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THE PROSTHETIC GROUPS OF PIG BRAIN MITOCHONDRIAL MONOAMINE OXIDASE

K. F. TIPTON
Department of Biochemistry, University of Cambridge, Cambridge (Great Britain)

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SUMMARY

1. The fluorescence of a purified preparation of pig brain mitochondrial monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4) has been studied.

2. This fluorescent material has been extracted and identified with FAD and the minimum molecular weight of the enzyme per FAD has been shown to be close to the molecular weight of the enzyme estimated by gel filtration.

3. Addition of a substrate or a product has been shown to cause a reduction of the flavin fluorescence of the native enzyme.

4. Pyridoxal phosphate has been shown to be absent from the preparation and the copper content has been shown to be considerably less than the enzyme and FAD concentrations.

5. The inactive FAD-free apoenzyme has been prepared and shown to be reactivated by FAD.

INTRODUCTION

The intracellular enzyme monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4) differs from the extracellular amine oxidase in probably not requiring pyridoxal phosphate as a cofactor[1]. Inhibition of the enzyme by atabrine[2] and studies on riboflavin-deficient rats[3] have indicated that the enzyme may have a flavin prosthetic group. Recently NARA *et al.*[4] and ERWIN AND HELLERMAN[5] have confirmed the presence of a flavin in purified preparations of monoamine oxidase, but have been unable to identify it. The enzyme has also been reported to contain copper ions which are essential for activity[6], but metal analysis by ERWIN AND HELLERMAN[5] and YOUDIM AND SOURKES[7] have failed to detect the presence of significant concentrations of copper in the enzyme. In this paper the presence of FAD in pig-brain mitochondrial monoamine oxidase and studies on its role in the activity of the enzyme are reported. A preliminary account of this work has already appeared[8].

Abbreviation: PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Enzymes

Purified pig brain mitochondrial monoamine oxidase was prepared by the method previously described[9] and D-amino-acid apooxidase was prepared by acid ammonium sulphate treatment[10]. The L-tyrosine apodecarboxylase preparation was an acetone-dried powder of *Streptococcus faecalis* Dunn which has been grown in a medium free from vitamin B_6. Catalase was obtained from Biochemica Boehringer and pronase from Calbiochem Corporation.

Assay methods

The activity of monoamine oxidase was assayed at 30°, using tyramine hydrochloride as a substrate, by the method previously reported[9]. D-Amino-acid oxidase activity was assayed by measuring O_2 consumption[10]: the assay medium contained in a volume of 2.4 ml, 100 μmoles of pyrophosphate buffer (pH 8.3), 9 mg DL-alanine, 100 units of catalase and FAD or the monoamine oxidase extract. The mixture was incubated at 30° and the reaction was started by the addition of D-amino-acid apooxidase. L-Tyrosine decarboxylase activity was assayed at 30° by measuring the carbon dioxide evolution manometrically. The main compartment of each manometer contained in a total volume of 2.5 ml, 200 μmoles of acetate buffer (pH 5.5), 2 mg of the L-tyrosine apodecarboxylase preparation, pyridoxal *plus* ATP or a sample of the monoamine oxidase hydrolysate *plus* ATP. The reaction was started by tipping 0.5 ml of a 0.2 M L-tyrosine suspension from the side-arm.

Chemicals

FAD, FMN, and the bathocuproine copper test reagent combination were obtained from Biochemica Boehringer, chloropromazine was obtained from May and Baker Ltd., and *n*-amylamine from Koch-Light Ltd. All other chemicals were obtained from British Drug Houses Ltd. or Hopkin and Williams Ltd. and were of the highest purity available. Distilled water was passed through a Permutit mark II deionizer before use. Nitrogen ("white spot") was obtained from the British Oxygen Co.

Protein determination

Protein concentration was determined by the micro-biuret method[11] using bovine serum albumin as a protein standard.

