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A SENSITIVE ASSAY FOR ORNITHINE AMINO TRANSFERASE IN RAT BRAIN MITOCHONDRIA BY NINHYDRIN METHOD

H Ravi Kumar[1,2,4], S Ananda[6], K S Devaraju[3], B M Prakash[5], S Sampath Kumar[4], S V Suresh Babu[1], N Ramachandraswamy [2] and H P Puttaraju[4]

[1]Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Bangalore-560029, India.
[2]Department of Biochemistry, Central College Campus, Bangalore University, Bangalore-560001, India.
[3]Department of Biotechnology, Jnanabharathi Campus, Bangalore University, Bangalore-560056, India.
[4]School of Natural Sciences, Biological Sciences, Jnanabharathi Campus, Bangalore University, Bangalore-560056, India.
[5]Evolutionary biology laboratory, Jawaharlal Nehru Center for Scientific Research, Jakkur, Bangalore-560064, India.
[6]PES Institute of Technology (PESIT), 100 Feet Ring Road, BSK III Stage, Bangalore-560085, India

ABSTRACT

To establish/develop an assay method for measuring Ornithine Aminotransferase (EC.2.6.1.13) activity using rat brain mitochondria as a source of enzyme in presence and absence of Pyridoxal Phosphate (PLP). The modified method, with the improved sensitivity, is adopted for the assay of ornithine amino transferase activity in rat brain mitochondria. The enzyme activity was measured at 620 nm, the study showed that reaction was optimum at 37°C for 30 minutes. The assay is sensitive enough to detect activity at the order of nanomoles pyrroline-5-carboxylate/mg protein/minute and can be compared as an alternative to the radio isotopic method which is more cumbersome and aminobenzaldehyde method which is less sensitive. The K_m & V_{max} shows maximum activity in the presence of Pyridoxal Phosphate (Coenzyme) concentration at 0.05mM when compared with absence of Pyridoxal Phosphate as higher the concentration of Pyridoxal Phosphate affects the affinity of the enzyme to substrate. The OAT activity in different tissues of the rat was also studied and highest activity was found in liver and kidney.

KEY WORDS

Ornithine Aminotransferase, Pyridoxal Phosphate, Pyrroline-5-carboxylate-Ninhydrin complex, Transmission electron microscope.

INTRODUCTION

Ornithine Aminotransferase (OAT; L-Ornithine: 2-oxo-acid Aminotransferase; EC 2.6.1.13) is a Pyridoxal phosphate (PLP) dependent mitochondrial enzyme which catalyzes the reversible transamination of L-ornithine and α-Ketoglutarate to glutamate and glutamic –γ- semialdehyde. Glutamic –γ-semialdehyde cyclises non-enzymatically to form pyrroline-5-carboxylate (P5C). OAT is expressed in nearly all mammalian tissues, including liver, Kidney, brain, skeletal muscles and eyes. OAT plays a role in arginine catabolism, pyroline biosynthesis or denovo Ornithine biosynthesis, depending on the tissue and the physiological circumstances (2). Liver OAT is suggested to be involved in the Ornithine synthesis for urea cycle, while the kidney OAT participates in Ornithine degradation (3, 4). In humans, a genetic deficiency of OAT causes gigrate atrophy, an autosomal recessive degenerative disease of Choroid and retina of the eye that leads to blindness (5, 6).

OAT is known as a mitochondrial matrix enzyme and has been purified from various tissue sources (7) including human liver (8). The mitochondrial OAT has been shown to be synthesized as a large precursor molecule with N-terminal leader peptide on cytoplasmic ribosome which then processed and gets associated with the mitochondria (9). The amino acid sequence

Address for Correspondence :

