Localização Subcelular e Propriedades Cinéticas da Arginase Hepática da Raia *Zapteryx brevirostris* (Müller e Henle, 1841) (Elasmobranchii, Rhinobatidae)

Subcellular Localization and Kinetic Properties of Hepatic Arginase from Skate Zapteryx brevirostris (Müller and Henle, 1841) (Elasmobranchii, Rhinobatidae)

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Abstract

The argininolytic activity of the liver of Zapterix brevirostris was studied at a pH and temperature close to the physiological conditions using preparation of arginase in mitochondrial suspension and partial purification. The kinetic behavior is characteristic of enzymes with two active sites. For arginase in mitochondrial suspension, K_{mapp1} = 1,22±0,17 mM and K_{mapp2} = 64,29±14,77 mM, and for partial purified arginase with K_{m1} = 2,32±0,17 mM and K_{m2} = 561,50±899,40 mM were obtained. The manganese cation in concentrations up to 0.25 mM has an activation effect on the arginase activity and an inhibitory effect with concentrations above 0.5 mM in both preparations. For arginase in mitochondrial suspension and in partial purification, the values of IC_{50} for $MnCl_2$ were 4.16 mM and 3.07 mM respectively.

The studies indicate that the mitochondrial membranes are the probable sites that control the L-arginine metabolism.

Key-words

Skate. Elasmobranchs. Arginase. Nitrogen Metabolism. Arginine.

Introduction

The capacity to synthesize, excrete and produce urea for osmoregulation is the basis for the classification of fishes as ureogenic, ureotelic and ureosmotic respectively. The evolution of the nitrogen metabolism in vertebrates leads to changes in the genetic expression and the subcellular localization of the nitrogen metabolism enzymes, proportionating metabolic strategies that is species dependent. Some of these changes occurred even before the evolution of the present dipnoic fishes and involved the change of carbamoyl phosphate synthetase III to carbamoyl phosphate synthetase II along with the change from mitochondrial arginase to cytosolic (MOMMSEN; WALSH, 1989).

The arginases are a restricted group of enzymes, widely distributed in the biosphere, dependent on Mn^{2+} as a cofactor and are involved in the maintenance of the tissue level of L-arginine and in the biosynthesis of urea. Mammals have two isoenzymatic forms of arginase, called I and II, with distinct physic-chemical properties and localized in the subcellular cytosol and mitochondria, respectively. The arginase type I is functionally associated with the urea cycle and is present in the hepatic tissue, whereas type II is present in extra hepatic tissues and supposedly involved in maintenance of adequate levels of L-arginine (JENKINSON; GRODY; CEDERBAUM, 1996).

The nitrogen metabolism is considered one of the most sensitive physiological systems with a capacity to adapt to the environmental changes. Most teleosts fishes excrete ammonia as a result of a complex metabolic activity involved with the degradation of proteins and amino acids. Whereas in mammals, intoxication symptoms appear when the concentration of serum ammonia is above 0.05 mM, fishes have a greater resistance to ammonia toxicity and with stand up to 0.05 and 2.0 mM in the blood (SAHA; RATHA, 1998).

The nitrogen metabolic strategies, adapted by the

various species, are related to the kinetic properties and the subcellular localization of the arginases (RODRIGUES; ROSA; BACILA, 1996). These strategies become evident in animals that are typically ureotelic, which maintain elevated levels of urea in the blood as in the case of Antarctic birds (ROSA, et al., 1993). The high levels of protein in their diet involve the deamination and use of the carbon chain of the amino acids in the metabolic processes such as gluconeogenesis and in the maintenance of blood glucose levels. On the other hand, it is believed that low solubility of the uric acid in biological fluids may be an important limiting factor in the urea elimination processes. Investigations of the nitrogen metabolism of penguins show that tissue levels and kinetic behavior of hepatic arginase of these birds are similar to those of mammals, with $K_{m,}$ of L-arginine, about 12 mM, compatible with the metabolism of typical ureotelic animals (RODRIGUES; ROSA; BACILA, 1998).

The scientific interest of the elasmobranchs arises due to their primitive evolutionary stage; the low incidence of cancer and the ureosmotic strategy which can help retain elevated levels of urea in the blood which can reach as high as 680 mM. Their evolutionary position gives biologists an opportunity to study a crucial stage in the evolution of vertebrates (BALLANTYNE, 1997).

