The FAD binding site of human liver monoamine oxidase A (MAO A) has been investigated by mutagenesis of the amino acid side chain of MAO attachment (Cys-406) to an alanyl residue. Expression of the C406A mutant in Saccharomyces cerevisiae results in the formation of an inactive enzyme, as found previously with the rat liver enzyme. The activity of this mutant enzyme is labile to solubilization, thus requiring all experiments to be done with membrane preparations. C406A MAO A was expressed in a rib 5'-cotranslational complex of the enzyme with the absence of riboflavin. FAD but not FMN or riboflavin restores catalytic activity with an apparent Kd of 62 ± 5 nM. The results from both in vivo and in vitro reconstitution experiments show increased activity levels (up to ~7-fold higher) when the analogues exhibiting higher oxidation-reduction potentials than normal flavin and decreased activity levels with analogues exhibiting lower potentials. Analogues with substituents on the pyrimidine ring bind to C406A MAO A more weakly than normal FAD, suggesting specific interactions with the N(3) and N(1) positions. Analogues with substituents in the 7 and 8 positions bind to C406A MAO A with affinities compatible with that of normal FAD. These results are discussed in regard to functional significance of 8a-covalent binding of flavins to proteins.

The FAD cofactors of monoamine oxidases A and B (EC 1.4.3.4, MAO A and MAO B) have been known for more than 30 years to be covalently bound to their respective proteins. The work of Kearney et al. (1) demonstrates the nature of the covalent linkage in MAO B to be an 8a-S-cysteinyl-FAD. Subsequent studies on purified human placental MAO A also showed the same type of covalent linkage (2). More recent studies show that recombinant human liver MAO A expressed in Saccharomyces cerevisiae (3) and human liver MAO B expressed in Pichia pastoris contain an identical covalent flavin linkage. The molecular basis for the requirement of a covalent flavin linkage has been addressed in MAO as well as other enzyme systems containing covalent flavin coenzymes. Mutagenesis studies by Shih and co-workers (5) demonstrate that replacement of Cys-397 in MAO B with a seryl residue resulted in an inactive enzyme in a COS cell expression system. More recent work from Ito and co-workers (6) show that recombinant rat liver MAO A in which Cys 406 is mutated to an alanyl residue does exhibit catalytic activity at a level estimated to be 40–60% that of the wild-type enzyme (depending on the nature of the substrate) when expressed in S. cerevisiae (6).

Mutagenesis studies on other flavoenzymes containing 8a-substituted covalent flavin coenzymes have shown that mutations of the linking amino acid result in enzymes still able to bind FAD non-covalently and to exhibit some catalytic activity. Replacement of His-44 with Cys in fumarate reductase results in a mutant enzyme that contains non-covalent FAD that can be reversibly resolved and reconstituted with FAD to form an active enzyme (7). Recent studies on vanillyl-alcohol oxidase demonstrate that replacement of His-422 with Ala results in an enzyme that binds FAD non-covalently, exhibits ~10% of the activity of the native enzyme, and has essentially the same structure of the native enzyme as determined by x-ray crystallography (8). Expression of rat t-gulono-β-lactone oxidase (containing an N(1)-histidyl-FAD (9, 10)) in a baculovirus insect cell system where the medium is deficient in riboflavin results in the formation of an apoenzyme that is reactivated by the addition of FAD (11). The binding of FAD to this apoenzyme is apparently non-covalent even though the liganding histidyl residue is present.

These studies with enzymes containing 8a-histidyl-FAD demonstrate that covalent linkage of the flavin is not necessary for protein folding to a form capable of binding the flavin coenzyme by non-covalent interactions. Although the catalytic activities of these mutated forms are lower than those of the native wild-type enzymes, covalent binding of the flavin does not appear to be a requirement for functionality. In the cases tested, however, catalytic turnover is more efficient when the flavin cofactor is covalently bound.

This manuscript describes experiments on the C406A mutant form of human liver MAO A expressed in a riboflavin-requiring strain of S. cerevisiae described in a previous publication from this laboratory (3). The results demonstrate that the apo form of the enzyme is incorporated into the outer mitochondrial membrane in a folded form that can bind FAD and a number of FAD analogues. Catalytic activity is reconstituted upon binding of FAD and several of the FAD analogues tested, which allow the determination of binding constants. Although native, expressed MAO A can be liberated from the

* This work was supported by National Institutes of Health Grant GM-29433 (to D. E. E.). Preliminary accounts of this work were presented at the American Society of Biological Chemistry and Molecular Biology, San Francisco, California, May 16–20, 1999 and the 13th International Symposium on Flavins and Flavoproteins, Konstanz, Biology, San Francisco, California, May 16–20, 1999 and the 13th International Symposium on Flavins and Flavoproteins, Konstanz, Germany, August 29–September 4, 1999. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship from the Markey Foundation.

