CHAPTER III

EVIDENCE OF DIFFERENT FORM OF CYTOPLASMIC L-ALANINE:4,5-DIOXOVALERATE TRANSAMINASE FROM MITOCHONDRIAL FORM

INTRODUCTION

The fact that overall regulation of heme biosynthesis is at the level of ALA synthetase is now well established. According to the recent literature there are different ways by which ALA synthetase in concert with heme, the end product of the pathway, exert control on heme biosynthesis (12,122). The feedback regulation of ALA synthetase by heme are at transcriptional (64-66) and post-transcriptional steps (62,63,70,136-138) and at the level of enzyme transfer from cytosol into mitochondria (71,126). It was reported both by Kikuchi et al. (72) and Srivastava et al. (74) that the precursor enzyme was incorporated into mitochondria with attendant processing to the mature enzyme form when the precursor of liver ALA synthetase was incubated with the homologous liver mitochondria. However, addition of hemin in the incubation mixture leads to the inhibition of ALA synthetase
translocation in a dose dependent manner (72). It has also been observed that hemin inhibition of ALA synthetase translocation is specific to ALA synthetase only as hemin seems to be ineffective in bringing about an inhibition of the translocation of another mitochondrial enzyme pyruvate cocarboxylase (74).

The regulation of heme biosynthesis at the level of ALA synthetase has been studied in details by others. The recent discovery of the enzyme alanine: DOVA transaminase, prompted us to examine the role of this enzyme in heme biosynthesis. In the previous chapter, it was shown that alanine: DOVA transaminase is functionally located in the mitochondrial matrix. Moreover, we can show that hemin, the end product of the pathway inhibits alanine: DOVA transaminase activity in vivo and in vitro (139). It is important to mention here that increase in the level of heme in intact mitochondrial preparation (60) at at least 75 times the basal level occurring in vivo, does not inhibit ALA synthetase activity. Therefore, it was concluded that end product inhibition of ALA synthetase cannot be a physiological mechanism for regulation of hepatic heme biosynthesis. thus, considering the above reports and our recent observation, it may be reasonable to assume that potential may exist for the regulation of heme biosynthesis at the level of alanine: DOVA transaminase, the mitochondrial matrix enzyme.
It is generally accepted that most mitochondrial proteins are coded by nuclear genes (40,141) and are synthesised in the cytosol as larger precursor polypeptides having extra sequence at the N-terminal end.

In chapter I, where we have shown the subcellular distribution profile of alanine: DOVA transaminase, we have seen that besides a major activity peak of the enzyme appearing in the mitochondrial fraction, a minor activity peak was seen in the cytosolic fractions when the fractions were assayed at 65°C. The enzyme activity in the cytosolic fractions cannot be due to the breakage of mitochondria that let to the leaking of the enzyme into the cytosol, since the activity of glutamate dehydrogenase, the mitochondrial marker enzyme was not detectable in these fractions. Therefore, this cytosolic form of alanine: DOVA transaminase is expected to be the precursor of the mitochondrial form, which is finally translocated into the mitochondria. If it were true then there is a possibility of a regulatory function operating at the level of translocation of newly synthesized alanine: DOVA transaminase, as in the case of ALA synthetase.

In order to ascertain the nature of the cytoplasmic alanine: DOVA transaminase we have purified this enzyme from cytoplasm and have shown that it is possibly a precursor of the mitochondrial alanine: DOVA transaminase.
RESULTS

PURIFICATION OF CYTOSOLIC L-ALANINE: 4,5-DIOXOVALERATE TRANSAMINASE

All purification procedures were carried out at 4°C, unless stated otherwise. The results of the enzyme purification are summarized in Table VII.

Step 1. Preparation of cytoplasmic extract:

The cytosol was isolated from the kidneys of rats which were fed on standard diet from Hindustan Lever Ltd. (India). The kidneys were cut into very small pieces and were washed thrice with cold buffer and homogenized in 4 volumes of 0.25 M sucrose in 10 mM potassium phosphate buffer, pH 7.6. The crude homogenate was centrifuged at 500 x g for 15 min to sediment cell debris. The postnuclear fraction was centrifuged at 10,000 g for 20 min and the supernatant was retained. The mitochondrial pellet was washed with the same buffer and the supernatant was pooled. The supernatant was further centrifuged at 1,05,000 x g for 3 hrs. The pellet was discarded and the supernatant was used for further step of purification.

