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# ESR ANALYSIS OF THE FAD IN BOVINE LIVER MONOAMINE OXIDASE

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SUMMARY- Two hydrazine spin labels, 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carbonyl ethyl hydrazine and 1-oxyl-2,2,6,6-tetramethylpiperidino-4-hydrazine, were synthesized as probes of the FAD binding site of monoamine oxidase. The reporter nitroxide moiety showed an ESR spectrum classified as partially immobilized which is indicative of FAD near the surface of the enzyme.

Attempts to pick up flavin semiquinone or free radical intermediates during substrate oxidation with the spin traps 5,5-dimethyl-1-pyrroline-1-oxidase and phenyl-t-butylnitrone were not successful.

As inhibitors of mitochondrial monoamine oxidase (E.C.1.4.3.4), hydrazino compounds have been extensively studied for their potential as therapeutic agents for the treatment of hypertension and central nervous system depression (1,2), as well as for investigation of catecholamine and serotonin metabolism (3,4). Patek and Hellerman showed that phenylhydrazine oxidation in air led to the formation of a stable flavin-inhibitor adduct (5). Singer has proposed that the phenyl moiety of phenylhydrazine becomes attached to the 4a-position of 8a-cysteinyl-FAD (6).

In the present investigation, the formation of irreversible complexes with hydrazines was used to probe the flavin environment of MAO. Also, several "spin traps" were used to determine if flavin semiquinone formation could be detected. The results of these investigations are the subject of this report.

## EXPERIMENTAL PROCEDURES

Materials- Bovine liver monoamine oxidase was isolated as described previously (7). Specific activity of the enzyme was 8200. 1-Oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylic acid was purchased from Medimpex, Budapest, Hungary. 4-Amino-2,2,6,6-tetramethylpiperidinooxyl was purchased from Aldrich Chemical Company, Inc. Triethylamine and N,N'-dicyclohexylcarbodiimide were purchased from Pierce Chemical Company. Di-tert-butyldi-

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carbonate, 2-hydroxyethyl hydrazine, and 1-hydroxybenzotriazole hydrate were purchased from Aldrich Chemical Company. Hydroxylamine-O-sulfonic acid was purchased from Sigma Chemical Company. The other common reagents used were of reagent grade quality. The spin traps, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO)[1] and phenyl-t-butylnitrone (PBN)[1], were purchased from Aldrich Chemical Company.

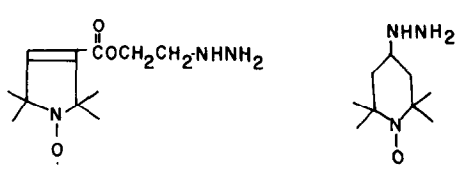
Methods- Electron spin resonance (ESR) measurements were recorded at room temperature with a Varian E4 Spectrometer. A modulation amplitude of 1 gauss was used throughout. Protein concentration was determined by the Lowry et al procedure (8). Enzyme activity was assayed by the spectrophotometric procedure of Tabor using benzylamine as the substrate (9).

The reaction of the enzyme with the two nitroxide spin labels was carried out as follows: 9.27 μmole of enzyme was solubilized in 2 ml of 0.02 M potassium phosphate buffer-0.1% Triton X-100 (pH 7.5). In separate experiments 41.3 mmol of the hydrazine spin labels was added to the enzyme and allowed to proceed at 4°C for 10-12 hours. The sample was then exhaustively dialyzed against a 0.02 M potassium phosphate buffer-0.1 Triton X-100 solution (pH 7.5), and the ESR spectrum was recorded.

For the spin trapping experiments 1 mM benzylamine was added up to 1000 units of enzyme in 0.5 ml of potassium phosphate buffer, pH 7.4 in the presence of either 7 mM DMPO or 120 mM PBN.