§ To whom correspondence should be addressed. Tel: 404-727-3592; Fax: 404-727-2738; E-mail: dedmond@bimcore.emory.edu.

1 The abbreviations used are: MAO, monoamine oxidase; WT, wild type.

outer mitochondrial membrane in an active stable form by
detergent extraction, the mutant enzyme is very unstable to
detergent solubilization, which suggests a role for covalent
flavin linkage in maintaining the structural stability of
the enzyme.

**EXPERIMENTAL PROCEDURES**

*Preparation of C406A Mutant of MAO A—* The gene encoding human
liver MAO A was obtained from Dr. P. Urban, CNRS, Gif-sur-Yvette,
France and was ligated into pUC18. Site-directed mutagenesis was
carried out using the transformer site-directed mutagenesis kit
from CLONTECH. Two primers were used, one carrying the desired
mutation and a selection primer that converts a unique NdeI restriction
site in pUC18 to an NcoI restriction site. This change in restriction site
was used in selection of the desired mutant. The pUC18 plasmid
containing the C406A MAO A gene was then subjected to polymerase chain
reaction using primers GAA GAT CTA TGG AGA ATC AAG AGA AGG CG
and CGA ATT CTC AAG ACC GTG GCA GGA GC. These two primers
were designed such that the polymerase chain reaction product
contains the C406A MAO A gene with BglII and EcoRI restriction sites at
the 5′ and 3′ ends, respectively. The resulting polymerase chain
reaction product was digested with the above two restriction enzymes,
ligated into a pETDP (60) expression vector, and transformed into XL-2
Blue ultracompetent cells. Confirmation of the correct mutated gene
sequence was performed at the University of Georgia, Athens. Positive colonies were screened for the C406A
MAO A insert by restriction digestion analysis. Plasmids were isolated
from positive colonies and transformed into S. cerevisiae strain RM2
(rib5–) using standard protocols as described previously (3).

*Expression of C406A MAOA in S. cerevisiae RM2 and Preparation of
Mitochondria—* Transformed yeast cells were grown in yeast extract/
peptone/dextrose media containing 0.5% (w/v) glucose and 200 μM ribo-
flavin in red glass culture flasks to prevent light destruction of the
flavin. After 16 h of growth, cells were collected by centrifugation,
washed with sterile distilled water, and resuspended in fresh medium
containing 0.4% (w/v) galactose for enzyme induction. For apoC406A
MAO A preparations, no riboflavin was added to the “induction” me-
dium. To produce holo C406A MAO A, 200 μM riboflavin was added
to the medium. For studies on the in vivo incorporation of riboflavin
analogues, the appropriate analogue was added to the induction me-
dium at a concentration of 200 μM in place of riboflavin. C406A MAO A
induction took place over a 16-h period of incubation of the culture
in the galactose medium at 30 °C.

After the induction period, cells were collected by centrifugation
and washed with sterile distilled water to remove any exogenous riboflavin.
Cell walls were disrupted by zymolase digestion (3), and mitochondria
were isolated as described previously in the presence of the protease
inhibitors: aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), pepstatin (1 μg/
ml), benzamidine (0.1 mM), and sodium metabisulfite (0.1 mM). The
mitochondrial preparation can be stored frozen as a suspension in 50
mM Tris HCl, pH 7.4, 2 mM EDTA, 0.6 M sorbitol, and 25% (v/v) glycerol
at −80 °C without loss of activity for 4 weeks.

*Analytical Assays—* C406A MAO A activity was determined fluoro-
metically by monitoring kynuramine oxidase activity at 30 °C (12).
Mitochondrial preparations were incubated with 1 mM kynuramine
in air-saturated 50 mM potassium phosphate, pH 7.5, for 30 min in the
absence of any detergent. The concentration of product formed is then
determined fluorometrically. Protein concentrations were determined
using the modified Bearden procedure as described previously (3). SDS-
polyacrylamide gel electrophoresis on mitochondrial protein samples
was performed as described by Laemmli (13) using 7.5% polyacrylamide
gels. Immunobots to detect MAO A or covalent flavin were performed
as described previously (3).

