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.) Protolysis 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 sulphydryl 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 isoalloxazine 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-100 (Bio-Rad Laboratories), 215-ml suspension, was diluted to 1.5 liters with 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, 100 ml of water, 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 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 water, 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 600 spectrophotometer equipped with a Sargent model SKL recorder or a Bausch and Lomb Spectronic 500 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, redisolved 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 two peaks with 1.2 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 50 p.s.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 18,000 units/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 412 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 (277:455 nm) of about 11.

**Reaction of Pargyline with Oxidase Flavin Component**—Serial additions of pargyline to monoamine 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 (11.9 nmol) determined with the use of sodium dithionite (4). Several 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 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 prepared here a yellow pigment, essentially the altered flavin, with a single absorption peak at 395 nm (ε max 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 photodissociated 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

<table>
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<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
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<tbody>
<tr>
<td>Fraction I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1</td>
<td>37,200</td>
<td>17,700</td>
<td>52</td>
<td>1.4</td>
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<tr>
<td>Fraction II&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>80,800</td>
<td>27,000</td>
<td>80</td>
<td>2.2</td>
</tr>
<tr>
<td>Fraction III&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.6</td>
<td>184,000</td>
<td>24,200</td>
<td>71</td>
<td>6.8</td>
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<tr>
<td>Fraction IV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8</td>
<td>33,200</td>
<td>11,800</td>
<td>34</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>See text for explanation.

<sup>b</sup>Before estimating monooamine oxidase activity, mitochondria were diluted in appropriate volumes of 0.05 M phosphate buffer, pH 7.6.

<sup>c</sup>Combined fractions containing enzyme eluted with 0.05 M phosphate; pH 7.6.

<sup>d</sup>Initial portion of enzyme eluted with 0.15 M phosphate, pH 7.6.

<sup>e</sup>Combined fractions containing major portion of enzyme eluted with 0.15 M phosphate buffer, pH 7.6.

<sup>f</sup>Enzyme eluted with 0.5 M phosphate buffer, pH 7.6.
hydrogenase. Our adduct derived from monoamine oxidase by Singer and Kearney (lo), n-amino acid oxidase, and lipoyl dehydrogenase. Three instances, namely succinic dehydrogenase as prepared by these authors, had involved an addition reaction to positions C(4a), N(5) of the flavin nucleus. It may be recalled that our isolated oxidase flavin-inhibitor adduct absorbed strongly at 398 nm. It is also found to serve as an inactivator of the oxidase (cf. 11).

Fig. 1 (left). Spectrum of monoamine oxidase after treatment with pargyline. Spectrum of oxidase (specific activity, 25,000; 13.3 μeq 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 μl of 4 × 10^{-4} M pargyline hydrochloride at 25°C. Final pargyline concentrations: Curve I, native enzyme; Curve II, 4.0 μM; Curve III, 7.8 μM; Curve IV, 11.2 μM; and Curve V, 15.2 μM.

Fig. 2 (center). Spectrum of peptide-flavin material derived from monoamine oxidase that had been inactivated by pargyline. The oxidase was treated with [7-14C]pargyline and the altered flavin coenzyme resulting was removed and isolated by chromatography precisely as described in Table II. Material dissolved in 6.9 ml of water (spectrum given in figure) contained 1.13 × 10^{6} dpm derived from 3.67 × 10^{-4} mole of pargyline. By assuming the altered coenzyme to be an adduct with [7-14C]pargyline (stoichiometry, 1:1 (see text)), the concentration of flavin derivative was considered to be approximately 5.3 μM and ε_{650}, approximately 29,000 cm^{-1} M^{-1}.

Fig. 3 (right). Titration of monoamine oxidase with pargyline. Discontinuous titrations were performed at 25°C by adding increasing quantities of pargyline to separate tubes each containing 15.7 μg of oxidase (specific activity 24,000) in a total volume of 40 μl 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 phosphate, pH 7.8, at 37°C.

Table II

<table>
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<tr>
<th>Sample</th>
<th>Total counts</th>
<th>14C bound per eq of enzyme</th>
</tr>
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<tbody>
<tr>
<td>Peptide-flavin after chromatography a</td>
<td>1.23 × 10^{6}</td>
<td>0.99</td>
</tr>
<tr>
<td>Peptide-flavin after gel filtration a</td>
<td>1.34 × 10^{4}</td>
<td>1.08</td>
</tr>
</tbody>
</table>

a Monoamine oxidase (specific activity 25,500; 13.3 μeq per liter), 5.03 mg, was incubated for 30 min with 270 nmoles of [7-14C]pargyline (3.08 × 10^{5} dpm per mmole) in a total volume of 3.6 ml of 0.05 M phosphate buffer, pH 7.6, 25°C. Following treatment enzyme was freed of excess pargyline by ultrafiltration on Sephadex G-25 (fine) (1 × 50 cm column equilibrated and developed with water) with collection of the protein in the void volume.

14C-Labeled protein prepared above was held at 0°C for 1/2 hour in 10% trichloroacetic acid and the precipitate resulting was collected by centrifugation, washed twice with cold 1% trichloroacetic acid, and resuspended in 2.0 ml of 0.1 M Tris buffer, pH 8.0. Proteolysis of the oxidase was accomplished at 37°C with the use of 0.6 mg of crystalline trypsin, 0.3 mg of crystalline α-chymotrypsin, and 1.1 mg of “1 300 trypsin” (all from bovine pancreas) in which pH 8 was maintained by addition of NH_{4}OH. After 2 hours additional proteases were added as described above and incubation continued for 2 hours. The mixture was chilled and 1 M HCl was added to give pH 6. The precipitate resulting was removed by centrifugation and the supernatant subjected to chromatography on a column (1 × 60 cm) of Sephadex G-25 (fine), equilibrated, and developed with water. The altered peptide-flavin was eluted as a single peak.

spontaneous interaction of the 2 molecules might nevertheless have involved an addition reaction to positions C(4a), N(5) of this flavin nucleus.

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 the flavin nucleus. It may be recalled that our isolated oxidase flavin-inhibitor adduct absorbed strongly at 308 nm.

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-

Table III

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Enzyme inactivated per 0.1 amole of pargyline</th>
<th>Catalytic center activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,400</td>
<td>319</td>
<td>570</td>
</tr>
<tr>
<td>15,800</td>
<td>304</td>
<td>540</td>
</tr>
<tr>
<td>20,300</td>
<td>314</td>
<td>560</td>
</tr>
<tr>
<td>26,800</td>
<td>293</td>
<td>520</td>
</tr>
</tbody>
</table>

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

b Expressed as molecules of benzylamine oxidized per min per catalytic center; calculated from titration data with use of ε_{650} benzaldehyde, 12.4 × 10^{3} cm^{-1} M^{-1} (1).

c The unit of enzyme activity is taken as a change in absorbance of 0.001 per min at 250 nm with 3 μm benzylamine as substrate in a 2.2 ml reaction mixture containing 50 mM phosphate buffer, pH 7.6, 37°C.

Propargylamines apparently do not apply to flavoproteins generally. We have observed absence of the pargyline effect in three instances, namely succinic dehydrogenase as prepared by Singer and Kearney (10), d-amino acid oxidase, and lipoyl dehydrogenase. Our adduct derived from monoamine oxidase by
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 end point 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 titrimetric agent found the equivalent weight of the enzyme from hog liver to be 115,000.4

Acknowledgment—The authors appreciate the technical assistance of Jill M. Collins.

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Mitochondrial Monoamine Oxidase: INACTIVATION BY PARGYLINE. ADDUCT FORMATION
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