Mechanistic Probes of Monoamine Oxidase B Catalysis: Rapid-Scan Stopped Flow and Magnetic Field Independence of the Reductive Half-Reaction

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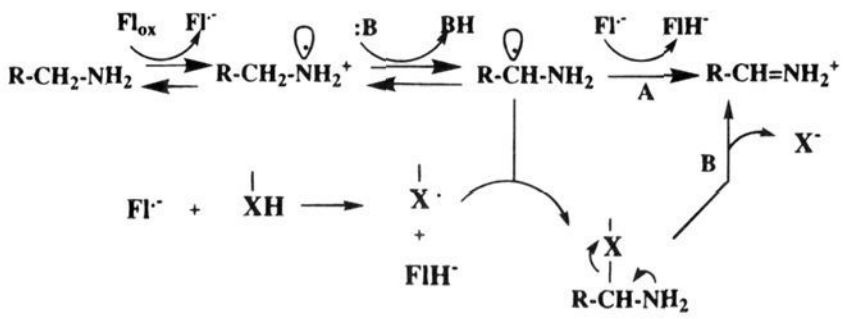
Monoamine oxidase (MAO, EC 1.4.3.4) is an outer mitochondrial membrane-bound flavoenzyme that catalyzes the oxidative deamination of biologically important amines. The B form (MAO B) has received extensive mechanistic attention by several groups,[1−4] and several proposed mechanisms have been suggested.[1−3] The most widely quoted mechanism is that originally proposed by Silverman[1] in which the initial event in catalysis is a single electron transfer (SET) reaction from the bound amine substrate to the covalent FAD resulting in an aminium cation radical substrate intermediate and an anionic flavin radical (Scheme 1). The proposed substrate radical then undergoes an α-proton abstraction and subsequent radical chemistry that results in the formation of the flavin hydroquinone and the protonated imine product.[2b] Several lines of experimental evidence have been obtained that are unsupportive of the SET mechanism. Perhaps the most direct negative evidence is the failure to obtain any spectroscopic evidence for the formation of any flavin semiquinone intermediates in anaerobic reductive half-reaction studies of the interaction of MAO B with a variety of unlabeled and [α,α-^{2}H]benzylamine analogues using absorbance-monitored stopped-flow kinetics.[2] These data were acquired using a single-wavelength instrument at regions of the visible spectral region where flavin radicals are known to absorb, and the possibility remains that transient spectral species might be missed. Improved rapid-scan stopped-flow instrumentation with 1 ms time resolution is now available to examine for the presence of any transient spectral intermediates in enzyme-catalyzed reactions.[5]

The anaerobic reductive half-reaction of MAO B[6] with several substrates was monitored for any spectral transients using the OLIS rapid-scan stopped-flow instrument and associated software. If the SET reaction mechanism were operative, this approach should provide evidence for any transient formation of either a neutral or an anionic flavin radical during the reductive half-reaction if the intermediate has a lifetime of 1 ms or greater. Experiments were performed using benzylamine, [α,α-^{2}H]benzylamine, and phenethylamine. All kinetic experiments were performed using substrate concentrations below the K_d value for substrate binding. This condition is expected to facilitate detection of any transient flavin radicals by slowing the apparently irreversible[2a] forward reaction toward flavin hydroquinone formation. The results with [α,α-^{2}H]benzylamine are shown in Figure 1. The spectral data show a monotonic decay of the spectral features of the oxidized flavoenzyme to the reduced form without formation of any flavin semiquinone intermediate or any other spectral intermediate with visible absorbance. This behavior is also observed in reductive half-reactions using unlabeled benzylamine or phenethylamine as substrates.

The rate of flavin reduction was determined by processing the rapid-scanning spectral data by Global Fit, the single-value decomposition algorithm (SVD) implemented by I. B. C. Matheson.[7] The SVD algorithm was applied to the absorbance vs time and wavelength file without assumption or prejudice.

Scheme 1



[a] Adapted from ref 1a. XH = amino acid residue.

