EFFECT OF SALTS ON MITOCHONDRIAL MONOAMINE OXIDASE FROM BOVINE LIVER

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Accepted August 23, 1979

Abstract—The effects of various salts on the activity of mitochondrial monoamine oxidase (MAO) from bovine liver were studied by measuring the initial and long-term enzyme reactions with an oxygen electrode and Warburg's manometer, respectively, using several monoamines as substrates. Salts with a -Cl- group, such as KCl and NaCl, or with a -NO3- group, such as KNO3 and NaNO3, increased the initial MAO activity with tyramine as substrate. NaCl strongly inhibited the long-term enzyme reaction. With tyramine as substrate, NaCl increased the activity only during the first 6 to 7 min of the reaction. Similarly with β-phenylethylamine or butylamine as substrate, NaCl increased the initial MAO activity, but decreased the activity in the long-term reaction. On the other hand, with serotonin or benzylamine as substrate, NaCl decreased both the initial reaction and the long-term reaction. The effect of NaCl on the enzyme with tyramine as substrate was reversible, while with serotonin as substrate it was irreversible. These results indicate that the effect of NaCl depends on the substrate used and suggest that bovine liver mitochondria contain at least two different forms of MAO.

There are many reports on multiple forms of mitochondrial monoamine oxidase (MAO) [monoamine: O2 oxido reductase (deaminating) EC 1.4.3.4] by studies on differences in enzymic properties in different animal species (1) and different organs of a single species (2) and the effects of oxygen (3), heat treatment (4), metal ions (5, 6) and various MAO inhibitors (7-11).

Recently, Shimamoto (12) reported that ammonium sulfate increased the initial activity of mitochondrial MAO from bovine liver, but decreased the long-term reaction of the enzyme. He also found that -SO4 groups increased MAO activity, whereas -NH4 groups decreased the activity.

This report describes the effects of various salts on the initial and long-term reactions of MAO from bovine liver mitochondria. Results showed that the effect of NaCl depended on the substrate used.

MATERIALS AND METHODS

1) Enzyme preparation: Fresh bovine liver obtained from a slaughter-house was rapidly frozen and stored at -20°C until use. After thawing, the liver was cut into small pieces and homogenized with 3 volumes of chilled 0.25 M sucrose buffered at pH 7.5 with 10 mM Tris-HCl buffer in a Waring blender or a Potter-type glass homogenizer at 4°C. The homogenate was centrifuged at 600 × g for 10 min at 4°C and the supernatant obtained was
recentrifuged at 8500 × g for 20 min. The resulting precipitate was suspended in 3 volumes of 0.1 M Tris-HCl buffer (pH 7.5) and used as the mitochondrial MAO preparation.

2) Measurement of MAO activity: MAO activity was determined by measuring oxygen consumption in the enzyme reaction. Oxygen consumption in the first one min after addition of substrate was measured with an oxygen electrode; oxygen consumption in the long-term reaction was measured manometrically for 60 min.

a) Measurement of oxygen consumption with an oxygen electrode: For measurement of the initial reaction, a Clark's oxygen electrode was used for measurement of oxygen consumption as described by Sho et al. (13) in medium of pH 7.5 at 38°C. Tyramine, benzylamine, β-phenylethylamine, butylamine or serotonin, at a concentration of 10 mM was used as substrate. Enzyme preparation of 62.5 mg dry wt. was used for this experiment. MAO activity was expressed as the amount of oxygen consumption in μM O₂ per min per mg dry wt. of enzyme preparation.

b) Manometric measurement: The long-term reaction was followed manometrically as described by Kamijo et al. (14). The reaction was carried out at pH 7.5 and 38°C for 60 min with the substrates listed above, and activity was expressed as oxygen consumption in μl O₂ per hr per mg dry wt. of enzyme preparation. The enzyme reaction was found to be linear during the incubation period and with the amounts of the enzyme preparation used. The ratio of MAO activity measured by oxygen electrodic and manometric method was 1 : 1.9–2.4.

RESULTS

Effects of various salts on mitochondrial MAO in bovine liver: The effects of NaCl, NaNO₃, KNO₃, and KCl at concentrations of 50 mM to 2 M on mitochondrial MAO from bovine liver were studied with 10 mM tyramine as substrate.

