Comparative Biochemistry and Physiology, Part B 164 (2013) 133–141

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Unusual hepatic mitochondrial arginase in an Indian air-breathing teleost, *Heteropneustes fossilis*: Purification and characterization

Shilpee Srivastava *, B.K. Ratha

Biochemical Adaptation Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

ARTICLE INFO

Article history:
Received 11 August 2011
Received in revised form 17 November 2012
Accepted 20 November 2012
Available online 27 November 2012

Keywords:
Arginase
Evolution
Heteropneustes fossilis
Teleost
Urea cycle

ABSTRACT

A functional urea cycle with both cytosolic (ARG I) and mitochondrial (ARG II) arginase activity is present in the liver of an ureogenic air-breathing teleost, *Heteropneustes fossilis*. Antibodies against mammalian ARG II showed no cross-reactivity with the *H. fossilis* ARG II. ARG II was purified to homogeneity from *H. fossilis* liver. Purified ARG II showed a native molecular mass of 96 kDa. SDS–PAGE showed a major band at 48 kDa. The native enzyme, therefore, appears to be a homodimer. The p*I* value of the enzyme was 7.5. The purified enzyme showed maximum activity at pH 10.5 and 55 °C. The K_m of purified ARG II for L-arginine was 5.25 ± 1.12 mM. L-Ornithine and N^{ω}-hydroxy-L-arginine showed mixed inhibition with K_i values 2.16 ± 0.08 and 0.02 ± 0.004 mM respectively. Mn^{+2} and Co^{+2} were effective activators of arginase activity. Antibody raised against purified *H. fossilis* ARG II did not cross-react with fish ARG I, and mammalian ARG I and ARG II. Western blot with the antibodies against purified *H. fossilis* hepatic ARG II showed cross reactivity with a 96 kDa band on native PAGE and a 48 kDa band on SDS–PAGE. The molecular, immunological and kinetic properties suggest uniqueness of the hepatic mitochondrial ARG II in *H. fossilis*.

1. Introduction

Enzymatic hydrolysis of arginine to ornithine and urea involves a trimeric metalloenzyme, arginase (L-arginine urea hydrolase, EC. 3.5.3.1) in ureotelic mammals (Kossel and Dakin, 1904). Each subunit contains a binuclear manganese for activity (Reczkowski and Ash, 1992). Arginase is traditionally considered as the final enzyme of the ornithine–urea cycle (OUC) located in highest concentration in the liver cytosol of ureotelic animals that synthesize urea for ammonia detoxification. Compared to other OUC enzymes arginase is widely distributed throughout the evolutionary spectrum in organisms, and has a wider tissue distribution in animals (Jenkinson et al., 1996; Cederbaum et al., 2004). Hence, it has been suggested to have important metabolic functions apart from urea synthesis. Even though several isoforms of arginase have been reported (Zamecka and Porembska, 1988), two major isoforms of arginase designated as arginase I (ARG I), predominantly found in liver cytosol, and arginase II (ARG II), found in mitochondrial compartment in non-hepatic tissues, have been characterized in several vertebrates including human (Ash, 2004). The extra-hepatic ARG II is catalytically similar to the hepatic ARG I, but has different house keeping functions such as production of ornithine, a precursor for polyamines, glutamate and proline biosynthesis (Jenkinson et al., 1996), urea biosynthesis for osmoregulation in marine fishes (Mommsen and Walsh, 1989; Withers, 1998; Steele et al., 2005), and controlling the concentration of tissue arginine for nitric oxide (NO) biosynthesis (Li et al., 2001). Recently evolution of urea biosynthesis has also been correlated with the freeze tolerance in wood frog, *Rana sylvatica* (Muir et al., 2008). Thus, the products of arginine catabolism have served various physiological and metabolic functions in various tissues and different organisms.