Step 2. Fractionation with heat treatment:

The supernatant was heated at 60°C for 10 min. The precipitate was removed by centrifugation at 10,000xg for 20 min.
TABLE VII: PURIFICATION OF ALANINE: DOVA TRANSAMINASE FROM RAT KIDNEY CYTOPLASM

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein (mg)</th>
<th>Total activity (units)*</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (factor)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cytosol</td>
<td>268</td>
<td>6.16</td>
<td>0.023</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>2. Heat treatment</td>
<td>203</td>
<td>5.40</td>
<td>0.027</td>
<td>1.1</td>
<td>87.6</td>
</tr>
<tr>
<td>3. Phenylsepharose CL-4B</td>
<td>24</td>
<td>3.20</td>
<td>0.13</td>
<td>5.7</td>
<td>51.9</td>
</tr>
<tr>
<td>4. Sepharose-6B</td>
<td>18</td>
<td>3.01</td>
<td>0.17</td>
<td>7.2</td>
<td>49.0</td>
</tr>
<tr>
<td>5. DEAE Cellulose DE-52</td>
<td>8</td>
<td>2.31</td>
<td>0.29</td>
<td>12.5</td>
<td>37.0</td>
</tr>
<tr>
<td>6. L-Alanine-Sepharose-4B</td>
<td>0.3</td>
<td>2.0</td>
<td>6.67</td>
<td>289.8</td>
<td>32.0</td>
</tr>
</tbody>
</table>

*One unit is the amount of enzyme that catalyzes the formation of 1 umol of δ-aminolevulinic acid in 30 min at 65ºC.
Step 3. Phenylsepharose CL4B column chromatography:

The supernatant obtained from the above was applied to a column of Phenylsepharose CL-4B (2.9 x 11 cm) using 10 mM potassium phosphate buffer, pH 7.6. The enzyme did not bind to the hydrophobic support and was eluted in the buffer wash. The fractions with high activity were pooled and concentrated using PEG 20,000.

Step 4. Sepharose-6B column chromatography:

The concentrated sample (4 ml) was then loaded onto Sepharose-6B column (2.5 x 42 cm) and eluted with equilibrating buffer at a flow rate of 6 ml/hr.

Step 5. DEAE Cellulose DE-52 column chromatography:

The active fraction from the previous column were pooled and further loaded on DEAE Cellulose DE-52 column (2.75 x 19 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6. The enzyme was eluted with 0 - 0.5 M KCl gradient of total volume (400 ml) at the flow rate of 20 ml/hr. The enzyme was eluted approximately in the middle of the gradient as shown in Figure 19.

Step 6. Alanine-Sepharose-4B column chromatography:

The pooled fraction of DEAE Cellulose DE-52 was applied finally to Alanine-Sepharose 4B column (1.32 x 4 cm), which was
Fig. 19: DEAE-Cellulose DE-52 column chromatography of rat kidney cytosolic alanine: DOVA transaminase. The column was run as described in the "Results" of chapter III.
previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol. The column was washed first with 100 ml of the same buffer and then the enzyme was eluted with 0 - 0.4 M KCl linear gradient of total volume 200 ml of the same buffer at the flow rate of 20 ml/hr (Fig. 20). The fractions having activity of alanine: DOVA transaminase were pooled and concentrated using Amicon-Ultra filtration cone and the enzyme sample was dialyzed against 10 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol, which was then stored at -20°C.

**Purity**

The enzyme appeared to be homogenous yielding a single band on polyacrylamide gel electrophoresis at pH 8.9 (Fig. 21).

**Optimal condition**

The cytoplasmic alanine: DOVA transaminase was shown to have pH optimum at 6.6 and this enzyme is also most active at 65°C.

**Molecular weight**

The apparent molecular weight of the cytosolic native enzyme was estimated to be 3,30,000 dalton by Sepharose-6B gel filtration as shown in Figure 22. The cytosolic enzyme contains six identical subunits of molecular weight 55,000 daltons, under
Fig. 20: L-alanine: Sepharose-4B affinity chromatography of rat kidney cytosolic alanine: DOVA transaminase. The column was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6. After extensive washing, the elution of the enzyme was done with potassium chloride gradient of 0-0.4 M in the same buffer. Fraction size of 4 ml was collected and the flow rate was maintained at 20 ml/hour.
Fig. 21: Polyacrylamide gel electrophoresis of the purified cytosolic alanine: DOVA transaminase 25 µg of the sample was electrophoresed at pH 8.9 in 7.5% gel.
Fig. 22: Determination of molecular weight of cytosolic alanine: DOVA transaminase on Sepharose-6B column (2.5 x 42 cm). The column was calibrated with 1 mg of ferritin (Mr. 440,000), 2 mg of thyroglobulin (Mr. 669,000), 2 mg of aldolase (Mr. 158,000) and 5 mg of catalase (Mr. 232,000).
denatured conditions as revealed by SDS-polyacrylamide gel electrophoresis (Fig. 23).

