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Characterization of Rat Monoamine Oxidase A with Noncovalently-Bound FAD Expressed in Yeast Cells

Iwao Hiro, Yukio Tsugeno, Isamu Hirashiki,[1] Fusahiro Ogata,[2] and Akio Ito[3]

Department of Chemistry, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka 812-81

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The FAD-binding cysteine of rat liver monoamine oxidase A (MAO A), Cys406, was converted to an alanine by site-directed mutagenesis of the cDNA. The wild-type and mutated enzymes were expressed in yeast cells and catalytic activities were assayed, using as substrates serotonin, tyramine, and kynuramine. Specific activities of the Ala-mutant for these substrates, calculated as the activities per pargyline-sensitive molecule, were about half of those of the wild-type enzyme. The K_m values of the mutant enzyme for the substrates were similar to those of the wild-type enzyme. An adduct between FAD and pargyline, a mechanism-based inhibitor, was attached to the apoprotein in the wild-type enzyme, while in the Ala-mutant it was detached from the apoprotein, thereby indicating the presence of noncovalently bound FAD in the mutant enzyme. The Ala-mutant rapidly lost activity during incubation, whereas the wild-type enzyme retained the initial activity. Partial protection from inactivation occurred in the presence of FAD, but not of FMN. Recovery of the enzyme activity was nil when FAD was added after the inactivation. Thus, while the covalent attachment of FAD in MAO A is not required for the catalytic activity, it may function as a structural core for the active conformation in the membrane.

Key words: covalently-bound flavin, flavoprotein, monoamine oxidase A, pargyline, site-directed mutagenesis.

Monoamine oxidase (MAO) is one of the enzymes responsible for the catabolism of various biogenic amine neurotransmitters as well as for the metabolism of certain exogenous amines, and is an intrinsic protein of the outer membrane of mammalian mitochondria (*1, 2*). Two forms of MAO, termed MAO A and MAO B, are defined by their substrate and inhibitor affinities (*1*). cDNA clones for MAO A and MAO B have been isolated from different species (*3-7*) and the primary structures of the proteins deduced from the cDNAs showed about 70% sequence identity between MAO A and MAO B. Using chimeric enzymes constructed from MAO A and MAO B, the regions of the molecule responsible for the substrate specificity of these enzymes were determined to be in the amino-terminal portion (*8, 9*). Each type of enzyme has an FAD covalently attached to a cysteine residue, Cys406 in MAO A and Cys397 in MAO B, *via* an 8α-(*S*-cysteinyl)-riboflavin linkage (*10*).

The prosthetic group of more than twenty flavoenzymes from bacteria to mammalian cells is covalently linked to the side chain of a particular amino acid, such as His, Tyr, or Cys *via* an 8α-*N*(1)-histidyl, 8α-*N*(3)-histidyl, 8α-*S*-cysteinyl, 6-*S*-cysteinyl, or 8α-*O*-tyrosyl FAD linkage (*11*). Despite the functional importance of flavin in these enzymes, little is known about the significance of the covalent binding of flavin to apoenzyme in relation to the catalytic activity, or the mechanism of covalent linking of the prosthetic group to a specific amino acid during intracellular transport and folding of the enzymes after synthesis on ribosomes.

In 6-hydroxy-D-nicotine oxidase (6-HDNO) and fumarate reductase, FAD is attached *via* an 8α-*N*(3)-histidyl linkage. Replacement of the FAD-binding histidine in these enzymes (*12, 13*) by other amino acids facilitated incorporation of FAD into the apoproteins in a non-covalent manner, and the mutant enzymes exhibited enzymatic activity, albeit at low levels, indicating that flavinylation is not an absolute requirement for catalysis. While the 8α-[*N*(3)-histidyl] linkage in 6-hydroxy-D-nicotine oxidase is formed autocatalytically, a certain conformational state of apo-6-hydroxy-D-nicotine oxidase was required for autoflavinylation (*14*). An unknown factor in the cytosol or in organelles is probably needed to maintain competency of the apoenzyme for incorporation of flavin.

Fumarate reductase (*12*), succinate dehydrogenase (*15*), and 6-hydroxy-D-nicotine oxidase (6-HDNO) (*13*) contain 8α-[*N*(3)-histidyl] FAD, and mutants in which the histidine is altered to other amino acid residues contain noncovalently bound FAD and show enzymatic activity. In the case of trimethylamine dehydrogenase, in which the cofactor FMN is covalently linked by a 6-*S*-cysteinyl FMN bond, its cysteine mutants showed enzymatic activity with non-covalently bound FMN, too. These observations raise the question of whether or not flavination is required for catalysis.

