*Biochemistry* **1996,** *35,* 11085−11091

# Observation of a Flavin Semiquinone in the Resting State of Monoamine Oxidase B by Electron Paramagnetic Resonance and Electron Nuclear Double Resonance Spectroscopy[†,‡]

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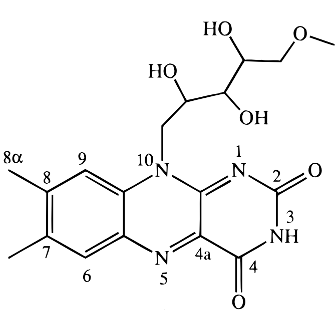
*Received March 28, 1996; Revised Manuscript Received June 25, 1996*[⊗]

ABSTRACT: Monoamine oxidase (MAO) plays an essential role in the regulation of various neurotransmitter and xenobiotic amines. Inhibitors of MAO have been employed in the treatment of depression and as adjuncts in Parkinson's disease therapy. X-Band and Q-band electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopic techniques are employed to characterize a signal assigned as a stable red anionic semiquinone radical in the resting state of MAO B. It is shown that the radical signal is not affected during substrate (either benzylamine or phenylethylamine) turnover, by anaerobic incubation with substrate, or by covalent modification of the active site flavin cofactor in the catalytically active dimer. Upon denaturation, however, the semiquinone absorbances and EPR signals are lost. Photoreduction of the native enzyme in the presence of ethylenediaminetetraacetate generates an EPR signal that is not the same as that obtained in the resting state and shows different proton ENDOR signals. These results suggest that the two flavin prosthetic groups that exist in catalytically active monoamine oxidase B are physically distinct.

Monoamine oxidase (MAO[1] ) (EC 1.4.3.4) is an integral membrane protein of the outer mitochondrial membrane (Schnaitman et al., 1967) that catalyzes the oxidative deamination of biogenic and xenobiotic amines to their corresponding aldehydes. Two isozymes of MAO exist, the A and the B forms, each displaying different substrate and inhibitor specificities (Dostert et al., 1989; Johnston, 1968). MAO A and MAO B inhibitors have been used clinically as antidepressant agents (Knoll, 1992) and in the treatment of Parkinson's disease (Birkmayer et al., 1975; Rinne, 1987), respectively.



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Both forms of MAO contain a covalently bound flavin adenine dinucleotide (FAD, **1**) cofactor (Kearney et al., 1971a,b) that is attached through the 8α-position of the flavin to a cysteine residue located in the C-terminal region of the protein (Bach et al., 1988). Catalytically active MAO B is believed to exist as a dimer (60 kDa per subunit) with one bound FAD molecule per monomer (Weyler, 1989). Mechanism-based inactivator studies of MAO B with *N*-cyclopropylbenzylamine (Silverman & Zieske, 1985a,b), *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984), 1-phenylcyclopropylamine (Silverman & Zieske, 1985a,b), (aminomethyl)trimethylsilane (Banik & Silverman, 1990), 5-(aminomethyl)-3-aryl-2-oxazolidinones (Gates & Silverman, 1990), and pargyline (Chuang et al., 1974) have shown that, on average, one inactivator molecule binds to each molecule of holoenzyme (120 kDa). These results indicate half-site reactivity. *N*-(1-Methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984), 1-phenylcyclopropylamine (Silverman & Zieske, 1985a,b), and pargyline (Chuang et al., 1974) were shown to become covalently attached to the active site flavin. The mechanism of amine oxidation catalyzed by MAO has been extensively studied, and there is much support for radical intermediates during the reaction (Silverman, 1995).

Spectroscopic evidence for a resting state flavin semiquinone in MAO B has recently been reported. The UV/vis absorption spectrum of MAO B displays two absorption maxima at 412 and 450 nm with the absorption at 450 nm corresponding to the oxidized form of flavin. The absorption at 412 nm was long assumed to be the result of contaminating heme proteins absorbing in the Soret region (Weyler & Salach, 1981), but it has been suggested, on the basis of

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[†] We are grateful to the National Institutes of Health (GM32634 to R.B.S. and HL13531 to B.M.H.) for financial support of this research.

[‡] Preliminary results were presented at the 6th Amine Oxidase Workshop and 5th Trace Amine Symposium at the University of Saskatchewan, Canada, in August 1994.

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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.

[1] Abbreviations: MAO, monoamine oxidase; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

S0006-2960(96)00749-0 CCC: $12.00

resonance Raman spectroscopy (Yue et al., 1993) and studies of the optical properties of partially reduced enzyme (Igaue et al., 1967), that the 412 nm absorbance may be due to the existence of a persistent anionic flavin semiquinone. Furthermore, we have recently reported fluorescence spectroscopic studies that show that two different chromophores are present in the resting state of MAO B (Woo & Silverman, 1994).