Mr. H. Ravi Kumar,
School of Natural Sciences, Biological Sciences,
Jnanabharathi Campus, Bangalore University,
Bangalore-560056, Karnataka, India.
Phone: +91-80-22961923
E-mail: ravikumarh79@gmail.com

of OAT precursor protein was predicted from the nucleotide sequence of c-DNA (10,11). In human kidney, OAT had the same nucleotide sequence as in human liver OAT (12) and rat kidney OAT had the identical amino acid sequence with that of rat liver OAT (13). Recently it has been reported that Ornithine amino transferase activity in WBC's/ Platelets lysate(s) have been shown in the human blood with presence and absence of PLP (14). In spite of importance of P5C in overall intermediate metabolism, little is known about the circulatory/ tissue levels and urinary excretion of P5C. The lacunae in our understanding of the role of the OAT in health and disease appear to be due to the metabolic constraint, in terms of insensitivity of the currently available assay methods. Nevertheless, methods based on o-aminobenzaldehyde(15) and ninhydrin (16) derivation of P5C have been successfully used to monitor the formation of the product (P5C) of OAT activity in tissues.

The present study deals with the isolation of mitochondria from the *Albino* rat brain (Strain *wistar*) and using it as an enzyme source to study the Ornithine amino transferase activity, assayed by modified ninhydrin method (substrate 35mM L-ornithine and 5mM α-ketoglutarate) in the presence and absence of PLP. Further the method was successfully adopted by varying the concentration of PLP as OAT is a PLP dependent mitochondrial enzyme, and higher concentration of PLP affects the affinity of the enzyme to the substrate. OAT activities in the different tissues of the Albino rat have also been assayed by modified ninhydrin method.

MATERIALS AND METHODS

Dissection of *Wistar albino* Rat: Rats were anesthetized using ether and decapitated along the neck region (the jugular vein of rat). The abdominal region was dissected and 20ml of normal saline (0.9%) was injected into the left ventricle of the heart to make the brain free from blood and to clean the external part of the brain. After injecting the saline the cadaver was cleaned and the neck was cut and dissected through the sides of the spinal cord. The top of the skull having a rounded region is called cranium (to protect the brain) was removed through scissor tip, to get inside and snipped along the seam where the skull bone is knit together. Caution was taken not to dig beneath the brain. After removing the skull, the clean and fresh brain was taken and kept in 0.9% saline and placed in an ice box. The rat brain was homogenized with glass homogenizer at 4°C in cold room. After preparing the homogenate, differential centrifugation (Sorvall: 873 WIDB) was done for the mitochondrial preparation.

Preparation of Mitochondrial Fraction: Tissues (0.5g) were homogenized at 4°C in 6ml buffer (Sucrose (300mM) HEPES (5mM) Dthiothrietol (3mM) & EDTA (0.2mM) pH 7. The homogenate was centrifuged at 600g at 4°C for 10 minute. The supernatant fraction was centrifuged at 12,000 g at 4°C for 15 minute. The pellet (mitochondria) was resuspended in 1.5 ml of fractionation buffer [Sucrose (300mM) HEPES (5mM) Dthiothrietol (3mM) & EDTA (5 mM) pH: 7] and centrifuged at 600g at 4°C for 4 minute. The supernatant fraction was centrifuged at 4000g at 4°C for 10 minute.The pellet (mitochondria) was suspended in homogenization buffer and stored in –80°C (28).

Transmission electron microscope (JEOL CXII 100) detection of Mitochondria in rat brain: The mitochondria obtained was analysed by electron microscopy to ascertain its purity for assay. After the centrifugation the pellet was dissolved in 3% glutaraldehyde and kept for 24 hour shaking. After 24 hours the fraction was fixed in 2.5% Glutaraldehyde / Karnovsky's (4% Paraformaldehyde+3% Glutaraldehyde) fixative in phosphate/Sodium Cacodylate buffer pH 7.2 –7.4 for 24 hours, the tissue was washed with buffer till the smell of the fixative was completely removed. It was then treated with 1% Osmium tetroxide for one and half hour, and washed with phosphate/cacodylate buffer, 70% alcohol – overnight or 1 hr, 80% alcohol - 1 hr, 90% alcohol - 1 hr. The pellet was treated with 2% Uranyl acetate in 95% ethanol (Enblock stain) - 1 hr, Absolute alcohol - 1 hr (Clearing) – Propylene oxide – 10-15min. – 2 changes (should be over by 30 min.) Infiltration with araldite: propylene oxide = 1:1 – overnight, Infiltrated with fresh araldite, with 3 changes in a gap of 3-4 hrs. Finally it was embedded and kept at 60°C for 48 hrs.