Copper determination

Two methods were used to assay the copper content of the monoamine oxidase preparation: (1) reaction with cuprizone[12] and (2) reaction with bathocuproine[13]. The enzyme preparation was dialyzed for 36 h against 0.01 M phosphate buffer (pH 7.6) before being used for copper determinations. 1 ml of the dialyzed preparation was made 2 M with HCl, and 0.5 ml of 20% (w/v) trichloroacetic acid was added and the mixture was centrifuged. The supernatent was then analyzed for copper content. Glassware used in these determinations was soaked in conc. HNO_3 for 14 days and exhaustively washed with deionized water before use.

Assay for pyridoxal 5'-phosphate

The presence of pyridoxal phosphate was assayed in a sample of the enzyme which had been subjected to pronase digestion followed by acid hydrolysis[14], by determining its ability to reactivate L-tyrosine apodecarboxylase in the presence of ATP[15].

Apparatus

Measurements of absorbance were made using a Unicam S.P. 500 spectrophotometer and 1-cm light-path quartz-glass cells, fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer, and measurements of pH were made using a Radiometer PHM22r pH meter. O_2 uptake was measured using a Clark oxygen electrode (Yellow Springs Instrument Co.) connected via a voltage divider to a Honeywell-Brown 1 mV strip-chart recorder. Buffer solutions were deoxygenated using a Nilox deoxygenator.

RESULTS

Fluorescence measurements

Fig. 1 shows the fluorescence spectrum of a sample of purified monoamine oxidase. The fluorescence maximum at about 520 mμ (uncorrected) when the enzyme was excited at 450 mμ is similar to the spectrum reported by ERWIN AND HELLERMAN[5] although other workers[4,16] have reported that their preparations of the enzyme do not fluoresce. When a sample of the monoamine oxidase was digested for 18 h at 30° with 2 mg of pronase, the fluorescence intensity of the preparation increased some 50%, indicating that the fluorescence of FAD bound to the native enzyme is in some way partially quenched. Comparison of the fluorescence of this pronase-digested enzyme

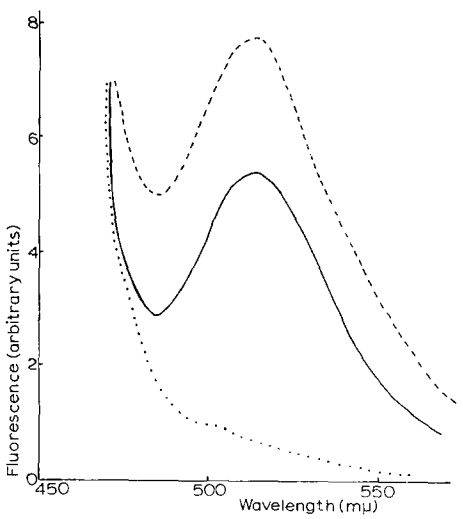

Fig. 1. The fluorescence emission spectrum of monoamine oxidase. The excitation wavelength was 450 mμ. All measurements were made in 0.01 M phosphate buffer (pH 7.6) at 30° with an enzyme concentration of 0.402 mg/ml. ———, native enzyme; · · ·, native enzyme plus excess dithionite; — — —, enzyme after pronase digestion.

with a sample of authentic FAD yielded a flavin content of 1 mole FAD per 118 000 g enzyme. The fluorescence of the native enzyme was almost completely abolished following the addition of an excess of sodium dithionite.

Extraction of flavin

Fluorescent material could be liberated from the enzyme either by heating an enzyme solution at 100° for 12 min or by treatment of an enzyme solution with trichloroacetic acid. In the former method, a solution of the enzyme in 0.01 M phosphate buffer (pH 7.6) was placed in a boiling-water bath in the dark for 12 min and then cooled in ice. The material was then centrifuged and the supernatant was used for identification of the extracted flavine. Trichloroacetic acid treatment was carried out by adding 0.2 ml of a 50% (w/v) solution of this acid to 0.8 ml of an ice cold solution of monoamine oxidase. The mixture was kept in ice in the dark for 15 min before the precipitated protein was removed by centrifugation; 0.2 ml of this supernatant was added to 2.0 ml of 1.0 M K_2HPO_4 and was used for flavin fluorescence assay. A further 0.2 ml of the supernatant was incubated in the dark at 38° before being treated in the same way as described above. Standard solutions of FAD and FMN were carried through the same procedures and care was taken to protect the flavin from light during these processes.

