Purification and Properties of Mitochondrial Monoamine Oxidase Type A from Human Placenta*

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A high yield purification scheme for monoamine oxidase A from human placental mitochondria is described. The enzyme is solubilized by a combination of treatment with phospholipase A and C and extraction with Triton X-100 and further purified by partitioning between dextran and polyethylene glycol polymers. The enzyme was obtained in 35% yield and high purity on DEAE-Sepharose CL-6B chromatography. This product, 90% catalytically active, showed a single major and several minor bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Further purification could be achieved by additional chromatography using Bio-Gel HTP, but concomitant loss of catalytic activity occurred (enzyme remained about 60% active).

The difference extinction coefficient for flavin$\text{max} - \text{flavin}_{456}$ nm was 10,800 ± 350 M$^{-1}$ cm$^{-1}$. A sulphydryl to flavin ratio of 7.5 was obtained when enzyme was denatured with sodium dodecyl sulfate, reduced with 2-mercaptoethanol, and titrated with 2,2'-dipyridyl disulfide. Anaerobic titration with 0.5 eq of sodium dithionite gave rise to the red anionic flavin radical, and full reduction was observed on further addition of reagent. The $K_m$ value for kynuramine was essentially the same for mitochondria (0.12 mm) and enzyme after DEAE-Sepharose CL-6B chromatography (0.17 mm). The concentration of clorgyline and deprenyl required for 50% inactivation also remained essentially unchanged. Incubation of the enzyme with 2,2'-dipyridyl disulfide caused inactivation in a bi-phasic manner with apparent second-order rate constants of 1230 M$^{-1}$ min$^{-1}$ and 235 M$^{-1}$ min$^{-1}$ for the rapid and slow phase, respectively. This inactivation was largely abolished by the inclusion of the competitive inhibitor amphetamine ($K_i = 20$ µM) in the incubation mixture. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a subunit molecular mass of 60–64 kDa, about 1.5–2.5 kDa higher than human liver monoamine oxidase B.

according to the scheme below.

\[
RCH_2NH_2 + O_2 + H_2O = RCHO + H_2O_2 + NH_3
\]

The enzyme has important functions in the metabolism of biogenic amines in the central nervous system and peripheral tissues. It has been linked to neuronal dysfunction in schizophrenia, parkinsonism, Down's syndrome, and other diseases. Most recently, the enzyme has been shown to oxidize 1-methyl - 4 - phenyl - 1,2,3,6 - tetrahydropyridine to 1-methyl-4-phenyl-2,3-dihydropyridine (Salach et al., 1984), a neurotoxin (Chiba et al., 1984) suspected of producing symptoms of parkinsonism in monkey (Burns et al., 1983) and man (Langston et al., 1983; Wright et al., 1984), and to a cytotoxic causing death of a cell line from rat (Denton and Howard, 1984). Inhibitors of monoamine oxidase have found clinical application in the treatment of severe depression (Paykel et al., 1983) and parkinsonism (Birkmayer et al., 1979) and may prove useful in the study and treatment of 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine poisoning.

In the last two decades, evidence has accumulated which led to the hypothesis that monoamine oxidase activity may be embodied in two proteins with differing substrate specificity. Contributing to this hypothesis was the development of the suicide inhibitors clorgyline (Johnston, 1968) and deprenyl (Knoll and Magyar, 1972), which at low concentrations inhibit specifically the oxidation of substrates classified as type A substrates (clorgyline) or type B substrates (deprenyl).

Evidence from immunological studies is in accord with the concept of two distinct enzymes (McCauley and Racker, 1973; Pintar et al., 1983; Denney et al., 1982; Kochersperger et al., 1985). Another hypothesis put forth to explain the separate reactivities, however, is that there is a single protein and that this protein's environment in a given tissue directs its properties. Specifically, lipids have been proposed to regulate enzyme activity since monoamine oxidase is a membrane-bound enzyme (Tipton et al., 1973), and numerous papers dealing with this proposal have appeared (Huang and Faulkner, 1981; Navarro-Welch and McCauley, 1982; Buckman et al., 1983).