*Flavin Analogues—* The following riboflavin analogues used in this
study were the generous gifts of Dr. John Lambooy (University of
Maryland): 7-nor-7-chlororiboflavin, 8-nor-8-chlororiboflavin, 8-nor-8-
bromoriboflavin, 7α-methylriboflavin, 8-α-methylriboflavin, 7-α,8-α-
dimethylriboflavin, 7-nor-7-bromo-8-α-methylriboflavin, 7-α-methyl-8-
nor-8-bromoriboflavin, 7-nor-7-chloro-8-α-methylriboflavin, and 7-α-
methyl-8-nor-8-chlororiboflavin. Dr. Sandro Ghisla (University of
Konzern, Germany) provided generous gifts of 8-nor-8-Fluororibofla-
vin, 7-nor-7-chloro-8-nor-8-chlororiboflavin, and 8-nor-8-amino riboflavin.
5-Deaza-5-carbariboflavin and N(3)-methyl riboflavin were previ-
ously synthesized in this laboratory. 8-Nor-8-cyanoriboflavin was
synthesized as described by Murthy and Massey (14). See Ref. 3 for a
listing of the structures of these riboflavin analogues.

FAD analogues were prepared enzymatically from the corresponding
riboflavin analogues using purified recombinant Corynebacterium am-
moniagenes FAD synthetase (a gift from Dr. W. McIntire, Veterans
Affairs Medical Center, San Francisco) (15). After incubation of the
riboflavin analogues (200 μM) with 3 μM ATP for 16 h in the presence
of the FAD synthetase in the absence of light, FAD was separated from
recombinant FAD using gel filtration on a Bio-Gel P-2 column using distilled water
as eluent. Purity of the FAD fractions was monitored by high perfor-
man l outspoken chromatography.

**RESULTS**

*Expression of C406A MAOA in S. cerevisiae—* Our initial
approach was to express the C406A mutant of MAO A in tens
of milligram quantities in the expression system described by
Weyler et al. (16) that has proven successful for the high level
expression of human liver MAO A (16–18). Activity is observed
in cell extracts as expected from the work of Ito and co-workers
(6). Attempts, however, to solubilize and purify the enzyme
were unsuccessful in our hands due to the extreme lability of
the mutant enzyme after extraction from the membrane. The
addition of exogenous FAD to cell extracts did not result in any
apparent stabilization of enzyme activity, which suggests the
lability of activity is more complex than simply the loss of FAD
on solubilization. These results suggest that the C406A mutant
form of MAO A appears to be stable in its membrane-associated
environment; however, is very unstable when extracted into an
aqueous detergent solution, conditions that are suitable for the
extraction and purification of the WT enzyme in a stable,
functional form. Therefore, the presence of covalent FAD ap-
ppears to impart structural stability to the enzyme.

Since the mutant enzyme is stable only in a membrane
environment, our alternative approach was to express the
mutant enzyme in the riboflavin auxotrophic strain of S. cerevisiae
(RM2), which has been described in a previous study investi-
gating the covalent incorporation of riboflavin analogues into
two recombinant MAO A and MAO B (3). This system also has
the advantage in that the Gal promoter is tightly regulated so
that no MAO expression is observed until galactose is added to
the medium. Therefore, a series of experiments were performed
similar to those done previously with WT enzyme. Cell cultures
were grown in the presence of riboflavin until reaching station-
ary phase. At this stage, no expression of MAO A or C406A
MAO A is detected either by catalytic activity or immunochemi-
cally. After washing the cells with distilled water to remove any
exogenous riboflavin, the cells were incubated in a fresh me-
dium (containing 0.4% w/v galactose) containing either 1) no
riboflavin, 2) 200 μM riboflavin, or 3) 200 μM riboflavin
analogues as done previously (3). After incubation for 16 h, the
mitochondria were isolated from different cultures and assayed
for MAO A activity. The results are presented in Table I, in
which specific activities are compared with a control sample
containing riboflavin.

*C406A MAO A Activity Levels in rib5 Yeast Expression System with Various Riboflavin Analogues—* Previous results
(6) have demonstrated that expression of rat liver C406A MAO
A results in an enzyme that exhibits −35 to −40% of the kynura-
mine oxidase activity exhibited by expressed WT enzyme under
identical conditions. Our results on the expressed human WT
and mutant enzymes are in good agreement with the data
published (6). The specific activity of C406A MAO A expressed
in the presence of riboflavin is 0.86 ± 0.13 milliunits/mg of
mitochondrial protein (average ± 17 determinations with a
range of 0.64–1.2 milliunits/mg). Expression of WT enzyme
under the same conditions results in a specific activity of 2.4 ±
0.5 milliunits/mg (7 determinations with a range of 1.8–2.9
milliunits/mg). Therefore, based on specific activity measure-
ments, our data suggest that C406A MAO A exhibits 36% that
of WT MAO A. Estimates of MAO catalytic sites in our mito-
chondrial preparations using clorgyline titrations result in a

