PURIFICATION AND SOME PROPERTIES OF L-ALANINE:4,5-DIOXOVALERIC ACID TRANSAMINASE FROM RAT LIVER MITOCHONDRIA

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SUMMARY: L-alanine:4,5-dioxovalerate transaminase (EC 2.6.1.44) has been purified to homogeneity from rat liver mitochondria. Molecular weight of the native enzyme is estimated to be 230,000 ± 3000 by gel filtration. Under denaturing condition, the dissociated enzyme has a subunit of approximately 41,000 ± 2000, indicating the enzyme apparently is composed of six identical subunits. The enzyme is heat stable and has optimal activity at pH 6.9. Km values for L-alanine and 4,5-dioxovalerate are 3.3 x 10^-3 M and 2.8 x 10^-4 M respectively. Excess dioxovalerate inhibits the enzyme activity. Pyridoxal phosphate and dithiothreitol also inhibit the enzyme activity.

INTRODUCTION:

The formation of 8-aminolevulinic acid by the condensation of glycine and succinyl CoA with the enzyme ALA synthetase (EC 2.3.1.37) is the first step of heme biosynthetic pathway (1-4). However, Verticoviski et al. (5,6) have reported recently the formation of 8-aminolevulinic acid via a transamination reaction in bovine liver. Furthermore, they showed that this dioxovalerate transaminase synthesizes 8-aminolevulinic acid more efficiently than aminolevulinic acid synthetase, isolated from the same source and suggested a significant role on the synthesis of ALA. Since ALA synthetase is known to regulate

Abbreviations: ALA, 8-aminolevulinic acid; DOVA, 4,5-dioxovaleric acid; PLP, Pyridoxal phosphate; DTT, Dithiothreitol.
the heme biosynthetic pathway (7-9), the enzyme DOVA transaminase might have some important role in the regulation of heme biosynthesis. As a part of our programme to study the physiological significance of this enzyme on heme biosynthetic pathway, we have purified the enzyme from rat liver and characterized partially.

**MATERIALS AND METHODS**

δ-aminolevulinic acid, L-Alanine, Pyridoxal phosphate, Dithiothreitol, Bentonite, Protamine sulphate were obtained from Sigma Chemical Co., St. Louis, Mo. Phenyl Sepharose CL-4B, Sepharose-6B, Molecular weight calibration kit from Pharmacia Fine Chemicals, Uppasala, Sweden. DEAE cellulose DE52 was obtained from Whatman Biochemicals Ltd., Maidstone, England. Polyethylene glycol 20,000 was from Fluka, Bucha Switzerland, Acrylamide, coomassie brilliant blue R-250 were obtained from Bio-Rad, Richmond, California. 3,5-Dibromolevulinic acid was a gift from Bruce F. Burnham, University College of Medicine, Utah, USA.

4,5-Dioxovalerate was synthesized from 3,5-dibromolevulinic acid as described by Varticovski et al. (5).

**Enzyme assay:** Alanine:4,5-dioxovalerate transaminase was assayed according to the method of Varticovski et al. (5) δ-Aminolevulinic acid formed was converted into 2-methyl-3-carbethoxy 4-(3-propionic acid) pyrrole by the method of Mauzerall and Granick and Ehrlich chromophor measured at 553 nm.

Protein was determined by the method of Bradford (11).

**Electrophoresis:** The purity of the enzyme was checked by gel electrophoresis which was performed by the discontinuous pH 8.9 method of Davis (12) using 7.5% polyacrylamide gels. SDS-PAGE using 10% acrylamide gel was done according to the procedure of Laemmli (13).

**Purification of L-alanine:4,5-dioxovalerate transaminase:** All purification procedures were carried out at 4 °C unless stated otherwise. The mitochondria were isolated from the livers of 48 hr fasted male Wistar rats (175-200 g) by the method of Johnson and Lardy (14). The packed mitochondria were suspended in 5 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol, freeze-thawed, sonicated and centrifuged at 10,000 x g for 30 min.