Modifications in the use of many metabolic pathways and enzyme functions have provided adaptability in the organisms to the changing environment during evolution. As compared to prokaryotes and lower eukaryotes, the arginine biosynthetic pathway is modified in higher eukaryotes to convert ammonia to urea through urea cycle. These include certain invertebrates (Campbell, 1973) such as land planarians (Campbell, 1965), earthworms (Bishop and Campbell, 1965), snails (Tramell and Campbell, 1972) and leech (Natesan et al., 1992), and vertebrates such as ureo-osmotic marine elasmobranchs, coelacanth and two semi-marine amphibians, ureotelic lungfishes, aquatic reptiles, amphibians and mammals. In eukaryotes including plants, ARG II activity has been reported in the mitochondrial compartment. However, in ureotelic vertebrates, ARG I activity has been detected in hepatic cytosolic fraction (Soberon and Palacios, 1976).

Cloning and sequencing of the two arginase genes (ARG I and ARG II) demonstrated that they were different from one another, but with homology in more than 50% of their total amino acid residues and 100% homology in areas critical to enzymatic function (Gotoh et al., 1996; Vockley et al., 1996; Joerink et al., 2006). The two isoforms also differ in their immunological cross reactivity (Spector et al., 1994; Cama et al., 2003). Mitochondrial ARG II has been suggested to be the ancestral gene, with cytosolic ARG I evolving by gene duplication some time before the emergence of amphibians when OUC became the

* Corresponding author at: Department of Biotechnology, Assam University, Silchar-788011, Assam, India.
E-mail address: shilp.vastava@gmail.com (S. Srivastava).

1096-4959/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpb.2012.11.007

predominant pathway for ammonia detoxification in land dwelling animals (Patterton and Shi, 1994; Jenkinson et al., 1996; Iyer et al., 1998; Perozich et al., 1998). In marine elasmobranchs (Casey and Anderson, 1985) and most ammoniotelic teleosts (Wright et al., 2004) arginase activity has been mitochondrial and in lungfishes arginase was reported to be cytosolic in liver (Gotoh et al., 1996). Mommsen and Walsh (1989) analyzed the sub-cellular location of OUC enzymes in a variety of fish species, and proposed that cytosolic arginase first appeared in the lungfish beginning the monophyletic ureotelic evolution. Griffith (1991) while reviewing the ureo-osmotic and ureotelic adaptations in different vertebrates such as marine elasmobranchs, primitive holocephalans, coelacanth (*Latimeria*), lungfish, aquatic and terrestrial amphibians and mammals suggested that the enzymes of the OUC evolved independently several times in vertebrates under environmental pressure to provide different physiological adaptations.

Presence of a full complement of OUC enzymes in both liver and kidney with the presence of both carbamoyl phosphate synthetase (CPS) I and III activities and glutamine synthetase (GS) activity in liver and kidney mitochondria (Saha and Ratha, 2007) and, arginase activity in both mitochondrial (60%) and cytosolic (40%) compartments (Dkhar et al., 1991) have been unique features reported in the freshwater air-breathing teleost, *H. fossilis* suggesting its evolutionary uniqueness (Srivastava and Ratha, 2010). *H. fossilis* also showed ureotelic adaptation during hyper-ammonia stress (Saha and Ratha, 2007), water deprivation (Saha et al., 2001) and ureo-osmotic adaptation during hyper-osmotic stress (Saha and Ratha unpublished). Presence of both cytoplasmic and mitochondrial arginase activities has also been reported in the liver of a marine toadfish *Opsanus* spp. (Walsh, 1995). Although arginase has been purified and characterized from the liver of a marine elasmobranch, *Squalus acanthias* (Casey and Anderson, 1982), and a fresh water teleost, *Clarias batrachus* (Singh and Singh, 1990), specific mitochondrial arginase has not yet been purified and characterized from any teleost fish. Therefore, an attempt was made to purify and characterize the mitochondrial arginase from the liver of *H. fossilis* and compare with the data available from other organisms to find out its evolutionary significance.