Covalent bond formation requires the expenditure of

Present addresses: [1] Central Research Laboratory, Mitsubishi Electric Corporation, Amagasaki, Hyogo 661; [2] Department of Biochemistry, School of Medicine, Kurume University, Kurume, Fukuoka 830. [3] To whom correspondence should be addressed. Tel: +81-92-642-2350, Fax: +81-92-642-2607, e-mail: a.itoscc@mbox.nc.kyushu-u.ac.jp

Abbreviation: MAO, monoamine oxidase.

free enthalpy. From a chemical point of view, activation of the 8α-methyl group appears essential for coupling of FAD. 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 phosphorylation. A study by Zhou *et al.* (*16*) using a riboflavin-depleted COS-7 cell line supports this hypothesis. Insight into the mechanism of the synthesis of the covalent apoenzyme-flavin bond has been gained for the histidyl (*N*3)-8α-FAD linkage in 6-HDNO (*14*). While 6-HDNO undergoes a self-catalytic addition of the cofactor, a certain conformational state of apo-6-HDNO is required for autoflavinylation. Whether or not the same principle of synthesis is valid for other enzymes of the same type or even for other covalent cofactor-apoprotein linkages is still unknown. Despite the functional importance of flavin in these enzymes, little is known of the significance of covalent binding of flavin to apoenzyme for the catalytic activity, or the mechanism of covalent linking of the prosthetic group to a specific amino acid during intracellular transport and folding of the enzymes after synthesis on ribosomes.

Wu *et al.* (*17*) reported that substitution of the FAD-binding cysteine residue of human MAO A and MAO B for serine resulted in a complete loss of the catalytic activity. This suggested that the covalent binding of FAD to this enzyme is essential for catalytic activity or for the formation of a proper conformation. We now report that when the cysteine of rat liver MAO A is replaced by alanine, the resultant Ala-mutant retains significant MAO activity and FAD is noncovalently bound to the enzyme.

MATERIALS AND METHODS

Materials—An oligonucleotide used for introduction of the mutation was synthesized using an Applied Biosystems 380B DNA Synthesizer. Restriction endonucleases and DNA modifying enzymes were purchased from Nippon Gene (Toyama), Toyobo (Osaka), Takara Shuzo (Kyoto), and New England Biolabs (Beverly, USA). An oligonucleotide-directed *in vitro* mutagenesis system was from Amersham (Buckinghamshire, UK). Zymolyase 20T, BCA protein assay kit, and HRP-conjugated goat anti-rabbit IgG were from Seikagaku Kogyo (Tokyo), Pierce (Rockford, USA), and Zymed (California, USA), respectively. Clorgyline and pargyline were obtained from Sigma (St. Louis, USA) and Research Biochemicals (USA), respectively. The radiochemicals, 5-[2-^{14}C]hydroxytryptamine (serotonin) bioxalate (55.8 mCi/mmol), [ring-^{3}H]tyramine hydrochloride (43.8 mCi/mmol), and [^{3}H]pargyline hydrochloride (phenyl and benzyl labeled; 714 GBq/mmol) were from New England Nuclear (USA). Plasmid YEp51 and yeast strain *Saccharomyces cerevisiae* YSA-1C (*MATa leu2 pep4*) were kind gifts from Drs. H. Tanaka and Y. Jigami. All other chemicals used here were of the highest grade commercially available.

Construction of Mutant cDNA in Expression Vector—The cDNAs for rat MAO A (*7*) were used for construction of a mutant protein. A site-directed mutation of the FAD-binding cysteine (^{406}Cys) to alanine was introduced, using a 27mer oligonucleotide, GTAGGCTGTGTAGGCGCCCCCGGAGTA. The mutation was verified by DNA sequencing. The original and mutant cDNA so constructed were inserted into a yeast expression vector, YEp51, downstream of the GAL10 promoter.

Expression of MAO A in Yeast Cells—Transformation of yeast cells, *S. cerevisiae* strain YSA-1C was done according to a published method (*18*) and transformants were selected on complete minimal leucine dropout medium (*19*). The transformed cells were cultured in the same medium, except glycerol was used instead of glucose, induction was with 1% galactose at an OD of about 0.4, followed by culture for an additional 48 h.