A flavin semiquinone would be expected to give rise to a radical EPR signal. X-Band EPR studies employing spin-labeled substrates and inactivators of the enzyme (Buckman & Eiduson, 1980; Zeidan et al., 1980; Zeidan, 1988), radical traps of turnover products (Yelekci et al., 1989), and partially reduced enzyme (Igaue et al., 1967; Yue et al., 1993) have been reported. An EPR signal from the resting state enzyme has not yet been reported, however. Results of our EPR and ENDOR spectroscopic studies of MAO B in the resting state are reported here.

## MATERIALS AND METHODS

*General.* Beef liver MAO B was isolated as described previously (Weyler & Salach, 1981) and stored as a concentrated solution (15−25 mg/ml) in sodium phosphate buffer (50 mM, pH 7.2) at 4 °C. The specific activity varied among preparations, ranging from 3.5 to 7 units/mg, where a unit of activity is the conversion of 1 $\mu$mol of benzylamine to benzaldehyde per minute at pH 9.0 and 30 °C. Enzyme was prepared in ambient light except for one preparation where all operations were performed in a dark room equipped with a 15 W Kodak red safety light. Pargyline was obtained from Aldrich Chemical Co. and further purified by recrystallization. *N*-(1-Methylcyclopropyl)benzylamine was prepared as previously described (Silverman & Yamasaki, 1984).

X-Band (9 GHz) EPR spectra were recorded on a modified Varian E4 spectrometer equipped with a $TE_{102}$ cavity and a quartz variable-temperature gas flow dewar. The spectrometer was interfaced to an IBM compatible PC, and the spectra were accumulated and averaged using data collection software developed by Morse (1987). All X-band spectra were recorded at −150 °C unless otherwise noted. EPR tubes were obtained from Wilmad Glass Co. (#707-SQ-250M and #727-SQ-250M) and were ∼4 mm outside diameter and ∼3 mm inside diameter.

Q-Band (35 GHz) EPR and ENDOR spectra were recorded on a modified Varian spectrometer as described previously (Werst et al., 1991). The ENDOR signals were observed as the change in intensity of the 100 kHz field-modulated, dispersion mode EPR signal (Hoffman et al., 1993). Proton ENDOR data were obtained with a broadened RF band width (Hoffman et al., 1994). These conditions improve intensity and the signal to noise ratio for resonances from strongly coupled nuclei, at the expense of resolution in the weakly coupled regime.

*Resting State MAO B.* Resting state X-band EPR samples were prepared by mixing MAO B with an equal volume of pH 7.2 sodium phosphate buffer (50 mM), loading into an EPR tube, and freezing in liquid $N_2$. Q-Band samples of resting state MAO B were prepared by centrifuging stock enzyme at 50 000 rpm (Ti55 rotor) for 90 min to concentrate the enzyme and carefully transferring the pellet with a Teflon spatula into an EPR tube. Minimal amounts of pH 7.2 sodium phosphate buffer were used to wash the enzyme pellet down to the bottom of the tube with the aid of a clinical centrifuge.

*Deuterium-Exchanged MAO B.* Stock MAO B (400 $\mu$L) was placed in a centrifuge tube, and the remaining volume (50 mL) was filled with pD 6.8 sodium phosphate buffer that was prepared in deuterium oxide instead of water. The sample was centrifuged at 50 000 rpm (Ti55 rotor). The pellet was homogenized into fresh deuterated phosphate buffer (50 mL) and allowed to equilibrate overnight. The sample was then centrifuged at 50 000 rpm (Ti55 rotor), and the pellet was loaded into the EPR tube using deuterated buffer to wash the pellet into the tube.

*Photoreduced Samples.* For photochemical reductions, samples were prepared by mixing equal volumes of MAO B and 100 mM EDTA (total volume of 200 $\mu$L) under nitrogen or argon using standard inert atmosphere techniques. Irradiations were conducted under argon, and the samples were placed 6 in. from a 250 W tungsten light bulb at 4 °C. The sample temperature never rose above 8 °C. Samples were irradiated for 4−267 h.

*Spin Concentration Determination.* MAO B EPR samples were prepared by mixing aliquots of stock MAO B (140 $\mu$M) with 50 mM pH 7.2 sodium phosphate buffer to give solutions of 35, 52, 70, and 88 $\mu$M MAO. A 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) stock solution was prepared (21.21 $\mu$M) in water. TEMPO EPR samples were prepared by mixing the TEMPO stock solution with 50 mM pH 7.2 phosphate buffer to give 1.1, 1.6, 2.7, 5.3, and 10.6 $\mu$M solutions of TEMPO. All of the EPR spectra were taken under identical conditions. The spectra were taken at low microwave power (0.5 mW) to safeguard against saturation. Double integration of the spectra of each of the MAO B samples and of the TEMPO radical was conducted to obtain a measure of the relative spin concentration in each.