A preliminary report has appeared (Gürne and Zeller, 1982) on the purification of monoamine oxidase A from human placenta, but yield was variable and low. To allow us to investigate the identity of the monoamine oxidase A and B isozymes, the effects of lipids on the activity of monoamine oxidase, and to study further their immunological properties, we developed a protocol for the purification of monoamine oxidase A from human placenta which gives reproducible and high yield. We report here on the purification procedure and on studies aimed at the physical and chemical characterization of the placental enzyme.
Purification and Properties of Monoamine Oxidase Type A

EXPERIMENTAL PROCEDURES

Materials—Bovine mitochondrial monoamine oxidase B was prepared as previously reported (Salach, 1979; Weyler and Salach, 1981). Human monoamine oxidase B was a generous gift from Dr. Creed Abell of the University of Texas, Medical Branch, Department of Human Biological Chemistry and Genetics, Galveston, TX. Glucose oxidase from Aspergillus niger in buffer solution was from Miles Laboratories. Phospholipase C from Clostridium perfringens came as the lyophilized powder, bovine liver catalase (suspension, 2 times recrystallized), rabbit muscle myosin, Escherichia coli galactosidase, rabbit muscle phosphorylase B, bovine erythrocyte carbonic anhydrase, polyethylene glycol (average M, 8000), dextran (average M, 500,000), triethanolamine hydrochloride, and kynuramine dihydrobromide were from Sigma. Bio-Gel HTP was from Bio-Rad. Hen egg ovalbumin, DEAE-Sepharose CL-6B, and Sephadex G-25 were purchased from Pharmacia. Triton X-100, purchased from Sigma, was treated for 4 h at 80 °C under aspirator vacuum on a rotary evaporator to remove volatile impurities. 2,2'-Dipirydyl disulfide, from Aldrich, was recrystallized once from ethanol. Other reagents and buffers were of the highest quality commercially available. Triethanolamine-HCl buffers were adjusted to desired pH with NH₄OH.

Phospholipase A was prepared from Naja naja siamensis snake venom1 obtained from Zootoxin Laboratories, Anthony, FL. The preparation was by a procedure modified from that reported by Cremon and Kearney (1964) as follows: after the treatment at 100 °C described therein, the cooled solution was centrifuged at 40,000 × g for 1 h, the supernatant was discarded, and 50 mM Tris base was added to the 1 M Tris base. The pH was adjusted to 7.5 with 3 N NH₄OH, the solution was centrifuged as before, and the supernatant was applied to a column packed with Bio-Gel P-30 (ratio of column volume to sample volume was 37) previously equilibrated with 50 mM Tris-HCl, pH 7.6. Protein was eluted at a linear flow rate of 1.36 cm/h. Phospholipase activity eluted as a broad complex peak and was assayed with a pH recording system according to Salach et al. (1971). We define the unit of activity as that amount of enzyme which liberates 1 υmol of protons/min under the standard assay conditions. It will be shown below that 1 unit of enzyme corresponds to about 8 υmol of active flavoenzyme.

Isoelectric focusing was carried out using a rotating paper strip and ionic strength 0.1, pH range 3.5 to 9.5, at 15 °C. Loading of gels was quantitated by flavin absorbance of the samples.

Monoamine Oxidase Type B—Monoamine oxidase B was pre-conditioned in 0.1 M triethanolamine-HCl buffer, pH 7.2, to give a final filtration of 20 mg of protein/ml. The homogenate was dialyzed 24 h against 0.1 M CaCl₂ and 1 M +(-)-amphetamine. Protein was eluted with a linear gradient from 0.8% octylglucoside to 1% d-amphetamine in a total volume of 1,220 ml. The buffers were made anaerobic, and anaerobiosis was maintained in the gradient chambers throughout the elution of the column. The flow rate was about 4 ml/min (linear flow rate 11.4 cm/h), and 6-ml fractions were collected. The enzyme was eluted toward the end of the gradient. Fractions containing the enzyme were combined into 4 pools, designated DEAE/P-1 through DEAE/P-4 for later reference (see Fig. 1), and concentrated

1 We recommend that phospholipase A prepared specifically from Naja naja siamensis be used in reproducing this procedure as it has come to our attention that others attempting to reproduce our procedure for the isolation of monoamine oxidase B from bovine liver encountered difficulties when phospholipase A from other sources was substituted.

