BEEF MITOCHONDRIAL MONOAMINE OXIDASE, A FLAVIN DINUCLEOTIDE ENZYME

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A previous communication has suggested that the beef mitochondrial amine oxidase is a flavo-enzyme, Nara et al., 1966. We have sought additional information concerning the nature of the flavin we have also tried to devise experiments which would implicate the flavin in catalysis. As mentioned previously (Nara et al., 1966), this is not a simple matter since the flavin is covalently attached to the enzyme and it has been difficult to obtain pure preparations of the enzyme.

EXPERIMENTAL PROCEEDURE

Enzyme - All preparations of the enzyme were homogeneous when the purity was checked by free boundary electrophoresis and also upon rechromatography on DEAE-cellulose, hydroxylapatite and Sephadex G-200. The enzyme had specific activities in the range of 8,000-9,000 when assayed at 25°. The spectrophotometric assay of Tabor et al., 1958 was used to assay the enzyme and the protein concentration was determined by the Lowry et al. (1951) method using bovine serum albumin as a standard.

Microbiological assays for flavin and adenine - Lactobacillus casei, ATCC No. 7469 was used for the determination of riboflavin as described by Snell and Strong, 1939. Purine was determined using a special mutant of E. coli which required purines for growth and was provided by Dr. John Hall of our Department.

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Chemical Determination of Ribose - For the experiment 4 mg of enzyme was first hydrolyzed with 0.5N KOH for 48 hours at 30°. The pH was then adjusted to 1-2 with 20% $HClO_4$. The procedure of Album and Umbreit, 1947 was used to determine ribose.

Epr spectroscopy - Epr measurements were taken in the Varian V 4500-10A spectrometer using 100 kc per sec field modulation. The experiments were conducted in an anaerobic epr tube. Quantitation was achieved by double integration of the spectrum and by comparison with a standard solution of the Cu-EDTA complex.

Anaerobic light irradiation experiments - The enzyme solution was prepared in 0.1 M potassium phosphate buffer, pH 7.4 containing 0.05 M EDTA. The reaction vessels were made anaerobic by degassing the solution with a vacuum pump and then by flushing nitrogen gas which had passed through an alkaline pyrogallol solution. This process was repeated 4 times. The anaerobic cuvette was then placed in a cold bath at 5°. The sample was about 30 cm from the 750-watt tungsten lamp and the spectrum was recorded at intervals in a Beckman model-DK-2 recording spectrophotometer.

RESULTS

Chemicals and Microbiological Determination of the FAD-like Components - The results of these analysis are summarized in Table I. The most accurate measurement of flavin dinucleotide content is believed to be the value obtained by sodium dithionite reduction of the enzyme. As can be observed in the table, the substrate benzylamine does not reduce all of the flavin present in the enzyme. The microbiological determination for riboflavin and purine are believed to be low due to the partial destruction or due to a incomplete liberation of all the components of FAD. Microchemical determination of ribose yielded values in close agreement with the spectral determination of the flavin dinucleotide content of the enzyme. Since FMN does not contain ribose, all of the flavin can be accounted for as a flavin dinucleotide.

TABLE I

Analysis of Enzyme for Flavin

Compound	Method	Moles per mole (Mol. Wt. of 300,000 assumed)
1. Riboflavin	Spectrophotometric	3.0[x]
	Microbiological	0.95[/]
2. Purine	Microbiological	1.1[']
3. Ribose	Microchemical	3.1["]

x The difference spectrum of the oxidized and reduced forms and the molar absorptivity index of 1.03 x 10^6 cm^2.mole at 450 mμ was used.

/ Lactobacillus casei, ATCC No. 7469 was used for the determination of riboflavin as described by Snell and Strong, 1939.

' E. coli, a special mutant requiring purines for growth was used to determine the concentration of purine and was obtained from Dr. John Hall of our department.

" The ribose content was determined by the orcinol procedure of Album and Umbreit, 1947. The enzyme of specific activity 8,000 was used for these analyses.

Isolation of a Flavopeptide- For the experiment, 10 mg of enzyme, specific activity 8,000, was heat denatured at pH 7.0. Then 3 mg of pronase was added and the reaction was allowed to proceed for 7 hours at 38°. After digestion, the sample solution showed 3 maxima at 266, 375 and 450 mμ and it was observed that the maximum at 410 mμ had disappeared. The sample was taken to dryness in vacuo. It was then dissolved in 1.5 ml of 0.1 M acetic acid and applied to a column of Sephadex G-15 (1 x 47 cm) which had been equilibrated with 0.1 M acetic acid, and then developed with same solvent. Fractions of 0.8 ml were collected in the dark and at 4°. The flow rate was 6 ml per hour. A typical chromatogram is shown in Fig. 1. The majority of the flavopeptide was found in Fraction B which was yellow. This fraction was evaporated in vacuo and then dissolved in 6.5 ml of 0.05 M phosphate buffer, pH 7.4.