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calculated $k_{\text{cat}}$ of 22 min$^{-1}$ for kynuramine oxidation by WT MAO A and 8 min$^{-1}$ for the C406A mutant. Using the published data in Hiro et al. (6) suggests the $k_{\text{cat}}$ for kynuramine oxidation by the WT rat liver enzyme is 9 min$^{-1}$, which is within the range of our value when consideration is given to variations in expressed activity among different preparations. However, titrations of clorgyline inhibition of enzyme activity results in overestimates of MAO A levels in crude preparations presumably due to nonspecific binding of clorgyline (19, 20). Since $k_{\text{cat}}$ for kynuramine oxidation by purified WT MAO A is 120 min$^{-1}$ (17), the level of WT MAO A in our preparations is overestimated by a factor of 5.5 from clorgyline titration data. Thus, our assumption is that $k_{\text{cat}}$ values for WT MAO A in both soluble form and in mitochondrial preparations are similar and taken to be 120 min$^{-1}$.

With this comparative data as a basis between the WT and C406A MAO A preparations, the influence of flavin analogue structure on C406A-expressed activity was determined (Table I). Of the flavin analogues tested, the 7-halogen-substituted flavin analogues exhibit highest activity (~5-fold higher than riboflavin), whereas the 8-halogenated flavins exhibit a 2–3-fold higher activity than riboflavin. The 8-nor-8-fluororiboflavin is an exception and has been found previously (3) to reconstitute less activity since it probably undergoes side reactions with cellular nucleophiles that lead to a lower effective concentration of this analogue. Those riboflavin analogues where an ethyl group replaces a methyl group in the 8 position are found to exhibit lower activities than riboflavin, possibly as a result of steric influences since the redox potential of these analogues are expected to be similar to that of riboflavin (21).

No catalytic activity is observed with 8-nor-8-aminoriboflavin or N(3)-methylriboflavin; as these two analogues are not converted to their respective FAD levels by the yeast enzyme (as will be shown below, the FAD forms of these analogues bind to apoC406A MAO A but do not reconstitute catalytic activity). The 5-deaza-5-carba-FAD also does not reconstitute catalytic activity as expected, since its reduced form does not react catalytically with oxygen.

To test whether the differences in observed activities of C406A MAO A in the presence of different flavin analogues are due to different levels of expressed C406A MAO A, mitochondrial proteins from each culture were separated on 7.5% SDS--polyacrylamide electrophoresis gels and blotted onto a nitrocellulose membrane. The blots were probed with polyclonal anti-MAO A antibodies. RF, riboflavin; Me, methyl.

![Image](http://www.jbc.org/content/275/17/20529/F1)

**FIG. 1.** Immunochemical detection of C406A MAO A. *S. cerevisiae* (RM2, transformed with expression plasmid containing C406A MAO A) was induced in the presence of the appropriate riboflavin analogue, and mitochondria were isolated. Digitonin-solubilized mitochondrial proteins (1.25 μg/mlane) were separated on 7.5% polyacrylamide gels and transblotted on to a nitrocellulose membrane. The blots were probed with polyclonal anti-MAO A antibodies. RF, riboflavin; Me, methyl.

**TABLE I**

<table>
<thead>
<tr>
<th>Flavin analogue</th>
<th>MAO A activity</th>
<th>% specific activity of Riboflavin control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>7-Nor-7-chlororiboflavin</td>
<td>539 ± 32</td>
<td></td>
</tr>
<tr>
<td>7-Nor-7-bromoriboflavin</td>
<td>560 ± 10</td>
<td></td>
</tr>
<tr>
<td>8-Nor-7-chlororiboflavin</td>
<td>19 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8-Nor-7-fluororiboflavin</td>
<td>22 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8-Nor-8-cloro-7-nor-7-chlororiboflavin</td>
<td>150 ± 26</td>
<td></td>
</tr>
<tr>
<td>8-α-Methyl-7-nor-7chlororiboflavin</td>
<td>17 ± 2</td>
<td></td>
</tr>
<tr>
<td>8-α-Methylriboflavin</td>
<td>11 ± 2</td>
<td></td>
</tr>
<tr>
<td>8-α-Methyl-7-α-methylriboflavin</td>
<td>8.4 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>8-Nor-8-chloro-7-α-methylriboflavin</td>
<td>140 ± 13</td>
<td></td>
</tr>
<tr>
<td>8-Nor-8-bromo-7-α-methylriboflavin</td>
<td>356 ± 38</td>
<td></td>
</tr>
<tr>
<td>8-Nor-8-aminoriboflavin None detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Carba-5-deazariboflavin None detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni3-methylriboflavin None detected</td>
<td></td>
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</table>