2 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPDS, 2,2'-dipyridyl disulfide; PEG, polyethylene glycol; DTH, dithionitrotetrazolium; HTP, hydroxylapatite. 13200
by ultrafiltration (Amicon PM-30 membrane) to give solutions 30–50 μM in enzyme (based on flavin content; see below). After addition of glycerol to give a final concentration of 50% glycerol, the pools were stored at −13 °C until further work.

Pool DEAE/P-3 containing the peak fractions from the previous chromatographic step was further purified by chromatography on Bio-Gel HTP (1.6 × 25 cm, 1.0 ml per column fraction, 10 ml fractions). The fractions eluted with 20–200 mM NaPi buffer, pH 7, containing 20% glycerol and 3 mM mercaptoethanol, in a total volume of 512 ml. Fractions of 7.2 ml were collected. Enzymatic activity was determined and active fractions combined to give 5 pools (HTP/P-1 through HTP/P-5, see Fig. 2); these were each concentrated (Amicon PM-30 membrane) to a volume of approximately 2 ml, this was dialyzed against 100 ml of equilibration buffer for 1 h (Spectra/Por 2 dialysis tubing) and applied to the column. Protein was eluted with a linear gradient of 20–300 mM NaPi buffer, pH 7, containing 20% glycerol and 3 mM mercaptoethanol, in a total volume of 512 ml. Fractions of 7.2 ml were collected. Enzymatic activity was determined and active fractions combined to give 5 pools (HTP/P-1 through HTP/P-5, see Fig. 2); these were each concentrated (Amicon PM-30 membrane) to a volume of 0.8–1.2 ml. Pools HTP/P-3 and HTP/P-4 were treated to remove Brij-35. The combined pools (2.1 ml, 51 mmoles of flavin) were dialyzed against 100 ml of 50 mM NaPi buffer, pH 7, containing 20% glycerol and 0.8% octyl glucoside for 1 h (Spectra/Por 2 dialysis tubing). The fractions containing enzyme eluting with a 1.5-m l fraction of DEAE-Sepharose CL-6B, in the chloride form, previously equilibrated with 15 ml of dialysis buffer, the major portion of the enzyme bound to the column.

The column was washed with 2 ml of the same buffer, and the enzy me was eluted with 250 mM NaPi buffer, pH 7, containing 20% glycerol and 3 mM 2-mercaptoethanol, in a total volume of 512 ml. Fractions containing enzyme were pooled (1.6 ml; 23 nmol of flavin), SDS was added to give a final concentration of 0.05%, and the solution was dia lysed against three changes of 100 ml each of 5 mM NaPi buffer, pH 7.0, containing 0.05% SDS for a total time of 3 h (Spectra/Por 2, 6 mm diameter). After dialysis, the sample was concentrated to 90 μl in the dialysis tubing, and stored at -20 °C for later use.

Cysteine Determination—Cysteine was determined spectrophotometrically by titration of sulfhydryl groups with DPD S by enzyme previously denatured with SDS (Tomich et al., 1981). Five to 10 μl of the protein, prepared by HTP chromatography and treated to remove detergent as described above (pools HTP/P-3 and HTP/P-4), were diluted with 0.20 ml of 0.1 mM triethanolamine- HCl buffer, pH 7.2, containing 1% SDS, and incubated at 37 °C for 15 min; 15 μl of 2-mercaptoethanol was added, and incubation was continued for 15 min longer. The samples were chromatographed yellow color was pooled (1.6 ml; 23 nmol of flavin), SDS was added to give a final concentration of 0.05%, and the solution was dia lysed against three changes of 100 ml each of 5 mM NaPi buffer, pH 7.0, containing 0.05% SDS for a total time of 3 h (Spectra/Por 2, 6 mm diameter). After dialysis, the sample was concentrated to 90 μl in the dialysis tubing, and stored at -20 °C for later use.