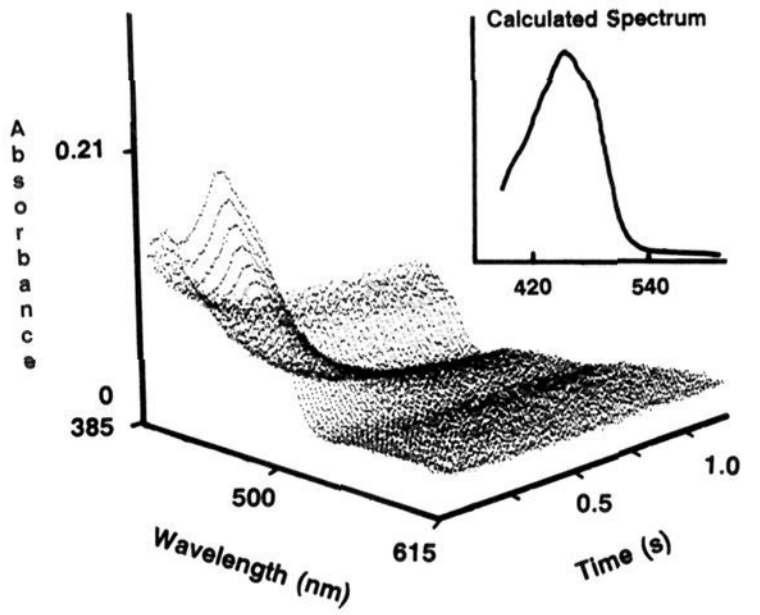


Figure 1. Rapid-scanning stopped-flow kinetic traces of MAO B after mixing with 100 μM [α,α-^{2}H]benzylamine. Conditions: The concentration of MAO B is 6.4 μM (after mixing) in 50 mM HEPES, pH 7.5, containing 5 mg/mL reduced Triton X-100. Solutions were degassed and placed under an argon atmosphere as described previously.[2] All kinetic runs were performed at 25 °C. Traces were acquired at intervals of 1 ms. The only time-dependent change in absorbance is the bleaching of oxidized flavin. Inset: Calculated spectrum of species undergoing bleaching. Spectrum is calculated with Global Fit (see text) assuming a time-dependent decrease in absorbance that follows first-order kinetics, as well as a static offset from the origin.

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(5) Stopped-flow kinetic studies were carried out with an OLIS, Inc., RSM-1000 rapid-scanning spectrophotometer and an OLIS, Inc., USA-SF stopped-flow mixing device with less than 2 ms dead time. The stopped-flow/spectrophotometer was modified in order to place the thermostated reaction cuvette (1.7 cm optical path) in the airgap of a GMW Associates electromagnet (7.5 cm cylindrical poles; model 5403). Absorbance spectra were recorded from 385 to 615 nm at a sampling rate of 1 kHz. On a time-averaged basis, the sample is illuminated for only 3% of the measurement time, which precludes any enzyme photoreduction.

(6) Enzyme was purified from bovine liver mitochondria using the procedure described by Salach (Salach, J. I. *Arch. Biochem. Biophys.* **1979**, *192*, 128) with further modifications: Weyler, W.; Salach, J. I. *Arch. Biochem. Biophys.* **1981**, *117*, 100.

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The best fits of the data and the most uniform distribution of residuals (defined as calculated minus experimental absorbance) were obtained by fitting the data with the first-order rate equation with offset. When other equations were applied following SVD, a nonsense calculated spectrum was obtained along with a grossly nonrandom distribution of residuals. When the first-order rate equation with offset was applied to the data set following SVD, the oxidized flavin spectrum was reproduced faithfully as the only chromogenic species that exhibits a time-dependent change in concentration. A chromogenic intermediate with a lifetime of less than 1 ms would not be observed with this technique.

The $^{D}k_{obs}$ value for benzylamine is reflected in the rate of flavin bleaching. No evidence was found for the formation of any intermediate spectral species that corresponds to a 4a-substituted flavin for any of the substrates tested. These results, therefore, do not support the proposal by Mariano's laboratory[3] that MAO catalysis proceeds by a nucleophilic mechanism in which electron transfer from the amine to the flavin and α-CH labilization occur *via* a 4a-flavin covalent intermediate.