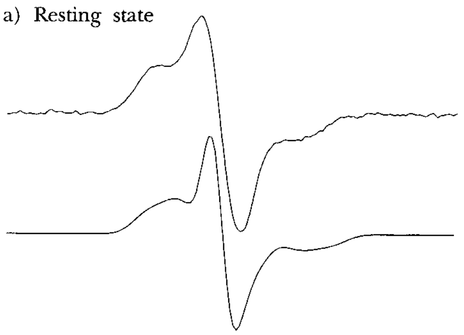
*Inactivated Samples.* Pargyline-inactivated samples were prepared by incubating 100 $\mu$L of MAO B with 100 $\mu$L of pargyline (2 mM) at 25 °C until no enzymatic activity was observed.

*Flavin Determination of N-(1-Methylcyclopropyl)benzylamine-Inactivated MAO.* An *N*-(1-methylcyclopropyl)-benzylamine-inactivated MAO sample was prepared by incubating 100 $\mu$L of stock MAO B and 150 $\mu$L of 5 mM *N*-(1-methylcyclopropyl)benzylamine at 25 °C until no enzymatic activity was observed. A control sample was prepared by dilution of stock enzyme (100 $\mu$L) with 50 mM Tris buffer (pH 9.0) to 250 $\mu$L. To each of the samples was added 250 $\mu$L of 20% w/v SDS, and each was heated at 100 °C for 1 h. The solutions were allowed to cool and their absorbance spectra recorded on a Beckman DU-40 UV/vis spectrophotometer.

*Denaturation of MAO B with Guanidine Hydrochloride.* MAO B (100 $\mu$L of a 180 $\mu$M solution) was added to 150 $\mu$L of 50 mM sodium phosphate buffer (pH 7.2). Guanidine hydrochloride (250 $\mu$L of an 8 M solution) was added, and the absorption spectrum was recorded.

## RESULTS

*X-Band EPR Spectra.* Low-temperature 9 GHz (X-band) EPR spectroscopy of resting state MAO B revealed the spectrum shown in Figure 1a (upper trace). The signal is narrow (∼12 G peak-to-peak for the inner transitions) with


a) Resting state

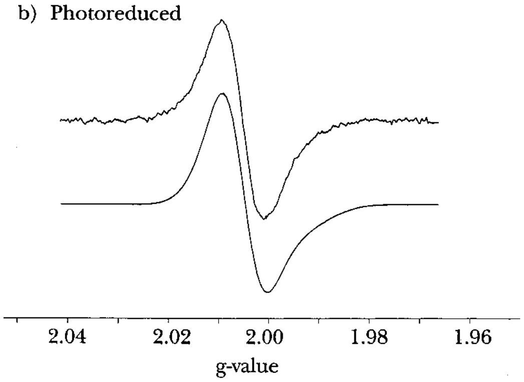

b) Photoreduced

FIGURE 1: X-Band EPR spectrum of (a, upper trace) resting state MAO B (600 $\mu$M) at pH 7.2. Spectrometer conditions: 9.1225 GHz, 0.2 mW, 77 K, 10 G modulation amplitude, 4 min scan time, and two scans. Parameters used in the simulation (a, lower trace) are listed in Table 1. X-band EPR spectrum of (b, upper trace) photoreduced MAO B (82 $\mu$M). Spectrometer conditions: 9.1245 GHz and the rest as in part a. Spectrum is simulated (b, lower trace) with parameters listed in Table 1. Spectrum a is multiplied by a factor of 40 over that of b.

additional resolved "wings" and is centered at $g = 2$. Power saturation studies (data not shown) show that all spectral features saturate at the same rate, indicating that they arise from a single species and that the resolved wings do not arise from anomalous saturation effects that have been observed in other flavin EPR spectra (Hyde et al., 1970; Palmer et al., 1971). The intensity of the signal is proportional to the enzyme concentration, and the same spectrum is obtained when the temperature is varied from 77 to 298 K, except for the expected decrease in signal intensity with increasing temperature. By comparison with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) standards, the spin concentration giving rise to the MAO signal was determined to be 0.02 spins per enzyme molecule (dimer). When heat-denatured enzyme was used, conditions where a flavin semiquinone would be rapidly oxidized, no signal was observed. Enzyme that was purified and loaded into the EPR tube completely in the dark exhibited the same spectrum (and spin concentration) as that prepared in room light; therefore, the signal is not the result of a photoactivation process. In addition, MAO that has been deuterium exchanged has a spectrum identical to that of nonexchanged enzyme. Neither aerobic nor anaerobic addition of substrate (benzylamine or phenylethylamine), followed by rapid quench freezing at liquid nitrogen temperatures, altered the EPR signal. Furthermore, inactivation with pargyline, a compound that is believed to form a covalent adduct with the active site flavin (Chuang et al., 1974), also does not alter the EPR signal, suggesting that the signal is not derived from the active site flavin (vide infra).