Assay for Ornithine Amino transferase (OAT: EC: 2.6.1.13) activity in rat brain mitochondria: Crude enzyme solution was prepared by homogenizing the brain of adult albino rat as described in preparation of mitochondrial fraction and treated with Triton X-100 (0.4% v/v), centrifuged at 5000 xg for 15 minutes. The supernatants were then used for protein estimation by Bradford's dye binding method. The assay mixture comprised substrates [35mM L-ornithine, 5mM α-ketoglutarate and cofactor 0.05mM pyridoxalphosphate] in 50mM Tris-HCl (pH-8.0) of a total volume of 1 ml enzyme. The reaction was pre-incubated for 10 minutes in substrate buffer cocktail and allowed to continue for 37°C for 30min .The reaction was stopped by adding 0.25ml of 3.6N perchloric acid and 0.25ml of 2% ninhydrin in water. Tubes were then kept in a boiling water bath for 15 min, a water-insoluble reddish pigment was precipitated by centrifugation (2500 xg/10 minutes) and dissolved with 0.5ml of ethanol and followed by

adding of 0.5 ml of 100 mM Tris HCl buffer, pH: 8.0 which transforms the colour to bluish and absorbance was measured at 620nm. The enzyme activity was expressed as nanomoles pyrroline-5-carboxylate/mg protein/minute (14).

Determination of K_m and V_{max} for ornithine: The K_m and V_{max} for ornithine for rat brain mitochondrial fraction were determined using varying concentration of Ornithine, in the presence and absence of 0.05mM PLP determined to be the optimum PLP concentration. A 700 mM ornithine stock (117.05 mg of ornithine in 1.5 ml) was prepared. The stock was diluted to prepare the ornithine series (17.5, 35, 70, 140, 280, 420, 560, 700). The assay was carried out as mention above. During the absence of PLP, the reaction volume was maintained by adding 100µl of water in the place of PLP. The values were calculated and plotted against substrate concentration using **GraphPad Prism V 3.0** to determine the K_m and V_{max}.

RESULTS AND DISCUSSION

Ornithine aminotransferase (OAT) is a Pyridoxal phosphate dependent enzyme. OAT is a mitochondrial matrix enzyme. The enzyme is coded by the nuclear gene and in humans the functional OAT gene is located on the 10^{th} chromosome and catalyses the reversible conversion of ornithine and α-ketoglutarate to P5C and glutamate (17). Glutamate semialdehyde is the initial product formed by the removal of the δ-amino group of ornithine; however, it cyclizes spontaneously to form P5C. P5C is the immediate precursor of proline, which can be synthesized either from the glutamate or from ornithine in the reaction catalyzed by P5C synthase or from ornithine in the reaction catalyzed by OAT. Physiological concentration of ornithine inhibit P5C synthase; thus, high concentrations should favour the OAT mediated pathway of proline synthesis (18). For example, in hormonally stimulated rat mammary gland, Arginase, OAT and P5C reductase activities increase co-ordinately in response to hormonal stimulation and arginine is a major biosynthetic precursor for the proline used in milk protein synthesis (19).