In order to further confirm the differences in the molecular weight of the cytosolic and mitochondrial enzymes, after purification from both sources, the two enzymes were loaded separately in Sepharose-6B column. The activity peaks at elution volume of 132 ml and 148 ml represent cytosolic and mitochondrial alanine: DOVA transaminase respectively (Fig. 24).

IMMUNOLOGICAL COMPARISON OF CYTOSOLIC AND MITOCHONDRIAL ALANINE: DOVA TRANSAMINASE

Ouchterlony double diffusion analysis showed single precipitin line, when anti-mitochondrial alanine: DOVA transaminase antibody was allowed to react with mitochondrial and cytosolic enzyme. This establishes an immunological identity between the cytosolic and mitochondrial alanine: DOVA transaminase (Fig. 25).

EFFECT OF INCUBATION OF HEMIN ON PURIFIED CYTOSOLIC ALANINE: DOVA TRANSAMINASE IN IN VITRO

Since hemin is shown to inhibit alanine: DOVA transaminase purified from mitochondria, we have examined the possibility of inhibition of cytosolic enzyme by hemin. Cytosolic alanine: DOVA
Fig. 23: SDS-PAGE of purified rat kidney cytosolic alanine: DOVA transaminase under denaturing condition. 10 μg of the standard marker proteins, viz. albumin (Mr. 67,000), ovalbumin (Mr. 43,000), carbonic anhydrase (Mr. 30,000) and 20 μg of the purified sample were electrophoresed simultaneously. Calculations of the mobilities of marker proteins as well as that of the sample are expressed relative to the bromophenol blue dye front marked before staining in coomassie brilliant blue R-250.
Fig. 24: Elution profile of purified cytosol and mitochondrial alanine: DOVA transaminase from Sepharose-6B column (2.5 x 42 cm), 0.5 mg of cytosolic and 0.5 mg of mitochondrial alanine: DOVA transaminase were run separately on the column. A constant flow rate of 8 ml/hr was maintained during each run.
Fig. 25: Ouchterlony double diffusion analysis of purified cytosolic and mitochondrial alanine: 4,5-dioxovalerate transaminase with antibody prepared against purified mitochondrial alanine: DOVA transaminase. The central well contained 50 μl of the antiserum and wells 1,3 contained 80 μg of cytosolic enzyme in each well and wells 2,4 contained 60 μg of mitochondrial enzyme in each well.
transaminase is inhibited only to a slight extent (Table VIII), compared to the mitochondrial enzyme; even with 50 μM hemin, the inhibition was only 20%.

DIFFERENTIAL ACTIVITY OF CYTOSOLIC AND MITOCHONDRIAL ALANINE:DOVA TRANSAMINASE IN HEMIN INJECTED RATS

To examine further the effect of hemin administration on the activity of mitochondrial and cytoplasmic alanine:DOVA transaminase, different doses of hemin (0.8 - 2.0 mg/kg body weight) were administered intravenously to rats and killed after 30 min. The activity of mitochondrial and cytosolic alanine:DOVA transaminase were assayed. As shown in Figure 26, the mitochondrial enzyme activity decreased progressively with increasing hemin doses, whereas the enzyme activity in the cytosolic fraction increased at lower doses; at higher dose of hemin (2.0 mg/kg body weight), the activity of both cytosolic and mitochondrial enzymes were inhibited. Our results show that due to hemin administration, the level of alanine:DOVA transaminase in kidney cytosol increased markedly, whereas the enzyme activity in the mitochondria decreased sharply.

DISCUSSION

The present study reports for the first time the occurrence of cytoplasmic form of alanine:DOVA transaminase, in addition to
TABLE VIII: EFFECT OF HEMIN ON THE PURIFIED CYTOSOLIC ALANINE: DOVA TRANSAMINASE.

<table>
<thead>
<tr>
<th>Conc. (uM)</th>
<th>Specific activity of Alanine: DOVA transaminase µmol ALA/mg protein at 65°C</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>0.2 (100)*</td>
</tr>
<tr>
<td>25</td>
<td>0.2 (100)</td>
</tr>
<tr>
<td>50</td>
<td>0.15 (75)</td>
</tr>
</tbody>
</table>

*Figures in the parenthesis are percent activity of the control.
Fig. 26: Effect of hemin treatment on rat kidney alanine: DOVA transaminase. Different doses of hemin were injected i.v. (as described in 'Materials and Methods'), and the animals were killed after 30 mins of injection. Alanine: DOVA transaminase activity is expressed as percent specific activity of the control.
mitochondrial enzyme, and also describes a procedure for the purification of rat kidney cytoplasmic alanine: DOVA transaminase using Alanine-Sepharose-4B affinity chromatography. Our results also indicate that the cytosolic enzyme is the precursor of mitochondrial enzyme.