As shown in Table 1, in the initial reaction, MAO activity increased with increase in the concentrations of these salts from 50 mM to 2 M. With 2 M of each salt, the activity

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>% MAO activity Initial reaction</th>
<th>Long-term reaction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>KCl</td>
<td>KNO₃</td>
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<tr>
<td>0.05</td>
<td>102.0</td>
<td>101.1</td>
</tr>
<tr>
<td>0.5</td>
<td>102.9</td>
<td>111.4</td>
</tr>
<tr>
<td>1.0</td>
<td>122.7</td>
<td>118.2</td>
</tr>
<tr>
<td>1.5</td>
<td>137.3</td>
<td>136.4</td>
</tr>
<tr>
<td>2.0</td>
<td>144.1</td>
<td>150.1</td>
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The initial reaction was determined with an oxygen electrode and the long-term reaction by Warburg's manometric method as described in the text, with 10 mM tyramine as substrate. MAO activity is shown as a percentage of the control activity (0.141 μM O₂/min/mg dry wt. by an oxygen electrodic method and 2.08 μl O₂/hr/mg dry wt. under 20% oxygen atmosphere by Warburg's manometric method, respectively) without added salt.
was about 145% to 160% of the control activity. The effects of NaCl on the initial and long-term reactions were examined and the results are shown on the right in Table 1. The activity in the initial reaction increased with increase in concentration of NaCl, and with 2 M NaCl, it was about 155% of the control activity. On the contrary in the long-term reaction the activity decreased with increase in concentration of NaCl and with 2 M NaCl it was about 66% of the control activity (Table 1).

*Time course of the effect of NaCl on the initial reaction:* The effect of NaCl on MAO activity was determined with an oxygen electrode at one min intervals after addition of 10 mM tyramine.

As shown in Fig. 1, the activity with 1.5 M NaCl was about 150% of the control activity for the first few min and then decreased to the control level after 6 or 7 min.

*Effect of NaCl on the substrate specificity of MAO in the initial reaction:* The effect of NaCl on the substrate specificity of MAO was tested using tyramine, benzylamine, \( \beta \)-phenylethylamine, butylamine and serotonin at concentrations of 10 mM. Results obtained with an oxygen electrode are shown in Fig. 2. With tyramine as substrate, the activity was not affected by 10 mM or 50 mM NaCl, but it gradually increased with increase in concentrations of NaCl from 50 mM to 2 M NaCl, the activity with 2 M NaCl being 133% of the control value.

With benzylamine as substrate, MAO activity was increased by low concentrations of NaCl, and with 10 mM NaCl it was 114% of the control. However, with higher concentrations of 100 mM to 2 M NaCl it decreased with increase in concentration of NaCl and

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**Fig. 1.** Time-course of effect of NaCl on initial reaction of mitochondrial MAO. Activity with 10 mM tyramine as substrate was determined with an oxygen electrode once a min from the start of the reaction. ○—○: control MAO activity. ●—●: MAO activity with 1.5 M NaCl.

**Fig. 2.** Effect of NaCl on substrate specificity of mitochondrial MAO. The initial activity with 10 mM tyramine, benzylamine, \( \beta \)-phenylethylamine, butylamine or serotonin as substrate, was determined using an oxygen electrode. Substrate: ○—○: tyramine. △—△: benzylamine. ●—●: \( \beta \)-phenylethylamine. ○—○: butylamine. ▲—▲: serotonin.
with 2 M NaCl it was 25% of the control activity. With β-phenylethylamine as substrate 50 mM to 1 M NaCl increased the activity less than 10%, but 2 M NaCl increased the activity to 141% of the control. The activity with butylamine as substrate was not affected appreciably by 50 mM to 100 mM NaCl but was increased by 0.25 M to 2 M NaCl, the activity with 2 M NaCl being 152% of the control. The activity with serotonin as substrate was decreased slightly (less than 10%) by 50 mM to 100 mM NaCl, but was decreased appreciably and progressively by increase in NaCl concentration from 100 mM to 2 M NaCl.

Thus with tyramine, β-phenylethylamine or butylamine as substrate, MAO activity was increased by addition of NaCl, whereas with serotonin or benzylamine as substrate it was decreased; that is, the effect of NaCl on the activity depended on the substrate used.

Effects of NaCl and oxygen concentration on the long-term reaction: The effect of NaCl on MAO activity in the long-term reaction was studied manometrically with various substrates and atmospheres of 20% and 100% oxygen.

As shown in Table 2, under 20% oxygen, the activity decreased with increase in NaCl concentration with all the substrates tested and the activity with tyramine or β-phenylethylamine was 75% of the control, that with butylamine was 50% of the control, and that with benzylamine or serotonin was 30% to 40% of the control activity. Similar results were obtained in reactions under 100% oxygen.

Thus, in the long-term reaction, MAO activity with all substrates tested decreased with increase in NaCl concentration both under 20% and 100% oxygen.