**Interaction of Flavin Analogues with C406A MAO A**

**Relative levels of expressed C406A MAO A activities in mitochondria isolated from *S. cerevisiae* induced in the presence of different flavin analogues**

To test whether the differences in observed activities of C406A MAO A in the presence of different flavin analogues are due to different levels of expressed C406A MAO A, mitochondrial proteins from each culture were separated on 7.5% SDS--polyacrylamide electrophoresis gels and blotted onto a nitrocellulose membrane. The blots were probed with polyclonal anti-MAO A antibodies. RF, riboflavin; Me, methyl.

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<td>560 ± 10</td>
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<td>8-Nor-7-chlororiboflavin</td>
<td>19 ± 0.1</td>
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<tr>
<td>8-Nor-8-cloro-7-nor-7-chlororiboflavin</td>
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<td>8-α-Methylriboflavin</td>
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<tr>
<td>8-Nor-8-chloro-7-α-methylriboflavin</td>
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<tr>
<td>8-Nor-8-bromo-7-α-methylriboflavin</td>
<td>356 ± 38</td>
<td></td>
</tr>
<tr>
<td>8-Nor-8-aminoriboflavin None detected</td>
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<tr>
<td>5-Carba-5-deazariboflavin None detected</td>
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<td>Ni3-methylriboflavin None detected</td>
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</tbody>
</table>
activity (data not shown), as observed previously (6).

With the ability to reconstitute apoC406A MAO A catalytic activity with FAD, it was of interest to examine the ability of FAD analogues to reconstitute the enzyme activity as well as to determine their relative binding affinities. In addition, FAD analogues can be prepared from several of the riboflavin analogues that are not converted by the yeast FAD synthetase system. Twelve FAD analogues were prepared and purified from their respective parent riboflavin forms by recombinant C. ammoniagenes FAD synthetase, which exhibits a broad substrate specificity.

The results in Table II show the relative levels of kynuramine oxidase activities relative to an FAD control as well as the relative binding affinities upon reconstitution experiments similar to that shown in Fig. 2. Those FAD analogues with halogen substituents in either the 7 or 8 positions exhibit limiting activities ranging from 3- to 7-fold higher than FAD. In addition, the high potential analogue 8-nor-8-cyano-FAD ($E_{m,n} = -50 \text{ mV}$ (14)) exhibits a limiting activity ~4-fold higher than FAD ($E_{m,n} = 208 \text{ mV}$ (23). Five FAD analogues (8-$\alpha$-methyl; 5-deaza-5-carba; 8-nor-8-amino; 1-deaza-1-carba; and N(3)-methyl-FAD) do not reconstitute any detectable activity.

The relative binding affinities were determined for those analogues that reconstitute MAO A activity as described for FAD (Fig. 2). The relative binding affinities for those FAD analogues not reconstituting MAO A activity were estimated by competition experiments with one of the FAD analogues exhibiting high catalytic activity. An example of this competition experiment is shown in Fig. 3, where 8-$\alpha$-methyl-FAD (which exhibits less than 5% the activity of FAD when incubated with apoC406A MAOA) markedly influences the observed apparent $K_d$ for 7-nor-7-chloro-FAD reconstitution of the enzyme. From this data, an apparent $K_d$ for 8-$\alpha$-methyl-FAD binding to apoC406A MAO A is determined to be 90 ± 12 nm. Using the same approach, the binding affinities of other FAD analogues that do not reconstitute MAO A activity were also determined (Table II). As shown in Table II, most of the FAD analogues bind apoC406A MAO A with binding affinities similar to that of normal FAD, with the exception of 1-deaza-1-carba, 8-nor-8-amino, and N(3)-methyl-FAD analogues. The tight binding of 5-deaza-5-carba-FAD is reasonable since this analogue has similar steric features as FAD. The absence of activity is attributed to the inability of the reduced form of this analogue to react with oxygen and may also be due to a slow rate of reduction by the substrate since it has a low oxidation-reduction potential ($E_{m,n} = -310 \text{ mV}$ (24). The inability of other analogues to reconstitute catalytic activity even at saturating concentrations provides some new insights into the FAD binding site of MAO A, which has heretofore been inaccessible due to the absence of structural data on this enzyme and to the covalent binding of the flavin coenzyme.

