Mitochondrial Monoamine Oxidase

INACTIVATION BY PARGYLINE. ADDUCT FORMATION*

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SUMMARY

Pargyline (N-benzyl-N-methyl-2-propynylamine), known to react stoichiometrically and irreversibly with the mitochondrial monoamine oxidase of bovine kidney, involving simultaneously the enzyme's flavin component, (HELLERMAN, L., AND ERWIN, V. G. (1968) J. Biol. Chem. 243, 5234-5243), has been shown to inactivate by forming a stable adduct with the flavin residue. Thus, the reaction of pargyline with an equivalent quantity of oxidase produced "bleaching" of the flavin at 455 nm and the appearance of a strongly absorbing species at 410 nm. Use of excess [7-14C]pargyline gave a stable 14C-protein product (1.1 residues of inhibitor bound per enzyme equivalent.) Protocols of the 14C-labeled protein and chromatographic fractionation of the peptides resulting revealed that most of the 14C label was associated with the fraction containing the altered coenzyme now absorbing maximally at 398 nm with a molar absorptivity of approximately 29,000 cm⁻¹ M⁻¹.

Given here also is a procedure for the preparation of monoamine oxidase with twice the activity of the enzyme obtained previously (ERWIN, V. G., AND HELLERMAN, L. (1967) J. Biol. Chem. 242, 4230-4238). Based on estimates of enzyme concentration obtained by discontinuous titrations of the enzyme with pargyline, the respective values for the equivalent weight and the catalytic center activity (turnover) were calculated to be 109,000 and 525 for oxidation of benzylamine in air at pH 7.6 and 37°.

In an investigation of certain inhibitors of the enzyme, mitochondrial monoamine oxidase (EC 1.4.3.4) (1), it was shown that the action of pargyline (2), N-benzyl-N-methyl-2-propynylamine, with this flavoenzyme involves the flavin component. In addition, the inhibitor combines with the enzyme irreversibly and covalently in a 1:1 stoichiometric fashion. Enzyme sulfhydryl groups persisted in the modified protein.

It was found also that this stoichiometry permits the use of pargyline as a titrimetric reagent for the oxidase, leading to the approximation of 10⁴ for the equivalent weight of the bovine kidney enzyme.

In this communication we have used a highly active monoamine oxidase preparation in studies designed to elucidate further the mechanism of pargyline-induced inactivation. Such inactivation has been correlated with evidence (cf. 3) that the adduct formed in the interaction of pargyline with the oxidase involves an addition reaction with the isoxazolone nucleus of the flavin component.

EXPERIMENTAL PROCEDURE

Materials and Methods

Digitonin (Calbiochem, A grade) "solutions" were made immediately before use by dispersion of the solid in hot 0.25 m sucrose. Pargyline (N-benzyl-N-methyl-2-propynylamine) and [7-14C]pargyline were supplied to us as hydrochlorides. Bio-Gel P-10 mixture (hydroxyapatite for chromatography, Bio-Rad Laboratories), 215-ml suspension, was diluted to 1.5 liters with water and added to 120 g of cellulose powder (Whatman, CF11) in a suction flask, mixed well, and placed under a vacuum for 10 min; Alternatively, 50 ml of calcium phosphate gel (Sigma), 30 mg per ml of water, were mixed with 12.5 g of cellulose powder in 100 ml of water. DEAE-cellulose (Bio-Rad, Cellex D, exchange capacity 0.60 meq per g), 20 g, was washed sequentially in a large sintered glass funnel with 150 ml of 0.5 M NaOH, 200 ml of water, 100 ml of 0.6 M HCl, 100 ml of water and equilibrated to pH 7.6 with 0.02 M phosphate buffer.

Monoamine oxidase activity was assayed by a modification (4) of the spectrophotometric method of Tabor et al. (5). Here, a unit of enzyme activity is defined as the amount of enzyme catalyzing change in absorbance at 250 nm of 0.001 optical density unit per min at 37° in a 2.2-ml reaction mixture containing 3 mm benzylamine in 0.05 M NaK phosphate, pH 7.6. Protein (6) and radioactivity were determined by standard procedures. Spectra were recorded with either a Bausch and Lomb Spectronic 900 spectrophotometer equipped with a Sargent model SKL recorder or a Bausch and Lomb Spectronic 505 recording spectrophotometer.