The metabolic pathways in the synthesis of urea in elasmobranchs appear similar to that of the ureotelic mammals, and the magnitude of serum levels of L-arginine do not differ significantly from that observed in the blood of other vertebrates. It is, therefore, believed that the metabolic control of L-arginine must attend to similar metabolic necessities (CAMPBELL, 1961; ANDERSON; CASEY, 1984). Studies of the nitrogen metabolism of *Squalus acanthias* show that the excretion of nitrogen occurs preferentially in the form of urea across the gills, with a discrete participation of renal pathways. It was also clear that the urea synthesis is related to the osmoregulation and the nitrogen excretion, without participation in the acid-base balance (WOOD; PÄRT; WRIGHT, 1995).

Studies of *Mustelus canis* showed that the liver, kidneys and the rectal glands are the principal ureogenic tissues with decreasing order of argininolitic activity, with K_m values of L-arginine closer to those found in other vertebrates (CAMPBELL, 1961).

On the other hand, the mitochondrial localization of arginase in elasmobranchs involves the transport of L-arginine across the mitochondrial membranes, and can represent an important site of metabolic control of this amino acid. Therefore, the objective of the present work is to compare the kinetic behavior of the partially purified arginase in the liver of *Z. brevirostris*, with that of the arginase present in mitochondrial suspension to clarify the role of the mitochondrial membrane in the hydrolysis of L-arginine.

Material and Methods

Specimens of *Z. brevirostris* (n=14), females, weighing between 450 and 770 g, were captured in Ubatauba, north coast São Paulo state using a dragnet at a depth from 30 to 60 m, in the months from July 2003 to February 2004. The skates were kept in the aquarium of the Marine biology laboratory- Instituto Básico de Biociências – Campus de Ubatuba – UNITAU.

After 48 hours of acclimatization in the aquarium, and within a period of 5 days, the skates were killed by the cervical column section, the liver extracted, washed in cold physiological solution and the subcellular fractions extracted.

The hepatic tissue was then homogenized in Potter-Elvehjem fitted with a Teflon pistol, in a compartmentalization buffer (TC) containing HEPES 20 mM (pH 7.0), Trimethylamine-N-oxide 1.0 mM, KH_2PO_4 5.0 mM, EDTA 0.5 mM and Sucrose 250 mM, in the proportion of 1 gram of tissue to 10 volumes of solution.

The homogenate was filtered with a double layer of gauze and centrifuged at 1500g for 10 minutes to eliminate the cells and the non homogenized tissues. In this step, the sediment was discarded (debris fraction) and the supernatent centrifuged at 14000g for 10 minutes to obtain the cytosolic fraction and the mitochondrial sediment. The mitochondrial fraction was washed twice with TC, centrifuged at 14000g for 10 minutes and the sediment of the last wash resuspended in a small volume of TC without EDTA.

Triton X-100, was added to the mitochondrial suspension obtaining a final concentration of 0.1% (v/v), sonicated for 30 seconds and centrifuged at 14000g for 10 minutes. The sediment was discarded and the supernatant (mitochondrial extract) used for the partial purification of hepatic arginase of Z. brevirostris heating the preparation at 55°C for 2 minutes in the presence of Mn^{2+} 1 mM. The denaturated proteins were eliminated through centrifugation at 14000g for 10 minutes. The clear supernatant was gel filtered in a sephadex G-25 column, previously calibrated with a

buffer HEPES 10 mM, pH 7.4. The fractions with the argininolytic activity were combined and used in the kinetic experiments. The entire process was done at 4ºC.

The kinetic studies with the isolated mitochondria were conducted in TC without EDTA, pH 7.4 with adequate concentrations of $MnCl_2$ and L-arginine, and maintained under shaking at a temperature of 25ºC. The kinetic studies with the partially purified arginase were conducted with a buffer HEPES 10 mM, pH 7.4 containing adequate concentrations of L-arginine and $MnCl_2$. The reaction was initiated by the addition of arginase. All the kinetic experiments were conducted at 25.0±0.1 ºC.

The urea resulting from reactions catalyzed by arginase was quantified by spectrophotometer using the method TDMU, as described by Geyer and Dabich (1971), with modifications.

