Monoamine Oxidase

II. COPPER, ONE OF THE PROSTHETIC GROUPS OF PLASMA MONOAMINE OXIDASE

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The nature of the prosthetic groups of monoamine oxidase has been the subject of much conjecture, and definitive experimental results have not been obtained. The results of Tabor et al. (1) suggest that pyridoxal phosphate may be the coenzyme of the enzyme, and Gorkin and Romanova (2) have suggested that the enzyme is a metalloenzyme. Others (3, 4) have proposed that monoamine oxidase is a flavoenzyme.

In a previous paper (5), we reported on a procedure for obtaining crystalline plasma monoamine oxidase. The availability of the crystalline enzyme has opened the way to a critical examination of the prosthetic groups of monoamine oxidase. In this paper, we wish to show that plasma monoamine oxidase is a copper enzyme and that the copper is involved in enzyme activity. While this work was in progress, studies similar in some respects were reported by Mann (6), who used a partially purified preparation of plant amine oxidase.

EXPERIMENTAL PROCEDURE

Materials—Partially purified and crystalline plasma monoamine oxidase preparations were prepared as previously described (5). Hydroxylapatite was prepared by the method of Tiselius, Hjerten, and Levin (7). Sodium diethylthiocarbamate and 2,2'-bipyridyl were purchased from Eastman Organic Chemicals. Cuprizone, neocuproine hydrochloride, and o-phenanthroline were obtained from the Sigma Chemical Company. Distilled and deionized water was used during the enzyme purification as well as in all experiments described in this paper.

Methods

Enzyme Assay and Protein Determination—The enzyme assays and the protein determinations were performed spectrophotometrically as previously described (5). One unit was defined as the amount of enzyme catalyzing a change of 0.001 absorbancy per minute under the standard assay conditions (5).

Metal Determination—In all analytical work to be described, all glassware were carefully washed with cleaning solution and rinsed with distilled and deionized water. Analytical grade metal salts were used as standards for the metal determinations.

The qualitative determinations of metal content were made spectrophotographically. The enzyme preparations were dialyzed for 6 hours against 0.03 M ammonium phosphate buffer, pH 7.0, containing 0.003 M EDTA and then dialyzed for 24 hours against the ammonium phosphate buffer alone, since the enzyme precipitated in the absence of salts (5). The dialyzed enzyme preparations at a concentration of 20 to 25 mg of protein per ml were analyzed with a Beckman model DU spectrophotometer with a flame, photomultiplier, and spectral energy-recording attachments (8).

The quantitative determinations of metal content were carried out colorimetrically. The copper content was determined by two methods: (a) as the cupric-cuprozine complex according to the method of Peterson and Bollier (9); and (b) as the cuprous-neocuproine complex according to the method of Griffiths and Wharton (10). The two methods showed good agreement. The valency state of copper in the enzyme was determined by the method of Griffiths and Wharton (11). The iron content was determined by the method of Mahler and Elowe (12), the molybdenum content by the method of Johnson and Arkley (13), the manganese content by the method of Sandell (14), and the zinc content by the method of Heinzen and Benne (15).

Spectrophotometric Determination—The enzyme assays, the protein determinations, and the metal determinations were made with a Beckman model DU spectrophotometer. Spectra of the enzyme were taken with a Beckman model DK-2 spectrophotometer. Special Thunberg type cuvettes with side arms were used for the anaerobic experiments. The cuvettes were evacuated and flushed with oil-pumped nitrogen repeatedly before the reactants were added together.

Dialysis Experiment—All the dialysis experiments were performed at 4° with continuous stirring. Visking cellulose tubings were used.

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The abbreviations used are: cuprozine, bis-cyclohexanone oxalylhydrazone; neocuprine, 9,9-dimethyl-1,10-phenanthroline; DEAE-cellulose, diethylaminoethyl cellulose.

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RESULTS

Relationship of Copper Content to Enzyme Activity—During the purification of plasma monoamine oxidase, there was an increase in copper content that was proportional to the increase in specific activity. Table I shows the typical result obtained with one preparation. In the initial steps of the purification procedure, the enzyme was contaminated with considerable amounts of ceruloplasmin, which has a high content of copper (16). This contaminant was completely separated from the monoamine oxidase by the first and second DEAE-cellulose chromatography steps. Then, a direct relationship between copper content and the enzyme activity was observed during the final five steps, as shown in Fig. 1. Fig. 1 illustrates the results obtained with two different preparations.