Preparation of Monoamine Oxidase—Mitochondria were obtained from the cortex of 7 kg of fresh bovine kidney and monoamine oxidase extracted with use of digitonin by methods described earlier (4). The extracted enzyme was precipitated from

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ammonium sulfate solution (55 to 100% saturation at 0°C), collected by centrifugation at 100,000 × g for 2 hours, redissolved in 5 mM phosphate, pH 7.6, and dialyzed (4°C) for 15 hours against 5 mM buffer with three changes of 2 liters each. The enzyme, in a final volume of 40 ml, was applied to a column of hydroxypatite (5 × 30 cm), washed successively with 50 ml of 5 mM phosphate, pH 7.6; 500 ml of 0.25 M phosphate, pH 7.6; 250 ml of 0.5 M phosphate, pH 7.6, and then eluted with 2.2 liters of 0.5 M phosphate, pH 7.6, containing 1% digitonin. The second peak containing most of the enzyme was collected and concentrated under pressurized nitrogen, 40 to 80 ps.i., in an Amicon ultrafiltration apparatus with an XM-50 membrane, to approximately 50 ml. The protein was dialyzed against two 2-liter portions of 5 mM phosphate, pH 7.6, for 4 hours and 16 hours. The dialyzed fraction was placed on a column of DEAE-cellulose (2.5 × 10 cm), washed with 50 ml of 5 mM phosphate, pH 7.6, and then with 70 ml of 0.05 M phosphate, pH 7.6. The oxidase was eluted in 0.075 M phosphate, pH 7.6, containing 1% digitonin, and collected in a single broad peak with specific activities ranging from 12,000 to 19,000 units per mg of protein. The enzyme was concentrated as above to 20 ml (final volume) and dialyzed as before and then applied to a column of calcium phosphate gel (1.5 × 20 cm). The column was developed with successive additions of 0.05 M, 0.15 M, and 0.50 M phosphate, pH 7.6, 100 ml each. A single peak of enzyme was eluted by each buffer, the more highly purified enzyme being eluted with the 0.15 M buffer in a single peak which contained fractions varying in specific activity from 23,000 to 27,500. Fractions were pooled according to their specific activity and the enzyme concentrated as described above to approximately 1.0 mg per ml before storage at −10°C.

RESULTS AND DISCUSSION

Active Bovine Kidney Mitochondrial Monoamine Oxidase—As shown in Table I, enzyme of specific activity 20,000 to 27,500 was isolated in 10% yield from the mitochondria. The absorption spectrum of purified enzyme exhibited maxima at 350 and 455 nm, the absorbance ratio 350:455 nm was found to decrease as the enzyme was purified. It was approximately 0.95 for the best preparations. Contamination of the purified oxidase by cytochrome like material was evidenced by Soret band absorption at 410 nm. Occasionally an almost complete removal of this contaminant was achieved (see Fig. 1). Absorption of the oxidase in the ultraviolet occurred maximally at 277 nm with an absorbance ratio (277:435 nm) of about 11.

Reaction of Pargyline with Oxidase Flavin Component—Serial additions of pargyline to monooxidase oxidase, all at 25°C, resulted in both irreversible inactivation of the enzyme and an alteration of the oxidase flavin component as indicated by a "bleaching" at 455 nm and formation of a new chromophore absorbing at 410 nm (Fig. 1). Completion of the reaction, estimated by the technique described in Fig. 3, was observed when 13.3 nmol of pargyline had been used, i.e., equivalent to the quantity of enzyme flavin initially present in the system, 13.9 nmol (determined with the use of sodium dithionite (4)). These results indicated that pargyline reacts specifically with the flavin component at the enzyme active center.