Preparation of Cell Extract and Mitochondria—Unless otherwise mentioned, all the procedures were carried out at 4°C. After harvesting and washing of the cells with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and 1 M sorbitol, these cells were incubated with Zymolyase 20T in the same buffer for 60 min at 30°C. The resultant spheroplasts were homogenized in a glass-Teflon homogenizer in 10 mM HEPES-KOH buffer, pH 7.4, containing 0.65 M sorbitol, 1 mM EDTA, and 1 mM PMSF to obtain cell extracts, which were then centrifuged at 2,500×*g* for 10 min and the supernatant was further centrifuged at 10,000×*g* for 10 min to obtain mitochondrial fractions. The mitochondrial pellet was resuspended in the same buffer and stored at −80°C until use.

Assay of MAO Activity—MAO activity was determined fluorometrically or radiometrically using kynuramine (*20*) or serotonin and tyramine (*21*) as the substrate, respectively. Kynuramine-oxidizing activity of the cell extract was measured in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM kynuramine. After incubation of the reaction mixture for 20 min at 30°C, the reaction was terminated with trichloroacetic acid and the product was measured fluorometrically. When radioactive substrates were used, the mitochondrial fraction was incubated in a total volume of 60 μl of 50 mM phosphate buffer, pH 7.4, containing 0.5 mM serotonin or tyramine, for 20 min at 30°C, and the reaction was stopped by adding 40 μl of 2 M HCl. The product was extracted with water-saturated ethyl acetate-toluene (1 : 1 v/v) and the radioactivity transferred into the organic solvent phase was estimated in a Packard Tri-Carb liquid scintillation spectrometer. Specific activity was expressed as nmol/min/mg of protein.

Labeling of MAO A with Pargyline—The mitochondrial fraction was incubated at 0°C in the suspension buffer, 10 mM HEPES-KOH buffer, pH 7.4, containing 0.65 M sorbitol, 1 mM EDTA, and 1 mM PMSF, in the presence of 10 μM [^{3}H]pargyline (*22, 23*). The reaction was terminated by adding SDS-PAGE sample buffer and boiled for 1 min at 100°C. Pargyline-conjugated proteins were separated by SDS-PAGE and the radioactivity was detected with fluorography.

Other Analytical Procedures—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (*24*). Immunoblot analysis was done using the method described by Towbin *et al.* (*25*), with rabbit anti-MAO A and HRP-conjugated goat anti-rabbit IgG for the primary and secondary antibody, respectively. Protein content was estimated using bicinchoninic acid, with bovine serum albumin as a standard (*26*).

RESULTS

Expression of Mutant MAO A in Yeast Cells—The FAD-

binding cysteine of rat liver MAO A, Cys406, was changed to an alanine residue by site-directed mutagenesis of the MAO A cDNA. The wild-type and mutated cDNAs were introduced into the yeast cell, *S. cerevisiae YSA-1C*, and the cell extracts were prepared as described in "MATERIALS AND METHODS." Expression of the recombinant MAOs in yeast cells was verified by Western blot analysis, using the specific antibody against rat MAO A (Fig. 1A). Kynuramine-oxidizing activity of extracts from the cells expressing the wild-type and mutated enzymes under various culture conditions was measured (Table I). The extract from the mutant cell exhibited a substantial level of the activity, about a half that of the wild-type cell extract, in contrast to the serine-substituted mutant, which had no activity (*17*). Subcellular fractionation experiments revealed that the activity in the mutant cell was localized in the mitochondria, as was that in the wild-type cell (data not shown). More than 95% of the activity in the mutant cell was resistant to proteinase K digestion, as was the case in the wild-type cell. Thus, the Ala-mutant was inserted into the mitochondrial membrane in the same manner as the wild-type enzyme.