Summary of purification scheme for mitochondrial monooamine oxidase A from human placenta

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>A424/A450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial suspension</td>
<td>8241 (100)</td>
<td>155 (100)</td>
</tr>
<tr>
<td>Phospholipase digestion</td>
<td>3472 (42)</td>
<td>145 (94)</td>
</tr>
<tr>
<td>Triton extraction</td>
<td>5728 (21)</td>
<td>103 (66)</td>
</tr>
<tr>
<td>Polymer partitioning</td>
<td>271 (3.2)</td>
<td>76 (49)</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>32 (0.4)</td>
<td>55 (35)</td>
</tr>
<tr>
<td>Bio-Gel HTP</td>
<td>12.6</td>
<td></td>
</tr>
</tbody>
</table>

This step was carried out to obtain an analytically clean sample, and no attempt was made to maximize yields. About 40% inactivation was observed in this chromatography.
and activity of amine oxidase A from PEG precipitation step described under "Experimental Procedures." Shown are absorbance (280 nm, —) and activity (●, ◦) on left ordinate; activity (units/ml) was normalized by division by 3 to accommodate ordinate numbering. Phosphate gradient is shown on the right ordinate (— —). Fractions of about 6 ml were collected. Pools referred to in text are as follows: DEAE/P-1: fractions 100–117 and 152–165; DEAE/P-2: fractions 118–129; DEAE/P-3: fractions 130–139; DEAE/P-4: fractions 140–151.

Properties of Monoamine Oxidase A—The partially active enzyme from the Bio-Gel HTP chromatography was used for determination of the cysteine content. Protein was denatured with SDS, reduced with 2-mercaptoethanol, and titrated with dithionite to accommodate ordinate numbering. Phosphate gradient is shown on the right ordinate (— —). Fractions of about 6 ml were collected. Pools referred to in text are as follows: DEAE/P-1: fractions 10–19; DEAE/P-2: fractions 20–29; HTP/P-3: fractions 30–35; HTP/P-4: fractions 36–42; HTP/P-5: fractions 43–56.

tuting 82% of the recovered enzymatic activity, is almost homogeneous. The peak fraction from the HTP chromatography shows only a trace contaminant.

Properties of Monoamine Oxidase A—The partially active enzyme from the Bio-Gel HTP chromatography was used for determination of the cysteine content. Protein was denatured with SDS, reduced with 2-mercaptoethanol, and titrated with DPDS as described under "Experimental Procedures." This method indicated that there are 7.5 cysteine residues/mol of flavin.

A range of 80,000–64,000 Da for the subunit molecular mass was obtained from SDS-PAGE when purified enzyme was compared to standards in gels prepared at several polyacrylamide concentrations in the range of 7.5–12.5%. When ammonium persulfate was used to polymerize the running gel, a band was observed just below the major protein band; this band appeared to be an artifact, since it could be avoided by pre-electrophoresing the running gels as described under "Experimental Procedures." This artifact was also absent when the catalyst for polymerization was riboflavin and light. With the latter method, however, protein bands were not as sharply defined as when ammonium persulfate was used. We were not able to determine the native molecular weight of the enzyme by gel filtration using a high performance liquid chromatography TSK 3000SW column since enzyme eluted in the excluded volume (data not presented). Attempts to determine the native molecular weight by the method of Hedrick and Smith (1968) also failed since the enzyme traveled as a broad smear rather than as a discrete band in native gels, even in the presence of 0.2% Brij-35 (data not shown). Similar non-discrete behavior was observed in attempted isoelectric focusing experiments in 1% agarose gels with various amounts and types of detergent; here, multiple bands, superimposed on a smeared background, were observed when gels were stained with nitro blue tetrazolium stain in the presence of tyramine (data not shown). A molecular mass of 73,500 Da, based on nanomoles of flavoprotein/mg of protein as determined by the biuret method, was obtained for pool HTP/P-3 and P-4. These data suggest that there is one flavin/subunit.

