MAOA and MAOAB (Fig. 2). To test whether they play any role in the enzyme properties, Tyr402, Tyr407, and Tyr410 in rat MAOA were changed individually to alanine by site-directed mutagenesis. These artificial enzymes were purified and their catalytic properties were measured. The yields of Y402A and Y407A were much lower than that of the wild type. Because of the low activities, the titration of some active mutant MAOAs with clorgyline was difficult. As an alternative, the molecular concentrations were determined by FAD absorption at 456 nm, as described in "MATERIALS AND METHODS." The K_m and k_{cat} values were measured using serotonin, PEA, tyramine, and tryptamine as substrates. The mutant enzymes showed significant drops in catalytic activity as expressed in k_{cat} (Table I). Among them, Y407A completely lost activity toward serotonin, PEA, and tyramine, and showed little activity toward tryptamine. The K_m for tryptamine also increased about 100 fold in Y407A. Y402A also showed a dramatic decrease in activity for all substrates, but the K_m value did not change significantly. The activity of mutant Y410A decreased to some extent, but not as significantly as in the case of Y407A and Y402A. Because these sites are near Cys406, the FAD binding site, the decrease in the catalytic activity of these mutant enzymes might derive from a change in the structure around FAD. Considering the possibility of an interaction between tyrosine residues and FAD due to their aromatic rings, mutant enzymes were prepared in which the tyrosine residues at 402 and 407 were replaced by phenyalanine, which has an aromatic ring similar to tyrosine. As a result, these mutant enzymes showed little change in their catalytic properties compared to the wild type. The results indicate that aromatic rings at these sites are necessary for maintaining the catalytic activity of the enzyme.

Fig. 1. **Purification of mutant enzymes.** Wild type and mutant enzymes were purified as described in "MATERIALS AND METHODS." 0.5 μg of protein was applied to each lane and the SDS-PAGE gel was stained with CBB. Lane 1, wild type MAOA. Lane 2, Y402A. Lane 3, Y402F. Lane 4, Y407A. Lane 5, Y407F. Lane 6, Y410A.

Human MAOA	- ^{400}E Q Y S G G C* Y T A Y F P^{412} -
Rat MAOA	- ^{400}E Q Y S G G C* Y T A Y F P^{412} -
Human MAOB	- ^{391}E Q Y S G G C* Y T T Y F P^{403} -
Rat MAOB	- ^{391}E Q Y S G G C* Y T A Y F P^{403} -

Fig. 2. **Amino acid sequences around FAD binding site of MAOA and MAOB.** Amino acid sequences of human and rat MAOA and MAOB are presented. C* denotes a cysteine residue to which FAD is covalently attached. Tyrosine residues are shown in bold characters.

TABLE I. **Kinetic constants of mutant enzymes using serotonin, PEA, tyramine, and tryptamine as substrates.** MAO activity was assayed with purified enzymes using radioactive substrates, as described in "MATERIALS AND METHODS." The values are the averages of two or three experiments. The k_{cat} values were calculated in terms of moles of FAD.

Substrate/ Enzyme	K_m (mM)	$k_{cat} \times 10^{-3}$ (min^{-1})	$k_{cat}/K_m \times 10^{-1}$
Serotonine			
WT	0.44 ± 0.05	0.76 ± 0.14	17.2 ± 1.4
Y402A	0.55 ± 0.08	0.01 ± 0.004	0.18 ± 0.07
Y402F	0.59 ± 0.15	0.30 ± 0.08	5.1 ± 7.3
Y407A	NC	NC	–
Y407F	0.95 ± 0.41	0.64 ± 0.09	6.7 ± 2.1
Y410A	0.51 ± 0.08	0.11 ± 0.01	2.2 ± 0.5
PEA			
WT	0.57 ± 0.06	0.12 ± 0.01	2.1 ± 0.1
Y402A	1.98 ± 0.52	0.002 ± 0.001	0.01 ± 0.005
Y402F	0.78 ± 0.40	0.05 ± 0.03	0.64 ± 0.21
Y407A	NC	NC	–
Y407F	1.42 ± 0.24	0.07 ± 0.04	0.49 ± 0.23
Y410A	1.13 ± 0.12	0.04 ± 0.01	0.35 ± 0.09
Tyramine			
WT	0.60 ± 0.14	0.41 ± 0.08	6.8 ± 1,2
Y402A	4.6 ± 1.6	0.02 ± 0.01	0.04 ± 0.01
Y402F	0.63 ± 0.16	0.20 ± 0.02	3.2 ± 0.05
Y407A	NC	NC	–
Y407F	1.42 ± 0.17	0.29 ± 0.01	2.5 ± 0,18
Y410A	1.13 ± 0.49	0.12 ± 0.04	2.0 ± 0.3
Tryptamine			
WT	0.033 ± 0.004	0.21 ± 0.01	64 ± 9
Y402A	0.055 ± 0.022	0.004 ± 0.001	0.70 ± 0.16
Y402F	0.035 ± 0.013	0.108 ± 0.011	31 ± 14
Y407A	3.1 ± 0.9	0.011 ± 0.002	0.04 ± 0.01
Y407F	0.028 ± 0.005	0.144 ± 0.009	51 ± 8
Y410A	0.038 ± 0.013	0.072 ± 0.019	19 ± 5