Non-Covalent Binding of FAD to the Ala-Mutant—To determine whether FAD involved in the activity is attached covalently or noncovalently to the mutant protein, mitochondrial fractions from the wild-type and mutant cells were incubated with radio-labeled pargyline, a mechanism-based irreversible inhibitor, followed by SDS-PAGE and fluorography. Since a preliminary experiment showed that activities in both mitochondria were completely inhibited by 10 μM pargyline, this concentration of the inhibitor was used in this experiment. Figure 1B shows a fluorogram of the radioactive pargyline bound to MAO proteins through covalently bound FAD. In the wild type mitochondria, a single band appeared at the position corresponding to MAO A protein (Fig. 1B, lane 1), whereas no band was observed in the case of Ala-mutant (lane 2). Although we could not detect the FAD-pargyline conjugate even in the low molecular weight region on SDS-PAGE, since pargyline inhibited the activity of the Ala-mutant, the inhibitor could bind to FAD in a mechanism-based manner. When the labeled materials were subjected to thin-layer chromatography, the radioactivity remained at the original position because pargyline was attached to the protein (data not shown). However, the radioactive spot was detected at around 0.4 R_f in the Ala-mutated sample, suggesting the presence of the free pargyline-FAD adduct, though the R_f value of the pargyline-FAD adduct is not known because the authentic compound is not available. The adduct seems to be more hydrophilic than free pargyline owing to the presence of the nucleotide moiety and to have an intermedi-

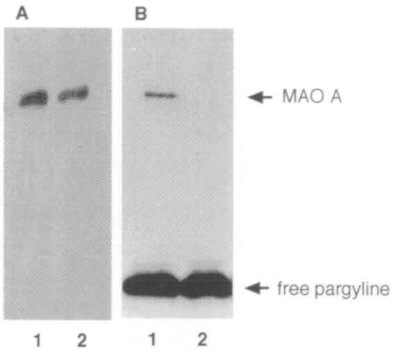

Fig. 1. **Expression of the wild-type and mutant MAO proteins in yeast cells and detection with [^{3}H]pargyline of the FAD covalently bound to the protein.** A: Mitochondrial fractions (5 μg protein) from the cells expressing the wild type MAO A (lane 1) and the Ala-mutant (lane 2) were subjected to SDS-PAGE and the MAO proteins were detected by Western blot analysis using anti-MAO A antibody. B: Mitochondrial fractions (1.8 μg protein) from the cells expressing the wild-type MAO A (lane 1) and the Ala-mutant (lane 2) were incubated with radioactive pargyline as described in "MATERIALS AND METHODS."

TABLE I. **Kynuramine-oxidizing activity of the wild-type and Ala-mutant enzymes expressed in yeast cells.** Analysis was done using whole cell extracts as described in "MATERIALS AND METHODS." Activities were determined by incubation (20 min at 30°C) in the presence of 1 mM kynuramine as the substrate.

Culture conditions	Activity (nmol/min/mg of protein)	
	Wild-type	C406A
25°C 48 h	1.45	0.30
30°C 24 h	2.62	1.02
30°C 48 h	3.66	1.30

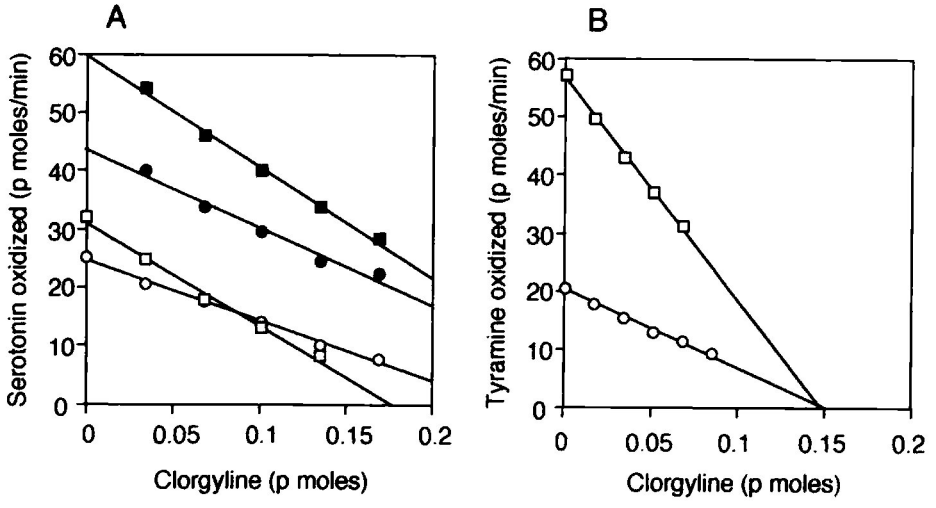

Fig. 2. **Titration of active enzyme with clorgyline.** The enzyme preparations were preincubated with various concentrations of clorgyline at 0°C overnight. Activities were assayed with serotonin (A) and tyramine (B) as substrates. A: Wild-type 0.8 μg of protein (□), wild-type 1.6 μg (■), Ala-mutant 1.0 μg (○), Ala-mutant 2.0 μg (●). B: Wild-type 0.36 μg (□) and Ala-mutant 0.54 μg (○).

ate R_f value between those of free and protein-bound pargyline.