The purification procedure used for pig-brain mitochondrial monoamine oxidase[9] involves an alcohol fractionation procedure as the final step. Attempts to extract fluorescent material by trichloroacetic acid treatment from less pure preparations of the enzyme prepared without alcohol fractionation were largely unsuccessful, negligible amounts of flavin being extracted. This finding is of interest since other workers[4,5] who have attempted to liberate flavin from their preparations of monoamine oxidase have been unsuccessful.

Identification of the extracted flavin

The nature of the flavin component from the boiled extract of monoamine oxidase was identified in two ways.

(1) Ascending chromatography on Whatman No. 1 chromatography paper developing with 5% aqueous Na_2HPO_4 (ref. 17). Freshly prepared solutions of FAD and FMN as well as a sample of the boiled enzyme extract were applied to the paper in a dark room illuminated by a Kodak Wratten No. 1 Safelight. After development the chromatogram was dried and examined under ultraviolet light. The boiled enzyme supernatant yielded a single major fluorescent spot (R_F 0.35) which ran alongside the FAD marker. No material running with the FMN marker (R_F 0.47) could be detected.

(2) The flavin was identified and estimated by titration with the FAD-specific D-amino-acid apooxidase. A callibration curve was produced by measuring the reactivation produced by a series of different amounts of FAD. From the reactivation produced by samples of the boiled monoamine oxidase supernatant, the molecular weight of the enzyme per FAD molecule was calculated to be 120 000.

Estimation of FAD content by fluorescence

The fluorescence emission spectrum of the material extracted from monoamine oxidase by trichloroacetic acid treatment was determined using an exciting wavelength of 450 mμ, and found to be identical to that of a sample of authentic FAD. Calculation of the FAD content of the enzyme from the fluorescence of this trichloroacetic acid

supernatant gave a value of 1 mole FAD per 114 000 g protein. After heating a sample of the trichloroacetic acid supernatant at 38° for 18 h, the fluorescence of the extract had risen and the FAD content of the enzyme, calculated on an FMN basis, was 1 mole per 110 000 g.

The effect of substrates on the fluorescence of the native enzyme

A study of the effect of substrates on the fluorescence of the FAD component of the enzyme could be complicated by the quenching of the flavin fluorescence due to the substrate being bound close to it. Thus tyrosine has been shown to be a strong quencher of flavin fluorescence[18], and so its derivative tyramine was avoided in these

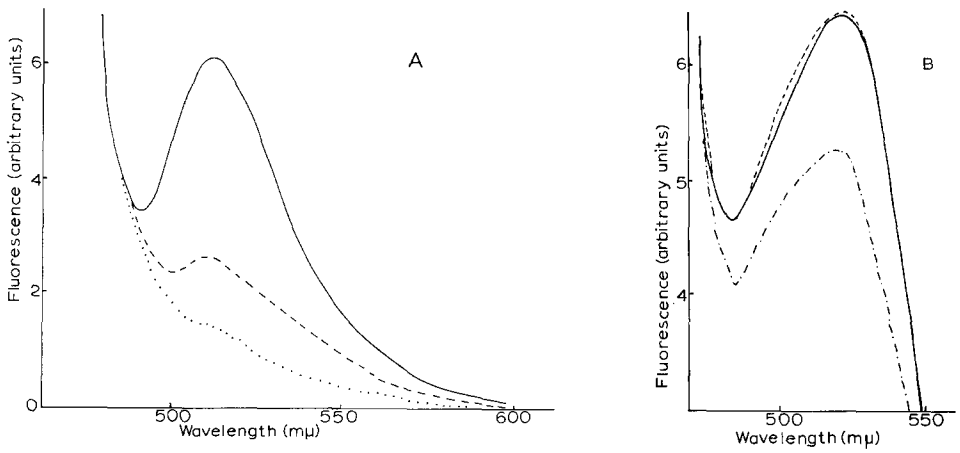

Fig. 2. The effect of substrate and product on the fluorescence of monoamine oxidase. The conditions were as described in the legend to Fig. 1, except that the samples were deoxygenated prior to use. The samples in Fig. 2B contained 1.0 mM KCN. A: ———, native enzyme; — — —, native enzyme in the presence of 1 mM *n*-amylamine; · · ·, apoenzyme (buffer not deoxygenated). B: ———, native enzyme; —·—·, native enzyme in the presence of 5 mM H_2O_2; — — —, the H_2O_2-treated sample of the enzyme after the addition of 100 units of catalase and shaking the sample.