TABLE II. **Molecular ratio of FAD to protein in the wild type and mutant enzymes.** FAD contents and protein amounts were measured with the purified enzymes as described in "MATERIALS AND METHODS." The values are the averages of two or three experiments.

Mutant	Protein Conc. (mg/ml)	ABS at 456 nm	FAD/MAOA
WT	1.33 ± 0.12	0.196 ± 0.022	0.75 ± 0.03
Y402A	0.42 ± 0.05	0.024 ± 0.005	0.29 ± 0.03
Y402F	0.45 ± 0.09	0.047 ± 0.01	0.54 ± 0.01
Y407A	0.65 ± 0.02	0.032 ± 0.01	0.25 ± 0.01
Y407F	0.69 ± 0.04	0.083 ± 0.01	0.61 ± 0.02
Y410A	0.50 ± 0.06	0.038 ± 0.01	0.40 ± 0.06

FAD, 11,800/cm/M at 456 nm.

Role of Tyrosine Residues Near the FAD Binding Site in FAD Incorporation—In calculating the molecular ratio of FAD and the apo-enzyme, we found that Y402A, Y407A, and Y410A had less incorporated FAD than the wild type or Y402F and Y407F, as showed in Table II. The wild type his-MAOA had a FAD:MAO of 0.75:1, rather than 1:1. This can be explained by an error in protein determination, so that the ratio in the wild type would be near 1. The mutants Y402A, Y407A, and Y410A showed ratios less than 0.4:1, although the ratios for mutants Y402F and Y407F were higher (0.54:1 and 0.61:1, respectively). In Fig. 1, the purified mutant enzymes Y402A and Y410A showed two bands with little difference in molecular mass on SDS-PAGE. Y407A also showed the same pattern in some other preparations. The lower protein band seems to be the apo-enzyme without FAD. Since the molecular mass of FAD is about 0.8 kDa, its absence would be enough to produce a difference in migration in SDS-PAGE. The wild type and phenylalanine mutant exhibited absorbance spectra with a peak at 456 nm, while no clear typical FAD absorbance spectrum was observed for the alanine mutant (data not shown). These data suggest that a residue with an aromatic ring at these sites is able to facilitate the covalent binding of FAD during enzyme maturation *in vivo*.

Effect of Mutations on Enzyme Structure—To test if there is a conformational change in these mutants that might cause the decrease in enzyme activity, the trypsin sensitivity of the enzymes was examined. Ten micrograms of the wild type and mutant forms of his-MAOA were digested with trypsin at a concentration of 10 or 100 μg/ml for 30 min at 30°C. The wild type enzyme, Y402F, and Y407F were essentially resistant to digestion by 10 μg/ml trypsin, and some degradation could be recognized by Western Blot at 100 μg/ml trypsin (Fig. 3). However, the mutant enzymes Y402A, Y407A, and Y410A were easily digested by 10 μg/ml trypsin. When the concentration of trypsin was increased to 100 μg/ml, these proteins were completely digested.

Taken together, these data suggest that MAOA with substitutions at Tyr-402, Tyr-407, or Tyr-410 with alanine, but not with phenylalanines show decreases in enzyme activity. Mutations at Tyr402 and Tyr407 have more dramatic effects on enzyme activity than mutation at Tyr-410. This effect may be derived from a conformation change in the enzyme because of the loss of interaction between tyrosine and FAD through their aromatic rings. The loss of this interaction also causes a decrease in the coupling of FAD to the enzyme during the maturation process.

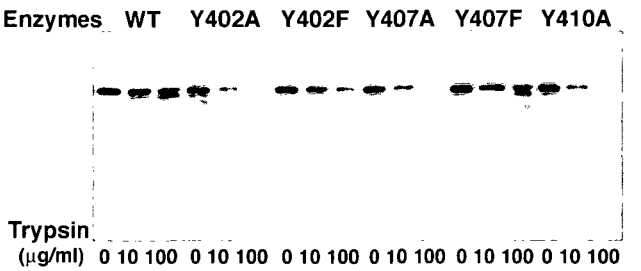

Fig. 3. **Trypsin sensitivity of purified MAOA and mutant enzymes.** Purified MAOA or its mutants were treated with 10 or 100 μg/ml of trypsin in buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.85 mM dodecyl maltoside at 30°C for 30 min. 0.5 mg of protein was applied to SDS-PAGE and Western Blot was done using anti-MAOA antibody.