Molecular Activity of the Ala-Mutant—To assess the role of the covalently bound FAD in the catalytic activity of MAO, we first compared the molecular activity of the wild-type and Ala-mutated enzymes. The number of active molecules in the mitochondrial preparations was estimated by titration of the activities with clorgyline, an irreversible mechanism-based inhibitor specific to MAO A (*27*). After incubation of the mitochondrial fraction with various amounts of clorgyline at 0°C overnight, the remaining enzyme activity was measured using serotonin or tyramine as the substrate (Fig. 2, A and B, respectively). The enzyme activity decreased as a linear function of the amount of clorgyline added. In the figure, the amount of clorgyline required to give 100% inhibition of the activity is directly related to the number of active MAO molecules and the slope of the line is related to the molecular activity. From Fig. 2A, the molecular activities of the wild-type and Ala-mutant enzymes for serotonin were calculated to be 182 and 118 min^{-1}, respectively, using their initial activities and the amounts of clorgyline required for complete inhibition. Several determinations showed average activities of 167 ± 21 and 106 ± 17 min^{-1} for the wild-type and Ala-mutant enzymes, respectively. Thus, the mutant MAO has about 70% of the molecular activity of the wild type enzyme. When tyramine was used as a substrate instead of serotonin, the average molecular activities of the wild-type and Ala-mutated enzymes were calculated to be 162 ± 4 and 63 ± 4 min^{-1}, respectively, so that the activity of the mutant enzyme was about 40% of that of the wild-type one.

The effect of mutation on affinity for substrates was assessed in terms of K_m values of the wild-type and Ala-mutated enzymes for the two substrates. The K_ms for serotonin were calculated to be 43 and 59 μM for the wild-type and Ala-mutated enzymes, respectively. Those for tyramine were 138 and 140 μM, respectively. Thus, there was no significant difference in affinity for the substrates between the two enzymes.

Inactivation of the Ala-Mutant and the Protection by FAD—In the course of titration experiments, we noticed that the enzyme activity of the Ala-mutant rapidly fell with incubation time at 30°C. We thus measured the time course of inactivation of the enzyme activity at various temperatures (Fig. 3). The Ala-mutant rapidly lost serotonin-oxidizing activity during incubation and only about 30% of the initial activity remained after incubation at 30°C for 8 h, whereas the wild-type enzyme retained its initial activity, even after 8 h. As shown in the figure, practically no decrease in the activity was observed for the Ala-mutant at 0°C. Thus, covalently bound FAD probably plays an important role in maintaining the active conformation of the MAO molecule.

Figure 4 shows the effects of addition of FAD during the incubation. The presence of 5 μM FAD clearly protected

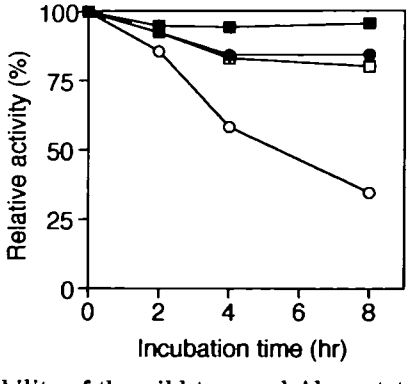

Fig. 3. **Stability of the wild-type and Ala-mutated enzymes.** Samples of the mitochondrial preparation were incubated at 0°C (solid symbols) or 30°C (open symbols) prior to estimation of MAO activity with serotonin as the substrate. Enzyme activity was measured at 30°C as described in the text. Data are plotted as percent remaining activity (relative to the initial activity) of wild-type (squares) and Ala-mutated (circles) MAO A.

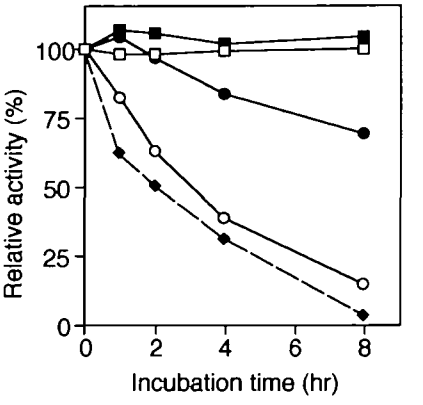

Fig. 4. **Effect of FAD and FMN on inactivation of the wild-type and Ala-mutant enzymes.** Samples of the mitochondrial preparation were incubated at various times, with or without 5 μM FAD, prior to estimation of MAO activity with serotonin as the substrate. Symbols are: wild-type enzyme (□, ■), Ala-mutant enzyme (○, ●). Closed symbols represent the addition of FAD. On the other hand, FMN was used with Ala-mutant instead of FAD (◆). Assays were performed as described in "MATERIALS AND METHODS," except that mixtures contained FAD or FMN.