Several radiochemical assay methods for orinithine Aminotransferase has been devised (20) since the initial colorimetric assay using o-aminobenzaldehyde (15) but o-aminobenzaldehyde method is still used for conventional assay because radiochemical methods require complicated steps such as column chromatography (20) or trapping of radiolabeled CO_2 (21) to separate the product from the reaction mixture. In ninhydrin method, stable ninhydrin replaces o-amino bnezaldehyde which is very unstable and rapidly polymerizes at room temperature. The ninhydrin method was found to be six times more sensitive than o-aminobenzaldehyde method under the same volume conditions, sensitive enough for tissue such as muscles. A water-insoluble reddish pigment was formed with P5C-ninhydrin complex and the sensitivity was further increased by increasing the volume assay mixture and/or decreasing the volume of the dissolving solvent. This method can be adapted to either low or high substrate concentrations and has high substrate concentrations and greater sensitivity, specificity than the spectrophotometric method (16). In normal individuals, fasting plasma ornithine concentrations range from 40 to 120µM, with a mean of 60 to 80 µM. Two distinct entities are associated with significant increase in plasma ornithine concentration (22). They are, gyrate atrophy, of the choroid and retina with symptoms limited mainly to the eye and the Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome, with symptoms resulting from ammonia accumulation and protein aversion (23). The former conditions due to a deficiency of the mitochondrial matrix enzyme ornithine amino transferase (OAT), while the basic defect in the latter is in the transporter that mediate ornithine entry into mitochondria (24,25). In the earlier study (14) we have increased sensitivity for the assay of OAT activity from WBC's and platelet lysate (s) and noticed the differential degree of enzyme activation from two sources., viz., WBC's (five fold increase) and platelets (nine fold increase) in presence of exogenous Pyridoxal phosphate, though the basal activates in the absence of Pyridoxal phosphate of two tissue sources. The observed differential degrees of OAT activation by exogenous Pyridoxal phosphate from different tissue sources, viz., WBC's and platelets, appear to suggest their differential affinity to cofactor is because of different isoenzymes or heteromers.

The present study involved isolation of mitochondrial fraction

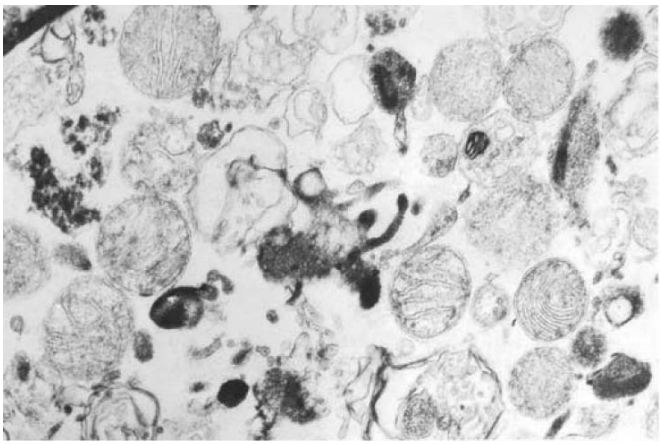

Fig 1: Transmission electron microscope (JEOL CXII 100) studies: for purity check of rat brain mitochondria

from albino rat brain. After having established the purity, based on transmission electron microscope as shown in the Fig 1 the Ornithine amino tansferase from rat brain mitochondria was assayed and the absorbance was measured at 620 nm as shown in Fig 2. OAT activity was measured in various tissues from rat as shown in the Table 1. Liver showed the highest activity while the activity in kidney and small intestine were about a half of that in liver. The OAT activity from rat liver was also mainly distributed in the mitochondrial fraction. OAT is known to play a different role in arginine, Ornithine and proline metabolism depending on the tissue and their physiological needs. Highest enzyme activity in rat liver suggest that the possibility of the different metabolic needs between the species. The observed differential degree of OAT activation by exogenous PLP from rat brain mitochondria appears to suggest their differential affinity to the Co-factor because of the differential stimulatory effect of PLP on OAT activity. The study show that reaction is optimum at 37°C for 30 minutes and the reaction was also carried by different concentration of Ornithine and also different concentration of PLP (0.1mM to 1mM) which shows that there is no linear relationship between velocity and PLP concentration and good activity was observed at the concentration of PLP 0.05 mM. Because higher the concentration of PLP affects the affinity of the enzyme to substrate. The enzyme kinetic analysis indicated a K_m value of 5.089 mM and V_{max} value of 1.570 nmol/min in the presence of PLP (0.05 mM). The K_m value of 2.089 mM and V_{max} value of 0.565 nmol/min in the absence of PLP. These values were calculated from the Lineweaver-Burk plot Fig 3. The results indicates that it has more affinity to the substrate than in the absence of the PLP, which in turn implies the critical role played by PLP as cofactor during the catalysis of Transamination reaction The assay is sensitive enough to detect activity at the order of nmol/ml and can be a practiced as an alternative compared to the radioisotopic