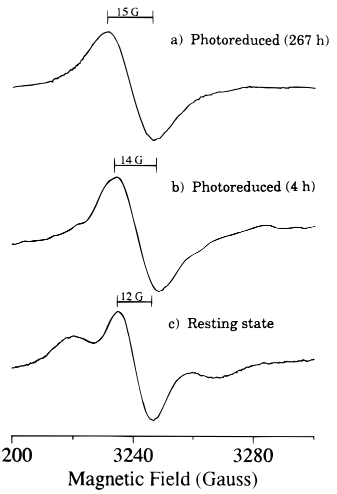


FIGURE 2: X-Band EPR spectra of MAO B following various times of illumination in the presence of EDTA. (a) Maximal spin concentration of photoreduced MAO B (irradiation time of 267 h); peak-to-peak width = 15 G. (b) Irradiation for 4 h (approximately 0.04 spin per enzyme molecule); peak-to-peak width = 14 G. (c) Resting state MAO B; peak-to-peak width = 12 G. Spectra b and c are multiplied by factors of 20 and 40, respectively, over that of a.

It was noted earlier in the literature that upon reduction of MAO with light/EDTA an EPR signal characteristic of an anionic flavin semiquinone was observed; however, no spectrum was published (Igaue et al., 1967). When MAO was treated under these conditions, an isotropic signal centered at $g = 2.0065$ with a peak-to-peak line width of 15 G was observed (Figure 1b, upper trace) whose intensity was dependent upon irradiation time. Quantitation of the maximal spin concentration obtained upon photoreduction indicates that there is approximately one spin per enzyme molecule (dimer), corresponding to only 50% of the flavin content. While this signal has approximately the same total peak width as does the resting state signal, it does not show the same resolved features, indicating that the flavin that is being photochemically reduced is not the same as the species responsible for the resting state EPR signal. When the X-band EPR spectra of MAO B are compared at various states of photoreduction (Figure 2), it is observed that the spectrum obtained at an irradiation time of 4 h corresponds to about equal amounts of spin arising from the unresolved signal of the photoreduced flavin and the signal of the resting state flavin and is intermediate in peak-to-peak width (14 G; Figure 2b) between that of the fully photoreduced signal (15 G; Figure 2a) and the resting state (12 G; Figure 2c). This observation is consistent with the presence of two paramagnetic species in the enzyme molecule.

The overall line widths of both resting state and photoreduced EPR signals, and their invariance in $D_2O$, suggest that they both arise from anionic forms of the flavin semiquinone in MAO B. The ~15 G wide signal that arises from photoreduction is very similar to those observed for other anionic flavin semiquinones (Palmer et al., 1971; Yue et al., 1993) in both appearance and line width. The EPR signal from resting state MAO B has a similar overall line width but shows greater resolution of the wings than is usually seen for flavin EPR signals. The signal more closely resembles a flavin radical, however, than other potential radicals such as those characterized for oxidized tyrosine (Bender et al., 1989; Rigby et al., 1994) or tryptophan (Moan

Table 1: MAO B Resting State and Photoreduced Radical EPR Signals: EPR Simulation Parameters[a] and $^1$H ENDOR Results

| | $g_\perp$[b] | $g_\parallel$[b] | $A_\perp$ (N5)[c] | $A_\parallel$ (N5) | $A_\perp$ (N10) | $A_\parallel$ (N10) | $^1$H ENDOR[d] |
|---|---|---|---|---|---|---|---|
| resting state | 2.0062 (2.0058) | 2.0020 (2.0010) | 5 | 70 | 3 | 18 | 11, 6 |
| photoreduced | 2.0050 (2.0050) | 2.0000 (1.9980) | 8 | 40 | 4 | 18 | 14, 9 |

[a] The $g$-value and $^{14}$N hyperfine parameters used to simulate the EPR spectra of Figures 1 and 3 (9 and 35 GHz, respectively) are listed. [b] Parameters listed are for X-band EPR signal simulations; parameters used for 35 GHz EPR simulations are given in parentheses. [c] Hyperfine couplings given in megahertz. The same nitrogen hyperfine parameters were used for 35 GHz EPR simulations. [d] Major proton hyperfine couplings observed by using 35 GHz ENDOR spectroscopy.

& Kaalhus, 1974; Essenmacher et al., 1993) residues. Tyrosine radicals, for example, typically have more complex resolved structure because of strong hyperfine coupling to both ring and $\beta$-methylene protons. There are no reports of other unique cofactors or modified amino acids in MAO B. We therefore investigated the possibility that the MAO B resting state radical arises from a flavin with slightly altered EPR characteristics.