To aid in the quantitation of the enzyme, the difference extinction coefficient of flavin was determined by anaerobic titration with the substrate tyramine (Fig. 4). Since the enzyme was not fully active, the end point was determined by reduction with dithionite. The amount of substrate required for full reduction of the flavin was determined by extrapolating through the dithionite end point (inset, Fig. 4).
were compared using the standard assay. The values were with respect to kynuramine was 125/min.

0.12 and 0.17 mM, respectively, as determined by double
corresponds to

mM) and incubation at room temperature \( \text{or} \) for

effect on the extinction of the flavin, suggesting that native

reduction;

spectra after successive additions of 2-pl aliquots

difference extinction coefficient was calculated from three independ-

The time-dependent inhibition of monoamine oxidase as a function of the concentration of the suicide inhibitors clorgyline and deprenyl was employed to distinguish whether the enzyme is of the A or B type (Salach et al., 1979). To test if the isolation procedure could change the type of activity, we compared the behavior of the purified enzyme (pool DEAE/P-2) and mitochondria toward these two inhibitors. The purified enzyme showed about the same relative sensitivity to clorgyline and deprenyl as the enzyme in mitochondria. The absolute sensitivity toward both inhibitors is, however, decreased by about one-half of an order of magnitude in the purified enzyme over mitochondria: 50% inhibition is observed with clorgyline at 0.16 \( \mu \text{M} \) with mitochondria and 0.7 \( \mu \text{M} \) with purified enzyme, and 50% inhibition is observed with deprenyl at 2.0 and 7.5 \( \mu \text{M} \), respectively. These results indicate that the enzyme from placenta behaves characteristically as monoamine oxidase A in the crude and purified state.

Incubation of partially purified enzyme (pool DEAE/P-1) at 30 °C in 50 mM NaP, buffer, pH 7.2, containing 0.06% Brij-35 with the sulfhydryl reagent DPDS led to time-dependent inhibition. Fig. 5 shows that inactivation is biphasic. The rapid rate constants were determined by the graphical method of Ray and Koshland (1961). Pseudo-first-rate constants for both processes are plotted against DPDS concentration in inset of Fig. 5. Both lines pass through the origin. The second-order rate constants calculated from the slopes were 1230 and 235 min\(^{-1}\) \( \text{M}^{-1} \) for the rapid and slow phases, respectively. Fig.

6 shows that (+)-amphetamine, a competitive inhibitor of monoamine oxidase A with a \( K_i \) value of 20 \( \mu \text{M} \), retards both phases of the inhibition. Appropriate controls are also shown in the same figure.

On titration of native monoamine oxidase A under anaerobic conditions (Fig. 7) with dithionite, the sequential formation of the red anionic flavin radical and the fully reduced enzyme was observed. The reduction with the first equivalent of electrons, forming the anionic flavin radical, is nearly isoosbestic except for the first 10 or 15% of the titration. This initial nonisoosbestic behavior is probably due to the fraction of inactive enzyme which is reduced completely upon addition of the first aliquot of dithionite. Reduction with the first equivalent was fast relative to the acquisition time for the spectra. The second phase of the reduction also proceeded in an isoosbestic manner, but the rate of reduction was slower and an excess of dithionite was used to achieve full reduction. Addition of tyramine to the radical did not affect the spectrum.

Relative mobilities of subunits from human monoamine oxidase A, human monoamine oxidase B, and bovine monoamine oxidase B were compared by SDS-PAGE, shown in Fig. 8. Since the three enzymes could be separated on 7.5 or 10% Laemmli gels, unique apparent subunit molecular weights for each enzyme are likely. The difference in molecular weight between human monoamine oxidase A and human monoamine oxidase B was estimated at about 1500–2500. The difference between human monoamine oxidase B and bovine monoamine oxidase B is probably no more than 1000, human monoamine oxidase B having the higher mobility.