Earlier (1) it was shown that the use of [7-14C]pargyline led to

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>7,910</td>
<td>2,710,000</td>
<td>343</td>
<td>(1.0)</td>
<td>(100)</td>
</tr>
<tr>
<td>Fraction II</td>
<td>3,600</td>
<td>3,110,000</td>
<td>857</td>
<td>2.5</td>
<td>115</td>
</tr>
<tr>
<td>Fraction III</td>
<td>438</td>
<td>1,310,000</td>
<td>2,900</td>
<td>8.7</td>
<td>48</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>77</td>
<td>740,000</td>
<td>9,600</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Fraction V</td>
<td>31</td>
<td>540,000</td>
<td>17,600</td>
<td>51</td>
<td>20</td>
</tr>
</tbody>
</table>

*See text for explanation.

† Before estimating monoamine oxidase activity, mitochondria were diluted in appropriate volumes of 0.05 M phosphate buffer, pH 7.6.

‡ Combined fractions containing enzyme eluted with 0.05 M phosphate; pH 7.6.

§ Initial portion of enzyme eluted with 0.15 M phosphate, pH 7.6.

‖ Combined fractions containing major portion of enzyme eluted with 0.15 M phosphate buffer, pH 7.6.

* Enzyme eluted with 0.5 M phosphate buffer, pH 7.6.

actions upon the flavin concomitantly with binding of 1 eq of 14C-labeled inhibitor residue per eq of oxidase; the protein sulf-hydryl groups had been excluded as possible sites for inhibitor binding. In view of the apparent involvement of the oxidase flavin component it was pertinent to examine a possible binding of the inhibitor to the flavin itself. Here, inactive enzyme containing 1.1 eq of 14C label per eq of enzyme was prepared with the use of [7-14C]pargyline. The flavin component, which for monoamine oxidase is covalently bound through a thioether linkage to a cysteine residue of the peptide (7), was "extracted" by subjecting the protein to proteolysis (cf. 8) as described in Table II. Chromatography of the partially hydrolyzed peptide on Sephadex G-25 by a procedure that has been used to obtain a characteristic flavin-peptide residue provided here a yellow pigment, essentially the altered flavin, with a single absorption peak at 395 nm (ε295 approximately 29,000 cm−1 M−1) (Fig. 2). This was found to contain 92% of the 14C label originally bound to the protein. Chromatography on paper showed that the radioactivity and pigment could not be resolved. We conclude that pargyline inhibits this oxidase irreversibly most probably by forming a stable adduct with the oxidase flavin component.

The spectral properties of this adduct resemble those of a flavin derivative prepared by Zeller et al. (9) from a photochemically induced reaction of N,N-dimethyl-2-propynylamine (structurally related to pargyline) with 3-N-methylumiflavin. This photochemically-induced product was shown to involve the addition of the pargyline analogue to form a cyclic derivative involving positions C(4a) and N(5) of the 3-methylumiflavin nucleus. It may be stated that the spontaneous addition reaction involving

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hydrogenase. Our adduct derived from monoamine oxidase by
Singer and Kearney (lo), n-amino acid oxidase, and lipoyl de-
eral. We have observed absence of the pargyline effect in
propynylamines apparently does not apply to flavoproteins gen-
ernamente. Here, again, inhibition of the enzyme was accompanied by
bleaching of the enzyme at 455 nm and the formation of a peak
absorbing in the vicinity of 410 nm (not shown), and it is al,-
sorbed strongly at 398 nm.

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total counts</th>
<th>dpm</th>
<th>mole eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pargyline-treated oxidase after gel</td>
<td>1.34 X 10^4</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>filtration*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide-flavin after chromatography*</td>
<td>1.23 X 10^4</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

* Monoamine oxidase (specific activity 25,500; 13.3 \( \mu \)eq per liter), 5.03 mg, was incubated for 30 min with 270 pmols of
[\(^{[7-14}C\) pargyline (3.08 X 10^6 dpm per mmole) in a total volume of
3.6 ml of 0.05 M phosphate buffer, pH 7.6, 25°. Following treat-
ment enzyme was freed of excess pargyline by ultrafiltration on
Sephadex G-25 (fine) (1 X 50 cm column equilibrated and de-
veloped with water) with collection of the protein in the void
volume.