2. Materials and methods

2.1. Materials

Live fish (*H. fossilis*) weighing 20–30 g were purchased from the local fish market. Fishes were killed by decapitation. Liver tissue was dissected out, washed in cold saline, blotted dry and stored immediately at −20 °C. Arginase purification and enzyme assays were completed within 1 week of tissue collection. All chemicals used were of analytical grade and purchased from either Sigma-Aldrich Chemical Co. or from local suppliers. Separation media, ampholites, western blot reagents and protein markers used were from either GE Health Care Bioscience or Bio-Rad Laboratories. The polyclonal antibody against human ARG II raised in rabbit was a gift from Prof. T. Gotoh. PVDF membranes were purchased from Millipore. Double distilled water passed through a Milli-Q water purification system was used in all preparations.

2.2. Separation of hepatic mitochondrial fraction and preparation of mitochondrial extract

A 20% homogenate (w/v) of liver tissue was prepared in an isolation buffer containing 10 mM Tris–HCl (pH 7.5), 300 mM mannitol, 150 mM KCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM β-mercaptoethanol and protease inhibitor cocktail (Sigma-Aldrich), using a Potter–Elvehjem type glass homogenizer with a motor-driven teflon pestle. The mitochondrial fraction was separated as described earlier (Dkhar et al., 1991). Briefly, A 20% homogenate (w/v) of liver tissue was prepared in ice-cold isolation buffer, and the mitochondrial pellet was isolated by differential centrifugation. The mitochondrial pellet was washed and suspended in the above buffer without mannitol but containing 0.5% Triton X-100 and a few gentle strokes with the homogenizer were applied. After 30 min, the mitochondrial suspension was centrifuged at 14,000*g* for 20 min. The supernatant obtained was the mitochondrial extract containing the solubilized arginase and this was used for further purification. All purification steps were performed in a cold chamber maintained at 4 ± 1 °C except for the heat treatment step.

2.3. Purification of hepatic mitochondrial arginase

2.3.1. Heat treatment

The mitochondrial extract was maintained at 55 °C in a water bath with slow constant stirring for 10 min. It was then immediately cooled on ice. Denatured proteins were removed by centrifugation at 10,000*g* for 20 min, and the supernatant was used for separation by ion exchange chromatography.

2.3.2. Ion exchange chromatography

The above supernatant was applied to a 1×30 cm anion exchanger (Bio-Rad Macroprep DEAE) column equilibrated with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM $MnCl_2$ and 1 mM β-mercaptoethanol. Chromatography was performed in a cold chamber using a Bio-Rad Biologic LP automated gradient system. The column was washed with the equilibrating buffer containing 50 mM NaCl. A linear gradient of NaCl (50 to 250 mM) in equilibrating buffer was applied at a flow rate of 3 mL/min and 3 mL fractions were collected. The arginase activity was eluted as a single peak at around 200 mM NaCl concentration. The active fractions containing arginase specific activity of more than 650 units/mg protein were pooled for use in the next purification step.

2.3.3. Ammonium sulfate precipitation

In a pilot experiment it was found that 70% ammonium sulfate precipitated all the arginase activity. The pooled DEAE eluted fractions were adjusted to 70% saturation with gradual addition of solid ammonium sulfate and slowly stirred for 1 h. The content was centrifuged and the pellet containing the active enzyme was dissolved in a minimum volume of 10 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MnCl_2$.

2.3.4. Gel filtration

The dissolved ammonium sulfate fraction was applied to a Sephadex G-100 (1.5×50 cm) column equilibrated with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM $MnCl_2$. The column was run at a flow rate of 18 mL/h, and 1 mL fractions were collected. Enzyme activity was eluted as a single peak. Seven fractions with high arginase specific activity were pooled, and used for separation by affinity chromatography.

2.3.5. Affinity chromatography

The pooled active fractions from the Sephadex column was applied to an arginine Sepharose 4B (Amersham Biosciences) column (1×10 cm) previously equilibrated with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM $MnCl_2$. The column was washed with 30 mL of equilibrating buffer. The adsorbed proteins were then eluted by applying a linear gradient of 0–200 mM NaCl in equilibrating buffer at a flow rate for 0.3 mL/min and 1 mL fractions were collected. Six fractions with very high specific activity were pooled and used as the purified hepatic mitochondrial arginase (ARG II) from *H. fossilis* for further analysis.