The SET mechanism proposed by Silverman (Scheme 1) would result in the reversible formation of a pair of radical intermediates. The reaction rates of systems with kinetically significant radical pair intermediates can be altered by an exogenous magnetic field ($B = 10-3000$ G).[8,9] Recent steady-state and stopped-flow kinetic studies have shown that the rate of ethanolamine ammonia lyase can be altered by as much as 60% by a 500−1500 G magnetic field.[10] This is compelling evidence for a kinetically significant radical intermediate, as only reactions with radical pair intermediates can be altered by a nonzero magnetic field.[9a] Similar steady-state kinetic studies of MAO B oxidation of benzylamine showed no effect of magnetic field in the range 10−2700 G.[9a,11] In the current study, this approach was extended by using anaerobic rapid-scanning stopped-flow spectrophotometry to follow the flavin reductive half-reaction as a function of magnetic field. No influence of magnetic field was observed either in the nature of the spectral traces or on the rate of flavin reduction with benzylamine, [α,α-^{2}H]benzylamine, or phenethylamine up to a maximum achievable field of 6500 G (Figure 2).

The SET mechanism would generate a radical ion pair that undergoes one of two fates: (1) reverse electron transfer to regenerate the amine and oxidized flavin or (2) rearrangement to a carbon-centered radical followed by a second electron transfer to generate reduced flavin and the iminium cation. Both forward and reverse electron transfer from the radical ion pair require the singlet electron pair (i.e., antiparallel electron spin orientation) in order to form a covalent interaction. If the radical ion pair exists as the triplet (i.e., parallel electron spins), this enzyme form is unreactive to forward and reverse electron transfer until the spins are once again paired. The rate of electron spin interconversion between the singlet and triplet radical ion pair should be influenced by an external magnetic field if the lifetime of the radical pair is at least $10^{-9}-10^{-6}$ s. This value is another estimate of the upper limit for the lifetime of the radical ion pair in MAO B.

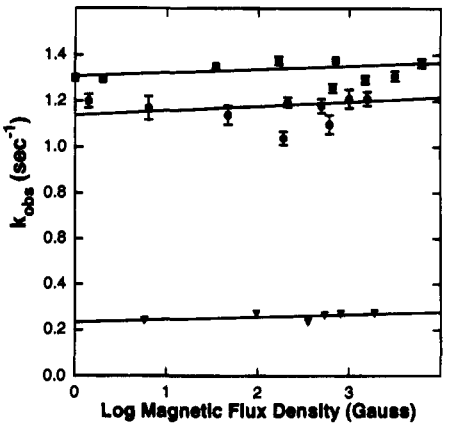

Figure 2. Influence of magnetic field on the rate of MAO B flavin reduction as measured by anaerobic rapid-scan stopped flow. Kinetic experiments were performed under an argon atmosphere at 25 °C in 50 mM HEPES (pH 7.5) and 5 mg/mL reduced Triton-X100. MAO B concentration (after mixing) was 6.4 μM. Residual oxygen was removed from the solutions by the addition of 20 nM glucose oxidase, 10 mM glucose, and 10 units/mL catalase. Substrate concentrations after mixing were 100 μM benzylamine (●), 100 μM [α,α-^{2}H]benzylamine (▼), and 40 μM phenethylamine (■).

In conclusion, the spectral data are best accounted for by reduction of oxidized flavin to the hydroquinone form without formation of any observable intermediates such as flavin semiquinone or an amine substrate 4a-flavin adduct. Additionally, a magnetic field in the range 10−6500 G has no influence on the rate of flavin reduction, thereby precluding the formation of any stable radical pair intermediates with a lifetime longer than 10^{-6} s. These data do not preclude other possible mechanisms with shorter-lived radical pair intermediates or irreversible radical pair chemistry that does not involve radical pair recombination in the forward direction.

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