**TABLE III**

<table>
<thead>
<tr>
<th>Titration of monoamine oxidase with pargyline*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>2,400</td>
</tr>
<tr>
<td>15,800</td>
</tr>
<tr>
<td>20,300</td>
</tr>
<tr>
<td>26,800</td>
</tr>
</tbody>
</table>

* Titrations as described in Fig. 3 were performed in a total
volume of either 20 or 40 ml.

The synthetic derivative of Zeller et al. (9) is stated to absorb
intensely at 390 nm (pH 7) because of the presence of an enamine
system involving position N(5) of this flavin nucleus.

2-Propynylamine, although less active than pargyline, was
also found to serve as an inactivator of the oxidase (cf. 11).
Here, again, inhibition of the enzyme was accompanied by
bleaching of the enzyme at 455 nm and the formation of a peak
absorbing in the vicinity of 410 nm (not shown), and it is ap-

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**FIG. 1 (left).** Spectrum of monoamine oxidase after treatment
with pargyline. Spectrum of oxidase (specific activity, 25,000;
13.3 \( \mu \)eq per liter), 1.4 mg in 1.0 ml of 0.05 M phosphate, pH 7.6, in
each case recorded 10 min after serial additions of 10 \( \mu \)l of 4 X 10^{-4}
m pargyline hydrochloride at 25°. Final pargyline concentra-
tions: Curve I, native enzyme; Curve II, 4.0 \( \mu \)m; Curve III, 7.8
\( \mu \)m; Curve IV, 11.2 \( \mu \)m; and Curve V, 15.2 \( \mu \)m.

**FIG. 2 (center).** Spectrum of peptide-flavin material derived from
monoamine oxidase that had been inactivated by pargyline. The
oxidase was treated with \( [7-14^C] \) pargyline and the altered
flavin coenzyme resulting was removed and isolated by chromatog-
raphy precisely as described in Table II. Material dissolved in 6.9
ml of water (spectrum given in figure) contained 1.13 X 10^5 dpm
derived from 3.67 X 10^{-4} mole of pargyline. By assuming the
altered coenzyme to be an adduct with \( [7-14^C] \) pargyline (stoi-
chiometry, 1:1 (see text)), the concentration of flavin derivative
was considered to be approximately 5.3 \( \mu \)M and \( \epsilon_{290} \), approximately
29,000 cm^{-1} M^{-1}.

**FIG. 3 (right).** Titration of monoamine oxidase with pargyline.
Discontinuous titrations were performed at 25° by adding in-
creasing quantities of pargyline to separate tubes each containing
15.7 \( \mu \)g of oxidase (specific activity 24,000) in a total volume of 40
\( \mu \)l of 0.05 M phosphate, pH 7.6. Reactions were terminated at 50
min and residual enzyme activity determined after addition of
benzylamine (final concentration, 3 mm) in 2.16 ml of 0.05 M phos-
phate, pH 7.0, at 37°.
parent that similarly to pargyline 2-propynylamine also forms an adduct with the oxidase flavin.

**Estimation of Monoamine Oxidase by Titration with Pargyline**—When monoamine oxidase solutions were allowed to remain 50 min at 25° with varying concentrations of pargyline in discontinuous titration, the activity was observed to decrease as a linear function with the amount of inhibitor added (Fig. 3; also Ref. 1). Since the inhibition reaction is stoichiometric, the titration endpoint can be related directly to enzyme concentration. Results appear to be valid for preparations of varying purity since end points that relate to enzyme activity were found to be similar regardless of enzyme homogeneity (Table III). Thus, pargyline, 0.1 neq, was found sufficient to inactivate 293 to 319 units of activity.

From titrations of six different preparations of oxidase with specific activities from 19,600 to 26,800 (296 ± 15 units of oxidase = 0.1 neq pargyline) the average value of the catalytic center activity for the oxidation of benzylamine in air (pH 7.6, 37°) was calculated to be 525. It was calculated that the equivalent weight of our best oxidase preparation of specific activity, 26,800, approximated 109,000. This value agrees with data for preparations of oxidase obtained from a variety of tissues and sources (8, 12). Oreland by use of pargyline as a titrametric agent found the equivalent weight of the enzyme from hog liver to be 115,000.4

4 In Lars Oreland, Dissertation 1972, Umea University Medical Dissertations.

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