We present spectroscopic evidence consistent with the presence of a stable tyrosyl radical in partially reduced human monoamine oxidase (MAO) A. The radical forms following single electron donation to MAO A and exists in equilibrium with the FAD flavosemiquinone. Oxidative formation of the tyrosyl radical in MAO is not reliant on neighboring metal centers and uniquely requires reduction of the active site flavin to facilitate oxidation of a tyrosyl side chain. The identified tyrosyl radical provides the key missing link in support of the single electron transfer mechanism for amine oxidation by MAO enzymes.

The mammalian monoamine oxidases (MAO) A and B are flavoproteins localized to the outer mitochondrial membrane. MAO catalyzes the oxidative deamination of neurotransmitters and exogenous alkylamines. The human enzymes are important pharmaceutical targets for antidepressants, and inhibitors of MAO B are used synergistically with L-DOPA in the treatment of Parkinson disease (1). Elevated levels of MAO B induce apoptosis in kidney (2) and neuronal cells (3) and are also associated with plaque astrocytes in the brains of Alzheimer patients (4). The anti-apoptotic action of a MAO B inhibitor is important in novel Alzheimer treatments (5). MAO is also implicated in the onset of Parkinson syndrome through inhibition of reduction potential by substrate binding (19), and alteration of the geometry of the substrate and/or flavin (18), perturbing electron transfer might not prevent the reaction in an enzyme-based model studies in reactions of amines with lumiflavins (11, 12). Studies of quantitative structure-activity relationships with MAO B have been used to support a second mechanism in which substrate α-C–H bond cleavage is via direct hydrogen atom transfer to a protein-based non-flavin radical followed by electron transfer to the flavin (13, 14). An organic radical species was originally reported in EPR spectra of resting bovine liver MAO B (15) but later was attributed to an artifact of purification of MAO B from bovine liver following EPR studies of highly purified recombinant sources of MAO A and MAO B (16). Edmondson and Miller (16) have proposed a concerted polar nucleophilic mechanism for MAO A involving a substrate-flavin C4a adduct and proton abstraction by the highly basic N-5 atom of the flavin. This mechanism is consistent with studies of quantitative structure activity relationships and kinetic isotope effects and with the apparent lack of an organic protein-based radical in EPR spectra of the resting form of the enzyme. The aminium cation radical mechanism for MAO proposed by Silverman et al. (17) involves single electron transfer from the substrate nitrogen lone pair to yield the substrate radical and flavin semiquinone. This mechanism is based on the susceptibility of amines to undergo single electron transfer chemistry during electrochemical and chemical oxidations (17) and is consistent with the results of mechanism-based inhibitor studies with a series of cyclopropyl inhibitors, which undergo rapid ring opening on formation of the cyclopropylaminal radical (reviewed in Ref. 9). The aminyl radical cation mechanism is consistent with electronic effects observed in quantitative structure activity relationships studies with a series of substituted benzylamines (16), but the identity of the 1-electron oxidant required for formation of the aminyl radical cation remains a major concern (16). Edmondson and Miller (16) argue that the ground state of flavin ($E_m \approx -0.2$ to $0$ V, where $E_m$ is midpoint redox potential) is unlikely to oxidize a primary amine ($E_m = +1.5$ V versus standard calomel electrode, although some oxidation occurs at much lower potentials), but based on a number of arguments, large apparent barriers to electron transfer might not prevent the reaction in an enzyme-active site (e.g. distortion of substrate by intrinsic binding to alter the geometry of the substrate and/or flavin (18), perturbation of reduction potential by substrate binding (19), and endergonic tunneling over short distances (20) followed by a thermodynamically favorable step in the reaction pathway). That a protein-bound oxidant is the 1-electron acceptor has been considered, but detectable EPR signals have not been found in purified recombinant MAO A or MAO B.