To 100 ml of mitochondrial supernatant, 20 ml of 2% (W/V) protamine sulphate was slowly added with stirring for 20 min. The mixture was centrifuged for 10 min at 10,000 x g. Then
Bentonite was added to the resulting supernatant to 1.25% saturation and stirred for 15 min. The precipitated proteins were removed by centrifugation at 10,000 x g for 10 min. The supernatant was passed through a column (2.5 x 11 cm) of phenyl Sepharose CL-4B using 5 mM potassium phosphate buffer pH 7.6 containing 10% glycerol. The enzyme did not bind to the hydrophobic support and was eluted in the buffer wash. Then this sample was loaded on DEAE cellulose DE-52 column (4 x 7.5 cm) previously equilibrated with same buffer. After washing with 150 ml of buffer, the enzyme was eluted with 500 ml linear gradient of 0 - 0.25 M KCl in the same buffer at the flow rate of 36 ml/hr. The enzyme was eluted approximately in the middle of the gradient (Fig. 1). The fractions of highest activity were pooled and concentrated with polyethylene glycol. Above concentrated sample was further applied to a column (2.0 x 37 cm) of Sepharose-6B equilibrated with the same buffer and flow rate was 16 ml/hr. The active fractions were pooled, concentrated and stored at -20°C.

RESULTS AND DISCUSSION

This is the first report on the purification of rat liver alanine: DOVA transaminase using a new procedure to apparent
homogeneity as demonstrated by polyacrylamide gel electrophoresis (Fig. 2). A typical purification procedure is given in Table 1. The enzyme was purified approximately 420 fold with overall yield of 20%. The final enzyme preparation has a specific activity of 59.5 U/mg protein. The specific activity is higher than previously reported values for homogenous bovine liver alanine DOVA transaminase (5,15). The purified enzyme in 5 mM potassium phosphate buffer pH 7.6 containing 10% glycerol is stable at -20°C for at least a month without loss of activity.

As in the case of bovine enzyme (5,15) the apparent molecular weight of the native enzyme was estimated to be 230,000 ± 3000 by sepharose-6B gel filtration (Fig. 3a). The enzyme contains six identical subunits of molecular weight 41,000 ± 2000 daltons, as revealed by SDS-polyacrylamide (Fig. 3b). This re-

Fig. 2. Polyacrylamide disc gel electrophoresis of the purified L-alanine:4,5-dioxovalerate transaminase. The purified enzyme preparation (protein 14 µg) was subjected to electrophoresis at pH 8.9 in 7% gel.
sult is in contrast to the value of bovine liver (15) where it was reported as homotetramer. As shown in Fig. 4 and Fig. 5, $K_m$ values for L-alanine and 4,5-dioxovalerate are $3.3 \times 10^{-3}$ M and $2.8 \times 10^{-4}$ M respectively. Furthermore, Lineweaver-Burk plots generated a series.

Table 1

Purification of L-alanine:4,5-dioxovalerate transaminase from rat liver mitochondria

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Alanine:4,5-dioxovalerate transaminase</th>
<th></th>
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<tr>
<td></td>
<td>protein</td>
<td>total activity</td>
<td>specific activity</td>
<td>purification</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>----------------</td>
<td>------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Mitochondrial extract</td>
<td>195.24</td>
<td>27.6</td>
<td>0.1413</td>
<td>1</td>
</tr>
<tr>
<td>Protamine sulphate</td>
<td>99.37</td>
<td>22.68</td>
<td>0.226</td>
<td>1.6</td>
</tr>
<tr>
<td>Bentonite</td>
<td>35.75</td>
<td>18.48</td>
<td>0.526</td>
<td>3.7</td>
</tr>
<tr>
<td>Phenyl Sepharose CL-4B</td>
<td>2.11</td>
<td>15.34</td>
<td>7.240</td>
<td>51.3</td>
</tr>
<tr>
<td>DEAE Cellulose D5-52</td>
<td>0.269</td>
<td>6.38</td>
<td>24.63</td>
<td>174.7</td>
</tr>
<tr>
<td>Sepharose-6B</td>
<td>0.094</td>
<td>5.6</td>
<td>59.5</td>
<td>421.9</td>
</tr>
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</table>