**DISCUSSION**

The data presented in this paper extend the previous study from Ito and co-workers (6) that documented the ability of C406A MAO A to bind FAD non-covalently and to function catalytically. A major conclusion from this study is the demonstration that MAO A can be incorporated into the outer membrane of the mitochondrion as an apoprotein, fold to a conformation that binds FAD, and reconstitute catalytic activity. No catalytic activity is observed on the addition of either riboflavin or FMN, which demonstrates the specificity of the apoenzyme to bind the dinucleotide form of the flavin cofactor. These results also demonstrate that covalent FAD is not required for correct folding of the protein or for import into its outer mem-

**TABLE II**

Activities of reconstituted apoC406A MAO A in the presence of different FAD analogues and the binding constants of the FAD analogues for apoMAO A

<table>
<thead>
<tr>
<th>Flavin analogue</th>
<th>Percent specific activity</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>100</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>7-Nor-7-chloro-FAD</td>
<td>475 ± 10</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>8-Nor-8-chloro-FAD</td>
<td>694 ± 14</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>8-$\alpha$-Methyl-FAD</td>
<td>$&lt;5$</td>
<td>90 ± 12*</td>
</tr>
<tr>
<td>5-Deaza-5-carba-FAD</td>
<td>$&lt;5$</td>
<td>69 ± 5*</td>
</tr>
<tr>
<td>1-Deaza-1-carba-FAD</td>
<td>$&lt;5$</td>
<td>377 ± 32*</td>
</tr>
<tr>
<td>8-Nor-8-amino-FAD</td>
<td>$&lt;5$</td>
<td>240 ± 84*</td>
</tr>
<tr>
<td>N(3)-methyl-FAD</td>
<td>$&lt;5$</td>
<td>920 ± 108*</td>
</tr>
<tr>
<td>7-Nor-7-chloro-8-nor-8-chloro-FAD</td>
<td>317 ± 4</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>7-$\alpha$-Methyl-FAD</td>
<td>47 ± 3</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>7-$\alpha$-Methyl-8-nor-8-bromo-FAD</td>
<td>323 ± 13</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>7-$\alpha$-Methyl-8-nor-8-chloro-FAD</td>
<td>393 ± 10</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>8-Nor-8-cyano-FAD</td>
<td>437 ± 38</td>
<td>61 ± 17</td>
</tr>
</tbody>
</table>

$a$ Determined by competitive binding experiment with 7-nor-7-chloro-FAD.
brane localization site. The results are consistent with the view that the covalent flavinylination reaction is autocatalytic, with the FAD binding to the protein initially in a non-covalent manner and subsequent nucleophilic attack by Cys-406 on the 8 position in the quinone-methide tautomer form of the flavin. The reduced flavin product is then proposed to be reoxidized by O$_2$, resulting in the covalent attachment of the FAD to Cys-406. This route differs from that reported with the C30A mutant of the 6-S-cysteinyl FMN containing bacterial trimethylamine dehydrogenase, which can bind FMN non-covalently during the folding of the protein and is catalytically active but does not bind FMN once the folding process is completed (25).

The results also show that, in agreement with the conclusions of Ito and co-workers (6) and with results from similar studies of other enzyme systems containing covalent flavins (7, 8, 11), that covalent flavin binding is not a requirement for producing a catalytically functional enzyme. The turnover numbers of enzymes in which the covalent binding is disrupted are found to be lower than those of their respective wild-type forms, suggesting an influence of covalent binding on the catalytic efficiencies of this class of flavoenzymes. The major alteration observed here in comparing C406A MAO A with wild-type MAO A is that the activity of the mutant form is very labile when the protein is removed from the membrane, whereas the native form is readily solubilized by detergents in a stable, functional form. These data suggest the covalent linkage of the flavin in MAO A has a role in maintaining the structural integrity of the enzyme.

One suggestion in a recent review for the role of covalent binding of flavins to enzymes is that it compensates for weak protein-flavin interactions that would result in the ready dissociation of the flavin coenzyme (26). The results presented here demonstrate that the binding affinity of FAD to apoC406A MAO A (−60 ± 10 mT) is comparable with binding affinities determined for many flavoenzymes containing non-covalent flavins. Comparable studies on the His-442 mutant of vanillyl-alcohol oxidase (8) show that the FAD cofactor is bound with an affinity that is too tight to be readily measurable. These results therefore suggest that compensation for weak protein-flavin interactions is not a valid reason to explain covalent binding of flavin to protein.

The binding of a number of FAD analogues to C406A MAO A provides a number of insights into the structure of the FAD binding site that have to date been elusive since the three-dimensional structure of this enzyme is not known. In those binding sites that have to date been elusive since the three-proto segment provides a number of insights into the structure of the FAD flavin to protein.