**DISCUSSION**

In this report, we present a method for the purification of monoamine oxidase A to near-homogeneity from human placenta (Table I) and the results of studies aimed at its partial characterization. The purification is based on our method for the purification of monoamine oxidase B from

3 W. Weyler and J. I. Salach, unpublished experiments.
Fig. 5. Semilog plot of rate of inactivation of monoamine oxidase A with DPDS. For these experiments, pool DEAE/P-1 was used (see "Experimental Procedures" and Fig. 1). This material was enriched in monoamine oxidase A as judged from the 277/456 nm ratio of 36 (the ratio prior to DEAE chromatography was about 100 and that of the most purified enzyme was 12,6). Enzyme was freed of glycerol as in Fig. 4, but the column was run in buffer containing 0.05% Brij-35 instead of Triton X-100. Enzyme solution was incubated at a concentration of 2.7 μM, with appropriate additions, at 30°C, in a final volume of 0.35-0.40 ml. When required, DPDS was added as a 10-μl aliquot from a methanolic solution of appropriate concentration. Aliquots (10 μl) of the incubation mixtures were removed for assay of remaining activity as a function of time. Symbols for DPDS concentrations are as follows: □—□, 0.20 mM; ■—■, 0.15 mM; ○—○, 0.10 mM; ○—○, 0.05 mM. Inset, plot of pseudo first-order rate constants (min⁻¹) for both phases versus DPDS concentration; fast phase, ■—■, slow phase, ○—○.

bovine liver (Salach, 1979), in which we employed a combination of standard protein purification techniques and the relatively novel technique of polymer-partitioning, developed by Albertsson (1971, 1973). Both purification schemes make use of digestion with phospholipase A and C, extraction with Triton X-100, and partitioning in a biphasic polymer system. The monoamine oxidase A and monoamine oxidase B fractionated similarly up to the polymer-partition step. With the monoamine oxidase B preparation (Salach, 1979), in which we used Ficoll, dextran, and PEG, the bulk of the enzyme activity was recovered from the solid interface between the two polymer liquid phases. In the monoamine oxidase A preparation, the bulk of the activity is recovered from the upper layer of the biphasic polymer system, in the presence or absence of Ficoll. This difference in behavior suggests differences in hydrophobic properties between these two enzymes. In contrast to monoamine oxidase B, monoamine oxidase A isolated from the polymer-partitioning step did not sediment on high-speed centrifugation; this may be due to differences in the state of aggregation of the two enzymes. Final purification of monoamine oxidase A was by ion exchange chromatography. The method reported here gives consistent yields and has been carried out with mitochondria obtained from one to six placentas. Although we have routinely used an anaerobic environment and 3 mM amphetamine in the ion exchange chromatography, only slightly lower yields were obtained in a single experiment where these conditions were not included.

As might be anticipated, a number of physical constants and structural parameters of monoamine oxidase A and monoamine oxidase B are similar. The difference extinction coefficient and the spectral ratio 277/456 for monoamine oxidase A was 10,800 ± 350 M⁻¹ cm⁻¹ and 12.6–12.9, respectively, compared to values of 10,500 M⁻¹ cm⁻¹ and 12–13 for monoamine oxidase B from bovine liver (our observation). The number of sulfhydryl groups detected by spectrophotometric titration of these enzymes denatured with SDS and reduced with 2-mercaptoethanol is 7 and 7.5/mmol of flavin in monoamine oxidase B and monoamine oxidase A, respectively.