Table 1: Distribution of OAT in rat tissues

Tissues	OAT activity units/g tissue
Liver	115.6 ± 1.4
Kidney	72.1 ± 6.1
Small Intestine	48.3 ± 5.8
Lung	28.4 ± 1.3
Spleen	22.5 ± 1.3
Brain	20.0 ± 1.8
Heart	16.8 ± 1.1
Muscle	5.2 ± 0.2

Homogenate of each tissues was prepared in 9 volumes of 0.25 M sucrose solution containing 0.2mM Pyridoxal phosphate using homogenizer. OAT activity of each homogenate was assayed by the modified ninhydrin method.

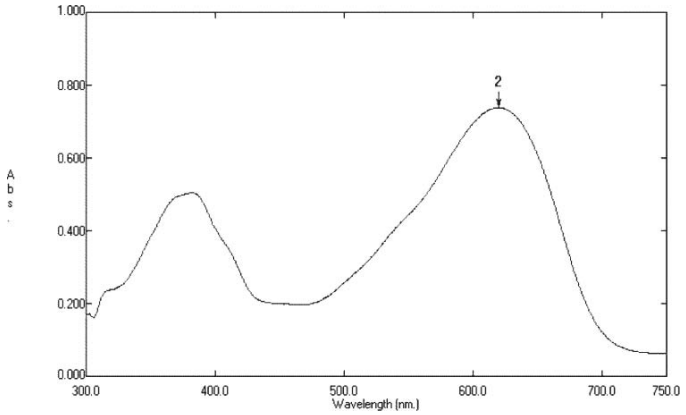

Fig 2: Absorption spectral of characteristics of OAT activity in rat brain mitochondria (Note the λ_{max} at 620 nm)

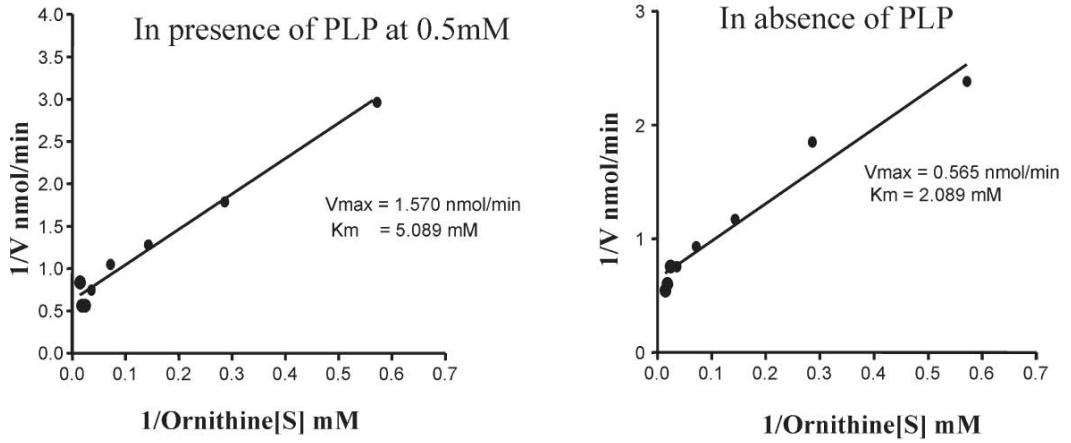

Fig 3: Lineweaver-Burk plot of 1/V versus 1/ Ornithine (S) in both presence and absence of PLP. Initial rates of reaction V were calculated from the A_{620nm} versus time by varying Ornithine concentrations

method which is more cumbersome and aminobenzaldehyde method which is less sensitive. These observations of differential stimulatory effect of PLP on OAT activity from different tissues opens new avenues to study the physiological relevance of these enzymes vis a vis interaction with PLP. The standardization of the enzyme kinetics can be extended to detect enzyme activity in patients of Gyrate Atrophy of Choroid and retina with hyperornithinemia by studying the OAT activity.

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