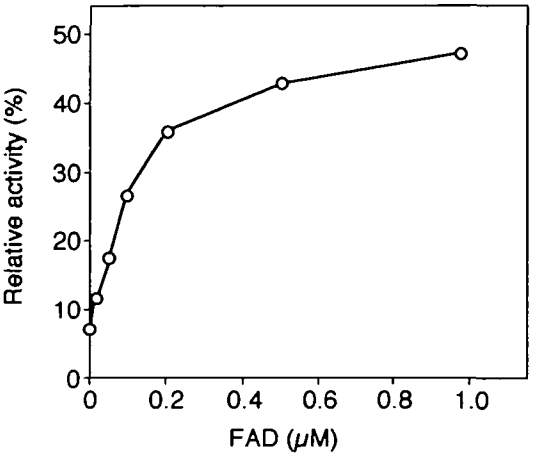

Fig. 5. **Effect of concentration of FAD on protection from inactivation of the Ala-mutated enzyme.** The mitochondrial preparations containing Ala-mutated MAO A were preincubated with various concentrations of FAD at 30°C for 13 h. Enzyme activity was assayed with serotonin as the substrate, as described in "MATERIALS AND METHODS." The activity was expressed as the value relative to the initial activity without preincubation.

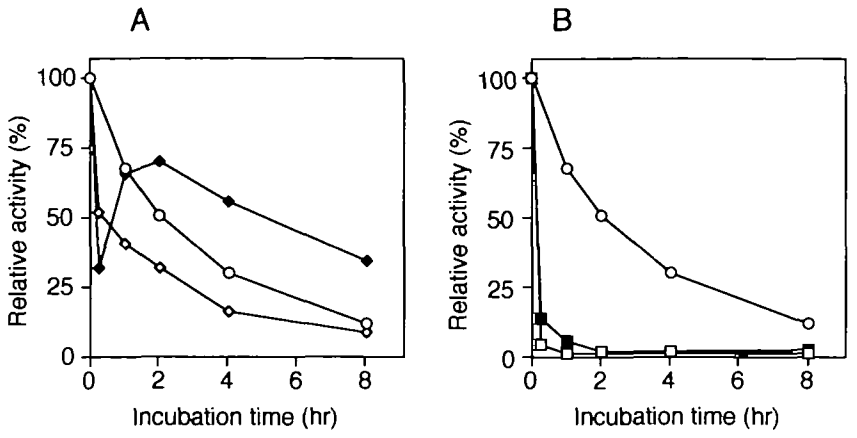

Fig. 6. **Reactivation of clorgyline-inactivated Ala-mutated enzyme by FAD.** Experiments were conducted essentially as described in "MATERIALS AND METHODS." The enzyme preparations of Ala-mutated MAO A were preincubated at various times with 0.5 (◆, ◇) or 2 equivalent (□, ■) of clorgyline to the active enzyme in the presence (closed symbols) or absence (open symbols) of FAD (5 μM) and then remaining activities were estimated by serotonin oxidation assay. The number of active molecules was previously estimated by titration of the activities with clorgyline. The enzyme activity of Ala-mutated MAO A incubated without FAD and clorgyline is shown (○).

the mutant enzyme from inactivation and about 70% of the activity remained after 8 h. No protection from inactivation was observed with FMN. The dose response of FAD for protection is shown in Fig. 5. Half-maximal protection from inactivation was obtained at as low a concentration as 0.1 and 1 μM FAD gave the maximal protection. The protection by FAD suggests that the observed inactivation is due to detachment of FAD from the enzyme protein and that the externally added FAD can be inserted into the resultant apoprotein. To examine whether FAD can be incorporated into apoprotein after complete depletion of the flavin, the mitochondrial fraction was incubated for 12 h at 30°C to completely inactivate the Ala-mutant and then 5 μM FAD was added to the incubation mixture. The recovery of the enzyme activity was nil. Thus, FAD addition might be effective only during the inactivation.

