Mitochondrial membrane-bound activity of arginase is independent of nitrogen excretion pattern in ureogenic and non-ureogenic vertebrates

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Arginase, that regulates metabolism of arginine, is widely distributed in organisms. The two major isoforms, cytosolic Arginase-I, and mitochondrial Arginase-II have been characterized well. However, reports also suggest another mitochondrial membrane-bound arginase which is extracted by washing the mitochondria with KCl. Here, we studied this mitochondrial membrane-bound arginase among vertebrates. Our observations support that arginase activity is predominant in cytosol which is designated as Arginase-I. The mitochondrial membrane-bound Arginase (mbArg) which resembles Arginase-II seems independent of nitrogen excretion pattern because of its presence both in ureogenic and non-ureogenic vertebrates.

Keywords: Arginase isoforms, Calotes versicolor, Garden-lizard, Gallus gallus, Heteropneustes fossilis, mbArg, Mus musculus, Rana tigrina, Reptiles

Materials and Methods

Animals and tissue collection
Tissues of representative types of Amphibians (Rana tigrina), Aves (Gallus gallus), Pisces (Heteropneustes fossilis), Reptiles (Calotes versicolor), and Mammals (Mus musculus), were either collected during practical classes or purchased from commercial resources. Fish and mice were maintained as per the Institutional Animal Ethical guidelines. Animals were sacrificed to dissect out liver, washed with saline and stored at −80°C. All the chemicals were of analytical grade purchased from Sigma, Himedia, SRL and other indigenous sources.

Isolation of mitochondria and mitochondrial membrane bound fraction of Arginase:
Homogenate (20%) of liver was prepared in homogenizing buffer containing 30 mM Tris HCl (pH 7.2), 1 mM EDTA, 250 mM Sucrose, 50 mM mannitol and protease inhibitor cocktail (Sigma-Aldrich) using a Potter–Elvehjem type glass homogenizer with a motor-driven teflon pestle. Cytosolic and mitochondrial fractions were separated according to previously described method. The mitochondrial pellet was washed twice with homogenizing buffer to remove the cytosolic contamination from mitochondrial pellet. For isolation of mitochondrial membrane bound fraction the mitochondrial pellet was washed with 100 mM KCl centrifuged at

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12000 × g for 15 min. The supernatant was considered mitochondrial membrane-bound fraction, and pellet was evaluated as washed mitochondrial fraction which was suspended in homogenizing buffer without mannitol. These fractions were used for enzyme assay and protein profile analysis.

Assay of succinate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase and arginase

Arginase assay was performed according to the method of Brown and Cohen with modification as reported by Saha and Ratha. The reaction mixture was consisting of 25 mM sodium glycinate buffer (pH 9.5), 2.5 mM MnCl₂, 25 mM L-arginine and suitably diluted enzyme extract in total volume of 2 mL which was incubated for 15 min at 30°C. The reaction was terminated by adding 10% perchloric acid, and protein was removed by centrifugation. Urea was estimated in the supernatant. A unit of arginase activity was defined as that amount of enzyme, which produced one µmole of urea per hour at 30°C. The extraction procedure of mitochondrial membrane bound form is checked by the mitochondrial and cytosolic marker enzyme assay. Glutamate dehydrogenase (GDH) activity was assayed by the spectrophotometric method of Olson and Anfinsen, and succinate dehydrogenase (SDH) by the method of Ells et al. However, enzyme specific staining of GDH and SDH were performed by the method of Okwumabua et al. as a marker enzyme of mitochondrial fraction. Enzyme specific staining of lactate dehydrogenase (LDH) was performed as described by Bharti and Mishra.

Protein profile and RT-PCR analysis

The protein concentration was estimated by the method of Bradford et al. using bovine serum albumin as the standard. Proteins from different fractions were resolved on 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were visualized by staining with Coomassie Brilliant Blue-R250 (CBB R-250). Total RNA was extracted from liver of all organisms using TRIzol (Invitrogen), and reverse transcribed using Oligo-dT primer according to suggested protocol for first strand cDNA synthesis kit (Fermentas). Expression of arginase-I (ARG I) and arginase-II (ARG II) was analysed through RT-PCR. β-actin was used as control experimental. The primer-sets used in this study have been summarized in Table 1.

Table 1—Forward and reverse primers used (5′–3′) to amplify genes of interest in RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Prod. size</th>
</tr>
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<tbody>
<tr>
<td>ARG I F</td>
<td>5′-GTCCAGAAGAACATTGGAAGGACTGAG-3′</td>
<td>300 bp</td>
</tr>
<tr>
<td>ARG I R</td>
<td>5′-GTCCCCAGGGCTAGCGTCG-3′</td>
<td>300 bp</td>
</tr>
<tr>
<td>ARG II F</td>
<td>5′-GGCTACGCTGTCCACATG-3′</td>
<td>286 bp</td>
</tr>
<tr>
<td>ARG II R</td>
<td>5′-CAGAGGCTCCACCTCCTCAAG-3′</td>
<td>286 bp</td>
</tr>
<tr>
<td>βACTIN F</td>
<td>5′-TGACCGGGAACCCACACTGTG-3′</td>
<td>600 bp</td>
</tr>
<tr>
<td>βACTIN R</td>
<td>5′-CTCAACAGCATTTGCGACGATGGAGG-3′</td>
<td>600 bp</td>
</tr>
</tbody>
</table>

Statistical analysis

The data were presented as mean ± SD. Data were analysed using one-way ANOVA followed by Tukey’s test.