The reaction systems used in the determination of the argininolytic activity was adjusted for a final volume of 1ml and the reaction interrupted by the addition of 1.5 ml of acid TDMU (aqueous solution of $FeCl_3$ 0.12 mM, H_3PO_4 0.0567%(v/v), H_2SO_4 20%(v/v)), followed by the addition of 1.5 ml of the color reagent (aqueous solution of thiosemicarbazide 3.6 mM and diacetylmonoxime 61.7 mM). The tubes were maintained in a boiling bath for 10 minutes and then rapidly cooled in running water. The absorbance readings were conducted in tubes of 10 mm, against a blank reagent, in a spectrophotometer FEMTO model 600S, adjusted for a wavelength 520 nm.

The argininolytic activity was expressed in International Units (U), defined as the quantity of arginase needed to catalyze the conversion of 1 mmol of L-arginine, in one minute at a temperature 25ºC.

The total protein in the suspension and the mitochondiral extract was determined by the method of Bradford (1976), using the bovine blood albumin as a standard.

All the experiments were repeated at least three times and the results expressed as mean ± Standard Deviation (SD). The values of IC_{50} were calculated from the inhibition curves, and defined as the inhibitor concentration required to reduce the enzymatic activity by 50%. The kinetic data was analyzed using the velocity equations and graphs as described by Segel (1993).

Results and discussion

The subcellular localization of hepatic arginase in *Z. brevirostris* was established by determining the total argininolytic activity, of the cytosolic and mitochondrial fractions (mitochondrial suspension), in a reaction system containing glycine buffer 20 mM, pH 9.5, L-arginine 250 mM and $MnCl_2$ 5 mM. In these conditions 99% of the total arginase activity was found in the mitochondrial fraction.

The use of ammonium sulphate and chromatogram of CM-cellulose in the purification of arginase of *Z. brevirostris* was followed by an accentuated loss of activity. The best results were obtained by heating the mitochondrial extract at 55ºC for 2 minutes, followed by chromatograph em sephadex G-25.

The activity of the partially purified arginase, analyzed in physiological pH and subsaturated conditions of the substrate, were directly proportional to the protein concentration in the range 20 to 70 mg (Figure 1).

The effect of L-arginine concentration on the arginase activity present in the mitochondrial suspension and on the partially purified arginase is presented in figure 2. The velocity equation that best fits the kinetic data of both the preparations which describe enzymes with two active sites, with the values of K_{mapp1} = 1.22±0.17 mM and K_{mapp2} = 64.29±14.77 mM, for the arginase in the mitochondrial and , K_{m1} = 2.32±0.17 mM and K_{m2} = 561.50±899.40 mM, for the partially purified arginase.

The effect of the Mn (II) cation on the arginase activity present in the mitochondrial suspension and the partially purified one reveals that the $MnCl_2$, activates the arginine hydrolysis in concentration of up to 250 mM, in both cases. Above this concentration, the Mn(II) acts as an inhibitor of the argininolytic activity with IC_{50} of 4.16 mM and 3.07 mM for the mitochondrial suspension and for the partially purified one respectively (Fig. 3).

The mitochondrial location of hepatic arginase for *Z. brevirostris* arises from the subcellular behavior of most of the teleosts fishes. The process of partial purification of arginase is done in two steps, heating at 55ºC and gel filtering with sephadex G-25. The procedures frequently used for the purification of arginases, precipitation with ammonium sulphate and chromatograph with CM-cellulose (CARVAJAL, URIBE;

Table 1. Steps in the purification process of hepatic arginase of Z. brevirostris. The activity was determined with a glycine buffer 20 mM, pH 9.5, containing L-arginine 250 mM and $MnCl_2$ 5 mM.

Steps	Total Volume (ml)	Total Activity (U)	Specific Activity (mU/mg)	Purification (times)	Yield (%)
Mitochondrial Extract	2,0	1,32	45,08	——	100
Heating at 55ºC	1,4	0,91	67,94	1,51	55
Sephandex G-25	2,0	1,26	294,39	6,53	95