The apparent subunit molecular weight of monoamine oxidase A obtained by SDS-PAGE analysis, in gels ranging from 7.5 to 12.5% acrylamide, was 60,000–64,000. This number agrees with the previously reported values, obtained by similar methods, of 60,000 and 67,000 (Cawthon et al., 1981; Brown et al., 1980, respectively). Comparison of the subunit molecular weight of purified monoamine oxidase A with that of human liver monoamine oxidase B shows that the A enzyme is 1,500–2,500 larger than monoamine oxidase B. Attempts to obtain the native molecular weight of monoamine oxidase A by molecular sieving or native polyacrylamide gel electrophoresis failed because of apparent aggregation of the enzyme (see "Results"). A molecular mass of 73,500 Da/mol of flavo-protein was obtained by measurement of flavin content/mg of protein (biuret method). This number would indicate that there is one flavin/subunit in monoamine oxidase A. In view of similarities with monoamine oxidase B, however, which
Fig. 7. Reduction of monoamine oxidase A with dithionite.
Monoamine oxidase A from pool DEAE/P-4 (see "Experimental Procedures" and Fig. 1) was prepared for anaerobic titration as in Fig. 4. Dithionite was standardized by monitoring reduction of riboflavin (extinction coefficient 12.5 mM⁻¹ cm⁻¹; Mayhew, 1971). Enzyme (15 nmol; 90% active) in 1 ml of buffer was titrated; the total amount of titrant added is indicated for each curve. Panel A, formation of anionic flavin semiquinone. Curve 1, initial spectrum; curve 2, 2.8 nmol of dithionite (DTH); curve 3, 7.0 nmol DTH; curve 4, 9.8 nmol DTH; curve 5, 12.6 nmol DTH. Panel B, reduction of semiquinone to fully reduced enzyme. The sample is that shown in panel A; reduction was continued by further addition of dithionite. Curve 1, same as curve 5 in panel A, curve 2, 15.4 nmol DTH; curve 3, 18.2 nmol DTH; curve 4, same as curve 3, but 10 min later; addition of 3 nmol of tyramine at this point caused no changes in the spectrum; curve 5, 25 nmol of DTH, spectrum taken 18 min after this addition; curve 6, 53 nmol DTH, spectrum taken 20 min after last addition.

include the same ratio of cysteine to flavin, the same covalent flavin attachment site (Kearney et al., 1971; Walker et al., 1971; Nagy and Salach, 1981; Yu, 1981), and similar apparent subunit molecular weights, but where there is one flavin/two subunits (Minamiura and Yasunohu, 1978), we feel that the ratio of flavin to subunit for monoamine oxidase A is still in doubt. It is clear that other methods of protein analysis and total amino acid analysis are required to establish the ratio of flavin to subunit and the minimum molecular weight for monoamine oxidase A.

The suggestion has been made that monoamine oxidase A and monoamine oxidase B are the same protein, the substrate specificity of which is determined by the membrane environment in a given tissue. It would appear reasonable, therefore, to suppose that purification of the enzyme from such a tissue might result in a change in its kinetic properties. Our experiments did not provide evidence to support the notion that the type of monoamine oxidase activity is dependent on membrane environment. Kᵣ values for kynuramine remained essentially unchanged for mitochondria and purified enzyme (0.12 and 0.17 mM, respectively). Furthermore, titration of mitochondria and the purified enzyme with the specific suicide inhibitors clorgyline and deprenyl revealed no qualitative change in kinetic properties. Finally benzylamine, a nearly exclusive monoamine oxidase B substrate, was oxidized at less than 1% of the rate found with kynuramine (data not presented). Although the type A/B activity does not appear to be affected by environment, it should be noted that the 40% inactivation during hydroxylapatite chromatography and the 10% inactivation observed on DEAE chromatography may be due to removal of residual bound lipids. The enzyme obtained from the hydroxylapatite chromatography has not been characterized with regard to substrate titration under anaerobic conditions, and Kᵣ parameters have not been evaluated. It is not possible, therefore, to say whether the observed decrease in activity is due to partial inactivation of the enzyme or change in the Kᵣ or Vᵣ parameters.