experiments. Model studies using solutions of FAD indicated that the quenching effect caused by adding *n*-amylamine to a solution of FAD was very small and since it is a good substrate for monoamine oxidase[19], this compound was used in these studies. As shown in Fig. 2A, the addition of *n*-amylamine to a solution of monoamine oxidase that had been largely freed from dissolved O_2 caused a significant decrease in the flavin fluorescence. When the mixture was shaken with O_2, the fluorescence increased almost to its original value. Histamine and spermidine, both of which are not substrates for monoamine oxidase, failed to cause an appreciable reduction in the flavin fluorescence.

Fig. 2B shows that H_2O_2, one of the products of the monoamine oxidase-catalyzed reaction, can cause a decrease in flavin fluorescence and that the addition of catalase to decompose the H_2O_2 to form O_2 reverses this. This unusual behavior of H_2O_2 acting as a reducing agent cannot be reproduced by adding it to solutions of free FAD. The kinetic mechanism for monoamine oxidase[20] indicates that a modified form of the enzyme is formed during the reaction and the overall reaction scheme can be represented in a simplified form as:

$$\text{Enzyme} + RCH_2NH_2 \rightleftharpoons \text{Enzyme}' + RCHO + NH_3 \quad (1)$$
$$\text{Enzyme}' + O_2 + H_2O \rightleftharpoons \text{Enzyme} + H_2O_2 \quad (2)$$

The modified form of the enzyme (Enzyme') presumably represents a reduced or partially reduced form of the enzyme; and since reduced flavins do not fluoresce when excited at 450 mμ (ref. 21), it is presumably the production of either the free reduced enzyme or a complex containing it that is observed fluorimetrically. The degree of reduction of fluorescence by H_2O_2 is considerably smaller than that produced by *n*-amylamine, but since in the overall Reaction 2 one molecule of O_2 would be produced per molecule of reduced enzyme produced, the amount of flavin reduction obtainable will depend on the overall equilibrium constant for the series of reactions represented by Reaction 2. The presence of CN^- was found to be necessary for maximum reduction of flavin fluorescence. This may be due to the contamination of the enzyme preparation with small amounts of catalase or peroxidase, although the presence of either of these enzymes could not be detected in the enzyme preparation.

The effect of inhibitors on flavin reduction

Preincubation of the purified enzyme with PCMB has been shown to cause inhibition[9], and it was found that after a sample of the enzyme had been incubated at 20° with 0.5 mM PCMB for 30 min, the ability of either *n*-amylamine or H_2O_2 to cause a reduction in flavin fluorescence was lost. After preincubation of the enzyme at 20° for 60 min with a 5 mM solution of either theonyl trifluoroacetone or 1,10-phenanthroline, the addition of *n*-amylamine to a solution of the enzyme under N_2 failed to produce a reduction in the flavin fluorescence but the addition of H_2O_2 caused a decrease in fluorescence similar in magnitude to that produced with the untreated enzyme. These results would indicate that the metal component of the enzyme functions in Reaction 1 rather than Reaction 2 of the simplified mechanism, although it must be borne in mind that it has not yet been unequivocally shown that a metal has an essential role in the activity of the enzyme.

Analysis for pyridoxal phosphate

Since monoamine oxidase is insensitive to inhibition by CN^- and semicarbazide, it would seem unlikely that it contains pyridoxal 5'-phosphate as a cofactor. The absence of pyridoxal or pyridoxal 5'-phosphate in preparations of the purified enzyme was confirmed by the failure of a hydrolysate of the enzyme to reactivate a preparation of *S. faecalis* tyrosine apodecarboxylase in the presence of ATP.