Synthesis of 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carbonyl ethyl hydrazine (HEHSL)[1]. Thirty-eight mg of 2-hydroxyethylhydrazine was reacted with 108 mg of di-tert-butyl-dicarbonate in 1 ml of acetonitrile in the presence of 50 mg of the catalyst triethylamine. The molar ratios of the hydrazine to blocking group to catalyst was 1:1:1. The reaction was allowed to take place with gentle stirring at 4°C. Fifty mg of the blocked 2-hydroxyethyl hydrazine was reacted with 45 mg of 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylic acid in the presence of 88 mg of N,N'-dicyclohexylcarbodiimide and 3 mg of 1-hydroxybenzotriazole hydrate in 1 ml of acetone. The reaction was allowed to proceed with gently stirring for 12 hours at 4°C. The precipitated dicyclohexylurea was removed by filtration and the blocked hydroxyethyl-hydrazine spin label was dried down by using a stream of nitrogen. Anhydrous ether was used to wash the product. It was then deblocked by the use of trifluoroacetic acid. Mass spectra of the blocked and unblocked hydroxyethyl-hydrazine spin label yielded molecular ions of 342 and 242, respectively. The blocked and unblocked HEHSL showed a single spot by silica gel thin layer chromatography ($R_f$ values of 0.32 and 0.25, respectively) using the solvents MeOH:chloroform (1:19, v/v). The structure of HEHSL is shown in Fig. 1

Synthesis of 1-oxyl-2,2,6,6-tetramethyl piperidino-4-hydrazine (HSL)[1]- A hundred mg of hydroxylamine-O-sulfonic acid was first reacted with 50 mg of potassium hydroxide to produce the salt (10,11). The salt was then reacted with 170 mg of 4-amino-2,2,6,6-tetramethylpiperidinooxyl at 65°C for 10 minutes in 5 ml of 65% methanol. The mole ratio of salt to spin label was 1.2 to 1. Chloroform was used to extract out the synthesized spin probe. The purity of




(I) (II)

Fig. 1. Structures of HEHSL (I) and HSL (II).

the reaction product was checked by thin layer chromatography in which the solvent was chloroform:ethylacetate (1:5, v/v). Ninhydrin spray or fluorescence was used to detect the compound. One spot was observed with a $R_f$ of 0.24. The structure of the product was checked by mass spectrometry (molecular weight of 186), infrared spectroscopy and NMR spectroscopy (after reduction of the nitroxide with sodium dithionite). Excellent agreement with theoretical values were obtained. The structure of HSL is shown in Fig. 1.

## RESULTS

Inhibition of Monoamine Oxidase by the Hydrazine Spin Labels HEHSL and HSL. Monoamine oxidase was incubated with HEHSL and HSL (Fig. 1) for various time intervals and aliquots removed for assay. The results obtained are shown in Fig. 2. The reactions were carried out at $4^{o}C$ in order to minimize enzyme denaturation.

ESR Spectra of the HEHSL and HSL-Monoamine Oxidase Adducts. Control experiments showed that the inhibition of monoamine oxidase by HEHSL and HSL were not reversible. The completely inactivated enzyme was extensively dialyzed against 0.02 M potassium phosphate buffer-0.1% Triton X-100 solution (pH 7.5) for at least 18 hours with numerous changes of dialysis fluid to remove unbound label. The spectra obtained using HEHSL and HSL are shown in Fig. 3.