Results

Arginase activity was observed in cytosolic, mitochondrial and mitochondrial membrane-bound fractions of fish (Fig. 1A), frog (Fig. 1B), chicken (Fig. 1C), garden-lizard (Fig. 1D), and mouse (Fig. 1E). The arginase activity was higher in ureogenic (mouse, frog and fish) vertebrate (Figs. 1 A, B and E) in comparison to non-ureogenic (chicken and garden-lizard) vertebrates (Figs. 1 C and D). However, the transcript of arginase was detected in all representative types [Figs. 1 A(i) and E(i)]. The ARG I and ARG II both transcripts were observed in fish [Fig. 1A(ii)], frog [Fig. 1B(ii)], chicken (Fig. 1C(ii)), and mouse [Fig. 1E(ii)], but only ARG II was detectable in garden-lizard [Fig. 1D(ii)]. The arginase activity in KCl associated fraction, supposed to be bound to mitochondrial membrane was higher in liver of fish (20%) followed by mouse (17%), frog (15%), garden-lizard (7%) and chicken (6%) as compared to arginase activity in crude extract (Fig. 2A). The activity of mitochondrial membrane-bound arginase was higher in mouse (65%), followed by fish (30%), frog (30%), garden-lizard (15%) and chicken (15%) as compared to the arginase activity of total mitochondrial fraction (Fig. 2B). On the basis of the spectrophotometric analysis [Figs. 3A(i) and B(i)], the mitochondrial membrane bound arginase seems as the different isoform but the enzyme-specific staining reveals the presence of enzymes from mitochondrial-matrix in membrane-bound fraction [Figs. 3 A(ii) and B(ii)]. However, cytosolic marker enzyme like LDH was negligible in the mitochondrial and membrane-bound fraction (Fig. 3C) as compared to the cytosol. The polypeptides of 98, 86, 54, 36, 27, 24 and 20 kDa were modulated in mitochondrial membrane bound fraction (Fig. 3D).
Fig. 1—Analysis of activity of arginase in different sub-cellular fraction of liver of (A) *Heteropneustes fossilis*, and (Ai) its expression; (B) *Rana tigrina*, and (Bi) its expression; (C) *Calotes versicolor*, and (Ci) its expression; (D) *Gallus gallus*, and (Di) its expression; (E) *Mus musculus*, and (Ei) its expression. [Values are plotted as mean ± S.D. *P* < 0.01, **P** < 0.05]

Fig. 2—The relative activity (%) of mitochondrial membrane bound arginase in five organisms as compared to (A) total activity of arginase present in crude; and (B) total activity of arginase present in mitochondria.

Fig. 3—Analysis of extraction procedure of mitochondrial membrane bound fraction from liver of *Mus musculus* (mammal) by described method. Activity of GDH in different sub-cellular fraction, by (Ai) spectrophotometric method, and (Aii) enzyme specific staining, and Integrated density value of GDH by enzyme specific method; Activity of SDH in different sub-cellular fraction, by (Bi) spectrophotometric method, and (Bii) enzyme specific staining, Integrated density value of SDH of enzyme specific method; (C) Activity of LDH in different sub-cellular fraction, by enzyme specific staining, Integrated density value of LDH of enzyme specific method; and (D) protein profile analysis of different sub-cellular fraction of liver of *Mus musculus*. 
Discussion

The presence of arginase-II along with arginase-I in ureogenic (mouse, frog and fish) vertebrate [Figs. 1A(i), B(i) and E(i)] in comparison to the non-ureogenic (chicken and garden-lizard) vertebrates [Figs. 1C(i) and D(i)] suggested specific physiological function dependent on physiological fate of arginine and ornithine. However, reports describe cytosolic arginase (arginase-I) in hepatic tissue and mitochondrial arginase (arginase-II) in extra hepatic tissue. It was also interesting to observe distribution of both isoforms in hepatic tissue of ureogenic as well as non-ureogenic vertebrates (Fig. 1).

In non-ureogenic vertebrates, arginase-II appears to regulate amino acid synthesis via glutamate and alpha-ketoglutarate. However, in marine fish mitochondrial arginase-II may be critical for production of urea for osmotic balance. In reptiles and birds, mitochondrial arginase-II supports the view of influencing the production of ornithic acid and uric acid along with glutamate, proline and polyamines metabolism. Reports on distribution and isoforms of nitrogen metabolizing enzymes support the findings because they have been influenced by the mode of the nitrogen detoxification pattern and environmental adaptation of organism in vertebrates. Results enlighten that both arginase isoforms (arginase-I and arginase-II) are present in liver of vertebrates. The presence of mitochondrial membrane bound arginase (mbArg) testifies its physiological significance (Fig. 4) in the transport of arginine and ornithine. The observation of active fraction of LDH in the mitochondrial membrane-bound fraction (Fig. 3C) appears negligible as compared to the cytosolic fraction but it may be due to mitochondrial-LDH associated with mitochondrial outer membrane. The presence of mitochondrial matrix protein in the mitochondrial membrane bound fraction (Fig. 3D) indicates the leakage of mitochondrial matrix proteins under influence of KCl.

The presence of transcripts of arginase-II transcripts [Figs. 1A(i), B(i), C(i), D(i) and E(i)] in all the five representative types (fish, frog, bird, reptiles and mammals), and arginase-I in fish, frog, chicken and mammals is in agreement that the different subcellular location of an enzyme critically enhance the efficiency of metabolism. However, the association of arginase with mitochondrial membrane was proposed to regulate the ureogenesis and the abundancy of arginase not to the nitrogen excretion pattern.

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