EPR simulations were carried out to characterize the resting state EPR signal in MAO B. The breadth of flavin EPR signals is due to unresolved hyperfine couplings to protons and nitrogen nuclei, and in particular to strong hyperfine couplings to N5 and N10 (Palmer et al., 1971, and references therein; Eriksson & Ehrenberg, 1973; Edmondson et al., 1990). A slight $g$ anisotropy also contributes to the overall line width. Couplings to $^{14}$N are expected to contribute the largest splittings and are highly anisotropic, and so these were explicitly included in the simulations. To simplify this comparison, proton hyperfine couplings were not included explicitly but were assumed to contribute to the line width. From previous EPR and ENDOR studies of anionic flavin radicals, isotropic hyperfine couplings to N5 and N10 are predicted to be $a_{\text{iso}} \sim 20$ and $\sim 9-10$ MHz, respectively (Weilbacher et al., 1988; Edmondson et al., 1990). The tensors are expected to have axial symmetry, and the relationships $A_\parallel(\text{N}) \sim 2.5$ ($a_{\text{iso}}$) and $A_\perp(\text{N}) \sim 0.25$ ($a_{\text{iso}}$) are expected to occur with coaxial $\mathbf{A}$(N)- and $\mathbf{g}$-tensors (Erikkson & Ehrenberg, 1973).

A model combining a slight $g$ anisotropy with hyperfine couplings from two strongly coupled nitrogen nuclei gave satisfactory simulations for both EPR spectra. The resulting simulations are shown under each experimental spectrum in Figure 1, and the parameters are given in Table 1. From the relationships given above, nitrogen hyperfine coupling parameters of $\sim 50$ and $\sim 5$ MHz would be expected for $A_\parallel$(N5) and $A_\perp$(N5), respectively, and these are matched reasonably well by the values of 40 and 8 MHz used in the simulation of the MAO B EPR signal generated by photoreduction. The enhanced resolution of the MAO B resting state EPR signal was reproduced by using a larger hyperfine coupling for N5 of $A_\parallel(\text{N5}) = 70$ MHz and slightly higher values for $g_\perp$ of 2.006 and $g_\parallel$ of 2.002 in comparison with values for $g_\perp$ of 2.005 and $g_\parallel$ of 2.000 for the photoreduced EPR signal.

*Q-Band Electron Paramagnetic Resonance Spectra.* To test the X-band analysis and further investigate the apparent differences between these signals, EPR spectra were obtained at the higher microwave frequency of 35 GHz ("Q-band"). At the higher frequency, $g$ anisotropy is enhanced, and indeed in the spectra of Figure 3, $g_\perp$ and $g_\parallel$ are now resolved. Because of the small values for $A_\perp$(N5, N10), the feature for $g_\perp$ is relatively narrow, whereas $g_\parallel$ is broadened by the larger $A_\parallel$(N) splittings. The simulations shown below each spectrum in Figure 3 employ essentially the same parameters as those used for the X-band simulations (Table 1), and these reproduce the perpendicular regions of the Q-band spectra quite successfully and confirm the observed differences in $g_\perp$ between the species. The $g_\parallel$ region of the spectrum was not as well simulated, most likely as a result of unusual relaxation effects leading to anomalous line shapes (Palmer et al., 1971). While these abnormalities preclude a definitive judgment as to the reliability of the simulation parameters for the parallel region of the spectrum, it is clear that the resting state EPR signal has a broader parallel feature than that of the photoreduced sample, in agreement with the higher value of $A_\parallel$(N5) used in both X- and Q-band simulations of the resting state signal.

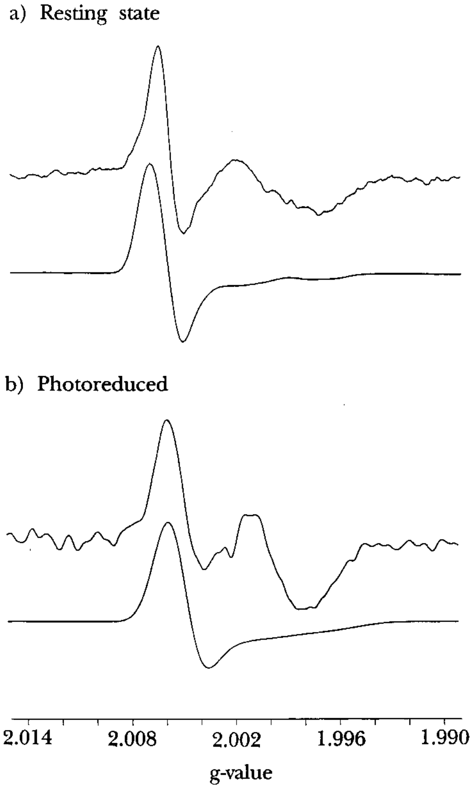


FIGURE 3: Q-Band EPR spectrum of (a, upper trace) resting state MAO B. Spectrometer conditions: 35.028 GHz, 0.05 mW, 4 K, 0.7 G modulation amplitude, 8 min scan time, and one scan. Parameters used in the simulation (a, lower trace) are listed in Table 1. Q-band EPR spectrum of (b, upper trace) photoreduced MAO B. Spectrometer conditions: 34.863 GHz, 0.1 mW, 4 K, 0.02 G modulation amplitude, 8 min scan time, and one scan. Spectrum is simulated (b, lower trace) with parameters listed in Table 1. EPR spectra are shown as analytical first derivatives of dispersion-detected signals.