Effects of NaCl on the pS curve of MAO: As described above, NaCl had opposite effects on the initial reactions with tyramine and serotonin as substrates. Therefore, we

<table>
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<tr>
<th>Substrate</th>
<th>O₂ (%)</th>
<th>NaCl (M)</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
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<tr>
<td>Tyramine</td>
<td>20</td>
<td>92.3</td>
<td>93.5</td>
<td>91.3</td>
<td>91.3</td>
<td>85.2</td>
<td>73.7</td>
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<td>100</td>
<td>93.2</td>
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<td>88.1</td>
<td>78.4</td>
<td>70.8</td>
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<td></td>
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<tr>
<td>Benzylamine</td>
<td>20</td>
<td>100.4</td>
<td>85.1</td>
<td>66.3</td>
<td>58.5</td>
<td>40.0</td>
<td>33.0</td>
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<tr>
<td></td>
<td>100</td>
<td>100.5</td>
<td>92.2</td>
<td>52.7</td>
<td>47.9</td>
<td>43.4</td>
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<td>S-Phenylethylamine</td>
<td>20</td>
<td>84.4</td>
<td>101.1</td>
<td>82.4</td>
<td>57.7</td>
<td>65.9</td>
<td>74.5</td>
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<tr>
<td></td>
<td>100</td>
<td>107.3</td>
<td>90.7</td>
<td>85.0</td>
<td>71.5</td>
<td>72.5</td>
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<tr>
<td>Butylamine</td>
<td>20</td>
<td>102.1</td>
<td>83.3</td>
<td>67.6</td>
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<td>100</td>
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<td>75.9</td>
<td>65.9</td>
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<tr>
<td>Serotonin</td>
<td>20</td>
<td>96.1</td>
<td>93.5</td>
<td>75.3</td>
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<td>58.3</td>
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<td>67.6</td>
<td>49.0</td>
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</table>

MAO activity in the long-term reaction was determined by Warburg's manometric method and is expressed as a percentage of the control activity (with tyramine as substrate 2.08 µl O₂/hr/mg dry wt. under 20% oxygen atmosphere and 7.08 µl O₂/hr/mg dry wt. under 100% oxygen atmosphere, with benzylamine 1.70 µl O₂ and 2.12 µl O₂, with β-phenylethylamine 0.71 µl O₂ and 1.56 µl O₂, with butylamine 2.23 µl O₂ and 4.84 µl O₂, with tryptamine 1.80 µl O₂ and 4.39 µl O₂, with serotonin 0.87 µl O₂ and 1.84 µl O₂, respectively) without salt.
examined the effect of 1.5 M NaCl on the pS curves of MAO with tyramine and serotonin as substrates.

As shown in Fig. 3 (left), in the absence of NaCl, MAO activity was maximal with 10 mM tyramine. Addition of 1.5 M NaCl, increased the activity with 10 mM tyramine by 60% and that with 1 mM tyramine by 48%; it had no appreciable effect on the activity with less than 0.1 mM tyramine (Fig. 3). As shown in Fig. 3 (right), in the absence of NaCl, MAO activity was maximal with 5 mM serotonin. Addition of NaCl decreased the activities with 5 mM and 10 mM serotonin 40% and 30%, respectively, but increased the activity with less than 1 mM serotonin (Fig. 3, right).

Lineweaver-Burk’s plots of mitochondrial MAO in bovine liver: Lineweaver-Burk’s plots of the initial MAO activity with tyramine or serotonin as substrate (1 mM–0.2 mM) in the presence and absence of 2 M NaCl were determined with an oxygen electrode.

As shown in Fig. 4, the Km value for tyramine was 0.17 mM. The Lineweaver-Burk’s plots of activity with and without 2 M NaCl crossed at a point on the abscissa, indicating that the presence of 2 M NaCl did not affect the dissociation constant of the enzyme-tyramine complex.
complex but increased the Vmax. Similar results were obtained with serotonin; namely, the Km value was 0.17 mM and the plots for activity with and without salt crossed at a point on the abscissa indicating same as with tyramine (Fig. 5).

Reversibility of the effect of NaCl on MAO: The reversibility of the effect of 2 M NaCl on MAO activity was studied by dialysis (Table 3). Activity with tyramine or serotonin was measured with an electrode before and after dialysis of reaction mixture containing 2 M NaCl for 16 hr in a cold room at 4°C. As shown in Table 3, with tyramine as substrate, the activity was increased to 128% of the control activity immediately after addition of 2 M NaCl but after dialysis for 16 hr, the activity was almost the same as that of the control. The activity of an undialyzed preparation that had stood for 16 hr in the cold room, was 114% of the control.

With serotonin as substrate, the activity decreased to 72% of the control immediately after addition of 2 M NaCl and 70% of the control after dialysis for 16 hr.