Table I
Distribution of copper during purification of plasma monoamine oxidase

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity</th>
<th>Copper content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg protein</td>
<td>mmoles/mg protein</td>
</tr>
<tr>
<td>1. Plasma</td>
<td>1.1</td>
<td>0.53</td>
</tr>
<tr>
<td>2. First ammonium sulfate</td>
<td>11.8</td>
<td>1.03</td>
</tr>
<tr>
<td>3. First DEAE-cellulose chromatography</td>
<td>38.3</td>
<td>1.28</td>
</tr>
<tr>
<td>4. Second ammonium sulfate fractionation</td>
<td>187</td>
<td>5.01</td>
</tr>
<tr>
<td>5. Second DEAE-cellulose chromatography</td>
<td>254</td>
<td>0.65</td>
</tr>
<tr>
<td>6. Hydroxylapatite chromatography</td>
<td>Fraction I</td>
<td>343</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fraction II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.18</td>
</tr>
<tr>
<td>7. Crystallization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First crystal</td>
<td>430</td>
<td>12.90</td>
</tr>
<tr>
<td>Second crystal</td>
<td>482</td>
<td>13.93</td>
</tr>
<tr>
<td>Third crystal</td>
<td>502</td>
<td>14.23</td>
</tr>
<tr>
<td>Fourth crystal</td>
<td>500</td>
<td>14.23</td>
</tr>
</tbody>
</table>

These steps have been fully described in a previous paper (5).

Fig. 1. Proportionality between copper content and specific activity during the purification of plasma monoamine oxidase.

C-----C represents results from Preparation 1; O-----O represents results from Preparation 2.

**Figure 1:** Proportionality between copper content and specific activity during the purification of plasma monoamine oxidase.

Copper and Other Metal Contents—Four times recrystallized preparations of plasma monoamine oxidase, which showed a single symmetrical peak in the ultracentrifuge, were found to contain 1 g atom of copper per 70,000 g of protein (Table I). Since the molecular weight was found to be 255,000 (5) by the Archibald method, there are 3.7 g atoms of copper per mole of enzyme.

Qualitative and quantitative determinations of the other possible metals known to be involved in oxidative processes, e.g., iron (12, 17–19), molybdenum (19, 20), zinc (21, 22), or manganese (6), were carried out with the recrystallized enzyme preparations. The results are summarized in Table II.

Effect of Cuprizone—The effects of various cuprizone agents on plasma monoamine oxidase are summarized in Table III. The recrystallized enzyme with a specific activity of 482 was used. The enzyme, 0.5 mg, was preincubated for 10 minutes with cuprizone agents before the addition of benzylic alcohol under the standard assay conditions.

Marked inhibition was observed with sodium diethyldithiocarbamate, cuprizone, sodium cyanide, 8-hydroxyquinoline sulfate, and o-phenanthroline. The observed inhibition occurred instantaneously, and longer preincubation periods did not affect the results to any appreciable extent.

No inhibition was observed on adding neocuproine, EDTA, and sodium azide, and slight inhibition was observed with sodium thioglycolate.

Effect of Cuprizone—The Lineweaver and Burk plots (23) with benzylic alcohol as the substrate in the presence and absence of cuprizone are shown in Fig. 2. The recrystallized enzyme with a specific activity of 482 was used. The enzyme, 0.5 mg, was preincubated for 10 minutes with cuprizone before the addition of benzylic alcohol under the standard assay conditions.

The concentrations of the cuprizone were (×10⁻⁶ M): I, 0.0; II, 1.5; III, 5.0. The result showed that competitive inhibition occurred. The Michaelis constant (Kₘ) and the inhibitor-enzyme dissociation constant (Kᵢ) were found to be 1.49 × 10⁻⁴ M and 1.4 × 10⁻⁵ M, respectively.