In chapter I, where we have shown the subcellular distribution of alanine: DOVA transaminase, the activity of this enzyme was detected both in cytoplasm and mitochondrial fractions. As a confirmation of the occurrence of a cytosolic form of alanine: DOVA transaminase, we have purified the cytosolic enzyme to homogeneity. We compared the molecular properties of mitochondrial and cytosolic enzymes and presented in Table IX. In our earlier paper (134), we reported the homogenous purification of mitochondrial kidney alanine:DOVA transaminase and the molecular weight of this enzyme is estimated as 2,25,000 daltons in its native form and under denaturing conditions, it showed a homogeneous subunit composition, each subunit having a molecular weight of 37,000 daltons. This shows that mitochondrial alanine: DOVA transaminase is a homohexamer. The molecular weight of bovine liver mitochondrial enzyme was reported to be of 2,40,000 daltons (29). However, the cytoplasmic alanine: DOVA transaminase has a molecular weight of 3,30,000 daltons in its native form. It is composed of six identical subunits, each having a molecular weight of 55,000 daltons. This result shows that the native cytoplasmic alanine:
<table>
<thead>
<tr>
<th>Properties</th>
<th>Alanine: DOVA transaminase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>Molecular weight in native form</td>
<td>225,000</td>
</tr>
<tr>
<td>Molecular weight under denaturing condition</td>
<td>37,000</td>
</tr>
<tr>
<td>Number of band(s) in SDS-PAGE</td>
<td>One</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>6.6</td>
</tr>
<tr>
<td>Optimal Temperature</td>
<td>$65^\circ$ C</td>
</tr>
<tr>
<td>Inhibition by Hemin</td>
<td>+</td>
</tr>
<tr>
<td>Immunological cross-reactivity with antimitochondrial alanine: DOVA transaminase antibody</td>
<td>+</td>
</tr>
<tr>
<td>Specific activity of the purified form (umol ALA/mg protein)</td>
<td>31.5</td>
</tr>
</tbody>
</table>
DOVA transaminase is a larger molecule than the native mitochondrial enzyme. Also, the subunits of cytoplasmic enzyme are of higher molecular weight than that of mitochondrial enzyme subunits. However, the rabbit antiserum raised against alanine:DOVA transaminase purified from the mitochondrial fraction reacted with cytosolic alanine: DOVA transaminase equally well indicating that alanine: DOVA transaminase in the cytosol and mitochondria are immunologically identical. This suggests that alanine: DOVA transaminase in the cytosol may be the precursor in transit to mitochondria.

Difference in molecular properties of alanine: DOVA transaminase, purified from cytosol and mitochondria is also supported by the variation of inhibitory role of hemin in vitro on the activity of enzyme. Table VIII shows that incubation of hemin with the purified enzyme has little inhibitory effect upon the enzyme. Mitochondrial form of the enzyme can be inhibited with hemin at a concentration as low as 5 μM and 60% inhibition was observed with 50 μM hemin (Table VI). However, cytosolic form of the enzyme remained intractible even at a dose of hemin (50 μM) which produced only 25% inhibition.

The different nature of mitochondrial and cytoplasmic enzyme is further elaborated by differential response to hemin injection. As reported earlier hemin injection inhibits the activity of mitochondrial alanine: DOVA transaminase. Inhibition
(60%) of the activity of mitochondrial alanine: DOVA transaminase is obtained with 0.8 - 1.2 mg/kg b.w. of hemin injection, whereas 150% stimulation in the activity of cytoplasmic form of alanine:DOVA transaminase is observed. Thus suggesting that there may be a possibility that alanine:DOVA transaminase synthesized in the cytosol may be transferred to the mitochondria and hemin may inhibit the intracellular translocation of this enzyme in kidney. Similar type of transfer of precursor of different mitochondrial matrix enzymes ornithine transcarbamylase (142, 143), carbamoyl phosphate synthetase (144,145) and ALA synthetase (71) to the mitochondria has also been reported. Further experiments are necessary to prove that the cytoplasmic alanine: DOVA transaminase is the precursor of mitochondrial enzyme so that this enzyme can be used as a model system to study the translocation of mitochondrial matrix proteins and also the biogenesis of alanine: DOVA transaminase in relation to regulation of heme biosynthesis.