**MATERIALS AND METHODS**

**Enzyme Purification**—Purified human liver monoamine oxidase A (heterologously expressed in Saccharomyces cerevisiae) (21) was prepared for use as in previous work in 50 mM potassium phosphate, pH 7.2, containing 0.05% Brij-35. MAO A in 50 mM potassium phosphate,
RESULTS AND DISCUSSION

The studies reported herein provide the first evidence for the presence of a protein radical, in the form of a tyrosyl radical, which is formed in partially reduced MAO. Reduction of an anaerobic solution of MAO A using sodium dithionite gives rise to the EPR spectrum shown in Fig. 1A, spectrum a. This spectrum exhibits features not observed for typical anionic flavosemiquinones (25). Typical anionic flavosemiquinone EPR spectra include those formed by electron-transferring flavoprotein or glucose oxidase at pH 9. When Fig. 1A, spectrum b, an example of these spectra, is subtracted, the result is a residual spectrum shown in spectrum c. This spectrum exhibits a $g_{av}$ of 2.0042, which is too high to arise from the flavosemiquinone

$^2$ The EPR spectrum of an anionic flavosemiquinone (both electron-transferring flavoprotein and glucose oxidase at pH 9.0 were used; both produced the same results) was subtracted from the MAO EPR spectrum in a proportion such that the minimum (smallest possible) residual spectrum remained. This represents the minimum proportion of the spectrum that cannot be accounted for by the formation of an anionic flavosemiquinone. Double integration (the standard method for quantification of EPR spectra) of this residual EPR spectrum, which arises from the tyrosyl radical, and comparison of this value with the double integral of the entire EPR spectrum before subtraction gives the proportion of tyrosyl radical formed.

The studies reported herein provide the first evidence for the presence of a protein radical, in the form of a tyrosyl radical, which is formed in partially reduced MAO. Reduction of an anaerobic solution of MAO A using sodium dithionite gives rise to the EPR spectrum shown in Fig. 1A, spectrum a. This spectrum exhibits features not observed for typical anionic flavosemiquinones (25). Typical anionic flavosemiquinone EPR spectra include those formed by electron-transferring flavoprotein or glucose oxidase at pH 9. When Fig. 1A, spectrum b, an example of these spectra, is subtracted, the result is a residual spectrum shown in spectrum c. This spectrum exhibits a $g_{av}$ of 2.0042, which is too high to arise from the flavosemiquinone
radical (2.0032) (25) but is consistent with assignment to a neutral tyrosine (tyrosyl) radical (26). This radical accounts for 18% of the total number of unpaired electrons observed. The EPR spectrum c of Fig. 1A also shows the partially resolved hyperfine splitting that is typical of such neutral tyrosine radicals as Y_D of photosystem 2 (27), Fig. 1A, spectrum d, but the MAO radical has a smaller line width than reported previously for a tyrosyl radical. Such narrowing of the EPR spectrum can occur when the unpaired electron is exchanging between two sites or species on a time scale that is rapid compared with the spectrometer frequency (28). Rapid exchange might occur between spatially close tyrosine residues in the active site of MAO (6). The rate of electron exchange between the two sites will be temperature-dependent, and thus the EPR spectrum of such a species should also be temperature dependent. The EPR spectra of the presumptive tyrosyl radical (formed by subtraction of an anionic flavosemiquinone spectrum recorded under the same conditions as shown in Fig. 1, except temperature), recorded at various temperatures, are shown in Fig. 1, except temperature), recorded at various temperatures, are shown in Fig. 1B. These spectra show clear evidence of temperature-dependent broadening, which is not seen for other known tyrosyl radicals (e.g., Y_D). Therefore, a possible explanation for the narrow line width of the presumptive tyrosyl radical is exchange narrowing. However, the relative proportions of flavosemiquinone and tyrosyl radical are the same at all the temperatures employed, so there is no temperature-dependent redistribution of the unpaired electron between these species.