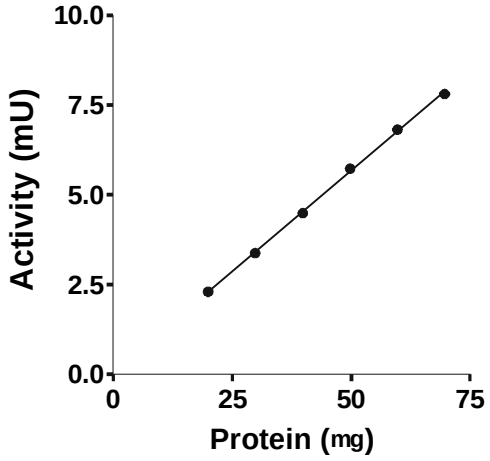
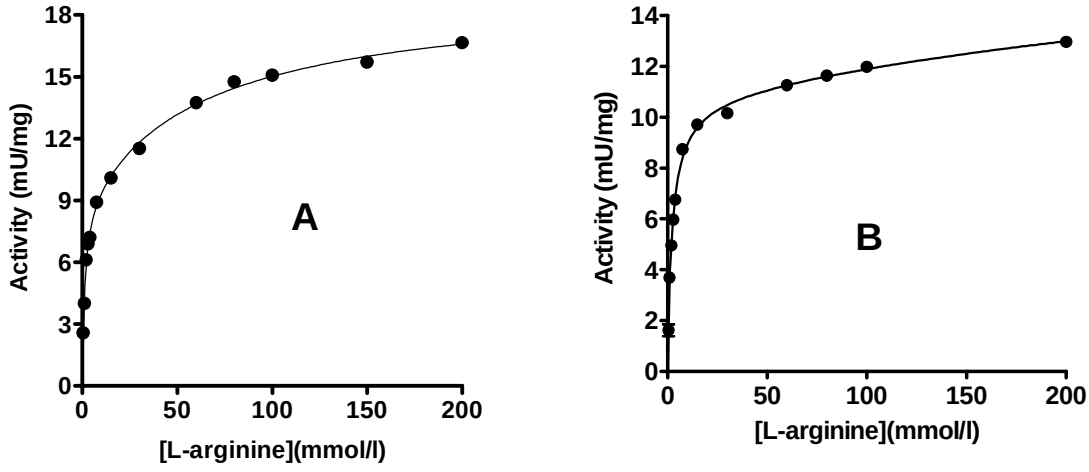


Figure 1. Effect of partially purified arginase on the argininolytic activity. The reaction was done on a HEPES buffer 10 mM, pH 7.4 with $MnCl_2$ 0,25 mM and L-arginine 1,5 mM.



igure 2. Effect of the L-arginine on the argininolyticactivity in the mitochondrial suspension (A), and of the partially purified arginase (B) from the liver of Z. brevirostris. The analysis conditions are (A) HEPES buffer 10 mM, pH 7.4, with Trimethylamine-N-oxide 0.5mM, KHPO 2.5 mM and Sucrose 125 mM at a temperature of 25ºC; (B) HEPES buffer 1'0 mM, pH 7.4, containing $MnCl_2$ 5 mM.

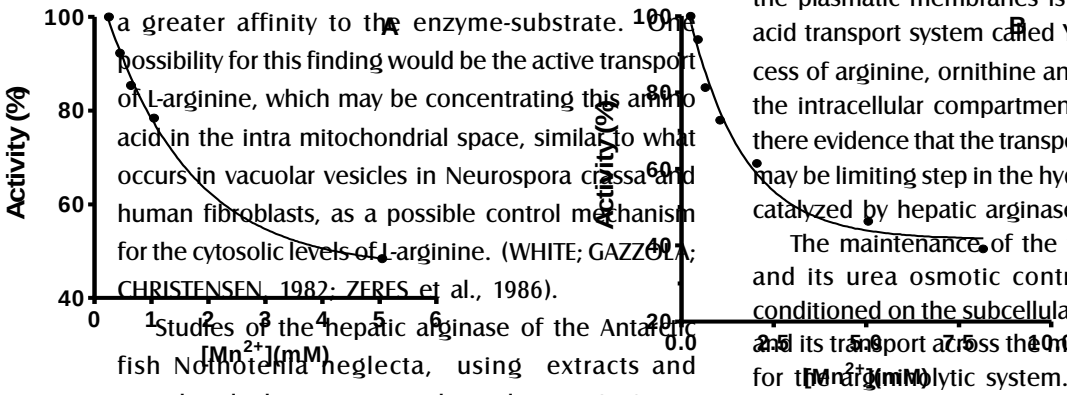


Figure 3. Effect of the Mn(II) cation on the argininolytic activity of the mitochondrial suspension (A) and on the mitochondrial extract (B) the liver of Z. brevirostris. The activity was determined at a temperature of 25ºC in a medium containing L-arginine 1.5 mM; (A) compartimentalization buffer without EDTA and (B) HEPES buffer 20 mM.