Valency State of Copper in Enzyme—Analysis of the valency of copper was performed by the method of Griffiths and Wharton (11). The results are summarized in Table IV. The conditions used for the experiments are described in the legend of Table IV. As shown in Table IV, copper of plasma monoamine oxidase...
Effect of chelating agents on plasma monoamine oxidase

The recrystallized enzyme, 0.5 mg, with a specific activity of 482, was preincubated for 10 minutes with chelating agents before the addition of benzylamine under the standard assay conditions.

<table>
<thead>
<tr>
<th>Chelating agents</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \times 10^{-3} )</td>
<td>%</td>
</tr>
<tr>
<td>Sodium diethyldithiocarbamate</td>
<td>3.0</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Cuprizone</td>
<td>3.0</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>97.7</td>
</tr>
<tr>
<td>Neocuproine</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>30.0</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>76.2</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>3.8</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>27.4</td>
</tr>
<tr>
<td>2,2'-Bipyridyl</td>
<td>3.8</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>9.7</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>3.8</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>40.8</td>
</tr>
<tr>
<td>EDTA</td>
<td>30.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>100.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium thioglycolate</td>
<td>100.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Dialysis against Acidic Buffer and Chelating Agent—Dialysis of plasma monoamine oxidase against buffers of pH lower than pH 5.0 resulted both in a decrease of the copper content and inactivation of the enzyme, as shown in Table V. The crystallized enzyme with a specific activity of 444 was used. Enzyme, 15 mg, was dialyzed against 500 ml of 0.2 M acetate buffer of pH 5.0, 4.0, or 3.0. Each aliquot of the dialyzed enzymes, which contained 4 mg of protein, was used for activity and copper determinations.

Valency state of copper in plasma monoamine oxidase

Valency state of copper in plasma monoamine oxidase was determined by the method of Griffiths and Wharton (11). The recrystallized enzyme with a specific activity of 482 was used. To 0.5-ml aliquots of the enzyme solution containing 5 mg of protein were added 30 µmoles of phosphate buffer (KH₂PO₄-Na₂HPO₄), pH 7.2; and the appropriate additions of benzylamine, sodium dithionite, or hydroxylamine hydrochloride to give a final volume of 0.8 ml. The copper was determined by first adding 0.2 ml of 0.2 M EDTA and 0.001 M p-chloromercuriphenylsulfonic acid followed by 1.4 ml of 0.1% neocuproine in glacial acetic acid.

\[ \text{State of enzyme} \quad \text{Copper reduction} \quad \% \]

1. Oxidized (bubbled with O₂) \quad ................. \quad 0
2. “Steady-state” after adding 6 µmoles of benzylamine \quad ................. \quad 0
3. Reduced (same as Line 2, under anaerobic conditions) \quad ................. \quad 0
4. Reduced form of Line 3, shaken with O₂ \quad ................. \quad 85-95
5. Reduced by adding 6 µmoles of dithionite under anaerobic conditions ... \quad 85-95
6. Reduced by adding 6 µmoles of hydroxylamine under anaerobic conditions \quad ................. \quad 85-95

Dialysis of plasma monoamine oxidase against acidic buffers

The crystallized enzyme with a specific activity of 444 was used. Of the enzyme solution, 4 ml containing 15 mg of protein were dialyzed against 500 ml of 0.00 M phosphate buffer (KH₂PO₄-Na₂HPO₄) at pH 7.0 or against 500 ml of 0.2 M acetate buffer at pH 5.0, 4.0, or 3.0. A 4-mg aliquot of the dialyzed enzyme was removed and used for activity and copper determinations.

Dialysis of plasma monoamine oxidase against acidic buffers

The cupric copper in the enzyme was reduced to cuprous copper by reducing agents such as sodium dithionite and hydroxylamine hydrochloride. The copper was found to be reversibly reoxidized by bubbling the solution with oxygen.

Dialysis against Acidic Buffer and Chelating Agent—Dialysis of plasma monoamine oxidase against buffers of pH lower than pH 5.0 resulted both in a decrease of the copper content and inactivation of the enzyme, as shown in Table V. The crystallized enzyme with a specific activity of 444 was used. Enzyme, 15 mg, was dialyzed against 500 ml of 0.2 M acetate buffer of pH 5.0, 4.0, or 3.0. Each aliquot of the dialyzed enzymes, which contained 4 mg of protein, was used for activity and copper determinations.