DISCUSSION

The catalytic oxidative reaction by MAOA is coupled to the reduction of an obligatory cofactor, FAD, which is covalently linked to the apo-enzyme at Cys406. In an earlier study, we found that substitution of Cys406 with alanine resulted in an active form of the enzyme, although there was no covalently bound FAD (*12*), suggesting that the covalent binding of FAD is not necessary for catalytic activity. The finding indicates that the enzyme forms a pocket in which FAD is incorporated. We do not know, however, how FAD is incorporated into the enzyme and forms the covalent binding, although several amino acid residues have been shown to be important for this process in MAOB. For example, Glu34 and Tyr44 are reported to be required for the initial binding of FAD (*24*). Mutagenesis at Glu34 and Tyr44 not only interferes with the covalent flavinylation and catalytic activity of MAOB, but also with the noncovalent binding of FAD. Substitution of tyrosine by phenylalanine had no effect on MAOB activity or the level of FAD incorporation compared with the wild-type enzyme (*25*), indicating that an aromatic ring, but not the hydroxyl group of the tyrosine residue, is essential at residue 44. Interestingly, the crystal structure of a flavoenzyme, monomeric sarcosine oxidase, shows it to be rich in aromatic amino acid residues around the FAD (*26*). This led us to investigate the role of aromatic residues around FAD for its incorporation into MAO.

The regions near the FAD binding sites are conserved between MAOA and MAOB in all species, suggesting that this region is of functional importantce. This region is rich in tyrosine residues, as is also the case in some other flavoenzymes. In MAOA and MAOB, the amino acid sequence around the FAD binding site is -Tyr-Ser-Gly-Gly-Cys(FAD)-Tyr-Thr-Thr-Ala-Tyr- (Fig. 2). The sequences of these regions in flavocytochrome C-552 and 6-hydroxy-D-nicotine oxidase are -Asp-Tyr-Tyr-Thr-Cys(FAD)-Tyr-leu-Ser-Asn-, and -Arg-Ser-Gly-Gly-His(FAD)-Tyr-Gly-Pro-Ala-, respectively. In the present study, the substitution of each of these tyrosine residues, especially Try407 and Tyr402, by alanine was seen to cause a dramatic decrease in the FAD incorporation and enzyme activity of MAOA. Since only the enzyme containing FAD exhibits the activity, a decrease in activity should be caused by a conformational change rather than a lack of FAD itself. Further, the mutant enzymes lost their correct folding, as indicated by the loss of resistance to trypsin. Wild type MAOA seems to fold tightly

because it is resistant to rather high concentrations of trypsin (Fig. 3), while the alanine mutants are quite sensitive to the protease. Although it is reasonable that the apo-enzyme lacking FAD would be more sensitive to trypsin treatment, we believe that mutant enzymes, even if FAD remains, lose their tight folding because they are digested almost completely by 10 μg/ml trypsin, while the wild type remains undigested. When the tyrosine residues were replaced by phenylalanine, the enzyme properties showed little change, and their resistance to trypsin was similar to the wild type enzyme (Table I and Fig. 3). These results indicate that residues with aromatic rings are important for maintaining the structure and catalytic properties of rat MAOA.

Among the tyrosine residues, Tyr-407 seems to be the most critical for enzyme activity, FAD binding, and enzyme folding. No activity for Y407A was detected using serotonin, tyramine, or PEA as substrates, and only low activity was seen with tryptamine. Unlike the other mutants, Y407A showed a more than 100-fold increase in the K_m value for tryptamine. This means that Tyr407 is responsible for substrate binding as well as for FAD binding and enzyme stability. The mechanism for this phenomenon remains to be studied. The residue may interact with the substrate directly, or be responsible for forming a specific structure for substrate binding. In any case, an aromatic amino acid residue seems to be helpful for substrate affinity, because Y407F showed little change in the k_{cat} or K_m values for substrates.

The attachment of FAD to the enzyme may require a pocket constructed partially of tyrosine residues (and may also include Tyr-53, which is corresponds to Tyr-44 in MAOB). Through the interactions among the aromatic rings of these tyrosine residues and that of FAD *via* a π-π cloud, the enzyme forms a catalytic center and adopts a stable conformation. Mutations at these sites may cause this structure to fail to form, resulting in drop in activity and loss of protease resistance.

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