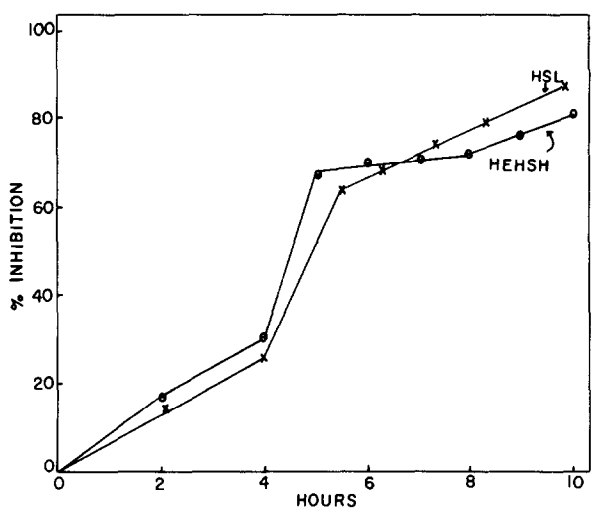



Fig. 2. Inactivation of monoamine oxidase by synthesized hydrazine spin labels. 9.27 μmoles of MAO was solubilized in 2 ml of 0.02 M potassium phosphate buffer-0.1% Triton (pH 7.5) and incubated with $4.13 \times 10^{-5}$ moles of HEHSL of HSL at about $4^{o}C$. Aliquots were removed to measure the enzyme activity at different time intervals.

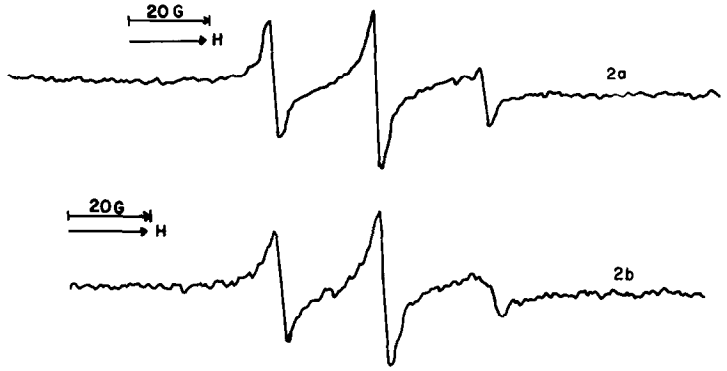



Fig. 3. ESR spectra of MAO inactivated with HEHSL and HSL. 9.27 μmoles of MAO was dissolved in 2 ml of 0.02 M potassium phosphate buffer-0.1% Triton (pH 7.5) and it was incubated with 4.13 x $10^{-5}$ moles of the synthesized spin label at 4°C for 10 hours. It was then dialyzed exhaustively against 0.02 M potassium phosphate buffer-0.1% Triton (pH 7.5). See "Methods" for details. (a) HEHSL. (b) HSL.

Influence of 5 M Guanidine HCl on the ESR Spectra. In order to confirm that the HSL was covalently attached to the monoamine oxidase, solid guanidine HCl was added to 5 M. The spectra obtained is shown in Fig. 4 and is indicative of nitroxide attached to a denatured protein.

Changes in the Visible Spectra Upon Reaction with HEHSL and HSL. Both Patek and Hellerman (5) and Singer (6) have reported the changes in the visible spectrum of monoamine oxidase when the enzyme is inhibited by phenylhydrazine. Since the newly synthesized spin labelled inhibitors have other reactive groups in addition to the hydrazino group, the spectral changes during the reaction of monoamine oxidase with HEHSL were recorded and are shown in Fig. 5. Similar results were obtained with HSL (spectrum not shown).

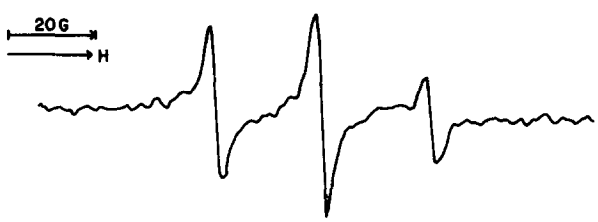



Fig. 4. Influence of 5 M guanidine hydrochloride on the ESR spectrum. About 0.4475 g of guanidine hydrochloride was added to 2 ml of the reaction mixture (9.27 μmoles of MAO and 4.13 x $10^{-5}$ moles of HSL). After incubation for 15 minutes at room temperature, the ESR spectrum was taken.