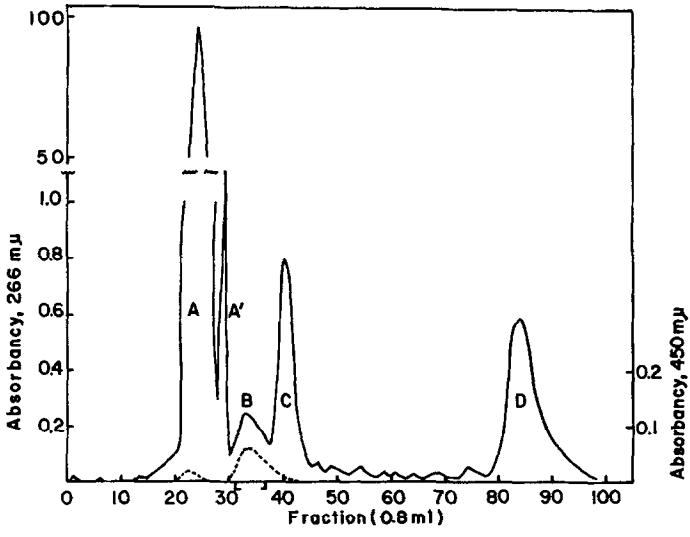

Figure 1. Chromatography of the pronase digest on a Sephadex G-15 column. The dry sample was dissolved in 1.5 ml of 0.1 M acetic acid and applied to a Sephadex G-15 column (1 x 47 cm) which had been equilibrated with 0.1 M acetic acid. The sample was eluted with the same solvent in the dark at 4°. Fractions of 0.8 ml were collected and the flow rate was 6 ml per hour. The solid line represents the absorbance of 266 mμ and the dotted line absorbance at 450 mμ.

The peptide showed maxima at 350 and 450 mμ in the visible wavelength region and a maximum at 263 mμ in the ultraviolet region. The difference

spectrum obtained by the addition of excess sodium dithionite showed two maxima at 360 and 450 mμ. The excitation spectrum of the flavopeptide showed maxima at 260, 375 and 450 mμ when the fluorescence emission maximum at 530 mμ was used. The fluorescence emission spectrum showed a maximum at 530 mμ when the excitation wavelength of 380 or 450 mμ was used. The R_fs of the major peptide in the solvent systems n-butanol-HOAc-H_2O (4:1:5); 0.1 M sodium phosphate buffer, pH 7.2; n-butanol-NH_4OH-H_2O (1:1:1); and pehnol-n-butanol-H_2O (160 g:30 ml:100 ml) were 0.04, 0.43, 0.60, and 0.20, respectively. The R_fs of FMN and FAD in these solvents were 0.13, 0.56, 0.72 and 0.17 and 0.05, 0.45, 0.16, and 0.23, respectively.

The flavopeptide was inactive in the D-amino acid apoenzyme reactivation experiments.

Implication of the Flavin Dinucleotide in Catalysis- Artificial one and two electron acceptors such as cytochrome c, 2,6-dichlorophenol indolephenol, methylene blue and tetrazolium salts cannot replace molecular oxygen as the electron acceptor. In order to show that the flavin is involved in catalysis, known specific inhibitors of the enzyme (Blaschko, 1963) shown in Table I were added to the enzyme to see if they would prevent the reduction of the 450 mμ absorption and by the substrate benzylamine. As can be seen from the table, each of the known potent inhibitor of the enzyme prevented the reduction of the 450 mμ peak.

Attempts to Detect the Flavin Semiquinone- The anaerobic addition of the substrate to the enzyme caused an apparent reduction of the flavin in the enzyme but no semiquinone could be detected spectrally. Recently, Massey and Palmer, 1966, have devised a procedure for converting the flavin in flavoenzymes to the semiquinone form in the presence of EDTA and light. When this procedure was applied to the purified enzyme at pH 7.4, what appears to be the semiquinone was detected. A gradual decrease of the 450 mμ peak resulted and the 410 mμ peak increased relatively to the 450 mμ peak. A new maxima appeared at 370 mμ as shown in Fig. 2. The color of the enzyme changed from

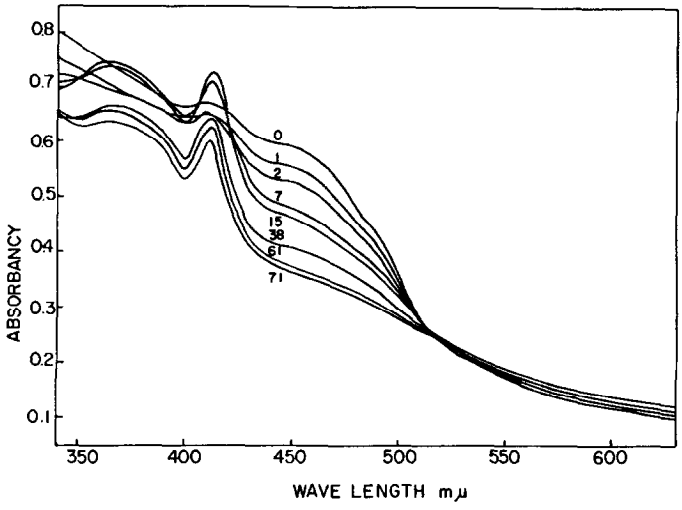

Figure 2. Spectral changes of the mitochondrial amine oxidase during photo-irradiation in the presence of EDTA. The enzyme concentration was 0.86 mg per ml and was dissolved in 0.1 M potassium phosphate buffer, pH 7.4 containing 0.06 M EDTA. The experiments were conducted anaerobically and the numbers above the curve represent the spectrum after definite (hours) intervals.