These results, summarized in Table 3, indicate that with tyramine as substrate, the activation by NaCl was reversible, whereas with serotonin as substrate, the inhibition by NaCl was irreversible.

DISCUSSION

Recently, Shimamoto (12) reported that ammonium sulfate, which does not affect the
activities of most enzymes, increased the initial activity of mitochondrial MAO from bovine liver, but decreased its activity in a long-term reaction. In the present work, we examined the effects of various salts (KCl, KNO₃, NaNO₃, and NaCl) on the initial and long-term reactions of mitochondrial MAO from bovine liver.

Results showed that the initial activity increased with increase in concentration of these salts, whereas the activity in the long-term reaction decreased with increase in concentration of NaCl. Activity in the initial reaction was increased by NaCl for only a few min and then returned to almost the control level. NaCl had similar effects on MAO activity in reaction mixtures under 20% and under 100% oxygen. The increase in MAO activity by NaCl in the initial few min of the reaction was neglected in studies on the long-term reaction, because activity in the first 10 min could not be estimated by the manometric method.

The initial MAO activity with tyramine or serotonin as substrate was increased by NaCl in the Lineweaver-Burk's plots. The Km values for these two substrates in the initial reaction were both 0.17 mM and NaCl increased the Vmax's but did not affect the Km values. These findings indicate that NaCl does not have a direct effect on the active center of MAO for these two substrates, and thus suggest that it affects other parts of the enzyme indirectly, resulting in increase in activity in the initial reaction and decrease in activity in the long-term reaction.

It has been reported that mitochondrial MAO from pig liver is mainly composed of two subunits and that the active form has a minimum M.W. of about 60,000 (15) and three fractions of mitochondrial amine oxidase from beef liver were isolated by purification procedure (16). Pargyline, a specific inhibitor of mitochondrial MAO, combines with the active center of the enzyme in a ratio of 1:1, inhibiting the activity irreversibly (17-19).

One possible explanation for the different effects of NaCl on MAO activities in the initial and long-term reactions is that MAO exists in several different forms with different activities which are in stable equilibrium with each other, and that NaCl changes the equilibrium between these forms, resulting in higher activity in the initial reaction and lower activity in the long-term reaction. The possibility that NaCl causes conformational changes of MAO is supported by the fact that a low concentration of NaCl reversibly activates choline acetyl transferase from human placenta and simultaneously renders the enzyme less stable to heat treatment and proteolytic enzymes (20).

We found that the decrease in MAO activity by NaCl was not affected markedly by the concentration of oxygen in the reaction mixture. Oxygen is considered to be a second substrate for MAO, because the reaction proceeds by a ping-pong mechanism (21, 22), but our finding that oxygen concentration does not influence the effect of NaCl suggests that NaCl does not participate in the step of combination of oxygen with MAO.

The following general mechanisms of activation of enzymes by salts have been proposed (23).

(1) Formation of an enzyme-salt complex that is the only catalytically active form.
(2) Formation of a substrate-salt complex that is a true substrate of the enzyme.
(3) Combination of the salt with the enzyme-substrate complex.
The fact that even purified MAO does not require any metal for activity (9), excludes possibility (1). However, possibility (2) or (3) is supported by the fact that NaCl had different effects on the initial reaction of MAO depending on the substrate: for example, with tyramine or β-phenylethylamine as substrate, NaCl increased the activity, whereas with benzylamine as substrate, it decreased the activity.

However, as stated above, NaCl did not have a direct action on the active site of MAO. Thus, with tyramine or β-phenylethylamine as substrate, NaCl must increase formation of the enzyme-substrate complex and/or form a substrate-NaCl complex that is the true substrate for the enzyme, resulting in increase in enzyme activity. Conversely, with benzylamine or serotonin as substrate, NaCl must decrease formation of the enzyme-substrate complex and/or decrease the concentration of substrate available by combining with it, thus decreasing the activity. With serotonin as substrate, the inhibition by NaCl was irreversible and thus other explanations should also be considered.

From the fact that NaCl which had opposite effects on MAO activity with different substrates must be explained by different mechanisms, it seems probable that mitochondrial MAO from bovine liver exists in more than one form, like MAO in other mammalian tissues (9, 10, 24) and that these forms are affected in different ways by NaCl.

We found that the other salts tested had similar effects to those of NaCl on MAO activity. Thus the effect of NaCl on MAO is not specific.

Finally, judging from the different responses to NaCl of mitochondrial MAO from bovine liver observed in both initial and long-term enzyme reactions with various substrates, it is concluded that the enzyme must exist in at least two different forms.

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