*35 GHz ENDOR Spectra.* Proton ENDOR spectra were obtained for both the resting state and photoreduced radicals of MAO B. Doublets, centered at the proton Larmor frequency ($\sim 55$ MHz) and corresponding to two relatively strongly coupled protons, are observed in both samples

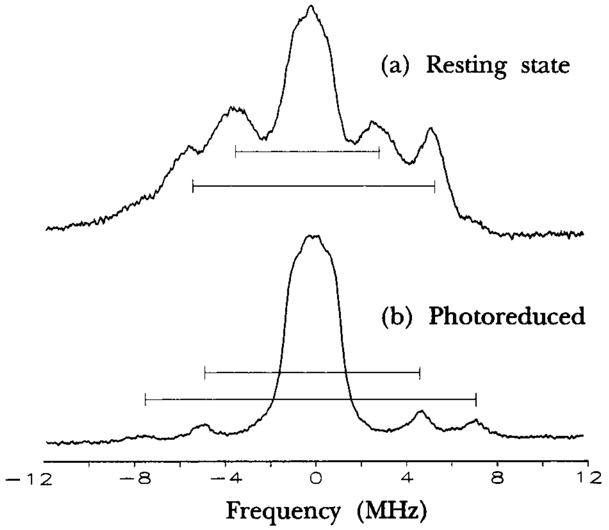


FIGURE 4: (a) Proton ENDOR spectrum of resting state MAO B. Conditions: 35.028 GHz, $g = 2.01$, 2 K, 1 MHz/s scan (negative sweep direction), 0.3 G modulation amplitude, 200 kHz RF band width, 0.05 mW, and 40 scans. (b) Proton ENDOR spectrum of photoreduced MAO B. Conditions: 35.021 GHz, $g = 2.00$, 18 scans, and the rest as in part a. Data were obtained under conditions that enhance stronger couplings (see Materials and Methods) and are shown centered at the proton Larmor frequency (~53 MHz). Horizontal bars indicate resonances separated by the proton hyperfine coupling A.

(Figure 4). The values of the observed proton hyperfine couplings in the resting state (6 and 11 MHz) are noticeably smaller than those in the photoreduced (9 and 14 MHz) samples (Table 1).

Although ENDOR data have not been reported for a C8α-cysteinyl, N10-ribityl flavin semiquinone anion such as found in MAO B, the proton ENDOR data for both EPR signals from MAO B are in a reasonable range with what is expected from reported hyperfine couplings of similar flavin semiquinones. The data displayed in Figure 4 were obtained under conditions that enhance strong couplings (Hoffman et al., 1994); by comparison with studies of anionic flavin semiquinones, such couplings are expected to be associated with protons at position C6 (8−11 MHz; Eriksson et al., 1969; Weillbacher et al., 1988) and from substituents at C8 and N10, with C8 (methyl) ~11 MHz and reported couplings of ~9−11 MHz for one of the $\beta$-methylene protons of the N10 ribityl group, ~9 MHz for N10 (methyl), and 4−6 MHz for $\beta$-methylenes of other substituents at N10 (Eriksson et al., 1969, 1970; Edmondson, 1985; Weillbacher et al., 1988). Anisotropic interactions will contribute to frozen-solution ENDOR patterns such as in Figure 4 and are expected to be significant for the C6 proton. In addition, the hyperfine couplings to protons at N10 and C8 substituents depend strongly on the angle between the flavin ring (the $\pi$-system) and the $\beta$-methylene protons.

The differences in the proton ENDOR between resting state and photoreduced MAO B could be the result of different protein conformations inducing altered conformations of the $\beta$-methylene protons on the C8 and/or N10 substituents and/or slightly different spin delocalizations for the two flavins. Consistent with the EPR spectral differences, these ENDOR data support the existence of two different species giving rise to radicals in MAO B.

*Flavin to Enzyme Stoichiometry.* Because of some earlier confusion in the literature regarding the actual flavin content in MAO, we sought to correlate the previously obtained stoichiometry of radioactively labeled inactivators to flavin content in the holoenzyme. MAO B was inactivated with *N*-(1-methylcyclopropyl)benzylamine followed by denaturation. It has been shown by UV/visible spectroscopy (Weyler, 1989) that enzyme inactivated under these conditions contains 1 equiv of inactivator per dimer (Silverman & Yamasaki, 1984). The content of oxidized flavin determined following denaturation of the inactivated enzyme was found to be $^1/_2$ of that found in denatured native enzyme. Since denaturation of the enzyme results in oxidation of all unmodified flavins (e.g., the flavin semiquinone), these results provide further evidence that there are two flavin cofactors per holoenzyme.