ENDOR spectroscopy allows the measurement of the hyperfine couplings to the protons of the tyrosyl radical, as shown in the spectrum of Fig. 2A, spectrum a, and tabulated in Table I. Only those features to high frequency of the Larmor frequency are shown, as the symmetrically displaced features to low field of the Larmor frequency are typically weak and difficult to discern (27). The experimental conditions employed were chosen to emphasize the contribution of the tyrosyl radical to the ENDOR spectrum at the expense of the radical of the overlapped flavosemiquinone anion. Fig. 2A, spectrum b, shows the ENDOR spectrum of Phormidium laminosum Y_D', the

![Fig. 2. A, ENDOR spectra of MAO A tyrosyl radical (a) and the tyrosyl radical Y_D of photosystem 2. Experimental conditions for a were microwave power 1.6 mW, rf power 100 W, rf modulation depth 200 kHz, temperature 100 K, average of 200 scans. Conditions for b were microwave power 10 mW, rf power 100 W, rf modulation depth 158 kHz, temperature 10 K, average of 160 scans. B, structure and carbon atom numbering scheme for the tyrosyl-neutral radical.](image)

**Table I** Assignment of hyperfine coupling constants to ENDOR spectral features

| Feature | Hyperfine coupling constant | Assignment |
|---------|-----------------------------|------------|
| 1       | 5.0                         | 2.6 H A_x  |
| 2       | 7.0                         | 2.6 H A_x  |
| 3       | -8.8                        | 3.5 H A_x  |
| 4       | 14.4                        | 3.5 H A_x  |
| 5       | 16.8                        | 3.5 H A_x  |
| 6       | -18.0                       | 3.5 H A_x  |
| 7       | -21.0                       | 3.5 H A_x  |
| 8       | 21.0                        | 24.5       |
| 9       | 24.2                        | 29.0       |
| 10      | -27.8                       | 3.5 H A_x  |
| 11      | -31.2                       | 3.5 H A_x  |

a Assignment of features being based on Ref. 27 and references therein.

b The line shape of this feature is disturbed by a small amount of the 8-Me flavosemiquinone feature that is not suppressed by the experimental conditions employed.

![Fig. 3. Equilibrium reductive titration of MAO A under anaerobic conditions. The starting spectrum of MAO A is shown as a solid black line (recorded at +35 mV versus standard hydrogen electrode). With progressive addition of sodium dithionite, the flavin is reduced further (absorption decrease at 456 nm, indicated by black arrow; selected spectra are shown as black dashed lines), whereas the intensity of the tyrosyl radical increases at 412 nm (blue arrows), reaching a maximum at ~9 mV (spectrum shown as solid red line). The red anionic form of the MAO A semiquinone also accumulates in this range, with a distinctive absorption at ~365 nm (red arrows). Further reduction by dithionite leads to progressive bleaching of the flavin and to gradual diminution of the radical signal (red dotted lines). The spectrum for the fully reduced MAO A (blue solid line; recorded at ~200 mV) indicates that the spectral contribution from both flavin and tyrosyl radicals are removed.](image)

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| Feature | Hyperfine coupling constant | Assignment |
|---------|-----------------------------|------------|
| 1       | 5.0                         | 2.6 H A_x  |
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| 5       | 16.8                        | 3.5 H A_x  |
| 6       | -18.0                       | 3.5 H A_x  |
| 7       | -21.0                       | 3.5 H A_x  |
| 8       | 21.0                        | 24.5       |
| 9       | 24.2                        | 29.0       |
| 10      | -27.8                       | 3.5 H A_x  |
| 11      | -31.2                       | 3.5 H A_x  |

a Assignment of features being based on Ref. 27 and references therein.

b The line shape of this feature is disturbed by a small amount of the 8-Me flavosemiquinone feature that is not suppressed by the experimental conditions employed.
known tyrosyl radical that exhibits spectra most similar to that of MAO A (27). Simulation of the EPR spectrum using these hyperfine coupling constants and G values for tyrosyl radicals from the literature produces a spectrum similar in shape to Fig. 1A, spectrum c, but without the exchange narrowing, as shown in Fig. 1A, spectrum e. The rotameric angle of the tyrosyl radical ring relative to the β-CH₂ group (see Fig. 2B) can be determined from the β-CH₂ hyperfine coupling constants using the Heller-McConnell equation (29),

\[ A_{iso} = B \cos^2 \theta \]  