Superscript aOne unit is the amount of enzyme that will catalyse the formation of 1 $\mu$ mol of 8-aminolevulinic acid/30 min at 37°C.
Fig. 3. Determination of the molecular weight of purified alanine:4,5-dioxovalerate transaminase in native (a) and denatured (b) form. a, Sepharose-6B (2.0 x 37 cm) column was calibrated with 1 mg of Ferritin (440,000), 2 mg of Thyroglobulin (669,000), and Aldolase (150,000), 5 mg of catalase (232,000) and 1 mg of enzyme was applied. b, SDS-polyacrylamide gel electrophoresis of alanine:4,5-dioxovalerate transaminase. 10 μg of each standard and the enzyme were treated and electrophoresed as described under "Experimental procedures". Calculation of mobilities of protein standards electrophoresed simultaneously are expressed relative to that of the bromophenol blue dye front.

Fig. 4. Lineweaver-Burk double reciprocal plots of initial velocity of alanine:DOVA transaminase activity against DOVA concentration at a series of fixed alanine concentrations.
Fig. 5. Lineweaver-Burk double reciprocal plots of initial velocity of alanine:DOVA transaminase activity against alanine concentration at a series of fixed DOVA concentrations.

of parallel lines indicating a ping-pong reaction mechanism which is characteristic of transaminase.

The purified enzyme when heated for 30 min. at 65°C lost 88% of its activity. The enzyme has optimal activity at pH 6.9 with a broad peak exhibiting 90% activity at pH 6.3 and at 7.4. The enzyme activity was markedly decreased by the addition of pyridoxal phosphate and dithiothreitol to the incubation mixture (Table 2). As it was reported in Rhodopseudomonas spheroides (16), even in our studies excess concentrations of dixovalerate inhibited the enzyme activity (Table 2).

The availability of large quantities of purified enzyme will allow the production of antibody. In future, we propose to study the role of specific 4,5-dixovalerate transaminase in different erythropoietic conditions to enlighten its role on heme biosynthesis.
Table 2
Inhibition of L-alanine:4,5-dioxovalerate transaminase by pyridoxal phosphate, dithiothreitol and 4,5-dioxovaleric acid

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Specific Activity (µ mol./30 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.63</td>
</tr>
<tr>
<td>PLP</td>
<td></td>
</tr>
<tr>
<td>2.5 mM</td>
<td>4.11 (78)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>2.03 (89)</td>
</tr>
<tr>
<td>DTT</td>
<td></td>
</tr>
<tr>
<td>2.0 mM</td>
<td>8.25 (66)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>1.88 (90)</td>
</tr>
<tr>
<td>DOVA</td>
<td></td>
</tr>
<tr>
<td>1.5 mM</td>
<td>13.91 (29)</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>11.88 (41)</td>
</tr>
<tr>
<td>4.0 mM</td>
<td>5.77 (70)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses are percent inhibition.

ACKNOWLEDGEMENT

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REFERENCES


Embryotoxicity of Styrene and Its Effect on Heme Biosynthesis

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Effect of styrene exposure on chick embryos during different developmental stages was studied, and an attempt was made to correlate its embryotoxicity with the process of heme biosynthesis at sublethal doses (0.25 - 5 μmole/egg). Styrene was injected directly into the yolk sac of embryo on 3rd, 7th or 14th day of incubation. Increased mortality was observed with higher concentrations of styrene, and the age of embryo was a critical factor in the manifestation of styrene toxicity. Styrene exposure to 14-day-old chick embryo increased the hepatic heme content whereas hepatic δ-aminolevulinic acid synthetase was inhibited. A possible alteration of heme biosynthesis with styrene exposure is suggested.

Styrene (vinyl benzene), the precursor of thermoplastic polymers, is extensively used in the manufacture of a variety of plastics, synthetic rubbers, resins and insulating materials. Thus, the possibility of styrene as a industrial pollutant is predominant. Recently styrene has been shown as mutagenic, carcinogenic and cytotoxic agent. Although embryotoxicity of styrene in higher concentrations (25-100 μmole/egg) has been reported in chick embryos, further studies are required to find embryotoxicity of styrene at low level, where the occupational hazard would be feasible.