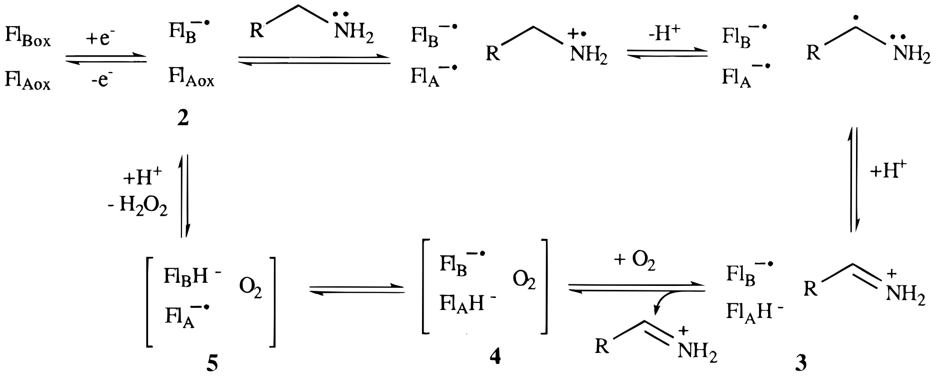
## DISCUSSION

We report here the observation and characterization of a new EPR signal associated with the resting state of MAO B. Both EPR and ENDOR spectra from reduced flavin species have been extensively characterized [reviewed in Palmer et al. (1971) and Edmondson (1985)]. The breadth of the flavin EPR spectrum largely results from unresolved $^{14}$N hyperfine couplings that occur because of appreciable spin density on N5 and N10 of the flavin ring; in addition, there can be substantial couplings to protons on the C8 substituents and, in the case of the neutral radical, to the N5 proton. The neutral and anionic flavin radicals can be readily distinguished because the loss of hyperfine coupling to the N5 proton in the anion results in a characteristic narrowing of the EPR line width from 18−19 G for the neutral radical to ~15 G for the anionic radical (Palmer et al., 1971; Yue et al., 1993).

The 15 G peak-to-peak line width and the overall appearance of the spectrum of photoreduced MAO B (Figure 1b) and its insensitivity to exchange in $D_2O$ are typical for an anionic flavin semiquinone. Resting state MAO B, however, shows an EPR spectrum (Figure 1a) that is similar to the photoreduced MAO sample but shows uncharacteristic resolution in the wings of the spectrum. This resolution is unusual and has only been observed in cases in which the unresolved line width of the spectrum has been reduced, such as by deuteration of C8 methyl protons, accompanied by an increase in the observed nitrogen hyperfine splittings by labeling with $^{15}$N (Edmondson et al., 1990). Substitution of *N*-histidyl at position C8 also results in a narrower peak-to-peak line width of 12 G (Edmondson et al., 1981). The resolution observed in the MAO B resting state EPR signal could be simulated by increasing the hyperfine coupling to $^{14}$N5 and decreasing the effective line width (Table 1, and Figure 1a). Thus, it is possible that the spectrum of resting state MAO B results from an anionic semiquinone flavin with some structural difference resulting in a reduced spin density at position C8, and increased density at N5. Consistent with this is the observed reduction in proton hyperfine couplings observed by ENDOR, relative to the anionic semiquinone flavin produced by photoreduction of MAO B (Table 1).

EPR spectra obtained at 35 GHz allow a further comparison between the two radical species found in MAO B. The increased microwave frequency results in the expected resolution of $g_{\|}$ and $g_{\perp}$ in both samples (Figure 3). The two EPR signals appear similar under these conditions, with the

Scheme 1: Hypothetical Functions for the FAD in Each Subunit of Dimeric MAO



resting state MAO B signal showing a slight increase in $g_\perp$ which is consistent with results from simulation of the X-band EPR spectra. For the relatively planar flavin radical, $g_\perp$ is parallel to the plane of the ring, and changes in $g_\perp$ may occur through perturbation of the ring structure.

Taken together, these results suggest that the resting state radical species in MAO B is an anionic flavin semiquinone radical with a structural perturbation leading to a slightly reduced spin density at C8, a slightly increased spin density at N5, and a slightly increased value of $g_\perp$. The EPR and ENDOR data further indicate that the flavin giving rise to the resting state EPR signal is structurally distinct from that obtained by photoreduction. This notion that two distinct flavin prosthetic groups exist in the holoenzyme is supported by the additional observation that neither pargyline nor substrate (benzylamine or phenylethylamine), both of which interact with the active site flavin, affects the resting state EPR signal. The weak semiquinone EPR signal from the resting state of MAO B is stable and so may arise from a flavin in a separate site (subunit) that is protected from oxygen as well as substrate. Previous results from fluorescence measurements provide further support for two distinct chromophores in MAO B (Woo & Silverman, 1994). In addition, both photoreduction (reported here) and dithionite reduction (Yue et al., 1993) appear to reduce only 50% of the flavin content of MAO B.