(Eq. 1)

where \( A_{iso} \) is the isotropic hyperfine coupling constant for the β proton, \( B \) is a constant (162 MHz), \( \rho \) is the unpaired electron spin density at C(1) of the tyrosyl radical, and \( \theta \) is the rotameric angle between the C(β)–H bond and the normal to the tyrosine ring plane. The \( \theta \) angles calculated for the two β-CH₂ protons are 54.6° and 61.1°. The structure of MAO B shows that tyrosine residues Tyr-60, Tyr-398, and Tyr-435 (equivalent to Tyr-69, Tyr-407, and Tyr-444 in MAO A) are in the vicinity of the active site (7). Of these three, Tyr-398 (Tyr-407 in MAO A) has \( \theta \) values closest to those measured for the tyrosyl radical studied here (50° and 70°), and the orientation looks similar in the MAO A structure. Mutation of Tyr-69 to alanine, serine, or phenylalanine had no effect on activity. Mutation of tyrosines 407 and 444 to serine (Y407S, Y444S) in MAO A leads to loss of activity (30); however, mutation to phenylalanine has a much larger effect at position 444 (Y444F) than 407 (Y407F). Although these data demonstrate a role for tyrosine residues, given the results of the temperature dependence studies (Fig. 1B), it may not be useful to think of the radical as being located on one identifiable tyrosine residue but rather as delocalized over a number of residues. Thus, mutation of individual tyrosine residues may not have a large impact on activity.

Previous EPR studies have not revealed the presence of the tyrosyl radical reported here (15, 31). However, the conditions employed in these studies were not optimized for the detection of the additional features shown in Fig. 1A, spectrum a, that provided our first evidence for the existence of the tyrosyl radical. Optical spectra of MAO reduced under anaerobic conditions using sodium dithionite (22, 31, 32) consistently show formation of the anionic flavosemiquinone, indicated by a band at ~365 nm and a narrow feature at ~412 nm (Fig. 3). Although the latter could arise from an anionic flavosemiquinone (33), it seems that the extinction coefficient of this feature in MAO A is not in keeping with those reported for other anionic flavosemiquinones (33). An alternative source of the 412 nm band is the identified tyrosyl radical, as such radicals are known to give rise to sharp bands at 410–415 nm (34). A similar 412 nm absorption band is also observed during equilibrium titration of human MAO B with sodium dithionite, consistent also with the presence of a tyrosyl radical as part of a similar redox equilibrium to that seen in MAO A (31). In MAO A, the optical data indicate that the 412 nm absorption is maximal at ~0 mV and is populated with the flavin semiquinone (Fig. 3). The absorption at 412

**Fig. 4.** Proposed schemes for the formation of a tyrosyl radical (Tyr*) in MAO (A) and oxidation of primary amines MAO A by radical-mediated homolysis of the substrate C-H bond (B).
nm disappears on further reduction of the enzyme, indicating that the tyrosyl and flavin semiquinone form a redox equilibrium in partially reduced MAO A.

The formation of a tyrosyl radical in sodium dithionite-reduced MAO A reported here provides the key missing link in support of a single electron transfer mechanism for amine oxidation. A variation on the aminoalkyl (cation) radical mechanism for amine oxidation employing a tyrosyl radical (Fig. 4) that was proposed by Silverman et al. (17), similar to that proposed previously by Edmondson (35), would account for our observations. This scheme requires that a redox equilibrium exists between the flavosemiquinone and a tyrosine radical in the active site. Formation of flavosemiquinone by titration with sodium dithionite establishes this equilibrium leading to the observed formation of the tyrosyl radical (Fig. 4A), and this accounts for why previous workers have not obtained evidence for an organic radical in the resting form of the enzyme. During catalytic turnover, reduction of the enzyme by single electron transfer from the substrate to flavin also generates a redox equilibrium with appearance of the tyrosyl radical and the aminyl radical cation intermediate (Fig. 4B). Upon establishing the tyrosyl radical, the reaction could proceed either by direct proton transfer and formation of a radical centered on the $\alpha$-carbon route. The latter route is consistent with the electronic effects seen in quantitative structure activity relationships analysis (16) of MAO A and with studies of mechanism-based inhibitors (36). We infer that the radical is short lived in partially reduced MAO A.

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A Stable Tyrosyl Radical in Monoamine Oxidase A
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