TORRES, 1994; RECZKOWSKI; ASH, 1994;), resulted in an accentuated loss of the arginase activity, and thus was of little use in the purification processes.

The values of K_{mapp} for the hepatic arginase of Z. brevirostris in mitochondrial suspension are smaller than the values of K_m obtained from partially purified arginase. In this case, the mitochondrial structure confers greater affinity to the argininolytic system present in the mitochondrial suspension, resulting in a greater affinity to the enzyme-substrate. One possibility for this finding would be the active transport of L-arginine, which may be concentrating this amino acid in the intra mitochondrial space, similar to what occurs in vacuolar vesicles in Neurospora crissa and human fibroblasts, as a possible control mechanism for the cytosolic levels of L-arginine. (WHITE; GAZZOLA; CHRISTENSEN, 1982; ZERES et al., 1986).

Studies of the hepatic arginase of the Antarctic fish Nothotenia neglecta, using extracts and mitochondrial suspension showed K_m = 2,40 mM and K_{mapp} = 8,42 mM, respectively (RODRIGUES et al., 2004). These values reflect the inverse kinetic behavior that found in the studies with Z. brevirostris. In case of N. neglecta, the mitochondrial structures in the liver reduce the affinity between arginase and its substrate L-arginine, hence K_{mapp} has more elevated value than K_m.

The hepatic arginase of Z. brevirostris needs the Mn(II) as an activator to reach its maximum activity.

The activation effect of Mn(II) increases up to a concentration of 0.25 mM and inhibits the activity of arginase in both partial purification and mitochondrial suspension at concentrations above 0.5 mM. The IC_{50} values of Mn^{2+} for both the mitochondrial suspension and the partially purified arginase are fairly close, indicating that the mitochondrial membranes participate discretely in the use of Mn(II) in the mitochondrial argininolytic system.

The transport of the cationic amino acids across the plasmatic membranes is restricted to the amino acid transport system called Y^+, which permits the access of arginine, ornithine and lysine amino acids in the intracellular compartments. On the other hand, there evidence that the transport of arginine in hepatitis may be limiting step in the hydrolysis of arginine when catalyzed by hepatic arginase (WHITE, 1985).

The maintenance of the tissue levels of arginine and its urea osmotic control in Z. brevirostris is conditioned on the subcellular localization of arginase and its transport across the mitochondrial membranes for the argininolytic system. The differences in the kineti behavior of partially purified arginase and the mitochondrial suspension, show the participation of the mitochondrial membranes in the metabolic control of L-arginine Z. brevirostris.

CONCLUSION

The hepatic arginase of Z. brevirostris, in mitochondrial suspension and partially purified, shows a kinetic behavior of two active site enzyme or two forms of isoenzymes present in the same preparation.

K_{mapp} values are lower than the K_m, this indicate that' the L-arginine transport through mitochondrial membranes envolves a type active process.

The hepatic arginase of Z. brevirostris is inhibited by Mn^{2+} concentrations above 500 mM, this characterizing presence of two binding sites, one as activator site and other as inhibitor site.

Resumo

A atividade argininolítica do fígado de *Zapterix brevirostris* foi estudada em condições de pH e temperatura próxima às condições fisiológicas, utilizando preparações de arginase em suspensão mitocondrial e parcialmente purificada. O comportamento cinético foi característico de enzimas com dois sítios ativos. Valores de K_{mapp1} =1,22:t0,17 mM e k_{mapp2} = 64,29:t14,77 mM foram obtidos com a arginase em suspensão mi tocondrial, e K_{m1} = 2,32:t0,17 mM e K_{m2} = 561,50 899,40 mM com a arginase parcialmente purificada. O cátion manganês, em concentrações de até 0,25mM, tem efeito ativador sobre a atividade da arginase e efeito inibidor em concentrações acima de 0,5 mM, em ambas as preparações. Os valores de IC_{so} do $MnCI_2$ foram de 4,16 mM e 3,07 mM, para arginase em suspensão mitocondrial e parcialmente purificada, respectivamente. Os estudos evidenciam as membranas mitocôndriais como um provável sítio de controle do metabolismo.

Palavras-chave

Raia. Elasmobranchii. Arginase. Metabolismo Nitrogenado. Arginina.

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