Styrene is known to bind predominantly to phenobarbital-induced cytochrome P-450, a heme protein in rat liver microsomes. Though the drug metabolising enzymes are absent or remain at very low level during embryonic development of normal chick liver, cytochrome P-450 linked microsomal monooxygenases can be induced with foreign compounds, which in turn enhance the synthesis of cytochrome P-450. The synthesis of cytochrome P-450 is regulated by heme. Further, it is well established that heme regulates its own synthesis by controlling δ-aminolevulinic acid (ALA) synthetase activity, the rate limiting enzyme of heme biosynthetic pathway. Therefore, the present study was undertaken to assess the embryotoxicity of styrene at sublethal doses (0.25 - 5.0 μmole/egg) and its effect on heme biosynthesis at the level of ALA synthetase and heme.

Eggs (0-day-old) of White Leghorn strain (obtained from Government Poultry Farm, Satbari, New Delhi) were incubated at 37°C with 65% RH and excess of fresh air. The eggs were rotated manually once in a day during incubation.

Styrene (purchased from SISCO, India) was dissolved in ethanol and subsequently diluted with olive oil to the desired concentrations. Different doses of styrene (0.25 - 5 μmole/egg) in a total volume of 50 μl were injected directly into the yolk sac through a small hole at different stage on 3rd, 7th or 14th day of embryonic development. The hole was covered with surgical tape to prevent infection. Control eggs were given the same amount of solvent injection and another group of eggs were maintained as uninjected controls. The mortality was monitored every day through candler.

ALA synthetase was assayed according to the method of Piedra et al. and ALA formation was determined by the modified method of Mauzerall and Granick. Heme extraction from liver was done according to Garber and Maes and estimated by the method of De Duve. Protein determination was performed by the method of Lowry et al.

Embryotoxicity of styrene is observed even at low doses like 0.25 to 5.0 μmole/egg (Fig. 1). A gradual increase in the rate of mortality is evident with increasing concentration of styrene injection. Maximum mortality is obtained at the earlier stages of development, which is already reported with higher concentrations of styrene (25-100 μmole/egg) by Vainio et al.

The present study further reveals that styrene exposure increases hepatic heme level and can affect its...
biosynthesis at the level of ALA synthetase. The hepatic heme level of styrene treated chick embryo on 14th day of development is shown in Table 1. It is evident that the heme levels are significantly elevated after exposure.

Since there is an alteration of heme level with styrene treatment, the enzyme ALA synthetase is also studied (Table 1). A significant inhibition in the activity of ALA synthetase is observed in styrene treated chick embryo. Moreover, the inhibition of hepatic ALA synthetase activity is increased proportionally with the concentration of styrene injection.

This is the first report that indicates styrene can inhibit ALA synthetase in vivo and it can also increase heme level. The increased hepatic heme levels may play an important role on regulation of ALA synthetase activity by feedback mechanism as suggested earlier. Further, endogenously derived glycine is known to be the major amino acid used for hippuric acid formation, which is the detoxified product of styrene. Being the substrate of ALA synthetase, the depletion of glycine may be a limiting factor with styrene exposure, which may ultimately decrease the ALA synthetase activity. Moreover, our result is supported by earlier reports of blockage of heme degradation as cytochrome P-450 binds with styrene in vivo and in vitro and causes an elevation of heme content.

The study reveals an interesting fact that styrene exposure increases heme level though it inhibits the enzyme ALA synthetase, which is responsible for the formation of ALA, the first committed precursor of heme. The biosynthesis of ALA in mammalian cells has been thought to occur exclusively through ALA synthetase until recently several lines of evidence have led to conclusion that ALA is also synthesized by the enzyme l-alanine, 4,5-dioxovaleric acid transaminase. This pathway is more efficient than ALA synthetase. The activity of this enzyme is higher than the ALA synthetase in chick embryo (unpublished data). Studies on alanine DOVA transaminase in styrene exposed chick embryos are needed to elucidate the mechanism of increased heme level.

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References