In the nomenclature of Hemmerich and Massey for flavoproteins, the "Dehydrogenases/Oxidases" can undergo one-electron reduction under artificial conditions (Hemmerich and Massey, 1979). Monoamine oxidase belongs to this classification group, and the placental enzyme indeed formed the red anionic flavin radical on partial reduction with dithionite under anaerobic conditions (Fig. 7). Addition of the first equivalent of electrons gave a spectrum closely resembling that of glucose oxidase reduced photochemically in the presence of 5-deazaflavin and EDTA at pH 9.2 (Stankovich et al., 1978). Further addition of dithionite led to full reduction of the enzyme. The observation of two isosbestic phases indicates that all of the enzyme is reduced to the radical state prior to full reduction. The radical thus appears relatively
stable toward further reduction as judged by its prolonged persistence in the presence of an excess of dithionite. Whether this stability is thermodynamic or kinetic cannot be determined from the present data. Addition of tyramine to the radical did not affect the spectrum, indicating that substrate is not capable of reducing the radical. This result is expected for a flavoenzyme oxidase which uses two electrons for the reduction of oxygen, the flavin going from the fully reduced to the fully oxidized state in the oxidative half-reaction. Similar behavior with dithionite was previously observed for bovine monoamine oxidase B (Weyer and Salach, 1981), but in that case reduction was slower for both phases, complete reduction failing even with a 3-fold excess of dithionite and incubation overnight at ambient temperature. This comparison suggests that either the redox potential of the monoamine oxidase A flavin is higher than that of monoamine oxidase B or that the FAD cofactor of monoamine oxidase B is less accessible to reagent than that of monoamine oxidase A.

Monoamine oxidase A was inactivated by incubation with the sulfhydryl reagent DPDPS. Plots of the inactivation data showed biphasic kinetics (Fig. 5). These data indicate that at least two groups are being modified and that the faster process leads only to partial inactivation. The plot of pseudo first-order rate constant versus inhibitor concentration for both phases passes through the origin, which implies that the associate rate constants are second-order. Any dissociation constant of an enzyme-inhibitor complex would have to be at least ten-fold higher than the highest concentration used in the inactivation experiment. The stepwise inhibition rules out the involvement of the sulfhydryl group modified at the faster rate in the catalytic mechanism; thus, the conceivable involvement of disulfide formation in the catalytic mechanism is not suggested by our results. (+)-Amphetamine, a competitive inhibitor, protects against both phases when used at 10 times its Ki value (Fig. 6). This observation suggests that the sulfhydryl groups modified may be near the substrate-binding site, or alternatively, the groups may be at a remote site, made inaccessible to sulfhydryl reagent due to a conformational change on amphetamine binding. Modification of two sulfhydryl groups by a number of sulfhydryl reagents was also suggested by spectral experiments with monoamine oxidase B from bovine liver in work of Yasunobu and co-workers (Yasunobu et al., 1979; Gomes et al., 1976), and we observed similar results with monoamine oxidase B and DPDPS. In our experiments with monoamine oxidase B, the inactivation kinetics did not show biphasic behavior but spectral analysis showed 2 mol of 2-pyridinediethol liberated per mol of enzyme inactivated. The latter results are consistent with the proposal of two independent modification reactions which proceed at similar rates, or a sequential mechanism in which the rate of the first group modified is slow relative to the second modification reaction. This difference in behavior between the two enzymes may reflect differences in the active site, as might be expected for enzymes having different substrate specificities, or it may be due to changes in primary sequence as a result of a species difference. Further studies of these observations are required for a more precise interpretation.

The demonstration of the efficacy of polymer-partitioning in the purification of this membrane protein and previously in the purification of monoamine oxidase B suggests that this technique should be incorporated into the repertoire of protein purification methods for membrane proteins. The enzyme isolated as described here should be useful in kinetic studies with substrates and inhibitors of pharmacologic interest and could play an important role in structural and mechanistic studies of the monoamine oxidase isozymes.

Acknowledgments—We thank Dr. Edna B. Kearney for helpful discussions, Donald V. Crabtree, Jr., for skilful technical assistance, Shelley Plant for checking the references, Valerie J. Storey for proofreading the manuscript, and the staff of Letterman Army Medical Center, San Francisco for collecting placentas.

REFERENCES


Purification and Properties of Monoamine Oxidase Type A