Analysis for copper

Analysis of the copper content of purified monoamine oxidase yielded a value of 1 gatom of copper per 590 000 g protein by the bathocuproine and cuprizone methods. These values indicate that the copper content of this preparation is considerably less than the flavin content of the enzyme, and since the molecular weight of the enzyme estimated by gel-filtration was 102 000 (ref. 9) there is considerably less copper in the preparation than there are molecules of enzyme.

This low copper content could be due to partial dissociation of the metal from the enzyme during purification, giving a preparation which had lost some 80% of its native activity. The ability of Cu^{2+} to increase the activity of the purified enzyme was investigated by preincubating the enzyme at 4° for 30 min with $CuSO_4$ solution at

final concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M. The incubation medium contained in a total volume of 2.4 ml, 100 μmoles of Tris buffer (pH 8.0), enzyme and $CuSO_4$. After incubation the activity was assayed in the normal way, using 10^{-3} M tyramine as the substrate. A control sample of the enzyme was treated in exactly the same way, except that $CuSO_4$ was omitted from the incubation medium. Some inhibition was observed at the highest copper concentration (12%) and no increase in the activity of the enzyme over that of the control was observed with any of these copper concentrations.

Production of apomonoamine oxidase

SWOBODA[22] has shown that it is possible to remove the FAD from glucose oxidase without irreversibly inactivating the enzyme, by the addition of the enzyme solution to acid ammonium sulphate, and this technique was found to be satisfactory with monoamine oxidase. The following procedure was used to produce a preparation of the apoenzyme: a 0.5-ml sample of the enzyme was added rapidly from a syringe to 5 ml of a saturated solution of ammonium sulphate in 0.1 M HCl which was cooled in ice. The mixture was allowed to stand for 30 min and the precipitate was removed by centrifugation and taken up in 0.5 ml 0.01 M phosphate buffer (pH 7.6). The precipitation procedure was repeated three times more, and the final precipitate taken up in 0.5 ml 0.01 M phosphate buffer (pH 7.6) and assayed. The best preparations of the apooxidase produced by this method had no detectable activity and had negligible flavin fluorescence (Fig. 2A), but with some preparations of the enzyme it has been found difficult to reduce the activity below some 15% of the original. The reason for these differences between preparations is uncertain.

The inactive apoenzyme could be partly reactivated by incubation with FAD, but not FMN. Fig. 3 shows a Michaelis curve for the reactivation process. Since the amounts of FAD required for reactivation are very low, the dissociation constant (K_d) for FAD binding to the enzyme cannot be accurately determined from the Michaelis

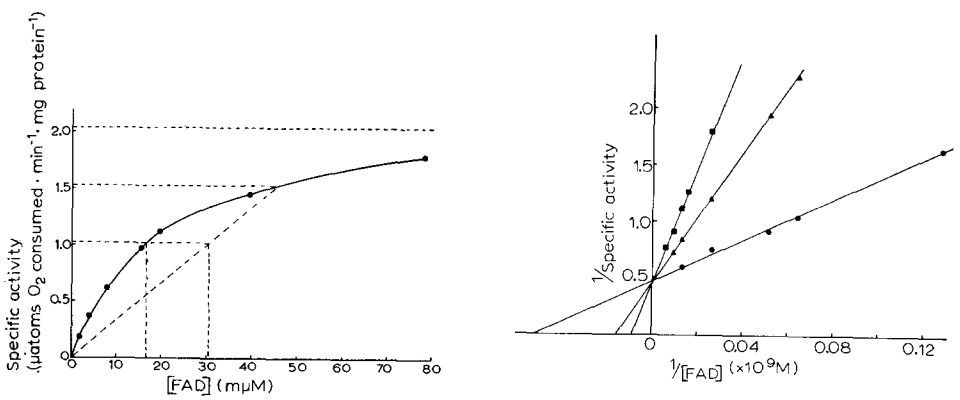

Fig. 3. The reactivation of monoamine apooxidase by FAD. Details of the method of preparing the apoenzyme and of the assay procedure are given in the text.