Since clorgyline is covalently bound to FAD in a mechanism-based manner and irreversibly inactivates the wild-type enzyme, we examined whether clorgyline inactivates Ala-mutant as fast as the wild-type enzyme and whether inactivation is protected by FAD, by incubating mitochondrial fractions with clorgyline in the presence of FAD (Fig. 6). When 0.5 equivalent of clorgyline to the active enzyme was used, the activity was more rapidly decreased than was the case without any addition. In the presence of the flavin, however, the activity first decreased to about 50% of the initial value in 15 min and then increased to about 70% for one to two hours, followed by a decrease (Fig. 6A). When two equivalents of clorgyline were used, the activity was almost completely inactivated within 15 min and recovery of the activity by FAD was nil (Fig. 6B). The reactivation of the partially inactivated enzyme by FAD clearly demonstrates the re-insertion of FAD into the clorgyline-inactivated enzyme.

DISCUSSION

We obtained evidence that when the FAD-binding cysteine of rat MAO A was replaced by alanine, the mutated protein expressed in yeast cells exhibited a level of activity similar to that seen in the wild-type enzyme. Molecular activities of the Ala-mutated enzyme, calculated based on the clorgyline-sensitive molecule, were about half those of the wild-type enzyme, for both serotonin and tyramine, though, the K_m values for these substrates were not altered by the mutation. The retention of the same affinity as the wild-type enzyme for substrates indicates that the structure of the enzyme, at least around the substrate binding site, was little changed by replacing cysteine with alanine. The difference in the activity between the wild-type and mutant enzymes is probably due to the state of FAD, *i.e.*, covalently linked *versus* non-covalently attached. Systematic studies on oxidation-reduction potentials of a number of 8α-substituted flavin analogues demonstrated that substitution of this position raises the potential by 0.02–0.03 V and that the nature of the substituents has little effect on electron affinity (*28*). Thus, the lowered activity of the Ala-mutant of MAO A could be explained by lowering of the redox potential of the flavin, as proposed for interpretation of the lowering of the activity of fumarate reductase by mutation of the histidine residue to which FAD is covalently attached (*29*). The decreased redox potential of the flavin in the mutant MAO A seems to be responsible for different effects of the mutation on the catalytic activity towards the two substrates. It is reasonable to surmise that the redox potential of substrates would be related to the rate of electron transfer rather than to affinity to the enzyme. In fact, the mutated fumarate reductases in *E. coli* showed activity to reduce fumarate, but no activity on succinate dehydrogenation (*12*). Thus, covalent linkage of FAD in MAO A is not essential for the catalytic activity or for the substrate binding.

As reported by Wu *et al.* (*17*), who used COS cells, substitution of the cysteine residues, Cys406 in MAO A and Cys397 in MAO B, to serines resulted in complete loss of the activity in yeast cells (Hiro and Ogata, unpublished results). The complete loss of the activity was also found in the mutant protein in which the residue was replaced with histidine (Hiro and Ogata, unpublished results). Lack of activity of the serine- or histidine-substituted mutants suggested that FAD was not attached to the apoprotein. It is interesting that both the wild-type and Ala-mutant enzymes contain FAD and that the serine mutant did not incorporate the cofactor. Since the nucleophilicity of the hydroxyl group is much weaker than that of the thiol group, it would not be sufficient for ether bond formation between the serine hydroxyl group and the 8α-methyl of FAD. Cysteine thiol seems to be a little larger than the serine hydroxyl group, although alanine methyl is much smaller. Taken together with the rapid inactivation of Ala-mutant, the results suggest that the FAD-binding pocket of MAO A is small and FAD would be tightly incorporated into the

pocket. The alanine mutant, but not the serine mutant, would have room for FAD incorporation. The wild-type enzyme has structural distortion around the FAD-binding region, but the structure of the enzyme is stabilized by the covalently bound FAD. In the Ala-mutant, once FAD is released from the protein, the apoprotein would no longer be able to retain a restrained structure and to incorporate FAD. Thus, in the wild-type enzyme, FAD might function as a structural core and as a catalytic center.