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The activity data demonstrate that those flavin analogues exhibiting higher oxidation-reduction potentials than FAD reconstitute a higher level of MAO A activity by factors of 3–7-fold. In contrast, those flavins with oxidation-reduction potentials lower than that of FAD (1-deaza-1-carba-FAD (Em = −280 mV (28)) and 8-amino-FAD (Em = −330 mV (29)) show no detectable activity. No direct correlations are apparent between the level of MAO activity and the two-electron oxidation-reduction potential of the free flavin analogue. Since these rate data are determined at single substrate concentrations, more quantitative interpretations cannot be made since it is not known what the effect of flavin analogue structure has on substrate $K_m$ values. Fraaije et al. (8) show that disruption of the 8a-N(3)-histidyl-FAD linkage in vanillyl-alcohol oxidase lowers the 2-electron oxidation-reduction potential of the bound FAD from +55 mV in WT enzyme to −65 mV in the H422A mutant (Em is the average of −17 mV for the oxidized/semiquinone couple and −113 mV for the semiquinone/hydroquinone couple). They suggest that the lowered oxidationreduction potential of the non-covalent FAD is responsible for the lowered catalytic efficiency of the H422A mutant relative to the WT enzyme. The observations reported here that higher redox potential flavins reconstitute higher activities in C406A MAO A lend support to their suggestion. The increase in activity observed for those high potential FAD analogues (8-nor-8-chloro-FAD (Em = −152 mV (4), 8-nor-8-bromo-FAD (Em = −148 mV (29), and 8-nor-8-cyano-FAD (Em = −50 mV (14)) relative to FAD (Table II) may be partially explained by the increase in flavin oxidation-reduction potential on 8-substitution with electron-withdrawing substituents to compensate for the lowering of the potential due to the disruption of the 8a-thioether linkage. Direct measurement of the oxidation-reduc-

**Fig. 4. Schematic depiction of the steric influence of 7a- and 8a-methyl substituents in the binding sites of wild-type and C406A MAO A.** The $k_{cat}$ values denoted are relative to that of unsubstituted FAD taken to be 120 min$^{-1}$. $A$, covalent FAD analogue binding (from Ref. 3); $B$, non-covalent FAD analogue binding (this study). Only the benzenoid portion of the isoalloxazine ring is shown.

Table II, similar levels of activities are observed. Analogues containing a variety of substituents in the 7 and 8 positions of the isoalloxazine ring and with replacement of N(3) with carbon are bound to apoC406A MAO A with affinities as high as that found with FAD (Table II). Therefore, the protein environment about the benzenoid portion of the flavin ring can accommodate more bulky substituents at either positions 7 or 8, such as replacement of the methyl groups with ethyl groups. Methylation of the N(3) position results in an ~15-fold decrease in affinity, which suggests specific protein-flavin interactions at N(3) in the pyrimidine ring. The 6-fold decrease in binding affinity for 1-deaza-1-carba-FAD shows that placing a hydrogen at the 1-position of the pyrimidine ring of the flavin has a measurable influence and suggests that the steric tolerance about this position is quite limited. Similar results have also been published on the binding of this analogue to flavodoxin (27).

The activity data demonstrate that those flavin analogues exhibiting higher oxidation-reduction potentials than FAD reconstitute a higher level of MAO A activity by factors of 3–7-fold. In contrast, those flavins with oxidation-reduction potentials lower than that of FAD (1-deaza-1-carba-FAD (Em = −280 mV (28)) and 8-amino-FAD (Em = −330 mV (29)) show no detectable activity. No direct correlations are apparent between the level of MAO activity and the two-electron oxidation-reduction potential of the free flavin analogue. Since these rate data are determined at single substrate concentrations, more quantitative interpretations cannot be made since it is not known what the effect of flavin analogue structure has on substrate $K_m$ values. Fraaije et al. (8) show that disruption of the 8a-N(3)-histidyl-FAD linkage in vanillyl-alcohol oxidase lowers the 2-electron oxidation-reduction potential of the bound FAD from +55 mV in WT enzyme to −65 mV in the H422A mutant (Em is the average of −17 mV for the oxidized/semiquinone couple and −113 mV for the semiquinone/hydroquinone couple). They suggest that the lowered oxidation-reduction potential of the non-covalent FAD is responsible for the lowered catalytic efficiency of the H422A mutant relative to the WT enzyme. The observations reported here that higher redox potential flavins reconstitute higher activities in C406A MAO A lend support to their suggestion. The increase in activity observed for those high potential FAD analogues (8-nor-8-chloro-FAD (Em = −152 mV (4), 8-nor-8-bromo-FAD (Em = −148 mV (29), and 8-nor-8-cyano-FAD (Em = −50 mV (14)) relative to FAD (Table II) may be partially explained by the increase in flavin oxidation-reduction potential on 8-substitution with electron-withdrawing substituents to compensate for the lowering of the potential due to the disruption of the 8a-thioether linkage. Direct measurement of the oxidation-reduc-
Interaction of Flavin Analogues with C406A MAO A

tion potential for the covalent FAD in MAO A and the influence of C406A substitution on that value remains to be done.