yellow to yellowish pink. The maximum concentration of the epr detectable free radical was found to develop at the earlier stage of illumination (1 hour) and it gradually decreased after 15 hours to about 1.2% of value based on the presence of 3 moles of a flavin dinucleotide (assumed molecular weight of 300,000). The free radical showed a g value of 2.00 characteristic of a semiquinone.

On admitting air carefully, the concentration of free radicals increased which may be due to back oxidation of the reduced flavin. The maximum amount of semiquinone which could be detected was 30% based on the presence of 3 moles of flavin dinucleotide per mole of enzyme (assumed molecular weight of 300,000). Although there are spectral differences, the spectrum observed resembled the red type observed by Massey and Palmer, 1966.

DISCUSSION

The flavin present in the mitochondrial monoamine oxidase is

TABLE II

Prevention of the Reduction of the 450 mμ Absorption Band by Inhibitors[1]

	Compound	Final concentration (μmole)	% Reduction of 450 mμ band
1.	D (+) Trans 2-phenyl cyclopropylamine	0.3	0
2.	P-Chloromercuribenzoate	0.3	0
3.	8-Hydroxyquinoline	0.3	32
4.	Iproniazid	0.3	0
5.	Benzylamine	60.0	62
6.	Sodium dithionite	6.0	100

[1] In experiments 1-4, the inhibitor was preincubated with 4,050 units of the enzyme (specific activity 8,000) for 15 minutes at 25° and then the substrate (benzylamine, 60 μmole) was added. In experiments 5 and 6 the compounds were directly added to the enzyme solution aerobically and the spectra were determined. The total volume in each case was 3.0 ml and all compounds were dissolved in 0.1 M potassium phosphate buffer, pH 7.4.

covalently attached to the enzyme. Thus it is not possible to remove the cofactor from the enzyme to identify the prosthetic group of the enzyme. Recently, we have obtained preparations of the beef liver enzyme (Yasunobu et al., 1967) which are electrophoretically and chromatographically pure. Using these preparations, we have attempted to settle the FMN or FAD question. Microbiological and microchemical tests have demonstrated the presence of ribose, purine and riboflavin in the enzyme. Also, we have demonstrated previously (Yasunobu et al., 1967) that during the purification of the

enzyme, there is a direct proportionality between flavin and the specific activity of the enzyme. At the present time, it appears that the correct number of moles of flavin dinucleotide present per mole of enzyme (based on an assumed molecular weight of 300,000) is 3 which is obtained by the spectrophotometric titration of the enzyme by sodium dithionite or from the ribose content of the enzyme. The low values observed for riboflavin which is covalently bonded to the enzyme have also been observed in the case of succinic dehydrogenase by Kearney (1960) where a recovery of 29% was observed. Confirmatory evidence for the presence of a flavin dinucleotide comes from the 260/450 mμ ratio observed for the flavopeptide. Both of these observations would suggest that flavin dinucleotide rather than FMN is the prosthetic group of the enzyme.

No acceptors of the electrons from the flavin dinucleotide·H_2 in the enzyme other than oxygen have been found (Yasunobu et al., 1967). Thus, it has not been easy to implicate the flavin as being catalytically involved. The evidence was found in the following manner. As mentioned previously, the spectrum of the enzyme is bleached by the substrate benzylamine as well as sodium hydrosulfite. Specific inhibitors of the enzyme were found to prevent the reduction of the visible maximum at 450 mμ. Moreover, compounds such as sarcosine, succinate and DL-amino acids were unable to reduce the flavin dinucleotide in the enzyme.

In conclusion, the present results suggest that the beef mitochondrial amine oxidase contains 3 moles of a flavin dinucleotide per mole of enzyme (based on an assumed molecular weight of 300,000). Erwin and Hellerman have also reported that the beef kidney mitochondrial amine oxidase contains 1 mole of FAD per mole (assuming a molecular weight of 100,000). However, since the flavopeptide is inactive in activating apo-D-amino acid oxidase, it is possible that the flavin adenine dinucleotide may not be FAD as reported for succinic dehydrogenase (Kearney, 1960) but a FAD-like substance. The flavin is involved in the oxidation of substrate but no semiquinone has yet been detected but it has been observed using the procedure of Massey and

Palmer, 1966. It is interesting that certain bacterial amine oxidases contain FAD (Campello et al., 1965 and Yamada et al., 1965) which is dissociable but in the animal enzyme, it contains a flavin dinucleotide which is covalently attached to the enzyme.

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