FAD of the Ala-mutated MAO A was non-covalently bound to the enzyme and was readily released from the apoprotein *in vitro*. Once the mutated enzyme had lost its catalytic activity by release of the prosthetic group, the activity could not be reinstated upon supplementation of external FAD. Since almost the same amount of the protein was detected by Western blot analysis (Hiro, unpublished data), the apoprotein remained in the membrane and failure of recovery of the activity by the addition of FAD was not due to degradation of the protein. The conformation of the apoprotein, or at least the FAD-binding pocket of the enzyme, seems to be rapidly denatured so that the prosthetic group can not be inserted into the apoprotein. When the mitochondrial fraction was incubated in the presence of FAD, inactivation of the catalytic activity was partially prevented. Since half-maximal protection of the inactivation was attained by about 10^{-7} M FAD (Fig. 5), the apparent dissociation constant of FAD to the Ala-mutant would be of this order of concentration. Compared with dissociation constants of non-covalently bound flavins in most flavin enzymes of the order of 10^{-7} to 10^{-10} (*30*), FAD is not firmly associated with the apoprotein of the mutant enzyme.

MAO protein is synthesized on free polysomes (*31*) and is transported to the outer membrane of mitochondria under the guidance of a targeting signal which is located at the carboxy terminal of the enzyme (*32*). When, where, and how the apoprotein covalently attaches FAD to the specific cysteine residue and forms an appropriate conformation in the outer mitochondrial membrane have to be considered. Since yeast and *E. coli* cells have no apparent enzyme containing this type of linkage and the presence of a linkage enzyme in both types of the cells is unlikely, the observation that the active enzyme with covalent FAD was formed in transformed cells (*33, 34*) suggests that the reaction occurs by autoflavinylation. Zhuang *et al.* (*35, 36*) reported that human monoamine oxidase A synthesized in a reticulocyte lysate translation system did not catalyze its own alkylation by the mechanism-based inhibitor, clorgyline, while the enzyme bound or inserted into the outer mitochondrial membrane catalyzed adduct formation, and that the enzyme acquired catalytically active conformation upon binding to the membrane, prior to its ATP and ubiquitin-dependent insertion (*36*). The present study also suggests that binding of FAD to the apoprotein leads to an active conformation. The FAD-binding domain of newly synthesized MAO apoprotein might be formed with the assistance of chaperones during or after transport into mitochondria, then FAD is incorporated into the pocket and autocatalytically linked to the specific cysteine residue *via* a thioether bond. Folding of at least some domains, including the active site, occurs and the active enzyme is inserted into the outer mitochondrial membrane after exposure of hydrophobic areas by mediation of ubiquitin and ATP. Zhou *et al.* (*16*) recently showed that MAO B was expressed equally in riboflavin-depleted COS-7 cells in the presence and absence of FAD, while no MAO B catalytic activity was observed when FAD was added *in vitro* after apoMAO B had been synthesized, suggesting that the apoprotein is extremely unstable and readily become incompetent for FAD incorporation, as expected from our present results. They also reported that Glu34 was essential for FAD binding and suggested that FAD binds to MAO B in a dual manner, first at Glu34 noncovalently and then at Cys397 covalently.

Recent observations on other flavoproteins suggest that attachment of flavin occurs during the folding of the enzyme. A small proportion of trimethylamine dehydrogenase is usually present as the deflavo enzyme in bacterial cells and no flavinylation product was obtained from the deflavo form, suggesting that in the fully folded form the apoenzyme is unable to recognize flavin (*37*). Covalent FAD attachment to the flavoprotein subunit of *p*-cresol methylhydroxylase absolutely requires the partner subunit, cytochrome *c* subunit (*38*). Yeast succinate dehydrogenase binds FAD after import and processing of the precursor protein in mitochondria, and the flavinylation is stimulated by one of the partner subunits, iron-sulfur protein, and TCA cycle intermediates (*39, 40*). Carboxy-terminal truncations of the apoprotein, which should not affect flavinylation if the reaction proceeds with unfolded protein, block the flavinylation. These results suggest that FAD addition occurs during or after the mature apoprotein folding and prior to its assembly. These findings suggest that protein conformation may be the signal for FAD attachment. As discussed above, apoMAO protein loosely bound to the outer mitochondrial membrane or trapped with a chaperonin might be the folding intermediate competent to be recognized by FAD, and the conformation of the apoprotein might irreversibly change after insertion into the membrane as an integral membrane protein.

MAO A with noncovalent FAD also provides a good system to study reaction mechanisms. We can change FAD coenzyme to its derivatives to gain insight into structural requirements for flavin and also to isolate adducts with mechanism-based inhibitors to analyze structures and reaction mechanisms of adduct formation.

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