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\[ \text{State of enzyme} \quad \text{Copper reduction} \quad \% \]

1. Oxidized (bubbled with O₂) \quad ................. \quad 0
2. “Steady-state” after adding 6 µmoles of benzylamine \quad ................. \quad 0
3. Reduced (same as Line 2, under anaerobic conditions) \quad ................. \quad 0
4. Reduced form of Line 2, shaken with O₂ \quad ................. \quad 85-95
5. Reduced by adding 6 µmoles of dithionite under anaerobic conditions \quad ................. \quad 85-95
6. Reduced by adding 6 µmoles of hydroxylamine under anaerobic conditions \quad ................. \quad 85-95

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Dialysis of plasma monoamine oxidase against acidic buffers

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Dialysis against sodium diethyldithiocarbamate removed copper from the enzyme with concomitant loss of activity as shown in Table VI. The recrystallized enzyme with a specific activity of 482 was used. Of the enzyme solution, 3 ml containing 60 mg of protein were dialyzed for 12 hours against 200 ml of 0.06 M phosphate buffer (KH₂PO₄-Na₂HPO₄), pH 7.0, containing 0.01 or 0.1 M sodium diethyldithiocarbamate and then dialyzed for 12 hours against 2 liters of the buffer alone to remove the excess chelating agent. To remove the fine, yellow precipitate of the copper-diethyldithiocarbamate complex, the dialyzed enzyme was applied at 4°C to a hydroxylapatite column (1 × 3 cm).

**Table VI**

**Dialysis of plasma monoamine oxidase against sodium diethyldithiocarbamate**

The recrystallized enzyme with a specific activity of 482 was used. Of the enzyme solution, 3 ml containing 60 mg of protein were dialyzed against 200 ml of 0.06 M phosphate buffer (KH₂PO₄-Na₂HPO₄), pH 7.0, containing the designated amounts of the chelating agent for 12 hours. The enzyme was dialyzed for an additional 12 hours against 2 liters of the phosphate buffer without chelating agent. The dialyzed enzyme was passed through a hydroxylapatite column (1 × 3 cm) that had been equilibrated with 0.06 M phosphate buffer, pH 7.0, and was collected in 1-ml fractions. Five-milliliter fractions of the resolved enzyme were used for activity and copper determinations.

<table>
<thead>
<tr>
<th>Concentration of sodium diethyldithiocarbamate</th>
<th>Specific activity</th>
<th>Copper content</th>
</tr>
</thead>
<tbody>
<tr>
<td>M units/mg protein</td>
<td>ppmoles/mg protein</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>482</td>
<td>13.93</td>
</tr>
<tr>
<td>0.01</td>
<td>11.0</td>
<td>1.84</td>
</tr>
<tr>
<td>0.10</td>
<td>5.7</td>
<td>0.53</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity</th>
<th>Copper content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>482</td>
<td>13.93</td>
</tr>
<tr>
<td>2. Dialysis against 0.1 M sodium diethyldithiocarbamate</td>
<td>9 0.63</td>
<td></td>
</tr>
<tr>
<td>3. Dialysis of Treatment 2 against cupric sulfate</td>
<td>341 12.64</td>
<td></td>
</tr>
<tr>
<td>4. Dia- lysis of Treatment 2 against cuprous sulfate</td>
<td>173 38.71*</td>
<td></td>
</tr>
</tbody>
</table>

*See Footnote 5. Chemical analysis indicated that all the copper was in the cupric state.

that had been equilibrated with 0.06 M phosphate buffer, pH 7.0. The complex precipitated on the top of the column, and the resolved enzyme, which passed through the column, was collected in 1-ml fractions. The resolved enzyme, which was in the orange-yellow fractions, was obtained in 70% yield (5 ml) and was used for activity and copper determinations.

The absorption spectra of the enzyme before and after sodium diethyldithiocarbamate treatment are shown in Fig. 3. The absorption maximum at 480 m previously observed in the original enzyme was abolished by the sodium diethyldithiocarbamate treatment, and the new maximum at about 380 nm was observed in the copper-free enzyme.