Fig. 4. Inhibition by chloropromazine of the reactivation of monoamine apooxidase by FAD. Details of the assay procedure are given in the text. ●—●, no inhibitor; ▲—▲, 0.2 μM chloropromazine; ■—■, 0.4 μM chloropromazine.

equation, but DIXON[23] has derived a method for determining the equilibrium constant from the Michaelis curve. The value for K_d calculated by this method as modified by KILROE-SMITH[24] was 14 mμM.

GABAY AND HARRIS[25] have shown that phenothiazines are competitive inhibitors of the reactivation of D-amino-acid apooxidase by FAD. The effect of chloropromazine on the reactivation of monoamine apooxidase by FAD is shown in Fig. 4, and as can be seen, inhibition is competitive with an apparent K_i of 0.85 μM.

DISCUSSION

The values calculated for the minimum molecular weight of monoamine oxidase per FAD molecule are in agreement with the value of 102 000 estimated for the molecular weight of the enzyme from gel-filtration data[9]. The average molecular weight value of the four methods of estimation (116 000) is also close to the value of 100 000 reported by ERWIN AND HELLERMAN[5] for the bovine kidney enzyme, although these workers calculated a considerably higher molecular weight for the enzyme from ultracentrifuge studies (290 000).

The relative ease with which the flavin can be removed from the enzyme is in contrast to results with monoamine oxidases purified from bovine liver[4] and kidney[5] which led to the suggestion that the flavin may be covalently bound to the enzyme. However, the properties of the enzyme with regard to its bound flavin seem to change considerably as the enzyme is purified; thus with the bovine-kidney enzyme, the flavin component does not fluoresce when excited at 450 mμ until the enzyme is considerably purified[5] and, in the case of the pig-brain enzyme, the FAD could not be extracted from a partially purified preparation by techniques which quantitatively extracted it from the purified enzyme. Thus the difficulty of extraction of the FAD from the preparations of bovine liver and kidney may be due to impurities, or to species or organ differences between the enzymes.

From the low concentrations of copper found in preparations of the pig-brain enzyme and the failure of Cu^{2+} to activate, it would appear that this enzyme probably does not require copper for activity. Similar results with purified preparations of bovine-kidney and rat-liver monoamine oxidase have lead to the same conclusion[5,7]. This is in contrast to work on the bovine-liver enzyme which suggests that copper is essential for activity[6], and to recent work with rat-liver mitochondria in which the inhibition of monoamine oxidase by prolonged dialysis was shown to be reversed by the addition of Cu^{2+} (ref. 26).

The absence of detectable amounts of pyridoxal phosphate from the purified preparations of the enzyme is in support of earlier conclusions that this compound is not involved in monoamine oxidase activity. These conclusions were, however, based on indirect evidence such as the lack of sensitivity of monoamine oxidase to carboxyl reagents and the lack of any effect of a pyridoxine-deficient diet on the monoamine oxidase activity in rat liver.

The production of the apoenzyme and its reactivation specifically by FAD can be regarded as definitive proof of the involvement of this cofactor in the activity of monoamine oxidase. Competitive inhibition of reactivation by chloropromazine is in line with the findings of GABAY AND HARRIS[25], who have suggested that phenothiazines might act by inhibiting flavoenzymes, and have carried out a thorough study of the

competitive inhibition of the reactivation of D-amino-acid apooxidase by a number of these compounds.

The relatively high value determined for the dissociation constant of the FAD-monoamine apooxidase complex is in contrast to the behavior of the native enzyme, which suggests a tighter binding. Thus activity is proportional to enzyme concentration at low concentrations of the latter[27], and enzyme activity is not lost during dialysis against 0.01 M phosphate buffer (pH 7.6) at 4° for 3 h. These findings suggest that the reconstructed enzyme may differ from the native enzyme in the strength of FAD binding. This may be due to changes in the enzyme protein brought about by the vigorous conditions used in the resolution procedure. A similar weakening of coenzyme binding following this resolution procedure has been reported for the cobamide-dependent enzyme methylmalonyl-CoA isomerase[28].

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