The results reported here agree with the resonance Raman study results of Yue et al. (1993), reporting an apparently altered flavin semiquinone radical in the resting state of MAO B, and further agree that reduction of MAO B results in an EPR signal representing only half of the flavin content. From resonance Raman studies, however, Yue et al. (1993) concluded that the resting state radical and dithionite-reduced radical flavins are the same species, whereas the EPR and ENDOR studies reported here show these radicals to be structurally distinct. The reasons for this, and the lack of an observable resting state EPR signal in the studies by Yue et al., are not clear at this time.

It has been reported that an 8% increase in the 450 nm absorption resulting from oxidized flavin was observed when the enzyme was incubated with potassium ferricyanide and that there was a 60% loss of enzymatic activity (Yue et al., 1993). This is consistent with one of the flavin prosthetic groups on the enzyme being at a lower oxidation state than the other in the resting state.

The intensity of the resting state signal corresponds to only 0.02 spin per enzyme molecule. Yue et al. (1993) have suggested that the inability to detect the semiquinone EPR signal easily in the resting state of MAO may be the result of spin−spin coupling to another radical present in the enzyme; if this is the case, the results of our experiments indicate that the other unidentified radical also must be in the non-active site subunit and be inaccessible to the medium. A more reasonable hypothesis is that there is a 2% equilibrium concentration of flavin semiquinone in the isolated enzyme and no other radical. Walker and Edmondson (1994) have carried out stopped-flow kinetics studies on the oxidation of substituted benzylamines by MAO B and concluded, from deuterium isotope effect results, that they were unable to detect the formation of an amine radical cation intermediate. As an alternative mechanism, it was suggested that direct hydrogen atom abstraction may occur, thereby bypassing the initial amine radical cation intermediate. Because of the strength of the benzylic C−H bond, it was noted that the only possible species capable of this hydrogen atom abstraction would be the hypothetical amino acid radical that was proposed to be spin coupled to the flavin semiquinone (Yue et al., 1993; Anderson et al., 1996); oxidized flavin would not be capable of such a hydrogen atom abstraction. The results presented here, however, indicate that the resting state radical species is not in the active site and is not directly involved in substrate oxidation. Furthermore, a hydrogen atom mechanism is not consistent with the numerous reactions of MAO B with cyclopropylamines and cyclobutylamines (Silverman, 1992; Silverman et al., 1993); these reactions cannot be rationalized in terms of a direct hydrogen atom abstraction because there is no α-hydrogen atom to abstract and several flavin adducts which were isolated could not arise from direct cyclopropyl or cyclobutyl cleavage by oxidized flavin.

To account for a fraction of the resting state enzyme containing a flavin semiquinone radical that is not affected by substrate turnover or pargyline inactivation, the hypothetical mechanism shown in Scheme 1 is proposed. By this mechanism, catalytically active MAO B contains one active site-oxidized flavin ($Fl_{Aox}$) and one flavin semiquinone ($Fl_B^{\bullet-}$) in the adjacent subunit (**2**). In the isolated enzyme, only 2% of the enzyme molecules is in the catalytically active form at any given time. Pargyline inactivation would result in attachment to $Fl_{Aox}$ but would not interfere with $Fl_B^{\bullet-}$. The proposed function of $Fl_B^{\bullet-}$ is to catalyze the oxygen-dependent reoxidation of the active site flavin cofactor after substrate oxidation is completed. As depicted in Scheme 1, this occurs as follows. As a result of substrate oxidation (active site flavin reduction) (**3**), a conformational change would occur allowing for oxygen binding (**4**).[2] Such a conformational change to allow oxygen binding is required because the flavin semiquinone appears to be protected from oxygen in the resting state. Subsequent to oxygen binding, the non-active site flavin semiquinone ($Fl_B^{\bullet -}$) would induce the transfer of electrons from the active site flavin to the non-active site flavin and from the non-active site flavin to oxygen (**4** to **5** to **2**). The flavins could communicate via electron transfer reactions, possibly mediated by an amino acid group. There is literature precedent to show that some oxidative enzymes containing two flavin prosthetic groups use one flavin for the reductive half-reaction and the other for the transfer of electrons to an exogenous oxidant to complete the catalytic cycle (Vermillion et al., 1981; Jorns, 1985; Ali et al., 1991). Recently, a flavin semiquinone radical was observed in the resting state of neuronal nitric-oxide synthase (Galli et al., 1996). The presence of bound substrate or inhibitor had no effect on the flavin EPR spectrum. It was proposed that an interdomain electron transfer occurs from the flavin to the active site heme. The mechanism shown in Scheme 1 is further supported by the observation that enzyme reduced to the semiquinone state is catalytically inactive,[3] which is consistent with the oxidized flavin acting as the initial electron acceptor.

## ACKNOWLEDGMENT

The authors thank Clark Davoust for technical assistance in the use of the instruments, Dr. Peter Doan for helpful discussions, and Dr. Joshua Telser for assistance in spectral simulations.

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BI960749F

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[2] Such a complex has not yet been detected (Husain et al., 1982).

[3] R. W. Von Korff and A. R. Wolfe, unpublished data.