The data, however, also suggest that other factors in addition to the oxidation-reduction potential of the flavin may be a determinant of the level of activity observed. For example, 8-α-methyl-FAD is bound quite tightly yet supports little or no MAO A activity even though the expected oxidation-reduction potential of this analogue (21) is similar to that of FAD (Em,7 = −208 mV (23)). This observation suggests that this flavin analogue is bound to the enzyme in a configuration altered from that of FAD in a manner that is not compatible for efficient catalysis. Similar conclusions might also be applied to the other FAD analogues that are bound to the enzyme with reasonable affinity but do not support catalytic activity.

It is instructive to compare the relative activities of flavin analogues that have been tested in wild-type MAO A (covalent binding) (3) and in the C406A MAO A (non-covalent binding) (Tables I and II). The 8-nor-8-halogen analogues have been shown previously to be covalently bound to MAO A in an 8-thioether linkage (3). The potentials of 8-thio-flavins are equivalent to normal flavins and are ~80 mV lower than those of the 8-halogen-substituted flavins (4). Previous results have demonstrated that 8-halogenated flavins are covalently incorporated into MAO A at Cys-406 in a thioether linkage with displacement of the 8-halogen substituent (3). The specific activities determined with either 8-nor-8-chloroflavin or 8-nor-8-bromoflavín are equivalent to that observed with riboflavin (3). With the C406A mutant, the 8-halogen substituent remains on the flavin ring, and the observed specific activities using either of the above analogues (Table I) is ~2.5 times higher than that observed with riboflavin control cultures. Thus, raising the oxidation-reduction potential of the bound flavin results in an increase in observed activity of the C406A mutant to an activity that is approximately equivalent to the WT enzyme. In fact, this increase in activity is probably higher since the data in Table II show increased activity with higher potential flavins to be 3–7-fold higher than the value observed with FAD. The experimental uncertainties of this system preclude a more quantitative comparison; however, the conclusions reached from the data are outside the inherent uncertainties. Large activity differences are observed on comparison of the 8-α-methyl flavin in a covalent versus a non-covalent linkage. When this analogue is covalently bound (presumably through an 8-α-thioether linkage) to MAO A, the specific activity observed is comparable with that of normal flavin (69%). In contrast, the non-covalently bound form of this flavin exhibits little or no activity (relative activity levels of 10% or less) even though it is bound to the enzyme rather tightly (Tables I and II), and the oxidation-reduction potential of this analogue is very similar to that of normal flavin. Thus, formation of the covalent linkage from Cys-406 to the flavin may be important in maintaining the bound flavin in the correct orientation in the active site for effective catalysis. Since the 7-α-methylflavín exhibits a similar specific activity compared with normal riboflavin control whether it is covalently bound or not, the protein environment about the 7-position is apparently able to accommodate the presence of a 7-α-methyl group. This accommodation, however, is lost when the 8-position is linked directly to Cys-406 in a thioether linkage, and the steric limitations about the 7-position become more severe with the loss of the intervening 8-methylene. Thus, 8-nor-8-halogen-7-α-methylflavins exhibit little or no catalytic activity when covalently bound (3); however, as shown in Tables I and II, they exhibit 3–4-fold higher specific activity than FAD when non-covalently bound to C406A MAO A. These interpretations are shown schematically in Fig. 4.

In summary, this system has demonstrated new insights into the binding of flavin to MAO A and into possible roles for the covalent linkage of the flavin to the protein. The general conclusions for this membrane-bound flavoenzyme appear to agree to those determined in other published studies on soluble flavo-enzyme systems (e.g. vanillyl-alcohol oxidase (8)) where crystallographic information is available. Therefore, the conclusions reached in this study on human liver MAO A may be cautiously applicable to human liver MAO B, which exhibits ~70% amino acid sequence identity and also has a covalently bound 8-α-S-cysteinyl-FAD.

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Ravi K. Nandigama and Dale E. Edmondson

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