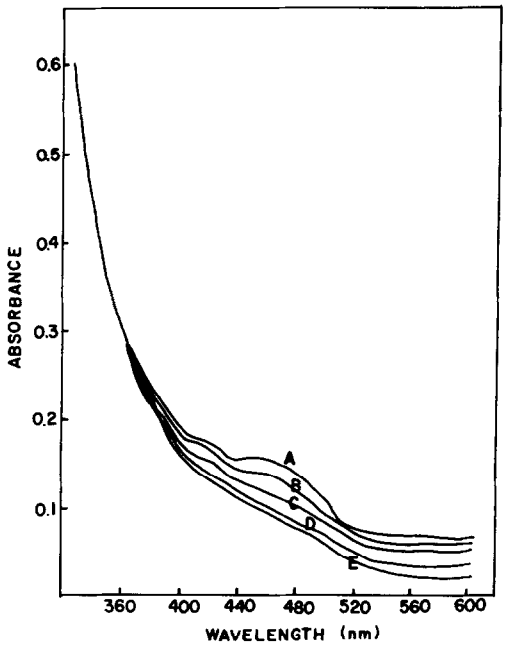



Fig. 5. Spectral changes of monoamine oxidase before and after inactivation by HEHSL. 9.27 μmoles of MAO was solubilized in 2 ml of 0.02 M potassium phosphate buffer-0.1% Triton (pH 7.5) and incubated with 4.13 x $10^{-5}$ moles of HEHSL at room temperature. A-E indicate spectrum taken at 0, 1, 3, 7 and 24 hours, respectively.

Search For Free Radical Intermediates. The newly developed spin trapping method (12) using DMPO or PBN makes possible the detection of short-lived organic free radicals and radicals of active oxygen (superoxide and hydroxyl radical). We applied this technique to see if any radical species could be trapped during the reaction of MAO with substrate. However, when highly purified monoamine oxidase and benzylamine were checked with DMPO or PBN, no free radicals were detected beyond what was observed in the control reaction mixture in the absence of enzyme. Incidently, autooxidation of the substrate was the least when N-methylated benzylamine type of substrate was used and is recommended for this type of study with monoamine oxidase.

## DISCUSSION

Nitroxide-containing compounds can be used as probes of protein structure as they contain unpaired electrons which yield different spectra depending on the environment in which they are located in proteins (13).

Two of the spectral types found in proteins are classified as partially mobilized and highly immobilized and often are indicative of a spin probe which is near the surface of a protein and one which is buried inside a protein, respectively.

It has been shown that it is most probable that monoamine oxidase is inhibited by certain hydrazine derivatives by reacting with the 4-α-position of the isoalloxazine ring of the 8-α-cysteinyl-FAD (14). Thus, a nitroxide-containing hydrazine compound HEHSL was synthesized first and the inhibition of monoamine oxidase and the ESR and visible spectra of the adduct were recorded (Fig. 3a). The ESR spectrum of the derivative suggested that the FAD was located near the surface of the enzyme since a partially immobilized nitroxide signal was noted. It was calculated that the distance from the free end of the hydrazine moiety to the nitroxide group was 9.74 Å and (15) thus a probe with a shorter distance between the free end of the hydrazine moiety and the nitroxide radical was sought. A successful synthesis of the HSL in which the distance was now 3.47 Å (15) also produced an ESR spectrum similar to that observed with HEHSL (Fig. 3b). Thus, it can be concluded that the FAD which exists as 8-α-cysteinyl-FAD is near the surface of monoamine oxidase.

Spin trapping (12), a technique for detecting free radicals of various types failed to detect any enzyme generated flavin semiquinone, superoxide anion, or carbon radicals.

The flavin environment in other flavoproteins have been studied by X-ray crystallography. Various portions of the isoalloxazine ring of the FMN in the Desulfovibrio vulgaris (16) and Clostridium MP flavodoxin (17) were exposed to solvent also suggesting a surface orientation.

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