Anaerobic addition of substrate benzylamine showed no effect on the spectrum of the copper-free enzyme. However, anaerobic addition of sodium dithionite bleached the maximum at 380 nm of the enzyme (Fig. 3, insert).

**Reconstitution of Plasma Monoamine Oxidase by Copper Ions**—When the sodium diethyldithiocarbamate-treated enzyme (50 mg) was dialyzed for 12 hours against 200 ml of 0.01 M Tris buffer, pH 7.0, containing 5 × 10⁻⁵ M cupric sulfate, and a second dialysis against 2 liters of the buffer alone to remove the excess copper was performed, 65 to 70% of the original activity was restored, and the copper content of the enzyme increased to about the original content. Dialysis against cuprous sulfate (5 × 10⁻⁵ M cupric sulfate + 1 × 10⁻⁴ M ascorbic acid) resulted in 30 to 35% restoration of the original activity (Table VII). It seems that some of the cuprous copper was autoxidized to cupric copper, thus accounting for the observed enzymatic activity.

It appears that more cuprous copper is incorporated than cupric copper initially and that all of the cuprous copper incorporated is autoxidized during dialysis and the subsequent treatments.
It was further shown that the restoration of the activity was
accompanied by an increase in the absorption at 480 mp and by
a decrease in the absorption at 380 mp in the spectrum of the
restored enzyme (Fig. 3).

Some other metallic ions, such as ferric, ferrous, zinc, and
manganese ions, were also tested to determine whether or not
they were able to restore the activity, but no appreciable acti-
vation of the enzyme was observed.

DISCUSSION

The results presented in this paper indicate that plasma mono-
amine oxidase is a copper protein. The results satisfied many
of the criteria for characterization of a metalloenzyme (24).
These points can be summarized as follows. (a) Copper is bound
to the enzyme, and the copper-enzyme bond is maintained
throughout the purification process. (b) The copper-to-protein
ratio increased proportionately with the increase in specific
activity of the enzyme during the purification procedure. (c)
The inhibitors of plasma monoamine oxidase, such as sodium
dithyldithiocarbamate, cuprizone, sodium cyanide, and
8-hydroxyquinoline, are all chemical reagents known to have a
high affinity for copper.

The copper in plasma monoamine oxidase was found to be
present in the cupric state. This conclusion was based on the
fact that direct determination of copper in the enzyme with
cuprizone accounted for all the copper; on the fact that the
enzyme was inhibited by cuprizone, which is specific for cupric
copper, and not by neocuproine, which is specific for cuprous
copper; and on the fact that cupric copper was more effective
in reactivating the copper-free enzyme. No valency change of
copper during the catalytic activity was observed by the method
of Griffiths and Wharton (11). This conclusion has been con-
firmed by electron paramagnetic resonance studies. 6

The absorption spectra of a number of copper proteins have
been reported (25). Enzymes such as ascorbic acid oxidase
(26), laccase (27), and ceruloplasmin (16) are blue and show an
absorption maximum in the region of about 600 mp. Other
enzymes, such as tyrosinase, do not show this absorption
maximum, but concentrated solutions show an absorption max-
imum in the neighborhood of 325 to 340 mp (28). Plasma
monoamine oxidase showed a main absorption around 480 mp,
and the spectrum does not resemble those of any other copper
proteins.

The results from the spectral studies of crystalline plasma
monoamine oxidase may be summarized as follows. (a) The
native enzyme exhibited an absorption maximum at about 480
mp. (b) The anaerobic addition of the substrate benzylamine
resulted in a bleaching of this maximum (5). (c) The copper-
free enzyme showed an absorption maximum at about 380 mp.
(d) The anaerobic addition of the substrate did not alter the
spectrum of the copper-free enzyme. (e) Addition of sodium
dithionite bleached the maximum of the native as well as of the
copper-free enzyme. Sodium dithionite reduces the copper
from cupric to cuprous state and also may react with the other
prosthetic group of the enzyme to form a colorless product. (f)
The reconstitution of the enzyme by cupric copper was accom-
plished by an increase of the absorption at about 480 mp and a
decrease of the absorption at 380 mp.

Acknowledgment—The authors are indebted to Dr. Laurence
H. Snyder for support of this project.

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